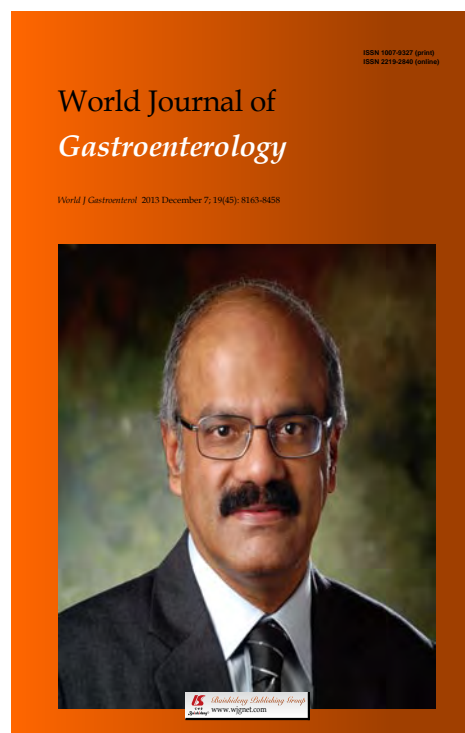
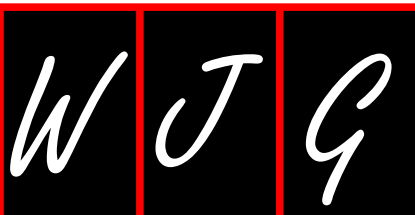


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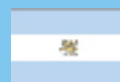
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## Are metabolic factors still important in the era of direct antiviral agents in patients with chronic hepatitis C?

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### Abstract

The high rate of sustained viral response (SVR) to boceprevir or telaprevir-based triple therapy in hepatitis C (HCV)-related, non-cirrhotic naïve patients or relapsers to previous antiviral treatment leads clinicians to believe that the impact of metabolic host factors on SVR is minimal when triple therapy is used, unlike what is observed with the peginterferon and ribavirin schedules. This concept is strongly expressed by some opinion leaders on the basis of the data derived from sub-analyses of registrative trials as well as from a post-hoc analysis of the phase II C208 clinical trial. The perception of unrestrainable therapeutic success with the use of newer, more powerful antivirals is now reinforced by the brilliant results obtained with sofosbuvir, an HCV NS5B polymerase inhibitor, as well as by the data from the phase II and III studies on the various combinations of second-generation NS3/4A inhibitors and NS5A and/or NS5B inhibitors. However, a great deal of concern has emerged from the real world scenario in which patients are often older and have more comorbidities than patients in the "world of trials". Furthermore, many of them have advanced fibrosis and previous failure with peginterferon and ribavirin treatment. Some

data from the recent literature suggest that the host metabolic factors may play a minor but non-negligible role in these difficult-to-treat patients, an issue that will hopefully be investigated in further studies. This editorial aims to provide a detailed analysis of the role that host metabolic factors played in the past and what role they may play in the era of direct antiviral agents.

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**Key words:** Metabolic factors; Insulin resistance; Direct antiviral agents; Chronic hepatitis C

**Core tip:** This editorial explores the past and present role of metabolic factors by analyzing the data that has emerged from the post hoc analysis of registrative trials of direct antiviral-based treatment. Low-density lipoprotein-cholesterol and statin use proved to be predictors of sustained viral response (SVR) in both boceprevir and telaprevir-treated patients, respectively. Furthermore, HOMA-IR negatively influenced SVR in prior partial and null responders treated with telaprevir-based schedules. By transferring these data to the real world scenario in which patients have comorbidities, advanced fibrosis and prior failure to antiviral treatment, we believe that metabolic factors might play a non-negligible role in influencing antiviral response, even in triple therapy.

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### INTRODUCTION

More than 170 million people are chronically infected

with the hepatitis C virus (HCV) worldwide<sup>[1]</sup>, thus HCV has become the main cause of chronic liver disease leading to death from liver failure or hepatocellular carcinoma (HCC) in many Western countries and in Japan<sup>[2]</sup>. Viral eradication resulting from antiviral treatment has led to a decrease in these complications. However, treatment with pegylated interferon and ribavirin, which has been the standard-of-care therapy for chronic hepatitis C for the last decade, has been able to cure only about 40%-50% of patients with hepatitis C genotype 1, the main strain in Europe and the United States<sup>[3-6]</sup>.

Genotype 1 is the main viral-related variable associated with treatment failure<sup>[3,4]</sup>. However, a number of other pretreatment variables related to the virus (high viral load) and to the host (interleukin 28b polymorphism, age, overweight, metabolic factors), as well as on-treatment variables (4-wk negativization of viral load) have been shown to affect the response to anti-viral dual therapy<sup>[7-10]</sup>.

## HCV INFECTION AND METABOLIC SEQUELAE: WHAT WE LEARNED FROM THE PAST

### Insulin resistance

Epidemiological data suggest that HCV interferes with glucose and lipid metabolism. Patients with diabetes show a greater prevalence of HCV as compared to the non-diabetic population<sup>[11]</sup>. Moreover, the prevalence of diabetes is significantly higher in chronic hepatitis C patients than in those with chronic liver disease other than HCV<sup>[12]</sup>. Furthermore, it is well known that HCV infection is an independent risk factor for developing diabetes<sup>[13]</sup>. These data suggest that HCV, rather than liver disease *per se*, predisposes patients to diabetes. The link between HCV and diabetes is the development of insulin resistance (IR), a metabolic prerogative of HCV (*i.e.*, IR is more than six fold higher in HCV patients than in those with HBV)<sup>[14]</sup>. IR, hepatic steatosis and body mass index (BMI) are related in a genotype-dependent fashion. In fact, HCV genotype 3 exerts a direct cytopathic and steatogenic effect on hepatocytes, thus resulting in a higher prevalence of steatosis and a lower prevalence of IR compared with HCV genotype 1, which very quickly induces IR and some steatosis<sup>[15-17]</sup>. IR occurs very early in transgenic mice expressing the HCV core protein and may even precede the occurrence of hepatic steatosis, thus indicating that IR is not a consequence of hepatic steatosis<sup>[18]</sup>, similarly to what is observed in humans<sup>[17]</sup>. Furthermore, IR is a pro fibro genetic stimulus<sup>[17]</sup>, thus patients with high IR show more advanced liver fibrosis than patients with low IR<sup>[19]</sup>. Finally, the association between IR and high HCV RNA levels<sup>[14]</sup> suggests a complex interplay between viral replication and insulin action. HCV may induce over-expression of the suppressor of the cytokine signaling-3 (SOCS3) gene in liver tissue. This gene is involved in the interferon signaling pathway and

is associated with poorer treatment outcome<sup>[20,21]</sup>. HCV core-induced SOCS3 may promote proteosomal degradation of the insulin receptor substrates 1 and 2 (IRS1/2), thus inducing severe hepatic IR<sup>[22,23]</sup>. Both SOCS3 over-expression and hepatic steatosis promote intra-hepatic and systemic lipid oxidation, thus leading to an imbalance of total glucose disposal in the muscles and resulting in peripheral (and not only hepatic) IR<sup>[24]</sup>.

Direct involvement of HCV in glucose metabolism has also been demonstrated "*in vivo*". In fact, there is robust evidence showing that IR improved significantly in patients with HCV genotype 1 who achieved SVR compared with patients who did not obtain viral clearance after treatment<sup>[25,26]</sup>. Furthermore, achieving viral clearance is demonstrated to significantly reduce the risk of both type 2 diabetes in retrospective cohorts<sup>[27]</sup> and of de novo IR in non-diabetic HCV patients<sup>[28]</sup>.

### Lipids

Hepatic steatosis is also related, in a genotype-specific manner<sup>[29]</sup>, to a decrease in serum levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (apoB), thus demonstrating the close link between HCV and lipids. A variable fraction of HCV in the serum circulates in lipo viro particles (LVP), with very-low-density-lipoprotein (VLDL) containing apoB and apoE. LVP reach the highest levels in the post prandial phase, suggesting that their formation is a dynamic process<sup>[30]</sup>. Very recently, a strong correlation between the maximum amount of LVP *in vivo* and both IR and metabolic syndrome was reported, suggesting that lipids may play a role in HCV-induced IR<sup>[31]</sup>. ApoE is considered the central component of the HCV-host lipid interaction, mediating HCV infectivity *via* lipoprotein receptors<sup>[32]</sup>. Lipoproteins (LP) are easily endocytosed, thus supporting the hypothesis that HCV can use this association with LP to adhere to the cell and subsequently enter the host cell by endocytosis<sup>[33]</sup>. Various cell surface receptors, including tetraspanin CD814, scavenger receptor class B member I, tight-junction proteins claudin-1 and occludin, and clathrin-mediated endocytosis have been proposed as entry factors for HCV, but the role each of them plays remains controversial. Recently, the Niemann-Pick type C1-like 1 (NPC1L1) gene receptor has come to the attention of researchers in the view of a potentially new therapeutic antiviral strategy since it is the possible target of the receptor-blocker drug ezetimibe<sup>[34,35]</sup>.

Few and inconsistent data have been reported on serum lipid level modifications during interferon therapy. Increased total cholesterol and triglyceride levels have been observed with interferon treatment, with a subsequent drop to pretreatment levels of both after discontinuing therapy, but with different trends depending on the HCV genotype<sup>[36,37]</sup>. In a small population of patients with genotype 2 and 3, viral clearance induced serum level modifications of lanosterol, a cholesterol precursor, suggesting a direct viral interference with the enzymes of sterol synthesis<sup>[29]</sup>.



### ***The effects of IR on antiviral response to dual treatment***

Patients with high IR show a slower decay of HCV viral load than patients with low IR, even in the very early phase of treatment (first 24 h), suggesting that hyperinsulinemia reduces the cellular response to pegylated-interferon<sup>[38]</sup>. Furthermore, high IR has been associated with a low rate of rapid viral response (RVR) in genotypes 1<sup>[39]</sup>, 3<sup>[40]</sup> and 4<sup>[41]</sup>.

Whether or not IR influences SVR rate has been a question of debate since 2005<sup>[9]</sup>. Two meta-analyses assessing the impact of IR on treatment outcome, both of which included fourteen studies with more than 2700 patients, were published in 2011<sup>[42,43]</sup>. However, among the studies which failed to find an association between IR and SVR, the main baseline HOMA value, an indirect measurement of IR<sup>[44,45]</sup>, was < 3 and the prevalence of advanced fibrosis or cirrhosis was also low or even absent<sup>[42]</sup>. This observation supports the hypothesis that the HOMA value is predictive of response to antiviral treatment mainly in patients with advanced disease stage. Liver fibrosis is an event which may occur as a consequence of HCV-related chronic necroinflammatory activity or via HCV related IR, or probably both. However, non-HCV related IR (genetic, or related to true metabolic syndrome) may also occur since almost 25% of the general population has the metabolic syndrome stigmata<sup>[46]</sup>. On the basis of these data, we can assume that a proportion of patients with prevalent virus-related IR (likely those with lower fibrosis as well as a lower incidence of cardio-metabolic comorbidities) have lower HOMA values and a higher likelihood of SVR after antiviral treatment, whereas other HCV patients with prevalent metabolic IR (likely those with the phenotype of metabolic syndrome) have a higher probability of advanced fibrosis as well as higher HOMA levels and a lower probability of achieving SVR<sup>[28,42,47]</sup>.

### ***The effects of obesity and lipids on antiviral response to dual treatment***

Obesity is another important metabolic cofactor that can affect antiviral response. It may induce IR and hepatic steatosis, both of which are associated with poor antiviral response either directly or by ultimately promoting liver fibrosis. However, it has been demonstrated that obesity is an independent negative predictor of response to antiviral treatment regardless of genotype and cirrhosis<sup>[7]</sup>.

Obesity is now considered an inflammatory condition, resulting in an abnormal immune response to therapy. Adipose tissue secretes many proteins, including adipokines, which regulate hepatic and peripheral glucose and lipid metabolism. One of the adipokines secreted by adipose cells is leptin, whose expression is regulated by interleukin-1 (IL-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and insulin. Although leptin secretion from adipocytes provides antiobesity signals, obese patients have elevated levels of leptin. This suggests an intrinsic leptin resistance in the obese, a complex phenomenon involving increased levels of SOCS3, which impairs post-receptor signaling

and leads to reduced adenosine monophosphate-activated protein kinase (AMPK) activation<sup>[48,49]</sup>.

Increased SOCS3 expression, which is associated with nonresponse to antiviral treatment<sup>[20]</sup>, has been demonstrated to be independently associated with obesity in patients with chronic HCV viral genotype 1<sup>[50]</sup>.

Regarding the role of circulating lipid levels on the efficacy of antiviral treatment, there are few, but concordant, data. Higher pretreatment total cholesterol and LDL-C<sup>[51,52]</sup>, as well as lower triglyceride levels are independent variables associated with higher SVR rates<sup>[53]</sup>.

### ***The interplay of Interleukin 28b polymorphisms and metabolic variables***

The relationship between IL28b polymorphisms and metabolic variables has been reported in several studies, thus emerging as a new and challenging issue. There is a close association between IL28b and lipid levels. In HCV genotype 1 patients, low apoE levels and higher LDL-C were associated with IL28b rs12979860 CC rather than CT/TT<sup>[54,55]</sup>. Both IL28CC and LDL-C were good predictors of SVR, but the predictive power of IL28CC was higher. Thus, the LDL-C level was found to be a significant predictor of SVR, mainly for IL28 heterozygous CT patients<sup>[56]</sup>.

Furthermore, lower steatosis<sup>[57]</sup> as well as lower IR<sup>[58]</sup> have been seen in genotype 1 patients with IL28b rs12979860 CC. In a recent, large cohort of genotype 1 patients from Italy, IL28b rs12979860 CC was associated with higher levels of total and LDL-C, lower levels of triglycerides, lower prevalence of IR and moderate-to-severe steatosis. However, only IR and steatosis were associated with IL28b rs12979860 CC after correcting for BMI and lipid profile, suggesting an indirect role of LDL-C status on IL28b polymorphism<sup>[58]</sup>. IR was found to have a predictive power for SVR which is independent of IL28 genotype<sup>[59]</sup>, although the likelihood of achieving SVR progressively increases from the lowest probability in patients carrying both negative predictors (IR and rs12979860 TT/TC; SVR = 20.7%) to the highest probability in patients with no IR and rs12979860 CC (SVR = 78.4%)<sup>[58]</sup>. These data give rise once again to the thorny question concerning the intimate pathogenetic interplay between metabolic and genetic host factors.

### ***Can pretreatment correction of the metabolic co-factor improve SVR to dual treatment?***

One of the most intriguing and challenging tasks was to transfer the information regarding the predictive power of a metabolic variable for SVR to a practical ground. This gave rise to a series of studies aimed at improving SVR by correcting the metabolic factors before or during antiviral treatment.

Some studies with very small cohorts have explored the effect of a lifestyle intervention in obese and insulin-resistant subjects with chronic hepatitis C. BMI, HOMA values and leptin levels, but not TNF- $\alpha$  and IL-6, decreased significantly after aerobic exercise<sup>[60]</sup>. A more

structured intervention based on 24-wk dietary and physical activity regimens significantly reduced BMI and HOMA values<sup>[61]</sup> and could be an interesting baseline strategy in difficult-to-treat chronic hepatitis C patients who are obese and insulin-resistant prior to starting peginterferon and ribavirin. However, to date, no studies have demonstrated the efficacy of this strategy. Tarantino *et al.*<sup>[62]</sup> demonstrated that a low-calorie diet for 3 mo before starting antiviral therapy in patients with genotype 1-chronic hepatitis C resulted in a significant improvement in IR as well as a 60% “end-of-treatment” response rate in the low-calorie diet group as compared to the control group (17.6%).

More data have been reported on insulin sensitizing agents used in combination with peginterferon and ribavirin. The randomized, double-blind TRIC-1 trial by Romero-Gómez *et al.*<sup>[63]</sup> analyzed 125 naive genotype 1 patients treated with peginterferon alpha-2a and ribavirin plus metformin or placebo on an intention-to-treat basis. Their final results showed that there was a significant decrease in both HOMA value and viral load during the first 12 wk, as well as an improvement in SVR rate in the metformin group as compared with the placebo group, but only in females<sup>[63]</sup>. While metformin failed to improve overall SVR, conflicting results have been obtained with other insulin sensitizing agents, mainly pioglitazone. Although comforting results have been obtained in genotype 4<sup>[64]</sup>, no improvement in SVR was observed by adding pioglitazone (30 mg/d) to peginterferon and ribavirin as compared with the standard of care of dual therapy<sup>[65,66]</sup>. Similar negative results have been shown by Harrison *et al.*<sup>[67]</sup> in a randomized controlled trial which compared pioglitazone plus standard of care *vs* standard of care alone. This study definitively demonstrated that even when pioglitazone was administered at an appropriate dose (45 mg/d), it failed to improve SVR, regardless of administration timing (*i.e.*, prior to starting the standard of care or during the peginterferon and ribavirin course)<sup>[67]</sup>.

## ADVENT OF DIRECT ANTIVIRAL AGENTS AND THE NEW SCENARIO

In recent years, several antiviral drugs that directly target HCV have been developed. These new drugs, known as direct-acting antiviral agents (DAAs), are designed to interfere directly with the HCV life cycle by inhibiting enzymes such as HCV NS3/4A protease and HCV NS5B polymerase, or other proteins such as NS5A. Two NS3/4A protease inhibitors, boceprevir and telaprevir, have become the first new drugs approved for the treatment of patients with genotype 1 HCV who have either not previously received treatment or who failed to achieve SVR with previous therapy<sup>[68]</sup>. These new drugs, however, must be given with peginterferon and ribavirin because of their low barrier to viral resistance when they are used as monotherapy, and this may limit efficacy. A further limitation is due to overall side effects resulting in

higher discontinuation rates. However, when candidates for this treatment are carefully selected, the overall SVR rates can almost double in naive and even triple in relapsers to previous double therapy<sup>[69-75]</sup>. Thus, triple therapy is the new standard treatment for HCV genotype 1 chronic liver disease.

One of the questions under debate is whether the predictors of treatment failure that are observed when using dual therapy in HCV genotype 1 also exert a negative influence in triple therapy.

### What we know about metabolic factors and triple therapy

Among the variables which have shown predictive power for SVR in dual therapy, some of them, such as IL28b polymorphism, fibrosis and the 4-wk viral response in both naive or previously treated patients, have also been highlighted in triple therapy on the basis of data emerging from registrative trials. Metabolic factors seem to play either no role at all, or only a minor one in influencing SVR in the context of triple treatment. However, some considerations have to be made when looking carefully at the post-hoc analysis of landmark phase-III trials.

In the boceprevir-based SPRINT-2 trial, two metabolic variables were associated with SVR, but only statin use proved to be an independent predictor of SVR (OR = 3.4; 95%CI: 1.1-10.7; *P* = 0.04), whereas BMI was not retained in the multivariate model after adjustment for other variables<sup>[72]</sup>.

In RESPOND-2, a boceprevir-based trial focusing on previously treated patients, both the response-guided treatment (RGT) group and the 48-wk triple treatment group had a significantly higher SVR rate compared with dual treatment. However, in obese patients (BMI ≥ 30), a 10% lower SVR rate was observed in the RGT group compared with the 48-wk triple treatment group (56% *vs* 65%)<sup>[73]</sup>.

A sub-analysis of baseline predictors in the SPRINT-2 and RESPOND-2 trials showed that a BMI ≤ 30 was significantly associated with SVR and with a ≥ 1 log10 HCV-RNA decline at week 4 in untreated patients but not in patients previously treated with peginterferon and ribavirin<sup>[76]</sup>.

In the two telaprevir-based studies carried out on untreated patients, both ADVANCE<sup>[77]</sup> and ILLUMINATE<sup>[75]</sup> showed a higher SVR rate in the RGT arms compared with the double treatment arm, regardless of diabetes and obesity. In the ADVANCE study, although a 30% greater improvement in the SVR rate of patients receiving telaprevir for 12 wk was achieved in all 3 BMI groups (< 25, 25-30 and > 30) as compared to the control group, a 12%-16% lower SVR rate was observed in overweight and obese patients compared to normal weight patients.

An important contribution was provided by Serfaty *et al.*<sup>[78]</sup> in their post-hoc analysis of the phase II C208 clinical trial<sup>[79]</sup>. In this study, which is the first to focus on the influence of baseline metabolic variables on SVR

in patients treated with triple therapy, only LDL-C was associated with SVR (even in multivariate analysis), thus confirming its predictive role for SVR even in the telaprevir-based triple regimen and not only in dual therapy. However, this is not the case for baseline IR measured by the HOMA index, which did not show any relationship with SVR. Furthermore, the HOMA index did not influence the 4-wk HCV RNA decline, nor were the rates of HCV RNA undetectability at week 4 found to differ among patients with or without IR. However, this is not surprising since baseline HOMA values were not found to be associated with SVR in many European studies in which the study population had a low prevalence of advanced fibrosis and a relatively low BMI<sup>[39,40,80,81]</sup>. This may suggest that a higher prevalence of “viral” IR, which can easily be counteracted by antivirals, actually does exist in this population of patients. The significant association between HCV RNA and HOMA values in the study of Serfaty *et al.*<sup>[78]</sup>, as well as the improvement of HOMA in patients who achieved SVR compared with those who did not, further confirm the direct “*in vivo*” involvement of HCV in IR pathways. On the other hand, a powerful, combined effect on suppressing HCV viremia and rapidly lowering IR was previously observed using a 14-d course of danoprevir monotherapy, *i.e.*, another powerful, selective inhibitor of NS3/4A HCV serine protease. Interestingly, overweight patients had a greater decrease in HOMA values than patients with normal BMI, despite a similar decrease in serum HCV RNA, suggesting a complex interplay between these two variables. The authors hypothesize an anti-inflammatory or insulin sensitizing effect of danoprevir<sup>[82]</sup>.

Other important data concerning IR were obtained from a post-hoc analysis of the REALIZE phase III study which was carried out to assess the impact of IR on virological response to a telaprevir-based regimen in previously treated patients. Baseline HOMA values were found to be associated with SVR at univariate analysis (TVR: OR = 0.76; 95%CI: 0.60-0.96) but not after adjustment for other baseline prognostic factors (TVR: OR = 0.95; 95%CI: 0.71-1.29)<sup>[83]</sup>. SVR decreased as HOMA values increased, both in the control group and in the pooled T12-PR48 group where, however, this trend was observed only in prior partial and null responders, but not in prior relapsers.

In summary, on the basis of all these data we can say that LDL-C and statin use proved to be predictors of SVR in telaprevir and boceprevir-treated patients, respectively. Obesity may negatively influence rapid virologic decline as well as SVR in previously naive patients treated with a boceprevir-based regimen. To the best of our knowledge, no sub-analysis regarding the impact of obesity on SVR has ever been carried out for telaprevir. No data on HOMA are available for boceprevir. HOMA values were reported as being univariately associated with SVR in a telaprevir-based trial on previously treated patients. In naive patients treated with a telaprevir-based regimen, the HOMA value

was not a predictor of SVR, nor was it found to be associated with rapid virological response.

However, some comments have to be made. First, we have no data on the real weight of baseline metabolic factors in patients with less favorable probability of response, such as those with advanced fibrosis and/or non-CC IL28b. It is reasonable to suppose that in patients with advanced fibrosis or cirrhosis, as well as in prior partial or null responders to dual therapy and in whom non-CC IL28b is highly prevalent, metabolic IR, metabolic syndrome and obesity may be significant cofactors of nonresponse to triple therapy. Hopefully, this will be an issue for further studies. Secondly, in a real world setting we have to face a change in the epidemiology of candidates to triple therapy compared with the “world of trials”. In the “real world scenario” we have to expect a higher prevalence of patients over 65 years of age who have often previously been treated with dual therapy and have a higher prevalence of comorbidities, including hypertension, dyslipidemia and diabetes. In this context, many patients might experience either a worsening in the sensitivity to interferon as well as a higher probability of side effects when a protease-inhibitor is added to therapy.

These points seem to have been taken into consideration by United Kingdom consensus guidelines for the use of protease inhibitors in the treatment of HCV genotype 1 infected patients. These guidelines recommend evaluating the presence of factors predictive of poor response to therapy, such as BMI and type 2 diabetes among others<sup>[84]</sup>.

Currently, an enormous effort is being made by researchers and companies to provide physicians with new and more powerful drugs with a high genetic barrier and able to work on several HCV genotypes. Recently, phase II and III studies on sofosbuvir, a new nucleotide analogue HCV NS5B polymerase inhibitor used in combination with peginterferon and ribavirin in genotypes 1, 4, 5 and 6 or in combination with ribavirin in genotypes 2 and 3, showed a high rate of SVR, up to 90% in untreated genotype 1 patients after a 12-wk regimen, with no additional side effects to those occurring with peginterferon and ribavirin<sup>[85-87]</sup>.

New combinations of drugs with interferon-free schedules are under evaluation in phase II or III studies<sup>[88-91]</sup>. Hopefully, by the end of the decade the holy grail of a pangenotypic oral association of highly powerful drugs will be able to cure virtually all patients. In this scenario, the role of predictors of SVR will rapidly fade, but we have to keep in mind that in the short amount of time that separates us from the availability of new and more powerful treatment schedules, many patients will have to be treated with boceprevir and telaprevir-based triple therapy. Furthermore, many patients around the world have no, or only limited access to DAAs. With this in mind, SVR predictors remain important tools that are available to us in order to assign patients to the best treatment schedules.



## CONCLUSION

Baseline metabolic factors seem to have a minor, though likely not negligible, role in influencing antiviral response to direct antiviral agent-based treatment in patients with genotype 1 chronic hepatitis C. Further studies aimed at clarifying their role in a subpopulation of unfavorable candidates to this treatment, such as patients with advanced fibrosis or prior partial or null responders to peginterferon and ribavirin, are needed.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Transmission of hepatitis C virus: Self-limiting hepatitis or chronic hepatitis?

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## Abstract

It has been suggested that hepatitis C virus (HCV) is selectively transmitted to a new host as an infectious clone from multiple HCV variants (quasispecies) in the donor. Most individuals with HCV infection develop chronic hepatitis, but approximately 15%-40% of them clear the virus spontaneously and the hepatitis is resolved in a self-limiting manner in the acute phase of infection. This difference in the outcome of acute hepatitis C is attributable to both viral characteristics and genetic regulation of infection. In particular, the evolutionary dynamics of the infecting virus and host genetic polymorphisms pertaining mainly to the immune system, including polymorphisms in the region of the Interleukin 28B gene encoding interferon- $\lambda$ -3, are associated with susceptibility to HCV infection.

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**Key words:** Hepatitis C; Spontaneous clearance; Interleukin 28B; Single nucleotide polymorphism; Interferon- $\lambda$

**Core tip:** Most individuals with hepatitis C virus (HCV)

infection develop chronic hepatitis, but in some the hepatitis is resolved in a self-limiting manner in the acute phase of infection. What factors are responsible for this difference in the outcome of hepatitis C? The evolutionary dynamics of the infecting virus and host genetic polymorphisms pertaining mainly to the immune system, including the Interleukin 28B gene, as well as susceptibility to HCV infection, are important in determining the outcome of infection.

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## INTRODUCTION

Hepatitis C virus (HCV) infection is a major threat to public health, and about 170 million people are estimated to be infected worldwide with a potential risk of progression to cirrhosis and hepatocellular carcinoma<sup>[1,2]</sup>. This review summarizes the two current topics of HCV study: the transmission mode of HCV with multiple variants (quasispecies) and the factors associated with susceptibility to HCV infection, with special reference to viral characteristics and host genetic variation.

## MODE OF HCV TRANSMISSION: HOW IS HCV WITH MULTIPLE VARIANTS TRANSMITTED?

HCV shows significant genetic heterogeneity among isolates, and the degree of variability is unevenly distributed throughout the viral genome: some regions are conserved and some are highly variable<sup>[3]</sup>. In particular, the hyper-

variable region 1 (HVR1) of the *HCV* E2 gene encoding a putative envelope glycoprotein mutates at a high rate, resulting in a wide spectrum of mutants referred to as “quasispecies” during infection<sup>[4,5]</sup>. Some virions may contain defective RNA genomes, which also affect the infectivity and replicability of the virus<sup>[6]</sup>. The mixture of clones present determines the biological and immunological properties of the virus.

How is HCV with multiple variants (quasispecies) transmitted to the new host? Does the status of transmitted HCV consist of multiple clones or a selected single clone? The transmission mode of HCV has been investigated by sequencing of the recovered viral genome from both donor and recipient<sup>[7,8]</sup>. HCV infection in human communities has occurred sporadically because no effective neutralizing vaccine against HCV has been developed. In particular, HCV infection in health-care workers through exposure to patient's blood due to a needle stick accident or accidental droplet transmission is a serious problem<sup>[9-12]</sup>. We previously reported a case of HCV infection resulting from a needle stick accident, and had an opportunity to investigate how HCV variants from the donor are transmitted to the recipient by comparing the HCV HVR1 genome encoding the envelope E2 protein recovered from the serum of both the donor and recipient<sup>[7]</sup>. In this case, we had observed the recipient before the onset of hepatitis and collected serum samples after obtaining informed consent. Thus, we were able to compare the HCV HVR1 genome between the donor's HCV at inoculation and the recipient's HCV just after onset of viremia. Interestingly, a minor subset of the donor's HCV clones was selectively transmitted to the recipient, and this selection determined the predominant clone in the new host. Several clones that appeared to stem from the recipient's predominant clone had one amino acid change within the HVR1 region during this short period. This particular case progressed to chronic hepatitis, and the same phenomenon has been demonstrated in the case of acute, self-limiting hepatitis<sup>[8]</sup>. These data suggest that a minor clone of the donor's HCV is transmitted and adapts to the new host. The precise mechanism of this viral selection in the initial phase of transmission has not been elucidated.

The simplicity of the transmitted viral strain in the initial phase of infection may explain some of the important clinical manifestations. Anti-viral therapy using interferon elicits a favorable response in the acute phase of HCV infection<sup>[13-16]</sup>. In addition, if a single strain is transmitted selectively in the initial phase of infection, this specific strain may be one of the factors determining disease activity. In fact, a study using a model of HCV transmission has demonstrated that a specific HCV strain recovered from a patient with fulminant hepatitis caused unusually severe hepatitis in a chimpanzee to which it was transmitted<sup>[17]</sup>. At present, the specific strain of HCV responsible for progressive liver disease cannot be discriminated from viral quasispecies in contaminated blood. Further investigation would be useful for clarifying the

specific viral strain responsible for the disease, and such efforts would be important for planning future strategies for the development of an effective therapeutic vaccine.

## SELF-LIMITING HEPATITIS OR CHRONIC HEPATITIS? HOW IS SUSCEPTIBILITY TO HCV DETERMINED?

### *The spontaneous clearance rate of HCV in the acute phase of infection*

Most individuals with HCV infection fail to clear the virus and develop chronic hepatitis with a risk of progression to cirrhosis and hepatocellular carcinoma. However, a small proportion of individuals are known to show resolution of the infection in a self-limiting manner. The rate of spontaneous viral clearance in acute HCV infection is reported to be approximately 15%-40% of all HCV-infected individuals<sup>[18-20]</sup>. Although differences in study populations such as race may influence the clearance rate in each cohort, a systematic review of 31 studies has estimated this rate to be 26%<sup>[20]</sup>. We have previously reported a Japanese population-based cohort study of the natural history of HCV infection in an area where community-acquired acute hepatitis C is endemic; here, the spontaneous viral clearance rate was estimated to be approximately 20%<sup>[21,22]</sup>. What is the difference between self-limiting resolution of hepatitis and progression to chronic hepatitis? Comparative studies of this issue have focused on both viral characteristics and genetic regulation.

### *Viral characteristics influencing the outcome of acute hepatitis C*

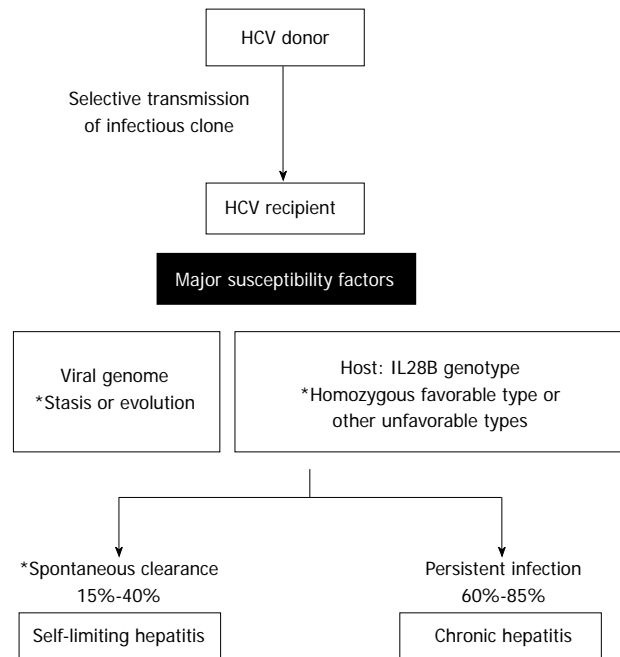
After the establishment of HCV infection, the viral genome mutates at a high rate, especially in the HVR1 of the HCV E2 region. The evolutionary dynamics of the infected virus are associated with the outcome of acute hepatitis C; genetic stasis and a high rate of evolution of HCV HVR1 are associated with resolution of infection in self-limiting hepatitis and progression to chronic infection, respectively<sup>[23]</sup>. The case we experienced progressed to chronic infection and 8 of 30 homogeneously predominant HCV HVR1 clones recovered from the recipient developed one amino acid mutation within this region during a short period of only 6 wk after infection<sup>[7]</sup>. As for the relationship between the viral load at the time of infection and the outcome of acute HCV infection, a recent study has shown that a high viral load in the initial phase of infection is associated with spontaneous viral clearance, leading to self-limiting resolution of hepatitis<sup>[24]</sup>. A high viral load may trigger strong innate immunity in the acute phase. However, it has also been reported that viral clearance may occur after a low infectious dose of HCV has been transmitted<sup>[25]</sup>. In addition, spontaneous viral clearance rarely occurs in the chronic phase of HCV infection where a low viral load is associated with spontaneous clearance<sup>[26]</sup>. The spontaneous clearance of HCV may thus depend on the immune system of indi-

viduals rather than the viral load. Further studies using a greater number of cohorts are needed to clarify the relationship between spontaneous viral clearance and the initial viral load, as well as the degree of induction of the innate immune response.

### Genetic regulation of HCV infection

HCV-specific humoral and cellular immune responses are detectable in infected individuals, and a strong immune response against HCV favors viral clearance<sup>[18,27]</sup>. Genetic variation in host genes involved in immune response is likely to account for the difference in outcome. In particular, induction of natural killer (NK) cells in the innate immune response during the acute phase of infection plays a crucial role in resolving HCV infection. We have previously reported differences in genetic variations between HCV-infected individuals with and without viremia in the Japanese population<sup>[22]</sup>, where a single nucleotide polymorphism (SNP) of transforming growth factor (TGF)- $\beta$ 1, which suppresses the proliferation and cytotoxicity of NK cells (the -509CC genotype or -509C allele), was associated with high HCV clearance rates and low transcriptional activity of TGF- $\beta$ 1<sup>[28]</sup>. The killer cell immunoglobulin-like receptor (KIR) and its human leukocyte antigen (HLA) have been reported to influence the outcome of HCV infection. Combinations of genotypes involving genes encoding the inhibitory NK cell receptor KIR2DL3 and HLA-C1 ligand directly influence HCV clearance in Caucasians and African Americans with an expected low infectious dose of HCV<sup>[25]</sup>. These data suggest that a diminished inhibitory effect of NK cells resulting from such gene regulation confers protection against HCV.

In a recent genome-wide association study, SNPs in the region of the Interleukin 28B (*IL28B*) gene encoding interferon- $\lambda$ -3 were shown to be closely associated with the virologic response of HCV to antiviral therapy<sup>[29-31]</sup>. Patients carrying an *IL28B* homozygote for the major alleles of rs12979860 (CC genotype)<sup>[29]</sup> or rs8099917 (TT genotype)<sup>[30]</sup> show a greater propensity to achieve a sustained virologic response to pegylated interferon- $\alpha$  and ribavirin therapy than those carrying an *IL28B* heterozygote or homozygote for its minor allele. This SNP (rs12979860) also influences the outcome of HCV infection in the context of natural history; the CC genotype enhances resolution of HCV infection with spontaneous clearance among individuals of European and African ancestry<sup>[32]</sup>. This CC genotype has also been reported to be associated with a higher rate of spontaneous clearance in Asian populations<sup>[33]</sup>. In addition, a recent study has demonstrated that SNPs in the region of *IL28B* (rs12979860) and HLA class II (rs4273729) are independently associated with spontaneous resolution of HCV infection in individuals of European and African ancestry<sup>[34]</sup>. A prospective follow-up study of patients who developed acute hepatitis C also revealed a strong correlation between the *IL28B* C allele at rs12979860 and clearance<sup>[24]</sup>. Taken together, the SNP of *IL28B* (rs12979860) can be a marker



**Figure 1** Transmission of hepatitis C virus, and the significance of viral and host factors for predicting the outcome of infection. HCV: Hepatitis C virus; IL28B: Interleukin 28B.

for indicating whether immediate antiviral treatment needs to be started in patients with acute hepatitis C<sup>[35]</sup>. Recently, upstream of the *IL28B* gene, a dinucleotide variant ss469415590 (TT or AG), in which ss469415590 (AG) activates the *IFNL4* gene encoding interferon- $\lambda$ -4 protein through a genome frameshift, has been reported to be more strongly associated with HCV clearance in individuals of African ancestry than the SNP of *IL28B* (rs12979860), but comparable to that in Europeans and Asians<sup>[36]</sup>. This variant is in high linkage disequilibrium with rs12979860, and further investigations are expected to elucidate the functional role of ss469415590 (AG) that activates the *IFNL4* gene in association with the innate immune response to HCV.

## CONCLUSION

Both the viral characteristics of an infecting clone and genetic regulation of infection by the host determine differences in the outcome of acute HCV infection (Figure 1). The evolutionary dynamics of the virus and genetic polymorphisms in the host pertaining mainly to the immune system influence susceptibility to HCV. In particular, the discovery of SNPs in the region of the *IL28B* gene has led to the characterization of a novel genetic marker of hepatitis C that is able to predict self-limiting viral clearance in the acute phase of infection as well as the response to antiviral therapy.

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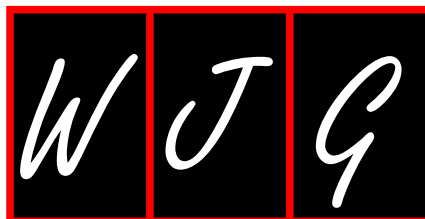
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## WJG 20<sup>th</sup> Anniversary Special Issues (13): Gastrointestinal endoscopy

# New progress in endoscopic treatment of esophageal diseases

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**Core tip:** The detailed indications and processes of minimally invasive endoscopic therapy based on endoscopic submucosal dissection for esophageal diseases are described.

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## Abstract

The technique of endoscopic submucosal dissection (ESD), which was developed for *en bloc* resection of large lesions in the stomach, has been widely accepted for the treatment of the entire gastrointestinal tract. Many minimally invasive endoscopic therapies based on ESD have been developed recently. Endoscopic submucosal excavation, submucosal tunneling endoscopic resection and laparoscopic-endoscopic cooperative surgery have been used to remove submucosal tumors, especially tumors which originate from the muscularis propria of the digestive tract. Peroral endoscopic myotomy has recently been described as a scarless and less invasive surgical myotomy option for the treatment of achalasia. Patients benefit from minimally invasive endoscopic therapy. This article, in the highlight topic series, provides detailed information on the indications and treatments for esophageal diseases.

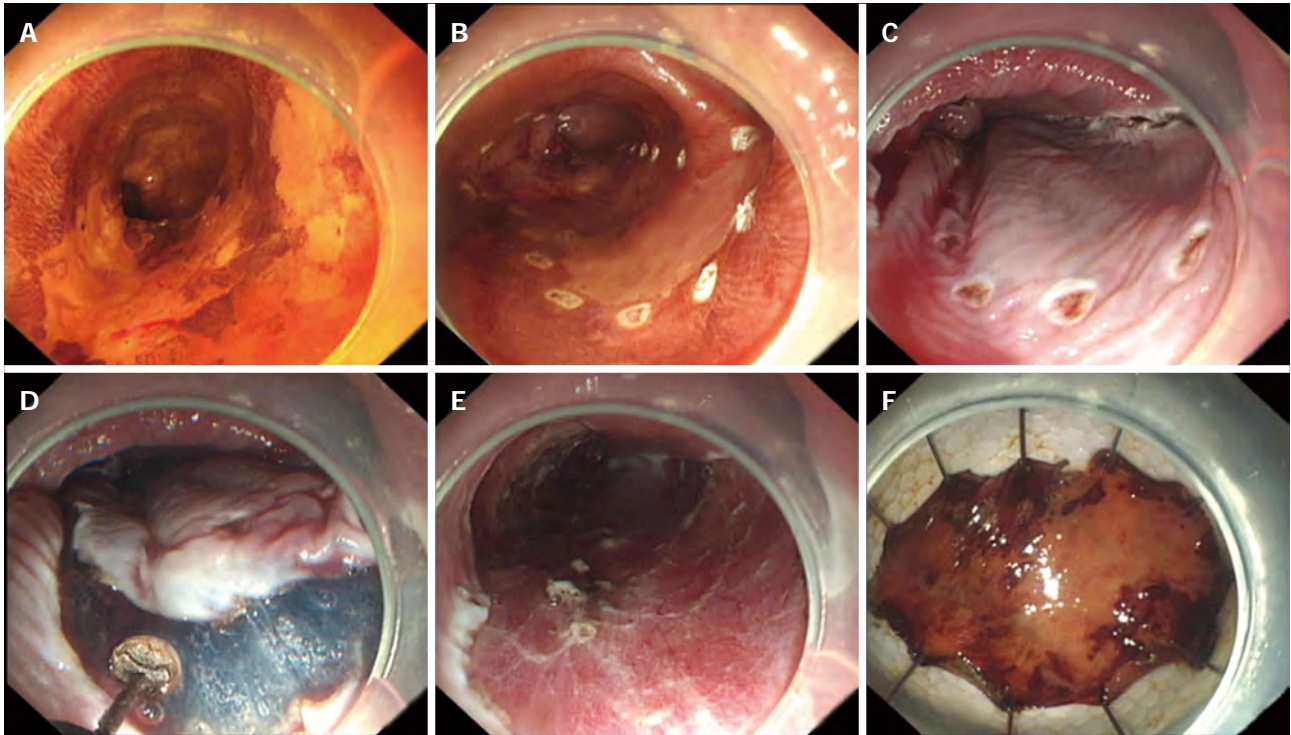
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## INTRODUCTION

Endoscopic submucosal dissection (ESD) was originally developed in Japan as a method of endoscopic “*en bloc*” resection of superficial gastric cancers. Today, ESD is increasingly used for superficial esophageal cancers and esophageal submucosal tumors (SMTs)<sup>[1,2]</sup>. With improved ESD technology, it has been used not only for tumors, but also for functional disorders, and has produced good therapeutic effects. In this review, an outline of the current status of minimally invasive endoscopic therapy based on ESD for esophageal diseases is described.

## ESD

As with candidates suffering from other gastrointestinal tract diseases, patients scheduled for esophageal ESD are assessed using two factors: a small likelihood of lymph node metastasis and technical resectability<sup>[3,4]</sup>. ESD for lesions in esophageal squamous epithelium is performed as outlined in Figure 1. The lesion is dyed with 2% Lugol's solution to obtain a clear margin, and



**Figure 1** Process of endoscopic submucosal dissection. A: The lesion is dyed with 2% Lugol's solution; B: Marking of the lesion by argon plasma coagulation probes; C: Submucosal injection; D: The mucosa is incised outside the marker dots, and then the submucosal tissue underneath the lesion is gradually dissected; E: The wound after resection; F: The lesion.

several marker dots are made approximately 5-10 mm from the margin of the lesion using argon plasma coagulation (APC). After injection of several milliliters of injection solution into the submucosal layer, the mucosa is incised outside the marker dots with an electric knife (IT knife, hook knife, or HybridKnife). The submucosal tissue underneath the lesion is then gradually dissected with the electric knife. The solution is injected repeatedly, when necessary, during the dissection. Exposed vessels on the artificial ulcer are coagulated with APC or coagulation forceps to prevent delayed bleeding, and metallic clips are always used to close the deeply dissected areas. Although numerous injection solutions have been proposed and tested, saline is the most commonly used in the clinic due to its low cost and ease of use. Indigo carmine is added to obtain a clear layer and epinephrine is used to reduce bleeding. Hyaluronic acid is also used to improve submucosal lift durations<sup>[5-7]</sup>.

## ENDOSCOPIC SUBMUCOSAL EXCAVATION

With improved ESD technology, ESD is not only used for mucous layer tumors, but also for muscularis propria tumors of the digestive tract. During this process, ESD is used to remove the submucosa muscularis propria of the tumor and to peel the tumor from the muscularis propria. We consider this application a new technology and named it endoscopic submucosal excavation (ESE). ESE is performed as outlined in Figure 2: (1) Marker

dots are made approximately 5 mm away from the lesion; (2) Using a 23-gauge disposable needle, several milliliters of submucosal injection solution are injected around the lesion to lift it off the muscularis propria layer, but only the mucosa is lifted, not the tumor. This step shows that the tumor has originated from the muscularis propria layer; (3) The mucosa is then incised outside the marker dots using the electric knife; (4) The submucosal connective tissue beneath the lesion is gradually dissected from the muscularis propria layer with the electric knife to show the lesion; and (5) The electric knife is used to peel the muscularis propria layer along the edge of the lesion. Finally, the lesion is resected completely from the muscularis propria layer using the electric knife<sup>[8,9]</sup>.

## SUBMUCOSAL TUNNELING ENDOSCOPIC RESECTION

ESE was developed to allow *en bloc* resection of SMTs which originated from the muscularis propria layer under direct vision. However, the surgical area in the esophagus is limited and the esophageal adventitia is very thin. If perforation occurs and the mucosal defect cannot be completely closed, serious complications such as esophageal fistula will occur. We performed a new endoscopic technique for the treatment of SMTs, called submucosal tunneling endoscopic resection (STER), which can reduce the risk of postoperative GI tract leakage and secondary infection. The procedure for STER is as follows: (1) Submucosal injection of diluted indigo carmine or methylene



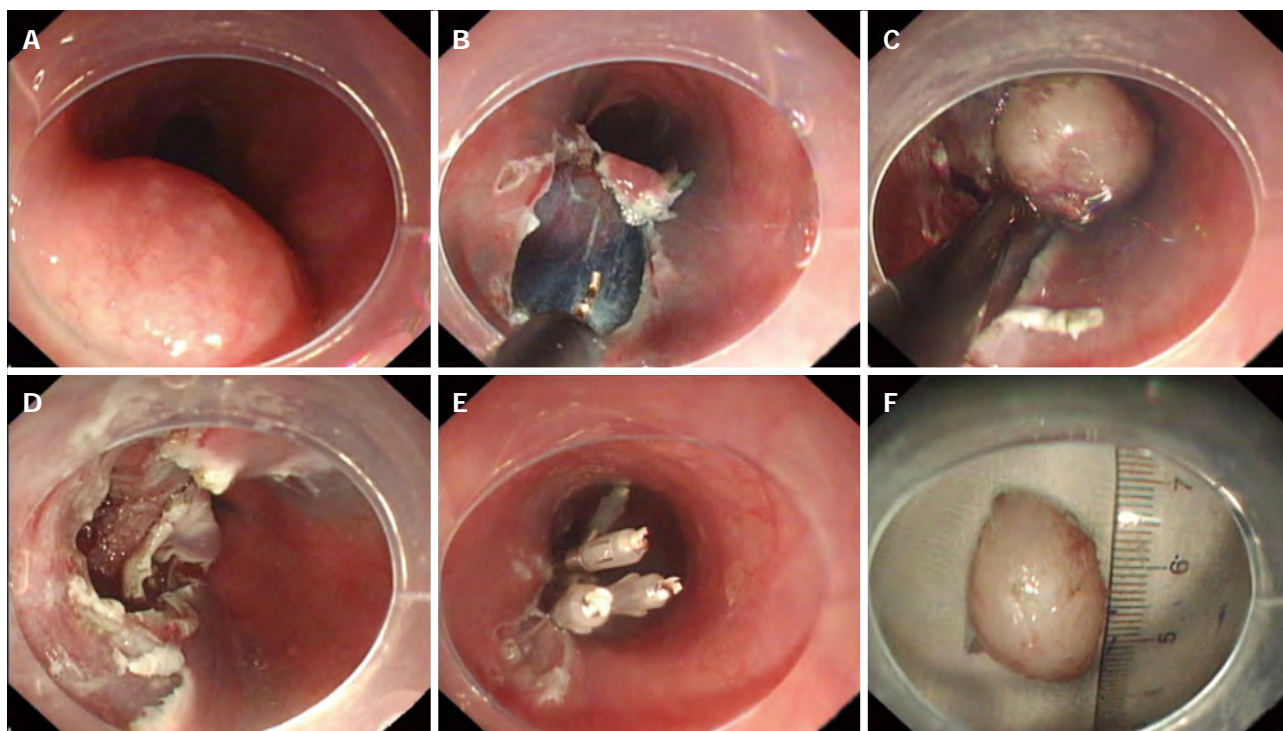


Figure 2 Process of endoscopic submucosal excavation. A: A round-shaped submucosal tumor; B: Removal of the mucosa; C: Dissecting the tumor from the muscular layer; D: The wound after resection; E: Sealing the wound with metallic clips; F: The lesion.

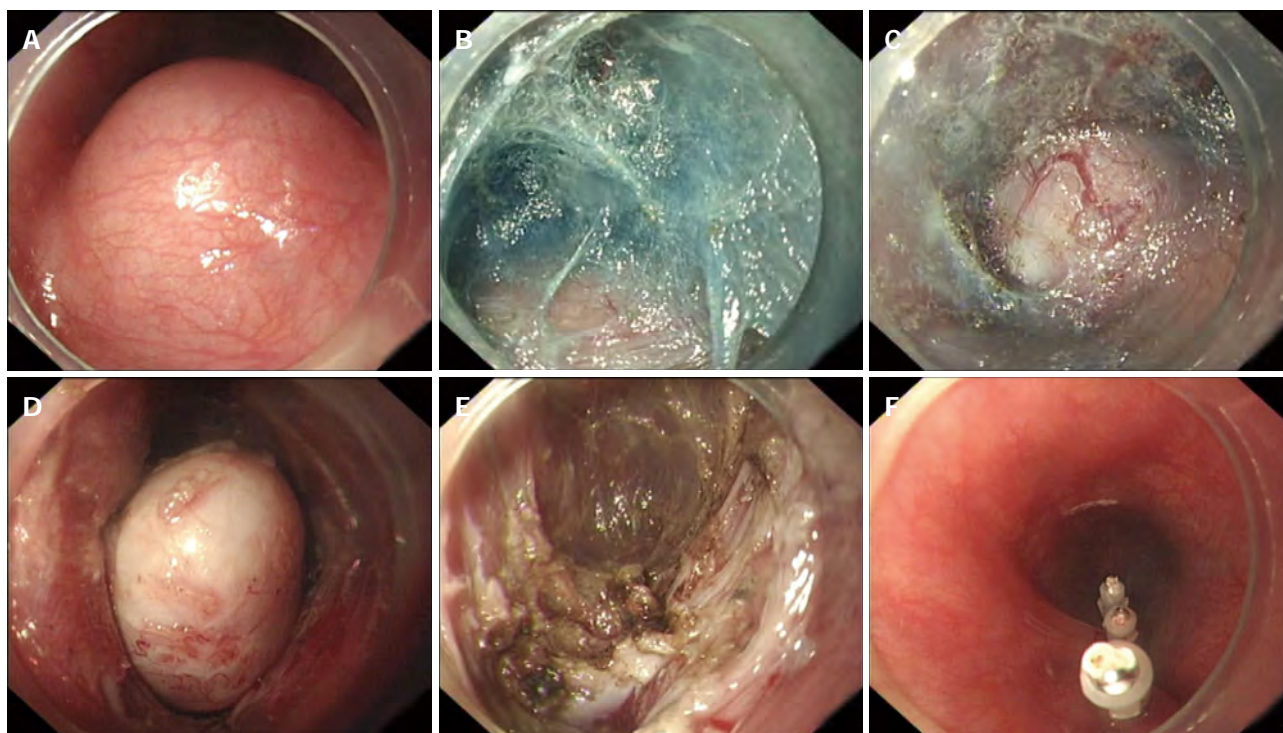
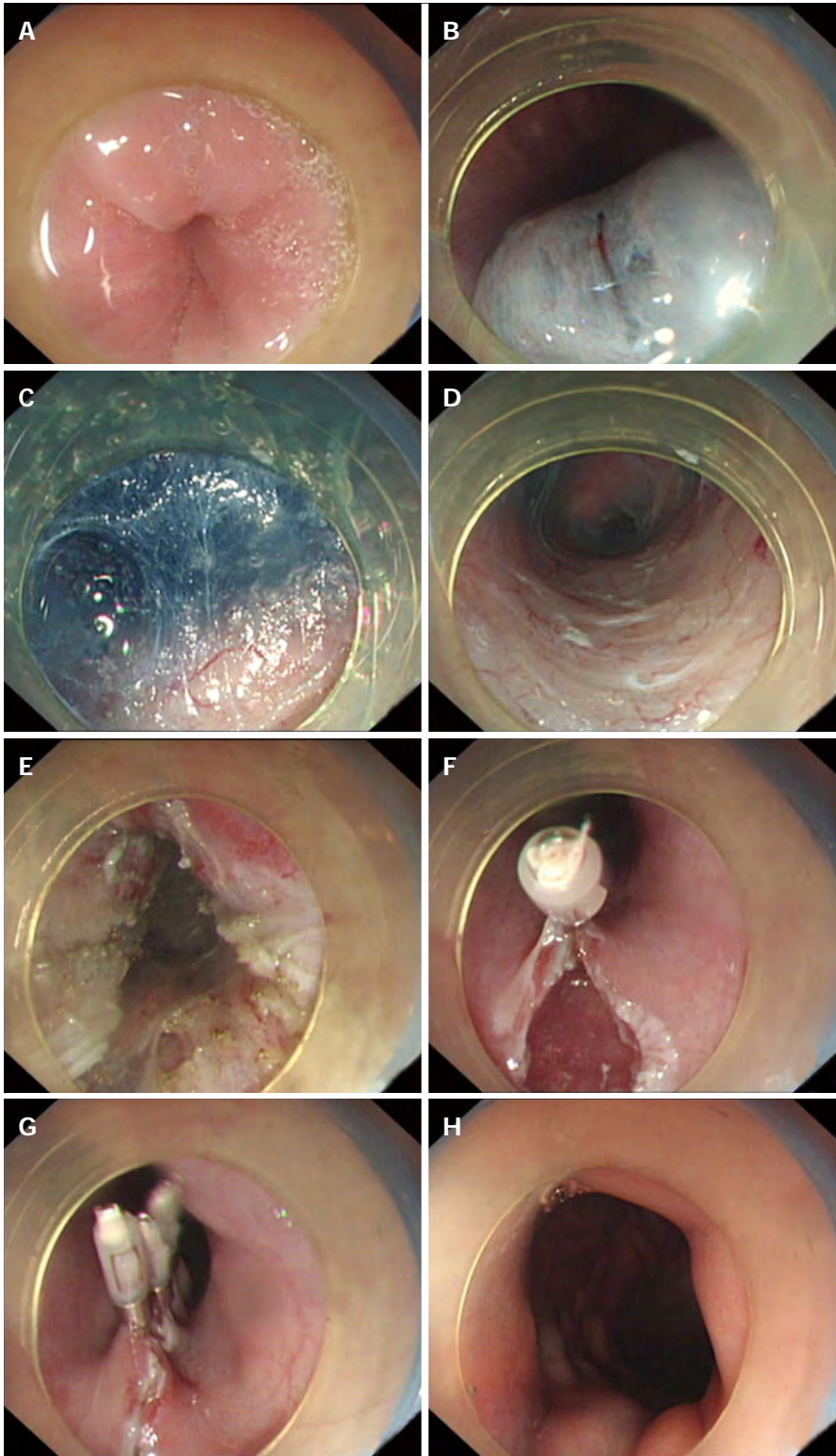


Figure 3 Process of submucosal tunneling endoscopic resection. A: The tumor; B: A submucosal tunnel was created; C: Revealing the submucosal tumor; D: Dissecting the tumor from the muscular layer in the submucosal tunnel; E: The wound after resection; F: Closure of the mucosal incision site with clips.

blue is needed to locate SMTs in the cardia when they are difficult to identify; (2) A submucosal tunnel is created to expose the tumor. A fluid cushion is obtained using an injection needle 5 cm proximal to the SMT. A 2-cm, lon-

gitudinal mucosal incision is made using the electric knife at the esophageal mucosa as the entry point. A submucosal longitudinal tunnel is created with the electric knife between the submucosal and muscular layers. Tunneling

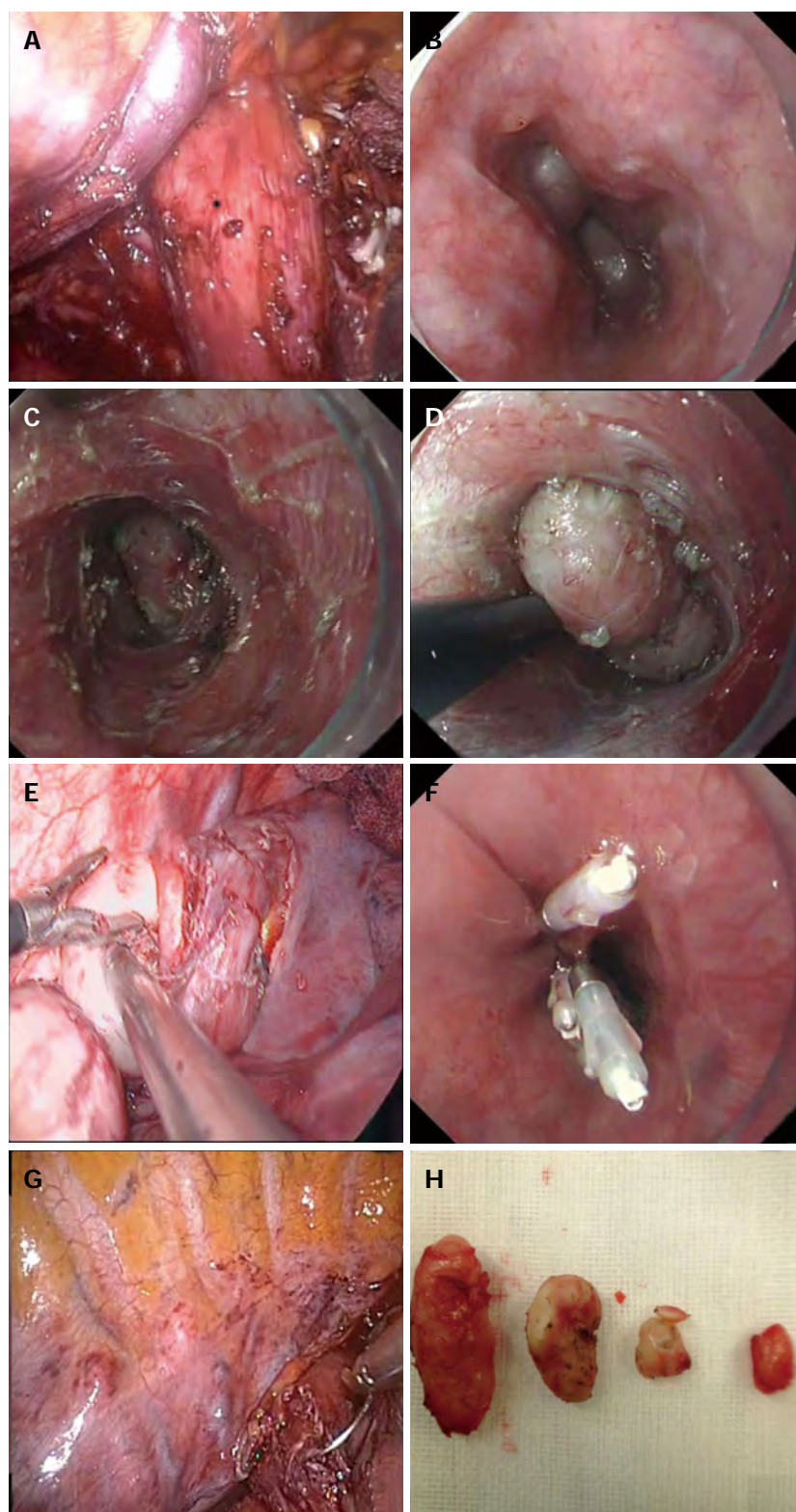




**Figure 4** Process of peroral endoscopic myotomy. A: The esophagogastric junction (EGJ) before surgery; B: Submucosal injection and an initial mucosal incision; C-D: A submucosal tunnel was created; E: The muscle was incised; F-G: The mucosal incision site was closed with clips; H: EGJ after surgery.

ends 1-2 cm distal to the tumor to ensure a satisfactory endoscopic view of the SMT and enough working space for resection; (3) Resection of the SMT is performed under direct endoscopic visualization. During the procedure, safe and complete resection of the tumor, without interruption of the tumor capsule, is the highest priority;

and (4) Closure of the mucosal incision site. After tumor resection and hemostasis with APC or hot biopsy forceps in the tunnel, the gastroscope is withdrawn through the natural orifice. Most commonly, the mucosal incision site is closed with 4 to 6 hemostatic clips<sup>[10,11]</sup> (Figure 3).



**Figure 5** Process of thoracoscope-assisted tunnel endoscopic resection. A: Thoracoscopic exploration of the tumor; B: Endoscopic exploration of the tumor; C-D: A submucosal tunnel was created to expose the tumor under endoscopy, which was the same as the submucosal tunneling endoscopic resection procedure; E: The thoracoscopic electric hook was used to separate the esophagus and open the muscular layer over the tumor by blunt dissection, we removed the large tumor from the chest; F: Use of metallic clips to close the mucosal entry; G: Use of thoracoscopy to suture the pleura mediastinalis; H: The lesions.

## PERORAL ENDOSCOPIC MYOTOMY

Peroral endoscopic myotomy has recently been described as a scarless and less invasive surgical myotomy option for

the treatment of achalasia<sup>[12,13]</sup>. Briefly, submucosal injection and an initial mucosal incision are first performed at the 5- to 6-o'clock position on the posterior esophagus, approximately 10 cm proximal to the esophagogastric



junction (EGJ). A submucosal tunnel is created passing over the EGJ and approximately 3 cm into the proximal stomach. The myotomy is initiated 2 cm distal to the mucosal entry point, approximately 6-8 cm above the EGJ, and is extended for a distance of 2-3 cm toward the stomach. After careful hemostasis, the mucosal incision site is closed with 4 to 6 hemostatic clips<sup>[14-16]</sup> (Figure 4).

## THORACOSCOPE-ASSISTED TUNNEL ENDOSCOPIC RESECTION

With the development of ESD technology, endoscopy is used to treat large esophageal SMTs. Thus, endoscopic treatment is risky and of limited value in patients with huge esophageal or extraluminal SMTs. Minimally invasive thoracoscopic treatment also has its advantages, however, for esophageal SMTs larger than 3 cm, use of the thoracoscope involves a risk of tearing the mucosa when separating tumors, causing serious complications such as GI leakage. Laparoscopic-endoscopic cooperative surgery has been introduced<sup>[17,18]</sup>. Recently, we developed combined thoracoscopic and endoscopic therapy to treat esophageal SMTs originating from the muscularis propria. The indications for thoracoscope-assisted tunnel endoscopic resection are: (1) Tumors larger than 5 cm; and (2) U-shaped tumors larger than 3 cm and which extend to more than 1/2 of the esophagus circumference. This type of tumor is difficult to treat by endoscopic or thoracoscopic treatment alone. The detailed steps of this procedure are as follows: The patient lies in the left lateral position, the thoracoscope is introduced and any adhesion or pleural effusion in the pleural cavity is checked; (1) Thoracoscopic exploration of the tumor; (2) Submucosal injection is administered and a submucosal tunnel is created to expose the tumor using the ESD technique under endoscopy, which is the same as the STER procedure; (3) If the tumor is so large that it can not be removed from the tunnel by endoscopy, the thoracoscopic electric hook is used to separate the esophagus and open the muscular layer over the tumor by blunt dissection. In addition, the submucosal "tunnel" is under surveillance by endoscopy to avoid esophageal mucosa injury; (4) Under endoscopic surveillance in the submucosal tunnel, the esophageal muscular layer is sutured by thoracoscopy, and insufflation in the tunnel is performed to observe whether there is any leakage in the thoracic cavity. The endoscope is withdrawn from the submucosal tunnel after hemostasis and metallic clips are used to close the mucosal entry; (5) Endoscopy is used to identify mucosal rupture. The esophagus is insufflated to observe any leakage in the thoracic cavity and then thoracoscopy is used to suture the pleura mediastinalis; and (6) A drainage tube is placed in the chest cavity if there is no obvious bleeding<sup>[19]</sup> (Figure 5).

The main complications consist of bleeding, perforation and esophageal stricture, most of which were treated by endoscopy and surgery was not required<sup>[20-22]</sup>. With the development of endoscopic techniques and the improvement in people's health consciousness, the model of the

diagnosis and treatment of some esophageal diseases is changing. Patients benefit from minimally invasive endoscopic therapy based on ESD, which results in reduced pain, rapid resumption of normal activity, and shorter hospital stay.

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## PNPLA3 I148M polymorphism and progressive liver disease

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### Abstract

The 148 Isoleucine to Methionine protein variant (I148M) of patatin-like phospholipase domain-containing 3 (PNPLA3), a protein is expressed in the liver and is involved in lipid metabolism, has recently been identified as a major determinant of liver fat content. Several studies confirmed that the I148M variant predisposes towards the full spectrum of liver damage associated with fatty liver: from simple steatosis to steatohepatitis and progressive fibrosis. Furthermore, the I148M variant represents a major determinant of progression of alcohol related steatohepatitis to cirrhosis, and to influence fibrogenesis and related clinical

cal outcomes in chronic hepatitis C virus hepatitis, and possibly chronic hepatitis B virus hepatitis, hereditary hemochromatosis and primary sclerosing cholangitis. All in all, studies suggest that the I148M polymorphism may represent a general modifier of fibrogenesis in liver diseases. Remarkably, the effect of the I148M variant on fibrosis was independent of that on hepatic steatosis and inflammation, suggesting that it may affect both the quantity and quality of hepatic lipids and the biology of non-parenchymal liver cells besides hepatocytes, directly promoting fibrogenesis. Therefore, PNPLA3 is a key player in liver disease progression. Assessment of the I148M polymorphism will possibly inform clinical practice in the future, whereas the determination of the effect of the 148M variant will reveal mechanisms involved in hepatic fibrogenesis.

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**Key words:** Alcoholic liver disease; Chronic hepatitis C virus hepatitis; Fibrogenesis; Genetics; Hepatocellular carcinoma; Liver disease; Nonalcoholic fatty liver disease; Patatin-like phospholipase domain-containing 3; Single nucleotide polymorphism; Steatosis

**Core tip:** The 148 Isoleucine to Methionine protein variant (I148M) of patatin-like phospholipase domain-containing 3 (PNPLA3) has recently been identified as a major determinant of liver fat content. Several studies conducted in different ethnicities confirmed that I148M influences the full spectrum of liver damage: from simple steatosis to nonalcoholic steatohepatitis and progressive fibrosis to hepatocellular carcinoma. Furthermore, I148M turned out to represent a major determinant of progression of alcohol related steatohepatitis, and to influence fibrosis progression and related clinical outcomes in chronic hepatitis C virus hepatitis, as well as other in liver diseases. All in all, studies suggest that the PNPLA3 I148M polymorphism may represent a general modifier of fibrogenesis in liver diseases.

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## INTRODUCTION

The clinical evolution of chronic liver diseases is highly variable, and genetic factors plays a key role in determining the inter-individual susceptibility towards end-stage liver disease and hepatocellular carcinoma. It is now established that all common diseases, including type 2 diabetes, atherosclerosis, and nonalcoholic fatty liver disease (NAFLD) among liver diseases<sup>[1]</sup>, exhibit a heritable component of susceptibility accounting for 30%-50% of risk. Indeed, also liver diseases can be considered complex traits that result from environmental exposures, *e.g.*, diet and physical inactivity for NAFLD, excessive alcohol intake for alcoholic liver disease (ALD), or infection for chronic viral hepatitis, acting on a susceptible polygenic background comprising multiple independent modifiers<sup>[2]</sup>. These are generally represented by common genetic variants with a mild effect or rare variants associated with a more marked phenotype<sup>[3]</sup>.

Despite initial hypothesis-driven, case-control studies identified some genetic loci associated with the progression of liver damage, the genetic determinants of NAFLD remained obscure until recently<sup>[1]</sup>. By 2008, the first genomewide association studies in the field of hepatic steatosis allowed to identify the rs738409 variant, by an hypothesis free drive approach, as the single major genetic determinant of hepatic fat content<sup>[4,5]</sup>. This sequence variation is a C > G single nucleotide change, encoding for the 148 Isoleucine to Methionine protein variant (I148M) of Patatin-like phospholipase domain-containing 3 (PNPLA3). The main purpose of this review is to provide an overview of the current knowledge of the PNPLA3 I148M polymorphism role in the progression of liver disease.

## NONALCOHOLIC FATTY LIVER

Ectopic fat accumulation in the liver related to systemic insulin resistance represents the prototypical manifestation of NAFLD<sup>[6]</sup>. With a prevalence of 20%-34% and rising, NAFLD is now the most frequent liver disease in industrialized countries<sup>[7,8]</sup>. In a minority of susceptible individuals, steatosis, that is excessive fat accumulation in the liver (> 5%), is associated with oxidative hepatocellular damage, inflammation and activation of fibrogenesis, *i.e.* nonalcoholic steatohepatitis (NASH)<sup>[9,10]</sup>, which can progress to cirrhosis and hepatocellular carcinoma<sup>[11,12]</sup>.

NAFLD is epidemiologically associated with obesity and metabolic syndrome. The pathogenesis is related to adipose tissue insulin resistance<sup>[13]</sup>, leading to an increased flux of free fatty acids to the liver<sup>[14]</sup>, increased lipogen-

esis induced by hyperinsulinemia, abnormal intra-hepatic lipid metabolism and dietary factors. Hepatic fat accumulation, in turn, worsens insulin resistance and liver damage, determining an increased risk of both cardiovascular and liver related mortality<sup>[15-17]</sup>.

Besides hepatocytes, two cell types play a key role in the pathogenesis of NASH. Kupffer cells are the hepatic macrophages that under basal conditions are involved in the maintenance of immune homeostasis, but during NASH become activated by intestinal bacterial products and oxidized lipids *via* Toll-like receptor-4 and secrete reactive oxygen species, chemokines and several cytokines, thereby orchestrating inflammation<sup>[18]</sup>. Hepatic stellate cells are hepatic pericytes localized between sinusoidal endothelial cells and the hepatocytes, which in quiescent conditions store lipids and retinoids, secrete extracellular matrix, and regulate blood flow. Upon activation in NASH, stellate cells release retinoids, undergo myofibroblast transition, secrete type 1 collagen and a variety of fibrogenic mediators thereby initiating the process of fibrogenesis<sup>[19,20]</sup>.

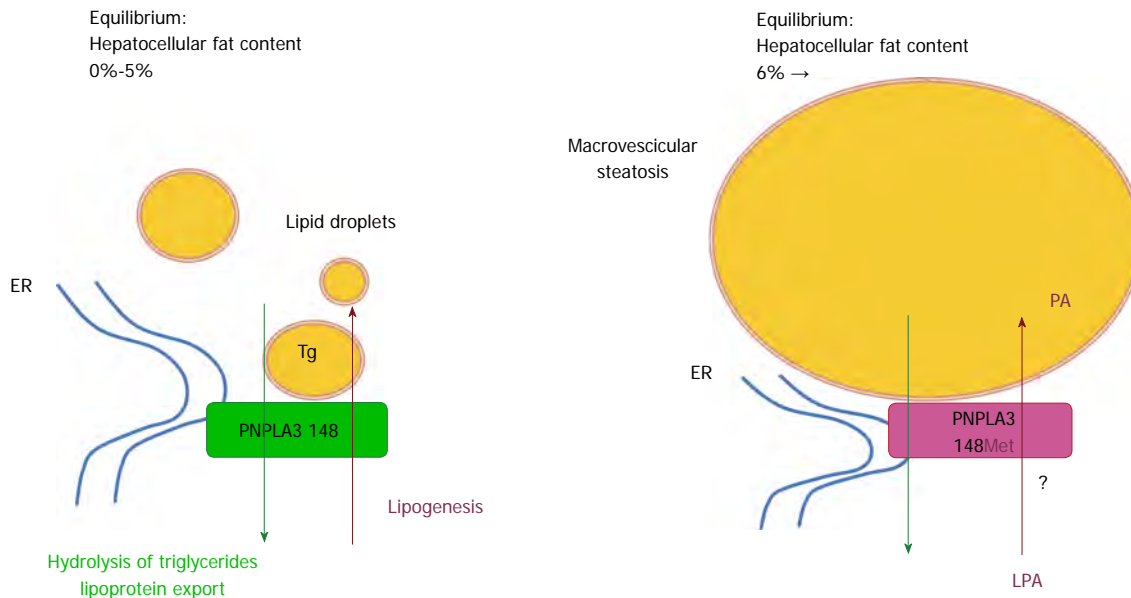
## GENETIC PREDISPOSITION TO FATTY LIVER

The risk of NAFLD is highly variable even in individuals with obesity and type 2 diabetes. Furthermore, even if the majority of obese subjects with metabolic syndrome develop simple steatosis, only about one third has NASH, and a minority progresses to more severe forms of the disease.

Epidemiological, familial and twin studies provide evidence for a component of heritability of liver fat content and NAFLD<sup>[21,22]</sup>. Indeed, NAFLD is more prevalent in Hispanics compared to Europeans, and less common in African-Americans, and this difference is not explained by diabetes and obesity<sup>[22,23]</sup>. Family studies demonstrated a strong heritability of NASH<sup>[21,24,25]</sup>. Accordingly, twin studies shown that, in subjects without viral hepatitis and alcohol abuse, alanine aminotransferases (ALT) levels and liver fat content are strongly heritable traits, with genetic factors explaining up to 60% of variability<sup>[26,27]</sup>. Overall, evidences indicate that about half of steatosis variability, determined by biochemical indices or noninvasive assessment of liver fat, is inherited<sup>[1]</sup>. Therefore, several hypothesis-driven studies tried to evaluate the role of candidate genetic variants in the susceptibility to NAFLD and progressive NASH, with the goal of identifying disease markers or potential drug targets, but with inconsistent results<sup>[1]</sup>.

## PNPLA3 I148M IS A MAJOR DETERMINANT OF FATTY LIVER

In 2008, two independent genome-wide association studies linked the common rs738409 polymorphism of PNPLA3 (I148M) with hepatic fat content and ALT levels<sup>[4,5]</sup>. In particular, a genomewide scan of the associa-



**Figure 1** Hypothetical mechanism of hepatic fat accumulation associated with the 148 Isoleucine to Methionine protein variant patatin-like phospholipase domain-containing 3 polymorphism. ER: Endoplasmic reticulum; LPA: Lyso-phosphatidic acid; PA: Phosphatidic acid; Tg: Triglycerides; ?: To be confirmed; PNPLA3: Patatin-like phospholipase domain-containing 3.

tion of non-synonymous sequence variations in the Dallas Heart Study revealed a very strong association of increased fat content with a single missense variant, I148M, in PNPLA3<sup>[4]</sup>.

Remarkably, the association between PNPLA3 I148M and liver fat was independent of major differences in body composition, diabetes and serum lipoprotein levels. Furthermore, the 148M at risk allele was more prevalent in Hispanics [minor allele frequency (MAF): 0.49] than in Europeans (MAF: 0.23), and less common in Afro-Americans (MAF: 0.17) explaining a consistent fraction of the inter-ethnic variability in NAFLD susceptibility<sup>[4,28]</sup>.

Since then, several studies and a recent meta-analysis have replicated the association between the I148M polymorphism and NAFLD in all ethnic groups, both in adults and in the developmental age<sup>[28-41]</sup>. The association of the I148M variant with hepatic lipid content is exposed in the presence of other risk factors, such as severe obesity<sup>[32]</sup>, visceral adiposity<sup>[42]</sup>, increased intake of sugars<sup>[43]</sup> or omega-6 poly-unsaturated fatty acids<sup>[44]</sup>, and other genetic factors<sup>[45,46]</sup>. Vice versa, weight loss results in a rapid decrease of intra-hepatic fat and of indices of liver damage in subjects homozygous for the 148M variant<sup>[47]</sup>. All in all, these data suggest that the 148M variant becomes a critical factor determining hepatocellular fat accumulation when stressing factors such as increased flux of free fatty acids related to adipose tissue insulin resistance in visceral obesity, increased lipogenesis stimulated by hyperinsulinemia and carbohydrates, or altered lipid metabolism intervene.

## FUNCTION OF WILD-TYPE AND MUTANT PNPLA3

PNPLA3, also called adiponutrin, encodes a 481 amino

acid protein with a molecular mass of approximately 53 kDa that in humans is mainly expressed in intracellular membrane fractions in hepatocytes<sup>[48]</sup>, and is induced in the liver after feeding and during insulin resistance by the master regulator of lipogenesis Steroid Regulatory Element Binding Protein-1c<sup>[49]</sup>.

Wild-type (148I) *PNPLA3* has lipolytic activity towards triglycerides<sup>[48,50]</sup>. The 148M mutation determines a critical aminoacidic substitution next to the catalytic domain, likely reducing the access of substrates and reducing the PNPLA3 enzymatic activity towards glycerolipids, thereby leading to the development of macrovesicular steatosis<sup>[48,50]</sup>. However, other reported a gain of lipogenic function associated with the 148M variant, which would acquire the ability to synthesize phosphatidic acid from lysophosphatidic acid<sup>[51]</sup>. In addition, results deriving from murine models gave contradictory results<sup>[52-55]</sup>. The issue of the functional consequences of the I148M polymorphism is therefore still intensively debated, and it may be hypothesized that PNPLA3 has additional physiological substrates. Human studies have also suggested a possible direct or indirect influence of *PNPLA3* genotype on adipose tissue biology<sup>[56,57]</sup>, which however awaits replication.

A model depicting hypothetical mechanisms of hepatic fat accumulation associated with the I148M *PNPLA3* polymorphism is shown in Figure 1.

## ASSOCIATION OF PNPLA3 I148M WITH PROGRESSIVE FIBROSIS IN STEATOHEPATITIS

Even if steatosis severity is a risk factor for NASH and progressive disease in NAFLD<sup>[58]</sup>, the association is not invariable, and hepatocellular fat is believed to represent

**Table 1** Studies evaluating the association between the 148M polymorphism of patatin-like phospholipase domain-containing 3 and liver fibrosis in patients affected by chronic hepatitis C virus infection, a leading cause of liver related mortality in Western countries

Study	Design	Patients	Outcome	OR	95%CI
Valenti <i>et al.</i> <sup>[75]</sup>	Cross-sectional	819	Cirrhosis	1.5	1.2-1.9
Müller <i>et al.</i> <sup>[76]</sup>	Cross-sectional	605	Cirrhosis	2.8	1.2-6.2
Trépo <i>et al.</i> <sup>[77]</sup>	Cross sectional prospective	537	Fibrosis stage	3.1	1.5-6.5
			Fibrosis progression	2.6	1.2-5.7

more a epiphenomenon of insulin resistance and altered lipid metabolism than the key driver underpinning liver damage progression. Indeed, accumulation of neutral lipids in cytoplasmic droplets is now retained to represent a protective response towards the increased burden of hepatotoxic free fatty acids and other lipids<sup>[14]</sup>. Therefore, the first question arising after the discovery of *PNPLA3* genotype as the major determinant of hepatic fat content, was whether the *I148M* polymorphism decreased liver damage favoring accumulation of fatty acids in lipid droplets or conversely increased the susceptibility to develop progressive NASH and fibrogenesis.

The answer came soon, as the *148M* allele was linked with NASH<sup>[30]</sup>, and our group first reported that homozygosity for the *148M* allele was associated with an 3.3-fold increased risk of both NASH and liver fibrosis in two independent cohorts of European subjects with histological NAFLD<sup>[31]</sup>. The association between *PNPLA3* *I148M* and the severity of fibrosis in NAFLD was almost contemporarily replicated by independent groups in adults<sup>[34,35]</sup> and in the pediatric population<sup>[59]</sup>, and confirmed by a recent meta-analysis<sup>[36]</sup>.

ALD shares many pathophysiological features with NAFLD<sup>[18]</sup>, most notably steatohepatitis being the key driver of fibrogenesis and liver damage progression. Indeed, candidate gene studies demonstrated that the *I148M* polymorphism is also strongly associated with the risk of developing ALD and with the susceptibility towards cirrhosis in alcohol abusers of different ethnic groups<sup>[60-62]</sup>. In one study in German subjects, the *I148M* polymorphism alone explained as much as 26% of cirrhosis variability in alcohol abusers<sup>[61]</sup>. Furthermore, the earlier the age of the increase at risk alcohol intake the stronger the effect of the *PNPLA3* *148M* mutation has on the cirrhosis susceptibility<sup>[63]</sup>.

## PNPLA3 *I148M* AND CHRONIC HCV HEPATITIS

The next natural question was clearly whether *PNPLA3* genotype represents a modifier of progression of other liver diseases in which steatosis plays a key role in the pathogenesis. Chronic hepatitis C virus (CHC), a leading cause of end stage liver disease and hepatocellular carcinoma in many Western countries<sup>[64]</sup>, is frequently characterized by steatosis, occurring in more than half of

patients. The presence of steatosis has been associated with more aggressive histological features, faster progression of fibrosis, and poorer response to therapy<sup>[65-68]</sup>. Hepatic steatosis favors hepatitis C virus (HCV) life-cycle<sup>[69]</sup>, and both viral and host factors are believed to contribute to its pathogenesis<sup>[67,70-73]</sup>. It became soon clear that the *I148M* polymorphism is a major determinant of the susceptibility to steatosis in also CHC, in particular in patients not infected by genotype 3 strains that *per se* strongly induces steatosis by altering very low density lipoproteins export<sup>[74-77]</sup>. This model is also consistent with the recent finding of a nonsense mutation of *APOB* in humans causing hypo-beta-alipoproteinemia and a massive history of severe steatosis associated with development of hepatocellular carcinoma in carriers of this mutation<sup>[78]</sup>.

Studies that specifically evaluated the association between the *I148M* *PNPLA3* variant and fibrosis progression in CHC are reported in Table 1. As in NAFLD and ALD, the effect of the *148M* mutation was not limited to predisposition to steatosis, but extended towards progressive fibrosis and cirrhosis development<sup>[75-77,79]</sup>. Interestingly, the size effect of the association of the *I148M* polymorphism with fibrosis appeared larger in subjects with at risk alcohol intake (> 30 g/d in males/females)<sup>[76,80]</sup>, suggesting the existence of an interaction between different triggering factors and *PNPLA3* genotype in fibrogenesis. Furthermore, genetic factors influencing immunological response towards HCV, *i.e.*, *IL28B* region polymorphisms, may influence the association between *PNPLA3* and steatosis<sup>[81]</sup>.

We could speculate that when steatosis inducing stressors such as obesity and insulin resistance, excess alcohol intake, and HCV infection stress the liver, in the presence of the “normal” *148I* *PNPLA3* allele the damage will result in simple uncomplicated steatosis, whereas the *148M* “at risk” allele will favor steatohepatitis and fibrogenesis, with progression towards cirrhosis and its complication in susceptible individuals<sup>[82]</sup>.

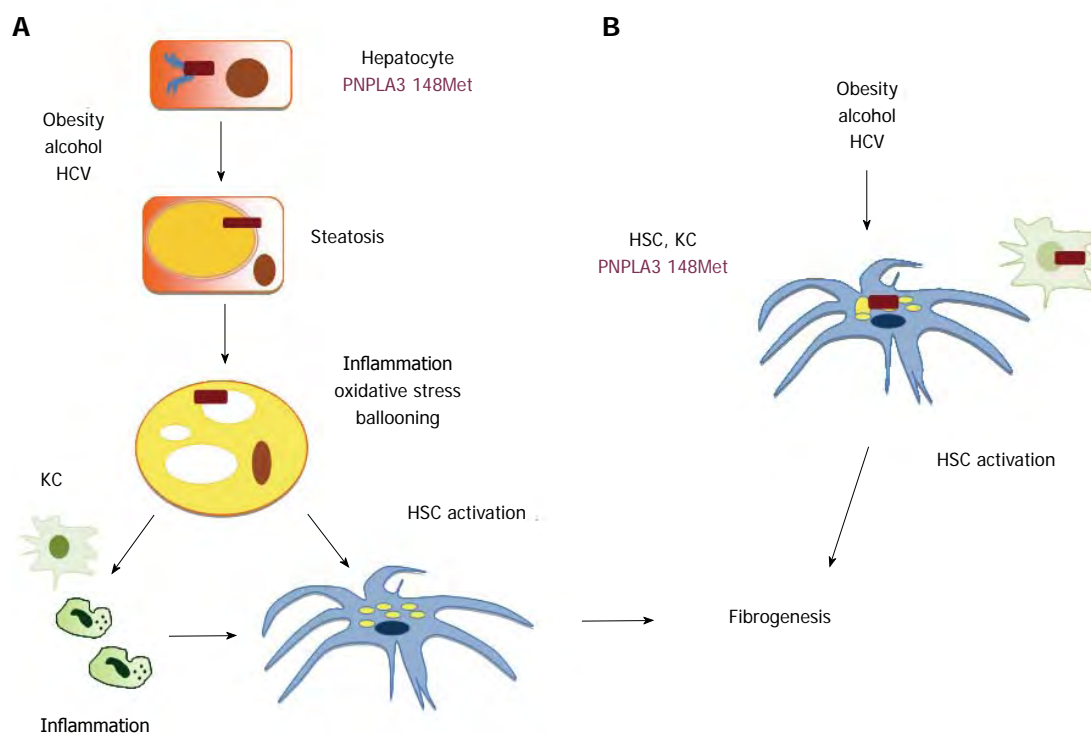
Finally, some studies indicate that during treatment with peg-interferon plus ribavirin the *I148M* polymorphism may affect sustained virological response (*i.e.*, cure rate)<sup>[75]</sup> and viral kinetics<sup>[83]</sup>, especially in difficult to cure CHC patients with advanced fibrosis<sup>[79]</sup>. However, the clinical impact of *PNPLA3* on the response to therapy will likely be modest in the new era of direct antiviral agents<sup>[79]</sup>.

## PNPLA3 *I148M* IN OTHER LIVER DISEASES

Having established that the *I148M* polymorphism is a modifier of the natural history of liver diseases associated with steatosis, *i.e.*, NAFLD, ALD and CHC, the possible role of *I148M* in determining the susceptibility to steatohepatitis and progressive liver damage in other liver diseases is becoming the subject of investigation.

As it affects more than 350 million people worldwide





**Figure 2** Hypothetical mechanisms linking the 148 Isoleucine to Methionine protein variant of patatin-like phospholipase domain-containing 3 polymorphism with hepatic fibrogenesis in the presence of triggering factors for steatosis (Obesity and insulin resistance, excessive alcohol intake and chronic hepatitis C virus infection). A: Direct effect of mutant 148 Isoleucine to Methionine protein variant patatin-like phospholipase domain-containing 3 (148M PNPLA3) on inflammation, oxidative stress, and cellular damage (ballooning) in hepatocytes with secondary activation of non-parenchymal cells, including Kupffer cells (KC) and hepatic stellate cells (HSC). Hepatocytes are shown in brown, KC in light green, neutrophils in dark green, HSC in blue. Nuclei are shown in darker shades of the cell color, whereas lipid droplets and steatosis in yellow, and ballooning (endoplasmic reticulum swelling) in white; B: Direct effect of the mutant 148M PNPLA3 on the activation of non-parenchymal cells. Mutant PNPLA3 (148M) is shown as a red box.

and is a leading causes of liver-related mortality<sup>[84]</sup>, chronic hepatitis B virus (CHB) infection represented the next disease in which the role of *PNPLA3* genotype had to be understood. Steatosis is indeed commonly observed also in CHB, and overall evidence suggests that it contributes to fibrosis progression<sup>[85-88]</sup>. Recent data from our group obtained in a relatively large cohort of European CHB patients with histological evaluation of liver damage indicate that the 148M variant predisposes to steatosis<sup>[89]</sup>. In patients with overweight or a positive history of alcohol intake, the I148M polymorphism predisposes also to severe steatosis, which in this population was associated with more severe fibrosis<sup>[89]</sup>. Additional studies will be required to test the interaction between the I148M genetic variant and acquired risk factors in the pathogenesis of progressive fibrosis and on related clinical outcomes also in CHB infection.

Hereditary hemochromatosis represents another interesting disease model. In fact, in a homogeneous genetic background in subjects homozygous for the C282Y mutation of the *HFE* gene, activation of fibrogenesis is caused by progressive hepatocellular iron overload *via* generation of oxidative stress<sup>[90,91]</sup> in the presence of precipitating factors, among which steatosis has a major role<sup>[92]</sup>. In a large series of Italian *HFE* C282Y homozygous patients with hemochromatosis, we showed that the I148M polymorphism is a strong predictor of the presence of steatosis and higher liver enzymes levels, and it is

also associated with the severity of fibrosis<sup>[93]</sup>. A possible interaction with other genetic forms of liver disease (*i.e.*, Wilson disease) may also be hypothesized and studies on this topic would help understanding the whole picture of *PNPLA3* gene interaction with liver stressors.

Finally, it has been reported that in primary sclerosing cholangitis, an autoimmune cholestatic liver disease characterized by inflammatory changes of major bile ducts, the 148M PNPLA3 variant is associated with increased mortality<sup>[94]</sup>. The effect of *PNPLA3* genotype was evident in the subgroup of patients with severe disease, *i.e.*, males with stenosis of the main duct, but unfortunately it could not be determined whether the association was mediated by steatosis and faster progression of fibrosis.

Although much work has clearly yet to be done in these and many other forms of liver damage, collectively these initial studies suggest that *PNPLA3* I148M is a promising candidate general modifier of fibrogenesis in liver diseases.

## ROLE OF PNPLA3 IN FIBROGENESIS

Intriguingly, the association between the I148M polymorphism and NASH appears to be independent of the severity of steatosis<sup>[31]</sup>, thus suggesting that this genetic variant not only influences the overall amount of hepatocellular fat, but by impacting on the concentration or subcellular localization of specific lipid species directly

modulates inflammation. It should not be forgotten that several lipids behave as inflammatory mediators acting through specific receptors<sup>[9]</sup>. Alternatively, if the *148M* mutation slows down triglycerides kinetics between cell compartments, it could be speculated that renders them more susceptible to lipoperoxidation, leading to oxidative stress, and in turn to hepatocellular damage and inflammation<sup>[9]</sup>. These hypothetical mechanisms linking the *PNPLA3 148M* mutation with hepatic fibrogenesis are shown in Figure 2. In the aforementioned scenario, shown in panel A, hepatocellular damage and the release of inflammatory mediators would lead to secondary activation of Kupffer cells amplifying the inflammatory cascade and cell death, and of hepatic stellate cells with initiation of fibrogenesis.

Even more striking is though the observation that patients the *I148M* polymorphism is associated in NAFLD with advanced fibrosis independently of NASH<sup>[31]</sup>, and in CHC with cirrhosis independently of steatosis, ALT levels, and hepatic necroinflammatory activity<sup>[75]</sup>. It could therefore be envisioned that the *148M* mutation also directly influences the activation of non-parenchymal hepatic cells in response to hepatotoxic insults, as shown in Figure 2B. This hypothesis needs to be addressed and the potential mechanisms investigated by experimental studies.

## PNPLA3 I148M AND HEPATOCELLULAR CARCINOMA

Last but not least, evidence is also accumulating that the *I148M* polymorphism predisposes to hepatocellular carcinoma, a common complication of cirrhosis and the fifth cause of cancer worldwide, with a clinically significant increment in risk, thereby representing a potentially useful biomarker<sup>[75,95-97]</sup>. We have recently reviewed elsewhere the clinical studies supporting such an association and the potential mechanisms involved<sup>[3]</sup>. To summarize, data indicate that the *148M PNPLA3* mutation favors hepatic carcinogenesis in steatohepatitis as well as in other liver diseases, and the mechanism is partly independent of the predisposition towards fibrogenesis and cirrhosis.

## CONCLUSION

In conclusion, *PNPLA3* is a novel key player in liver disease progression. Assessment of the *I148M* polymorphism will possibly inform clinical practice in the future, whereas the determination of the physiological role of wild-type *PNPLA3* and the *148M* variant will reveal mechanisms involved in hepatic fibrogenesis and carcinogenesis and hopefully identify novel therapeutic targets.

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## Peritoneal carcinomatosis

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### Abstract

Several gastrointestinal and gynecological malignancies have the potential to disseminate and grow in the peritoneal cavity. The occurrence of peritoneal carcinomatosis (PC) has been shown to significantly decrease overall survival in patients with liver and/or extraperitoneal metastases from gastrointestinal cancer. During the last three decades, the understanding of the biology and pathways of dissemination of tumors with intraperitoneal spread, and the understanding of the protective function of the peritoneal barrier against tumoral seeding, has prompted the concept that PC is a loco-regional disease: in absence of other systemic metastases, multimodal approaches combining aggressive cytoreductive surgery, intraperitoneal hyperthermic

chemotherapy and systemic chemotherapy have been proposed and are actually considered promising methods to improve loco-regional control of the disease, and ultimately to increase survival. The aim of this review article is to present the evidence on treatment of PC in different tumors, in order to provide patients with a proper surgical and multidisciplinary treatment focused on optimal control of their locoregional disease.

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**Key words:** Carcinosis; Peritoneal; Ovarian; Gastric; Colorectal; Hipe; Intraperitoneal chemotherapy; Cytoreductive surgery; Cancer; Advanced

**Core tip:** This review aims to present the evidence on treatment of peritoneal carcinosis in different tumors, in order to provide patients with a proper surgical and multidisciplinary treatment focused on optimal control of their locoregional disease.

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### INTRODUCTION

Several gastrointestinal and gynecological malignancies have the potential to disseminate and grow in the peritoneal cavity. This condition is often associated with disease progression and poor prognosis. The occurrence of peritoneal carcinomatosis (PC) has been shown to significantly decrease overall survival in patients with liver and/or extraperitoneal metastases from gastrointestinal cancer. Moreover, overall survival in patients with

PC is generally only slightly influenced by systemic chemotherapy, so that the occurrence of PC is traditionally regarded by the surgeon as a terminal condition.

In 10%-35% of patients with recurrent colorectal cancer (CRC) and in up to 50% of patients with recurrent gastric cancer (GC), tumor recurrence is confined to the peritoneal cavity: those patients have been shown to ultimately die from complications of locoregional tumoral widespread, in most cases without occurrence of metastases in other sites. This natural unfavorable evolution of recurrence is commonly observed in epithelial ovarian cancer (EOC) too, a condition always associated with PC and in which locoregional widespread of the tumor is the most common cause of death. However, while in EOC there is general agreement that complete removal of peritoneal seedings is associated with longer survival, in CRC and GC complete removal of peritoneal carcinomatosis is usually followed by short-term recurrence, so that patients are usually treated with limited palliative resection or gastrointestinal bypass without the intent for complete cytoreduction.

On the other hand, almost 15% of patients with colorectal cancer and almost 40% of patients with stage II-III gastric cancer present with PC at abdominal exploration: in these cases there are no standardized indications for surgery, and operations vary from simple exploration and biopsy to palliative resection of the primary tumor, the latter procedure being associated with wide interruption of the peritoneal integrity and further seeding of neoplastic cells.

Preoperative diagnosis of PC could be very difficult. Imaging techniques (mainly based upon computed tomography-scan and magnetic resonance imaging), could assist in planning cytoreduction but also in preventing unwarranted laparotomy in patients with unresectable disease. However, they are limited in their ability to visualize localized PC, having low sensitivity for small-volume disease. The gold standard in diagnosing PC continues to be the direct peritoneal visualization, either by laparotomy or laparoscopy.

During the last three decades, the understanding of the biology and pathways of dissemination of tumors with intraperitoneal spread, and the understanding of the protective function of the peritoneal barrier against tumoral seeding, has prompted the concept that PC is a loco-regional disease: in absence of other systemic metastases, multimodal approaches combining aggressive cytoreductive surgery (CRS), intraperitoneal hyperthermic chemotherapy (HIPEC) and systemic chemotherapy have been proposed and are actually considered as promising methods to improve loco-regional control of the disease and ultimately to increase survival. Even if evidence of efficacy of these multimodal approaches comes from several phase-II studies, a few phase-III studies have been published for CRC and GC, and other are ongoing for EOC. HIPEC privileges consist in increasing loco-regional drugs concentration limiting their systemic diffusion and consequentially their tox-

icities and adverse events. The role of peritoneal plasma barrier in promoting a loco-regional high-dose effect is very important. Indeed, peritoneum has the capability to limit the systemic drugs diffusion in the peritoneal space. Moreover, the hyperthermia enhances the efficacy and the penetration of many of the drugs employed.

The renewed interest on treatment of PC is going to change the attitude of the surgeon towards tumors with peritoneal seeding, thus new paradigms are focusing on the proper behavior that the surgeon should adopt when PC is encountered during operation. Consideration should be given to the different proposed approaches facing different degrees of peritoneal cancer dissemination, but, above all, the question should be: what should be done if PC is encountered? In presence of PC, therapeutic algorithms should be addressed, taking into account the different pathologies and the risk-benefit balance.

The aim of this review article is to present the evidence on treatment of PC in different tumors and to give indications to surgeons who deal with patients with PC, in order to provide patients with a proper surgical and multidisciplinary treatment focused on optimal control of their locoregional disease.

## ROLE OF CYTOREDUCTIVE SURGERY AND HIPEC IN THE TREATMENT OF ABDOMINAL CARCINOMATOSIS FROM DIFFERENT PRIMARY MALIGNANCIES

Biological research has identified three pattern of peritoneal cancer spread: (1) random proximal distribution (RPD), in which early peritoneal implantation is due to the presence of adherence molecules on cancer cell surface, even when ascites is present; this is typical of moderate-grade and high-grade cancers, such as adenocarcinoma and carcinoid of the appendix, non-mucinous colorectal cancer, gastric cancer and serous ovarian cancer; (2) complete redistribution (CRD), in which there is no adhesion to the peritoneal surface close to the primary tumor, due to the low biologic aggressiveness of tumor cells; this distribution is typical of pseudomyxoma peritonei and diffuse malignant mesothelioma; and (3) widespread cancer distribution (WCD), in which there is presence of adherence molecules on the surface of cancer cells that produce a great amount of mucus, interfering with cell adherence: this biological behavior is found in aggressive and undifferentiated tumors such as G2-G3 cistadenocarcinoma of the appendix, mucinous colorectal cancer and mucinous ovarian cancer.

Information about patterns of spread are very important to plan the best surgical treatment. In fact, while RPD should be treated by a selective parietal peritonectomy of the macroscopically involved regions, for CRD and WCD a complete peritonectomy and an extended cytoreduction are needed<sup>[1]</sup>.



## PERITONEAL MESOTHELIOMA

Malignant mesothelioma is an uncommon tumor arising from the serosal layer of pleura, peritoneum, pericardium and tunica vaginalis testis.

The incidence of this disease has been rising worldwide since 1970, due to widespread exposure to asbestos during previous decades, and it is not expected to decrease before the next 20 years. In the United States, approximately 2500 new cases of mesothelioma are registered each year. Diffuse malignant peritoneal mesothelioma accounts for 10% to 30% of all mesotheliomas.

Despite the absence of randomized studies, which are obviously difficult in a rare disease, clinical results obtained with the combination of CRS and HIPEC support the adoption of this procedure as a treatment of choice for peritoneal mesothelioma.

In historical series, standard therapy with palliative surgery and systemic chemotherapy is associated with a median survival of about one year, ranging from 9 to 15 mo. With such a classical approach, the disease tends to remain within the abdominal cavity throughout its clinical course; an autopsy study demonstrated that 78% of patients had died because of complications directly related to local-regional progression.

On the contrary, Yan *et al*<sup>[2]</sup> in a recent multi-institutional study examined 407 patients affected by peritoneal mesothelioma treated with CRS and HIPEC in 7 different surgical centers. The mean age of the patients was 50 years, 89% of the cases were epithelial mesothelioma while 11% were sarcomatoid or bifasic. CC0-CC1 rates was achieved in 46% of cases, lymph nodes metastases were found in 6% and distant metastases in 3% of the patients. After a mean follow up of 30 mo, the median survival was 53 mo. A multivariate analysis showed as independent prognostic factor the histological type of the mesothelioma, the level of cytoreduction achieved, lymph node metastases, and the possibility to perform HIPEC.

## PRIMARY PERITONEAL CARCINOMA

Primary peritoneal carcinoma (PPC), was described for the first time by Swerdlow<sup>[3]</sup>. Its pathogenesis has been controversial. Some Authors believe that PPC develops from a malignant transformation of embryonic germ cell nests cells<sup>[4]</sup>, other from the celomic epithelium lining the abdominal cavity (peritoneum) and the ovaries (germinal epithelium), manifesting a common response to an oncogenic stimulus<sup>[5]</sup>. A multifocal origin have been suggested by Muto *et al*<sup>[6]</sup> with clonality studies, while other authors suggest a unifocal origin<sup>[7]</sup>.

Even if from an histological and a clinically point of view PPC is similar to advanced epithelial ovarian cancer, it diffusely involves the peritoneum by papillary carcinoma in the absence of an obvious primary site and grossly normal ovaries<sup>[8]</sup>. It accounts for 10% of all pelvic serous carcinomas. Most reported cases of PPC have been described in women, usually elderly; however,

rare cases have been reported in children and males. Histologically most reported PPC cases are primary peritoneal serous papillary carcinoma, while rarely are they described as peritoneal mixed epithelial carcinoma and malignant mixed Mullerian tumor.

The prognosis of PPC is poor, the median survival time ranging between 7 and 27.8 mo; 5-year survival rates range from 0% to 26.5%<sup>[9]</sup>.

PPC diagnosis cannot be easily made preoperatively, being typically made by exclusion after both operative assessment and pathological study. In fact if ovaries seem normal with widespread disease elsewhere in the abdomen, PPC may be considered as a diagnostic possibility. However, because surface involvement of the ovaries is present in approximately 96% of the cases, the distinction between extra ovarian primary peritoneal cancer and epithelial ovarian carcinoma may only be made after histological examination to evaluate the extent of ovarian invasion by tumor<sup>[10]</sup>.

Therefore, surgery remains critically important for both diagnosis and therapy of PPC. Once the diagnosis has been established and the extent of disease documented, maximal cytoreduction becomes the primary goal of the procedure. Excision of all visible implants is the hallmark of cytoreductive efforts. To the best of our knowledge, no study has been conducted assessing the efficacy of CRS with HIPEC for this kind of carcinomatosis, even if it is reasonable that this approach should be taken in consideration in the context of clinical studies.

## PSEUDOMYXOMA PERITONEI

Pseudomyxoma peritonei (PMP), a syndrome firstly described by Rokitansky in 1842, is an enigmatic, often fatal intra-abdominal disease characterized by gelatinous ascites and multifocal peritoneal epithelial implants secreting copious globules of extracellular mucin. This condition is almost always due to a perforated epithelial appendix cancer. Three pathologic variants of PMP are known: disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis (PMCA), and peritoneal mucinous carcinomatosis with intermediate or discordant features.

The natural history of this disease has been drastically changed by the introduction of CRS combined with HIPEC. Ronnett *et al*<sup>[11]</sup> found a significant difference in the prognosis of patients affected by this three different forms of PMP. The most important prognostic variable affecting 10 years survival rates are the possibility to achieve CC0-CC1 (the more the complete cytoreduction, the longer the survival) and the pathological feature of PMP. Deraco *et al*<sup>[12]</sup> reported an overall 10 years survival rate of 78.9% in patients affected by PMP and treated with CC0-CC1 CRS, while no patient with CC2-CC3 CRS survived 10 years. Patients affected by DPAM had a 10 years survival of 67% while those affected by PMCA had no more than 40.7% 10 years survival rate. Baratti *et al*<sup>[13]</sup> recently published a study on prognostic

value of serum tumor markers in patients with PMP undergoing CRS and HIPEC. It is clear that pre-operative normal values of C385 were statistically related to the ability to perform an adequate cytoreduction and that elevated pre-operative values of C17.9 were associated with reduced progression-free survival. CEA has also been shown to have good sensitivity in case of progression. Chua *et al.*<sup>[14]</sup> published the results of a retrospective multi-institutional study. The purpose of this study was to evaluate outcome and long-term survival after CRS and HIPEC in PMP patients. The registry included 2296 patients from 16 centers with a mean PCI of 20, CCR0/1 was achieved in 83%, mortality was 2% and major morbidity was 24%. Median survival was 196 mo, disease free median survival was 98 mo, and 10 and 15 years survival was respectively 63% and 59%. Multivariate analysis have identified as negative prognostic factor for overall survival: previous chemotherapy courses, PMCA histological type, major postoperative complication, CCR 2/3, older age, while negative prognostic factor for progression free survival were all the above plus high peritoneal cancer index and not using HIPEC.

## PERITONEAL CARCINOMATOSIS FROM GASTRIC CANCER

Penetration of the gastric serosa and lymphatic spread are the two most important factors affecting prognosis in gastric cancer (GC)<sup>[15-17]</sup>. When the gastric serosa is infiltrated by tumor, PC becomes praverly frequent<sup>[18]</sup>. Subsequently, up to half of the patients with advanced gastric cancer (AGC) will develop a peritoneal carcinomatosis in spite a radical surgery<sup>[19-22]</sup>, and PC is quite common in gastric cancer, being already present in 5%-20% of patients explored for potentially curative resection<sup>[18,23]</sup>. There are several methods for detecting the presence of free peritoneal tumor cells (FPTC) with different sensitivity<sup>[24-26]</sup>. FPTC in the washing could be identified in up to 24% of stage IB and up to 40% of stage II or III GC patients<sup>[27]</sup>. Moreover, after radical resection, the peritoneum is the only site of recurrence in 10%-34% of cases, and one of the recurrences sites in 29%-44% of cases<sup>[28-32]</sup>.

The five-year survival rate in patients with peritoneal carcinosis from GC (PCGC) is lower than 3%<sup>[33]</sup>, with an overall mean and median survival of 6.5 and 3.1 mo, respectively<sup>[34]</sup>. Among the non-gynecologic malignancies, PCGC has a better prognosis than PC from pancreatic cancer but worse than PC from CRC<sup>[34]</sup>. Saito *et al.*<sup>[35]</sup> reported a 5-year survival rate of advanced GC with FPTC of 15.3%, similar to that of patients having macroscopical peritoneal metastasis (14.8%). As a counterpart, there are no 5-year survivors among patients with distant peritoneal metastases.

Systemic chemotherapy may improve median survival up to 12 mo in advanced/metastatic GC<sup>[36-39]</sup>, but a similart survival benefit has not been reported in macroscopic PC<sup>[40-43]</sup>. One possible explanation seems to be

that systemic chemotherapy inadequately reaches the abdominal cavity<sup>[44]</sup>. Yonemura *et al.*<sup>[45]</sup> demonstrated a survival benefit by treating patients with PFTC with radical resection followed by adjuvant systemic chemotherapy. Patients treated with adjuvant chemotherapy survived significantly longer than patients in control group: the 1 and 2-year survival rates were 88% and 44%, and 53% and 9%, in adjuvant group and control group, respectively. The mean overall survival was 21.1 and 9.1 mo for adjuvant and control group ( $P < 0.05$ ). The ineffectiveness of systemic chemotherapy in PC may be related to a number of factors, such as the peritoneal-plasma barrier, the intraperitoneal poor blood supply and oxygenation of cancer cells, and the low apoptotic potential of such hypoxic tumor cells<sup>[37,46-48]</sup>. Neoadjuvant chemotherapy (NACT) has been described to decrease the load of macroscopic PCGC<sup>[37,49]</sup>. Yano *et al.*<sup>[50]</sup> reported a small series of 4 out of 26 (15.4%) patients affected by PCGC with complete remission of peritoneal metastasis with after NACT. All these patients subsequently underwent curative resection. Inokuchi *et al.*<sup>[51]</sup> reported a partial response in 9 out of 13 patients (69%). However, one further study suggests that after NACT the detection of FPTC can change from positive to negative and vice versa. This change is not linked to the response to the systemic chemotherapy. Ten out of 42 (24%) patients with negative peritoneal cytology shifted into positive for FPTC during NACT, while 7 out of 19 (37%) with FPTC positive cytology at staging laparoscopy turned negative<sup>[52]</sup>.

GC peritoneal spread remains a major problem, and some Authors finally suggest that there is no role for surgery in PCGC<sup>[53]</sup>. Since the 80s, Japanese surgeons combined CRS, regional hyperthermia and intraperitoneal chemotherapy in a multimodal approach<sup>[54]</sup>. As for other types of PC, in GC HIPEC after CRS is accomplished to eliminate FPTC and to prevent or delay PC<sup>[53,55]</sup>. A number of studies have been conducted, with the aim to demonstrate a significant reduction in the rate of subsequent PC and an increase in survival of patients with AGC when radical surgery was combined with HIPEC<sup>[20,56-61]</sup>. Yonemura *et al.*<sup>[62]</sup> demonstrated that HIPEC could improve significantly the median survival from 15 to 48 mo and the 5-years survival rate from 12% to 42% in patients with PFTC. On the other hand, the combined CRS and HIPEC treatment of PCGC seems to be the one with less encouraging results in terms of survival and of morbidity and mortality when compared to other types of PC<sup>[63,64]</sup>. A French retrospective, multi-center study published in 2010 evaluated toxicity and significant prognostic factors after CRS and HIPEC (and/or early postoperative intraperitoneal chemotherapy, EPIC) for PC from nongynecologic neoplasms<sup>[65]</sup>. The study involved 1290 patients from 25 French institutions who underwent 1344 CRS procedures between 1989 and 2007. HIPEC was made in 1154 cases (86.4%). The principal origin of PC was CRC ( $n = 523$ , 40.5%), and no more than 159 GC cases were present in this series

(12.3%). The whole group overall 3- and 5-year survival rates were 49% and 37% respectively. The PCGC group showed the worse outcome with a 3- and 5-year survival rates of 18% and 13%, respectively. The overall median survival of the whole group and of the PCGC group were 34 and 9 mo respectively. Li *et al*<sup>[66]</sup> from China reported in 2010 a series of 128 patients with PCGC. Fifty-four (42.2%) underwent gastrectomy, and 10 underwent resection with HIPEC. The other 74 (57.8%) received non-resection surgery. The median survival in the unresected group was 6 mo compared to 11.8 mo of the resected patients. Moreover, they observed a significantly improved survival in the patients treated with surgery and HIPEC compared to those treated with surgery alone<sup>[67]</sup>. Post-operative complications were more frequent in the HIPEC than in the resection alone group (20.0% *vs* 13.2%,  $P = 0.34$ ). Yang *et al*<sup>[68]</sup> published the final results of a phase III randomized trial, performed in China in order to evaluate the efficacy and safety of CRS plus HIPEC for the treatment of PCGC. The median overall survival was 6.5 mo in CRS alone group and 11 mo in the CRS + HIPEC group ( $P = 0.046$ ). This outcome was even more significant in patients with synchronous PCGC ( $n = 51$ ), being the median overall survival 12 mo in CRS + HIPEC group ( $n = 24$ ) and 6.5 mo in the CRS group ( $n = 27$ ,  $P = 0.029$ ). The 1-, 2-, and 3-year survival rates were 29.4%, 5.9% and 0% for CRS group, and 41.2%, 14.7% and 5.9% for CRS + HIPEC group, respectively. The CC-score has been demonstrated to influence survival, but HIPEC obtained a significant advantage both in CC 0-1 and CC 2-3 patients. In the CRS + HIPEC patients, the median overall survival in CC 0-1 ( $n = 20$ ) and in CC 2-3 subgroups ( $n = 14$ ) was 12 and 8.2 mo respectively. In CRS patients, the median overall survival in CC 0-1 ( $n = 20$ ) and in CC 2-3 subgroup ( $n = 14$ ) was 11 and 4 mo respectively. Serious adverse events arose in 9 patients, 4 in the CRS group (11.7%) and 5 in the CRS + HIPEC group (14.7%) ( $P = 0.839$ ). Multivariate analysis recognized CRS + HIPEC, synchronous PC, CC 0-1, systemic chemotherapy and no serious adverse events as major independent predictors for better survival. HIPEC was about 2.6 times likely to increase survival.

Gill *et al*<sup>[67]</sup> published a systematic review analyzing survival, mortality and morbidity in the treatment of PCGC with CRS and HIPEC. Ten studies were included. Overall median survival was 7.9 mo. In the subgroup of patients with residual nodules after CRS, less than 0.25 cm in size, the median survival raised up to 15 mo. The 1- and 5-year survival were 43% and 13%. The treatment-related mortality rate was 4.8% and the morbidity was 21.5%.

Recently, Yonemura *et al*<sup>[61]</sup>, proposed a multimodal strategy which associates neoadjuvant intraperitoneal and systemic chemotherapy (NIPS), CRS + HIPEC and early postoperative intraperitoneal chemotherapy (EPIC). The rationale of this method is to reduce tumor burden before surgery with NIPS, a bidirectional chemotherapy that attacks PC from both sides of peritoneum (from the

peritoneal cavity and from sub-peritoneal blood vessels), and reducing macroscopic and microscopic PC with CRS + HIPEC. At the end, the use of EPIC is proposed to eradicate residual intraperitoneal cancer cells before fibrin and adhesion development. Authors recommend two cycles of NIPS to achieve a negative cytology status. Severe complication post-NIPS have been reported in 4 out of 79 patients. This strategy allowed to obtain a change in washing cytology from positive to negative in 41 out of 79 patients (63%).

Three recent meta-analysis of randomized trials analyzing patients with advanced GC (with or without PC) demonstrated the survival benefit offered by HIPEC<sup>[69-71]</sup>.

In the last ten years, a new drug for intraperitoneal treatment of GC has been developed in Germany. Catumaxomab (trade name Removab<sup>®</sup>) is a rat-mouse hybrid monoclonal antibody that is made up of one "half" (one heavy chain and one light chain) of an anti-Epithelial cell adhesion molecule (EpCAM) antibody and one half of an anti-CD3 antibody, thus finally binding both EpCAM and CD3. EpCAM is an epithelial differentiation antigen that is expressed on normal epithelial cells and on almost all carcinomas (especially gastrointestinal and ovarian carcinomas) and functions as cell adhesion molecule<sup>[72]</sup>. In addition, the Fc-region can bind to an Fc receptor on accessory cells like other antibodies, which has led to calling the drug a trifunctional antibody. Actually Catumaxomab is used to treat malignant ascites, because of the intraperitoneal application of this anti-EpCAM antibody has shown significant benefits in puncture-free survival (survival without repeated paracentesis) for patients with malignant ascites in a phase III randomized trial<sup>[73]</sup>. This study demonstrated no statistically significant increases in median overall survival for other cancers, while in patients with GC a small survival increase was associated with the use of Catumaxomab<sup>[73]</sup>. Progression-free survival has been analyzed in a phase II study with the use of intraperitoneal catumaxomab in gastrointestinal EpCAM<sup>+</sup> tumors<sup>[74]</sup>. Furthermore two phase 2 studies are ongoing (follow-up phase), evaluating resectable advanced GC patients treated with adjuvant intraperitoneal Catumaxomab.

In conclusion, in PCGC CRS and HIPEC proved with good evidence to improve survival with acceptable morbi-mortality. It is very important to obtain the diagnosis and the diffusion grade of PCGC before the CRS and HIPEC with the use of staging laparoscopy. The role of surgery is fundamental, complete cytoreduction demonstrated to be strictly related to an improvement in survival. In patients with PCGC, multimodal treatment should be mandatory, leaving a pivotal role to HIPEC after CRS.

## PERITONEAL CARCINOMATOSIS FROM OVARIAN CANCER

Nowadays, the treatment diagram for advanced EOC



has been universally accepted as a combination of maximal CRS and adjuvant chemotherapy, including cases with grossly peritoneal diffuse disease. Grade III C and IV are no longer considered as “lost”. Different studies demonstrated that a progressively more aggressive surgical effort is associated with improvements in disease-free and overall survival rates. It is suggested that aggressive surgery should be performed in dedicated centers with high volume of cases, offering in-hospital mortality significantly lower than low volume ones<sup>[75]</sup>. The more the surgeon became radical and increases his/her surgical volume, the more he/she prolongs the disease-free and overall survival and reduces the in-hospital mortality. As a counterpart, the tumor biology and the initial disease diffusion have been suggested as the most important factors in survival benefit of surgery<sup>[76-79]</sup>. It's still undefined how the intrinsic features of the tumor make intra-abdominal implants easier to remove<sup>[80]</sup>. In general, upper abdominal tumor implants are suggestive of an aggressive tumor biology<sup>[81]</sup>. Covens and Berman criticized the role of CRS in advanced EOC. They proposed that both survival and surgical resectability are mostly determined by tumor biology instead of the operative effort by the surgeon<sup>[82,83]</sup>. The retrospective review of data from the Scottish Randomized Trial in Ovarian Cancer revealed in a population of 889 patients with disease stage ranging from IC to IV that the benefit of optimal debulking surgery seems to depend from the extent of disease before surgery<sup>[79]</sup>. Hager *et al.*<sup>[84]</sup> analyzing 456 women with advanced stage III/IV ovarian cancer, demonstrated no correlation between nodal status and survival. Moreover in advanced EOC nodal status was not a prognostic factor for patients undergone to optimal cytoreduction.

Complete cytoreduction is reached when no visible tumor remains after the surgical procedure. Starting from this classification a number of prospective and retrospective studies have been conducted to investigate the feasibility and the impact on survival of CRS in advanced EOC.

Up to now, the majority of available series report cases treated with the standard systemic platinum-taxanes chemotherapy and CRS. Only one study analyzed cases treated also with intraperitoneal chemotherapy<sup>[85]</sup>.

Between 2003 and 2010, 15 studies have been published analyzing patients treated with CRS and systemic chemotherapy for advanced EOC. The overall survival (OS) ranges between 46.5 and 106 mo for patients with complete CRS (no residual disease) and between 12 and 39 mo for incomplete CRS (residual disease of more than 1 cm)<sup>[85-92]</sup>. All these papers demonstrated that CRS plays a central role in advanced EOC treatment. The necessity of adjuvant chemotherapy has already been demonstrated. Surgical effort must be absolute.

Between 2000 and 2010, 20 observational studies have been published about CRS + HIPEC in treating PC from advanced and recurrent EOC. The first was published by Cavaliere *et al.*<sup>[93]</sup> reporting about 20 patients with recurrent EOC. They reported a median OS of 25

mo with a 3-year survival of 50%.

De Bree *et al.*<sup>[94]</sup> and Chatzigeorgiou *et al.*<sup>[95]</sup> reported about 19 and 20 patients respectively with recurrent ovarian cancer. They found median DFS of 26 and 21 mo, respectively. De Bree reported a median OS of 54 mo and Chatzigeorgiou a median OS for optimally cytoreduced patients (considered as residual disease of < 1.5 cm) of 29 mo. De Bree found a 3 and 5 year survival of 63% and 42%. Both studies reported a perioperative mortality rate of about 10%.

Four studies have been published in 2004<sup>[96-98]</sup>. Zanon *et al.*<sup>[96]</sup> described a cohort of 19 patients with recurrent EOC. They reported a median DFS of 17 mo with a median OS and OS in optimally cytoreduced patients (residual disease < 0.25 cm) of 28 and 38 mo respectively. Three and 5-year survival were 35% and 12% respectively. Perioperative mortality rate was 3 % with grade 1 or 2 morbidity rate of 27% and 3% respectively and with grade 3 and 4 morbidity of 7%.

Piso *et al.*<sup>[97]</sup> reported a series of 19 patients with peritoneal carcinomatosis due to primary or recurrent EOC. The median DFS was 18 mo, with mean OS and OS in optimally cytoreduced patients (residual disease < 0.25 cm) of 33 and 44 mo respectively and a 5-year survival rate of 15%. Perioperative mortality rate was 3%, grade 1-2 morbidity rate was 10% and grade 3-4 morbidity of 10% and 15% respectively<sup>[97]</sup>.

Ryu *et al.*<sup>[98]</sup> reported a series of 57 patients with advanced EOC. The median DFS was 26 mo. Median OS in optimally cytoreduced patients (residual disease < 1 cm) was 41 mo. The OS at 5-year was 54%. The survival advantage has been found to be more pronounced in stage 3 disease. Multivariate analysis showed HIPEC as an independent prognostic factor. Perioperative mortality was 4 % with grade 1, 2 and 4 morbidity rate of 14%, 5% and 4%, respectively.

Gori *et al.*<sup>[99]</sup> and Reichman *et al.*<sup>[100]</sup> reported about 29 and 13 patients respectively with advanced EOC. Median DFS were 15 and 11 mo respectively, with a median OS in Gori's paper of 64 mo and a 3-year survival rate in Reichman's study of 55%. None of these two studies reported morbidity nor mortality.

Raspagliesi *et al.*<sup>[101]</sup> and Rufán *et al.*<sup>[102]</sup> published two reports with 40 and 33 patients respectively, with advanced and recurrent EOC. Median DFS and OS in the first paper were 11 and 32 mo, and median OS and OS in optimally cytoreduced patients (residual disease < 1 cm) were 48 and 66 mo respectively. Five-year survival in Raspagliesi's series was 15%; 3 and 5-year survival rate in Rufian study were 46% and 37%, respectively. Reported mortality for both papers was 0%. Raspagliesi reported 20% of grade 1 morbidity. Rufian reported grade 1 and 2 morbidity rate of 12% and 10% and grade 3 and 4 morbidity of 10% and 6% respectively.

Helm *et al.*<sup>[103]</sup>, Cotte *et al.*<sup>[104]</sup> and Bae *et al.*<sup>[105]</sup> published series of 18, 81 and 67 patients with recurrent (Helm and Cotte) and advanced EOC (Bae). Helm *et al.*<sup>[103]</sup> reported a median DFS of 10 mo and median OS and OS



in optimally cytoreduced patients (residual disease < 0.5 cm) was 31 and 31 mo respectively. Perioperative mortality was 6% and grade 1, 2, 3 and 4 complications have been reported in 11%, 50%, 40% and 13% of patients respectively.

Cotte *et al*<sup>[104]</sup> described a median DFS of 19 mo and an OS and OS in optimally cytoreduced patients (residual disease < 0.25 cm) of 28 and 55 mo respectively. Perioperative mortality was 3% and grade 1, 2, 3 and 4 complications have been reported in 6%, 1%, 5% and 2% of patients respectively.

Bae *et al*<sup>[105]</sup> reported a 5-year survival rate of 66%, with a 0% perioperative mortality and grade 1, 2, 3 and 4 morbidity rate of 14%, 13%, 0% and 0%, respectively.

Di Giorgio *et al*<sup>[106]</sup> published data about 47 patients with advanced and recurrent EOC. They reported a median DFS of 20 mo with an OS and OS in optimally cytoreduced patients (residual disease < 0.25 cm) of 24 and 26 mo respectively. Five-year survival rate was 17% and perioperative mortality 4%. Grade 2, 3 and 4 complication rate were 21%, 9% and 13% respectively.

Bereder *et al*<sup>[87]</sup>, Guardiola *et al*<sup>[107]</sup>, Fagotti *et al*<sup>[108]</sup>, Pavlov *et al*<sup>[109]</sup> described results of CRS + HIPEC in advanced and recurrent EOC and in recurrent EOC.

Guardiola *et al*<sup>[107]</sup> published a series of 47 patients with a median DFS of 14 mo and a 5-year survival of 63%. Perioperative mortality rate was 0% and grade 4 complication rate was 13%. Fagotti *et al*<sup>[108]</sup> reported a median DFS of 10 mo, with 0% perioperative mortality and grade 2, 3 and 4 complication rate 36%, 8% and 8% respectively. Pavlov *et al*<sup>[109]</sup> described 56 patients with a median DFS and OS of 26 and 38 mo respectively. Perioperative mortality was 2% and grade 1, 2 and 4 complication rate were 5%, 11% and 2% respectively. Bereder *et al*<sup>[87]</sup> published the widest series reporting about 246 patients with advanced and recurrent EOC. Median DFS was 13 mo, median OS and OS in optimally cytoreduced patients (residual disease < 0 cm) were 49 and 56 mo respectively. Three and 5-year survival were 60% and 35%. Reported intraoperative mortality was 0.4% and grade 3 morbidity 12%.

Lastly, Deraco *et al*<sup>[110]</sup> published a multi-institutional phase 2 study evaluating the impact of CRS + HIPEC as upfront treatment on PFS and OS in 26 women with stage 3-4 advanced EOC. All enrolled patients underwent CRS, followed by HIPEC. Patients were then treated with adjuvant systemic chemotherapy. Macroscopically complete cytoreduction was achieved in 57% of patients, with minimal residual disease ( $\leq 2.5$  mm) remaining in the other 43%. Five-year OS was 60.7% and 5-year DFS 15.2%. Excluding operative death, all the patients underwent a median of 6 cycles of systemic chemotherapy at a median of 46 d from combined treatment. Four patients experienced  $\geq$  grade 3 morbidity, with one post-operative death due to sepsis.

Globally, 7 randomized controlled trials evaluating the effectiveness of HIPEC in advanced and recurrent EOC have been proposed: five are already ongoing<sup>[111-115]</sup>

and two have been only proposed<sup>[116]</sup>.

Ansaloni *et al*<sup>[117]</sup> reported about 39 patients with advanced and recurrent EOC. The mean DFS was 14 mo. Grade 1-3 post-operative complications occurred in 18% of patients. Perioperative mortality was 0.3%.

In conclusion, despite the lack of high evidence data that will be brought from the ongoing randomized trials, HIPEC associated to complete CRS seems to give survival results comparable to the standard treatment. Data are still heterogeneous due to the different meaning given to the completeness of cytoreduction, as showed in all the aforementioned studies. Some centers consider cytoreduction complete when there is no macroscopic residual disease, others follow more permissive limits. Moreover, confusion exists about “optimal” and “complete” cytoreduction. However, data clearly show as in patients with no macroscopic residual disease CRS + HIPEC increases the survival rates. These results could be overcome in terms of surgical effort and morbidity rate reduction by the use of NACT.

## PERITONEAL CARCINOMATOSIS FROM COLO-RECTAL CANCER

The multi-disciplinary treatment of CRC is actually standardized up to stage III C<sup>[118-120]</sup>, while it is unclear and not supported by strong evidences for stages IVa and IVb. American guidelines from NCI recently consider liver resection as available treatment for IVa stage, but they don't mention nowadays HIPEC as treatment option for IVb CRC, including the peritoneal carcinomatosis (PCCRC).

Another way to assess the actual relationship between HIPEC for PCCRC and Evidence Based Medicine is to measure the percentage of ongoing trials from the NCI database: worldwide, among 239 active registered trials on IVb stage CRC, only eight include HIPEC as keyword (2 phase III, 4 phase II and 2 phase I trials: from www.cancer.gov, consulted 26<sup>th</sup> of June 2013). The only concluded randomised clinical trial comparing systemic chemotherapy with cytoreduction plus HIPEC is the Dutch trial published in 2003<sup>[121]</sup>: 105 patients with PCCRC without evidence of hematogenous metastases enrolled between 1998 and 2001 were randomly allocated to receive 5-fluorouracil and leucovorin with or without palliative surgery or “aggressive” cytoreduction plus HIPEC followed by the same chemotherapy regimen. They demonstrated a median overall survival of 22.3 mo for the HIPEC arm against 12.6 mo for the standard therapy, with a significant difference ( $P = 0.032$ ). Unfortunately, the value of this RCT is limited by several factors: it was based on a chemotherapy scheme that is not the actual gold standard (not including *i.e.*, Irinotecan and Oxaliplatin); appendiceal ( $n = 18$ ) and rectal ( $n = 12$ ) tumors were not balanced in the two groups; the HIPEC protocol was based only on mitomycin C in the perfusate; the role of surgery in the control arm was unclear and impossible to determine on available data. Another randomized trial was designed by Elias to com-

pare early postoperative intraperitoneal chemotherapy plus systemic chemotherapy with chemotherapy alone after complete cytoreductive surgery for the PCCRC treatment. In 2000, after 4 years and only 35 patients enrolled, the study was stopped and the partial results analysis did not demonstrate any advantage in term of survival<sup>[122]</sup>.

Another attempt to design a RCT comparing standard systemic therapy with CRS + HIPEC + chemotherapy is the USMCI8214/ACOSOG Z6091 trial<sup>[123]</sup>, a well designed study, trying to overcome the Dutch trial limitations, with a specific target population (peritoneal carcinomatosis only, colon cancer) and using advanced/state-of-the-art chemotherapy. This trial recently closed, failing to meet accrual and amplifying the concerning from Elias *et al*<sup>[122]</sup> about the feasibility of this kind of studies: basically, even if few trials are active nowadays (in particular the last could be the PRODIGE 7 French trial<sup>[124]</sup>, with 150/280 patients enrolled at January 2012), the idea to get a level of evidence I a/ I b in support of HIPEC for PCCRC is near to be abandoned.

Anyway, the Dutch study was the base for several other trials more adequate and focusing on singular aspect, but without the same level of evidence: in particular three case control studies (evidence IIIa) have been published between 2009 and 2011. Elias *et al*<sup>[125]</sup> had the merit to include the oxaliplatin at 460 mg/m<sup>2</sup> dose in the perfusate, plus Irinotecan in 18/48 patients, comparing the HIPEC group with a standard therapy based on 5-fluorouracile (5-FU), folinic acid and systemic postoperative oxaliplatin (OX) or Irinotecan (IRI). They reached the impressive median survival of 63 mo, with a 5-year survival rate of 51% for the HIPEC group patients with a complete CRS. Franko *et al*<sup>[126]</sup> compared 67 patients treated with mitomycin C-based HIPEC with 38 controls and all the 105 patients received 5-FU, IRI, OX and bevacizumab/cetuximab. Unfortunately they included patients with liver metastases and the use of OX and target therapies was greater in the HIPEC group (78% *vs* 18% and 59% *vs* 18% respectively). Chua *et al*<sup>[127]</sup> included 294 patients, comparing supportive care and palliation with postoperative systemic chemotherapy based on 5-FU, IRI, OX, Capecitabine and monoclonal antibodies, with or without HIPEC (low-dose mitomycin C) and EPIC (high dose 5-FU). The difference between curative or palliative therapy was based on preoperative assessment of the Peritoneal Surface Disease Severity Score.

Among not randomized, retrospective multi-institutional studies, the largest published series comes from the French registry, including 523 patients with PCCRC treated from 1990 to 2007 with CRS and HIPEC<sup>[128]</sup>. Even if a 16% of incomplete CRS, with macroscopic residual (CCR-1) makes it difficult to extrapolate data about survival and the great number of participants centres adds variability (relating in particular to learning curve and surgical standardization), the reported 30-d mortality was only 3%, absolutely lower than in the Dutch trial (8%).

The peritoneal cancer index (PCI) is a semi-quantitative powerful tool, easily reproducible and validated by several studies and expert consensus<sup>[129-132]</sup>, which aims at defining and measuring the peritoneal involvement. However, using PCI to select PCCRC patients and to guide the therapeutic strategy need some comments: a threshold value to get a formal contraindication to CRS + HIPEC is not available today; a PCI greater than 20 is associated with a worse prognosis, even if in a small series (24 patients). Elias *et al*<sup>[133]</sup> described a significant advantage in survival even when PCI was over 24; for PCI < 10, there is agreement about the usefulness of CRS + HIPEC, and the median survival for these patients ranges from 31 to 48 mo<sup>[128,134-136]</sup>; similar data are provided by Gilly *et al*<sup>[137]</sup>; in currently active trials a high PCI value is generally not an exclusion criteria; different studies from the same center stressed the difference between the PCI declared at the beginning and at the end of surgery, suggesting to systematically add 2 point at the preoperative score<sup>[138]</sup>; Sugarbaker *et al*<sup>[139]</sup> suggest to correlate PCI with patients demographic when deciding to add or not HIPEC to their therapeutic scheme.

Pioneering studies about chemotherapeutic agent penetration in the tumor were available since early 90s<sup>[140,141]</sup> and have recently been confirmed<sup>[142]</sup>, showing, for example, a diffusion depth of about 1-2 mm for Mitomycin C<sup>[143]</sup>. Nevertheless, even if the rationale is something more than the common principle of resect as more tumor as possible, the attitude to consider useful HIPEC only after an adequate CRS is a recent acquisition. Moreover, there is no accordance on the dimensional cut-off (1, 2.5 or 5 mm) and in different series the impact of CCR on survival varies enormously<sup>[129,131,136,144]</sup>. In particular, if the role of macroscopic residual (CCR-2) nodules seems clear and formally contraindicate HIPEC, it is unclear the difference between nothing (CCR-0) and very small nodules (up to 2.5 mm, CCR-1 in some series). Indeed, even in CCR-1 cases, CRS + HIPEC was reported to be related to a better prognosis<sup>[134,145-147]</sup>.

Surprisingly, the tumor progression during neo-adjuvant chemotherapy is not demonstrated to be an independent prognostic factor as for gastric cancer with PC and actually is not a formal contraindication to CRS + HIPEC<sup>[148,149]</sup>.

As for other organs and pathologies, in the treatment of PCCRC the acronym HIPEC correlate to a wide spectrum of possible variation in temperature, molecules, concentration and contact time<sup>[129,131,134,145,146,150,151]</sup>. The associated systemic chemotherapy is highly variable too. This great number of parameters makes a standardization difficult: the statement from Elias *et al*<sup>[124]</sup> on the necessity to follow the most experienced centers protocols is acceptable and functional, though not methodologically correct. Finally, the lack of evidence suggests the enrollment of as many patients as possible into well designed randomized trials.

Among the most significant HIPEC protocols, those based on Oxaliplatin in the perfusate have to be report-

ed. From first demonstrations of the rationale<sup>[152]</sup> and the pharmacokinetic<sup>[153]</sup> during hyperthermic application, few phase II trials included OXt in their protocols<sup>[145,154]</sup>. In particular, Elias *et al.*<sup>[133]</sup> published a series of 24 patients treated with Oxaliplatin in the perfusate at 460 mg/m<sup>2</sup> in 2 L/m<sup>2</sup>, during 30 min at 43 °C and later a revised protocol including 106 consecutive patients treated with lower dose of oxaliplatin (360 mg/m<sup>2</sup>) combined with irinotecan (360 mg/m<sup>2</sup>) in 2 L/m<sup>2</sup> of 5% dextrose, for the same time at 43 °C. The usefulness of the Irinotecan association is controversial and may be the cause of an increased toxicity<sup>[155]</sup>.

Starting from 1995, several attempts were done to clarify the relationship between the primary tumor pathology and the outcome of PCCRC: tumor site (appendix, colon and rectum), grading, nodal and liver metastases were analyzed<sup>[130,131,146,150,156-158]</sup>. Following the substantial failure of this search (no strong correlation at several multivariate analysis), researchers lost their attention on tumor demographic in more recent publication, maintaining some interest only for tumor size<sup>[159]</sup>. Moreover, earlier reports on HIPEC suffered from the very small number of included patients, making any stratification impossible. However, beside their role as independent prognostic factors, tumor characteristics are mandatory to get a better stratification, given that the only outcome parameter used is the overall survival, whereas only few studies considered quality of life and PC-free survival<sup>[160-162]</sup>.

The combined treatment of synchronous liver metastases in patient with PCCRC is beyond the scope of this review, but this topic is strongly related to HIPEC: a variable percentage of patients included in retrospective studies underwent at the same time liver resection and CRS<sup>[128-130,145,163,164]</sup>; the report of a different impact of liver metastasis in patient accordingly to the CCR (with a significant prognostic negative value only for CCR-0 patients) underlines the possible different meaning of these two types of tumor spread ("local" *vs* "systemic"); even if a liver metastasis is not considered an absolute but only a relative contraindication to HIPEC, it seems logical that all the randomized recently designed study on HIPEC should exclude cases with liver involvement.

Currently, the main research effort is forwarded to RCTs evaluating mandatory second-look surgery with CRS + HIPEC in patients at high risk of developing PCCRC versus standard of care (control arms)<sup>[165]</sup>. Background for this new field of interest mainly are: increasing importance assigned to metachronous PC in the natural history of the tumor; definition of parameters to estimate the risk of secondary PC<sup>[166]</sup>, including synchronous completely resected PC, ovarian metastases, perforated primary tumor and in some experiences pT<sub>4</sub> tumor, colon occlusion and positive peritoneal cytology<sup>[124,167]</sup>; a great percentage of asymptomatic and work up negative high risk patients were diagnosed to harbor macroscopic PC during second look laparotomy at one year<sup>[168]</sup>. As expression of the two main groups working on HIPEC

for CRC (American and French), two different RCTs are enrolling patients to demonstrate the usefulness of an early second look treatment for high risk patients to detect and treat (with CRS + HIPEC) metachronous PC, with acceptable morbidity and mortality<sup>[165]</sup>.

In summary, to date there is no level I or II evidence that HIPEC increases the survival of patients with PC-CRC when added to modern perioperative chemotherapy protocols. The role of a complete cytoreduction, even if well recognized as beneficial and mandatory to allow a rational use of HIPEC, is not supported by RCTs.

In this lack of evidence, there are two opposite attitudes: the NCI does not even mention HIPEC among the treatment options, while the French guidelines recommended it in the treatment of patients with PC from CRC.

Beside its role as prognostic factor, the PCI is a fundamental tool to guide toward a tailored therapy, shifting from the idea of a threshold value to a parameter integrating with every tumor biology data and the clinical status of the single patient.

The next goal will be the demonstration of the usefulness of the "second-look strategy" for high risk patients in terms of overall survival and PC-free survival.

## CONCLUSION

Peritoneal carcinomatosis is a real challenge for oncologists and surgeons, which treatment is very difficult. Many surgeons and oncologists are still use to raise the white flag in discovering them. The loco-regionality of PC and the real characteristic and barrier ability of peritoneum with its proper lymphatic system have not still sufficiently investigated.

Substantial differences exist in treating the different form of PC from different diseases among different centers and countries. Consequently different evidences in results still remain and are undoubtedly discussed. For this reason the chemosurgery (association of chemotherapy and surgery as one entity) is not yet considered as a definitive valid option.

The different forms of PC from different diseases should not continue to be treated in unique centers. Advanced diseases should be centralized in all countries, and centers performing chemosurgery should not continue to treat all diseases, but disease-specialized centers should start to apply chemosurgery to the different forms of PC. The major risk is to lose the link between the PC and the primary tumor: waiting for a better understanding of the peritoneal diffusion pathophysiology and trying to redefine its prognostic role, it would be prudent to mention the pathological classification of the primitive tumor, that is frequently missed.

Lastly, to increase knowledge and overcome the actual limits, we all need a big effort toward a multidisciplinary approach, selection and discussion of the different cases with a reciprocal knowledge increase. More importance and credit should be given to translational medicine.



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## Quasispecies structure, cornerstone of hepatitis B virus infection: Mass sequencing approach

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viral strains. In the present review, we update the information regarding HBV variability and present a summary of the various NGS approaches available for research in this virus. In addition, we provide an analysis of the clinical implications of HBV variants and their study by NGS.

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**Key words:** Hepatitis B virus; Next generation sequencing; Quasispecies; Linkage analysis; Gene overlapping

**Core tip:** We provide an update of hepatitis B virus (HBV) virology, focusing on its complex replication cycle which generates high genetic variability, which led HBV infection to evolve as viral quasispecies, complex distributions of variant populations that are closely related but not identical. We also discuss the clinical and virological implications of this population structure and the application of different next-generation sequencing approaches, which enable analysis of thousands of clonally amplified regions, to study these heterogeneous viral populations.

### Abstract

Hepatitis B virus (HBV) is a DNA virus with complex replication, and high replication and mutation rates, leading to a heterogeneous viral population. The population is comprised of genomes that are closely related, but not identical; hence, HBV is considered a viral quasispecies. Quasispecies variability may be somewhat limited by the high degree of overlapping between the HBV coding regions, which is especially important in the P and S gene overlapping regions, but is less significant in the X and preCore/Core genes. Despite this restriction, several clinically and pathologically relevant variants have been characterized along the viral genome. Next-generation sequencing (NGS) approaches enable high-throughput analysis of thousands of clonally amplified regions and are powerful tools for characterizing genetic diversity in

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### HEPATITIS B VIRUS INFECTION HAS SINGULAR VIROLOGY: IS IT A “PSEUDORETROVIRUS”?

Human hepatitis B virus (HBV; Hepadnaviridae family) causes acute and chronic infection in humans and chimpanzees. HBV infection is distributed throughout the world, and it is estimated that around 2000 million

people worldwide have been in contact with this pathogenic agent. Despite successful vaccination programs and effective antiviral therapies, there are over 350 million carriers of HBV surface antigen (HBsAg). Some 150 million of them have active infection and a high risk of progression to cirrhosis or hepatocellular carcinoma (HCC). HBV-infected individuals have a 30-fold higher risk of developing HCC than the remainder of the population, and it is estimated that 53% of liver cancers worldwide are associated with HBV<sup>[1]</sup>.

HBV consists of a spherical particle with a diameter of 42 nm. The outer envelope is comprised of a phospholipid/protein layer and the inner shell contains core antigen (HBcAg) with the viral genome. Based on differences in the nucleotide sequence of its genome, HBV virus is currently classified into 10 genotypes (A-J) and multiple subgenotypes, with characteristic ethnic/geographic distributions<sup>[2-4]</sup>. The viral genome is 3.2 kb in length, and within the double-shelled particle, the genome adopts the conformation of a circular, partially double-stranded DNA molecule (rcDNA), with a complete non-covalently closed minus strand and an incomplete plus strand.

The HBV genome has four highly overlapping open reading frames (ORFs) - S, P, C and X - that encode seven proteins. The S ORF encodes three different-sized surface antigens whose common region contains the main antigenic loop, the "a" epitope (amino acids 124-147). The a epitope is one of the main targets for neutralizing anti-HBs antibodies. The S ORF is completely overlapped by the P ORF, which encodes a polyprotein with different domains: the reverse transcriptase (RT), the polymerase, RNase H, and the terminal protein (TP). The catalytic center of RT is located at the C domain and is defined by four amino acids mapped at positions 203-206: tyrosine, methionine, aspartate, and aspartate (203YMDD206). The YMDD motif is highly conserved among viral polymerases/reverse transcriptases. The 3' region of C ORF is overlapped by P ORF and contains two start (ATG) codons. The region between these codons is called the preCore region, while the remaining region from the second ATG is known as the Core gene. The two proteins translated from these two ATG start codons are the viral capsid (HBcAg) and the e antigen (HBeAg); the latter is a soluble antigen that acts as an immunomodulator. Finally, the 5' end of the X ORF is overlapped by the P ORF, and the 3' end is overlapped by part of the preCore. The X ORF encodes the x antigen (HBxAg), which is a multifunctional transactivator protein. Furthermore, the HBV genome contains various elements that regulate replication. One important element is enhancer II (ENH II), located in the X gene sequence, which modulates the promoters of transcription of viral mRNA.

#### ***Viral cycle: a DNA virus with a retrotranscription step, similar to a retrovirus***

The HBV population is highly heterogeneous and is comprised of genomes that are closely related, but not identical; hence, it is considered a viral quaspecies, a term commonly associated with RNA viruses. The com-

plex viral replication cycle of HBV may be the cause of this quaspecies nature.

HBV replication occurs exclusively in hepatocytes, but the specific and highly effective viral determinants that direct HBV into hepatocytes are still unknown. It has been hypothesized that HBV hepatotropism is mediated through specific binding of the myristoylated N-terminal preS1 domain of the large HBV surface protein to a hepatocyte-specific receptor that has not been completely defined<sup>[5]</sup>. After interacting with the cellular receptor, HBV particles enter the liver cell<sup>[6]</sup> and release the outer envelope. HBcAg from the inner shell directs the particle to the nucleus, where the rcDNA genome is repaired by host and viral polymerases to a fully double-stranded covalently closed circular genome (cccDNA). This molecule remains in the nucleus of the infected hepatocyte as a minichromosome until the cell dies<sup>[7]</sup>. cccDNA also acts as a template for synthesis of five different viral mRNAs: preCore mRNA, pre-S2 mRNA, X mRNA, S1 mRNA, and the pregenomic RNA (pgRNA). The largest of these, pgRNA, is 3.5 kb in length and contains a redundant fragment of the preCore region at both ends. pgRNA yields the polymerase and core transcripts. In addition, the 5' and 3' ends of pgRNA adopt a hairpin stem-loop structure, named epsilon. The epsilon signal at the 5' end directly interacts with the polymerase and constitutes the first step in initiation of reverse transcription. The interaction induces recruitment of HBcAg monomers around the polymerase/pgRNA complex. These particles can be considered true previrions, with the HBV genome in RNA conformation. Within these particles, pgRNA serves as a template for reverse transcription to the minus strand of HBV DNA, which acts as a template for synthesis of the HBV DNA plus strand by the HBV polymerase. The plus strand is incomplete and yields a new particle with a new rcDNA genome<sup>[8]</sup>.

Thus, the HBV viral cycle includes a transcription and translation step, but also a reverse transcription step similar to what occurs in retroviruses, mediated by the different activities of HBV polymerase<sup>[2,3]</sup>. One potential explanation for the quaspecies nature of the virus occurs at the retrotranscription step: the viral enzyme lacks proofreading activity and the new genomes are highly prone to accumulate errors<sup>[9]</sup>. This fact, in addition to the extremely high viral replication rate results in a remarkably heterogeneous viral population, referred to as a quaspecies<sup>[10]</sup>. In the section *Variability only due to poor fidelity of the viral polymerase?* we discuss further potential sources of the virus quaspecies formation.

Nonetheless, quaspecies variability may be somewhat limited by the high degree of overlap between the HBV coding regions (67%)<sup>[11]</sup>. This restriction would not be very important in the Core region because it is not overlapped and contains variable epitopic domains that play a central role in the immune response against the virus<sup>[12,13]</sup>. In contrast, the viral reverse transcriptase is highly conserved, due to its important functions and overlapping with the S ORF<sup>[3]</sup>. Positions at or near the



initiation site for synthesis of the first viral strand are also highly conserved, and are homologous over 67 nucleotides to the U5 region; a region comparable to retroviral long terminal repeats<sup>[14]</sup>.

The Core is the most suitable region to study the natural evolution of the viral genome, keeping in mind that the immune system is the main driver of evolutionary pressure<sup>[15]</sup>. Certain specific mutations observed in viral variants may have implications in the pathogenesis of viral disease. For example a G to A substitution at nucleotide 1896 yields a *de novo* stop codon in the preCore region that is associated with HBeAg seroconversion and a high risk of fulminant hepatic failure in acute infection<sup>[16]</sup>. In conclusion, depending on the HBV region analyzed, different features of the quasiespecies will be examined.

### ***Singular HBV virology: viral genome integration and nuclear reinfection of nascent viral capsids***

One peculiarity of hepadnaviruses is that their genome is often found to be inserted in the hepatocyte genome<sup>[17-19]</sup>, a fact that could indicate a possible common origin with retroviruses. These integrations can induce chromosome changes, genome instability, or changes in the expression of human genes<sup>[20]</sup> and have been found throughout the different chromosomal sites in the host genome [*e.g.*, near the telomerase reverse transcriptase gene (*tert*) and fibronectin gene (*fn1*)<sup>[19,21-24]</sup>]. Integrated HBV DNA sequences and episomal HBV genomes have been found in 85%-90% of HBV-related cases of HCC<sup>[19,21]</sup>. Recent studies using various genetic approaches and microarray technology have reported that HBV-related HCC displays higher rates of chromosomal abnormalities than HCC due to other risk factors, and even generates chimeric oncogenic proteins<sup>[25,26]</sup>. Ding *et al.*<sup>[19]</sup> analyzed 286 unique integration sites from 40 pairs of HBV-related HCC and tumor-adjacent tissues by massive sequencing on the Illumina platform [see next-generation sequencing (NGS) methods in later sections], and seven HBV integrations per individual were detected. However, the relationship between HBV integration and HCC has not been completely elucidated.

Another peculiarity of viral replication is nuclear reinfection. The nascent capsids have the same structure and conformation as those that originally infected the hepatocytes and released the outer envelope, and this similarity enables nuclear reinfection. In this way, the new capsids can increase the amount and diversity of nuclear cccDNA during viral replication<sup>[27]</sup>. This leads to an increase in the intracellular and intranuclear viral quasiespecies.

Based on its singular virology, HBV can be considered a non-enveloped RNA virus at the intracellular level and an enveloped DNA virus at the extracellular level. In addition, it has “pseudoretroviral” behavior consisting of an ability to integrate into the host cell genome, and a highly variable genome that leads to a quasiespecies nature. In the next sections, the variability of the HBV genome will be discussed in the light of data obtained by recently developed massive sequencing technologies.

## **TOOLS FOR VIRAL QUASIESPECIES**

### **STUDY: NGS TECHNOLOGIES AS A NEW STRATEGY FOR A DEEP ANALYSIS**

Study of HBV quasiespecies evolution together with disease progression is important to clarify the pathogenesis of this condition and improve the available treatment strategies<sup>[28]</sup>. This type of study can be performed by standard methods or by new, more powerful approaches.

#### ***Standard approaches to describe genomic variability***

For the past 40 years, the most widely used method for sequencing DNA has been Sanger sequencing, which is based on a single sequencing reaction with fluorescent dideoxynucleotides, which act as chain terminators<sup>[29-31]</sup>. This produces a mixture of DNA fragments of different sizes, all ending with one of four terminators, each of which is marked with a different fluorochrome that can be detected when the fragments are separated by size in capillary electrophoresis-based sequencers. To obtain the maximum amount of product to be analyzed, polymerase chain reaction (PCR) is performed to amplify the region of interest. Analysis of a complex genomic sample (*e.g.*, a viral quasiespecies) by Sanger sequencing yields the most common nucleotide present in the quasiespecies, known as the consensus sequence, but individual sequences are not obtained. Thus, analysis of the HBV RT domain C (putative active center) covering codons 202 to 206 can yield positions with different nucleotides (A/GGC<sub>202</sub> TAT<sub>203</sub> A/GTG<sub>204</sub> GAT<sub>205</sub> GAT<sub>206</sub>), but it is uncertain whether the changes are present in the same sequence or in two different sequences, for example GGC<sub>202</sub>.TAT<sub>203</sub>.ATG<sub>204</sub>.GAT<sub>205</sub>.GAT<sub>206</sub> or AGC<sub>202</sub>.TAT<sub>203</sub>.GTG<sub>204</sub>.GAT<sub>205</sub>.GAT<sub>206</sub>. Minor nucleotide changes in the same position can only be detected as “inner small peaks” when their frequency is higher than 20% (*e.g.*, a clear G peak with a small A peak inside in codon 202). Therefore, Sanger sequencing of a complex genomic mixture presents two main limitations: low sensitivity for minor variant detection and inability to perform haplotype analysis.

In addition to such direct sequencing methods, some indirect molecular techniques, mainly focussed on detection of specific variants, have also been extensively used. The most common is reverse hybridization in Line Probe Assays format that can detect specific variants present as at least 5% of the viral quasiespecies<sup>[32,33]</sup>. Other, less commonly used methods for detecting specific variants include restriction fragment length polymorphism (RFLP) analysis<sup>[34-36]</sup>, 5'-nuclease assays<sup>[35]</sup>, melting point analysis<sup>[37]</sup>, mass spectrometry<sup>[38]</sup>, DNA chip technology<sup>[39]</sup> and real-time PCR using mutation-specific primers<sup>[40]</sup>. Although these methods can differentiate between population mixtures<sup>[41]</sup>, they do not allow simultaneous detection of different substitutions in the same viral genome. Therefore, complex variants with two or more amino acid changes, such as those related to entecavir resistance or those causing a fitness increase during lamivudine

treatment, cannot be properly studied by these indirect methods. Furthermore, they cannot be used to properly describe the composition of a quasispecies.

The traditional method used to analyze a heterogeneous genomic population, such as a viral quasispecies, is cloning and sequencing. This is a costly, labor-intensive process that requires multiple, complex experimental steps, and provides limited resolution regarding the mutation spectrum and frequencies. Analysis of a quasispecies by cloning requires a large number of clones (> 100) which significantly hampers its use.

## NGS

The genome sequencing scenario is now changing, mainly because of the development of NGS techniques<sup>[42]</sup>. NGS approaches enable high-throughput analysis of thousands of clonally amplified regions and are powerful tools for characterizing genetic diversity in viral strains<sup>[43]</sup>.

The most widespread platforms available are the 454 FLX (Roche)<sup>[44]</sup>, the Solexa genome analyzer (Illumina)<sup>[45,46]</sup>, the SOLiD system (Applied Biosystems)<sup>[47]</sup>, and recently, the Ion Torrent system (Life Technologies)<sup>[48]</sup>. These platforms apply different experimental strategies (enzymological, chemical) and complex engineering (high-resolution optics, hardware, and software), and provide significant time saving and a minimal requirement for associated equipment in comparison to cloning. NGS technologies seek amplification of single strands of DNA with specific sequences (added by chemical binding or by specific PCR primers) and perform sequencing reactions on the amplified strands. The presence of specific sequences enables selection of PCR-amplified fragments without requiring a bacterial cloning step. The yield of sequence reads and total bases per instrument run is significantly higher than that produced in a capillary sequencer run: from several hundred thousand reads (454 FLX and Ion Torrent) to tens of millions of reads (Illumina and SOLiD)<sup>[44]</sup>.

**FLX-454 pyrosequencing platform (Roche):** The 454 technology is based on ultra-deep pyrosequencing (UDPS), a DNA sequencing method that relies on chemiluminescent detection of pyrophosphate release during polymerase-mediated dNTPS incorporation. The pyrophosphate is converted into ATP, which allows generation of light by the enzyme luciferase in amounts proportional to the amount of ATP. UDPS is a fast method that can detect and quantify small viral subpopulations (present in < 5%)<sup>[43,49]</sup>; specifically, a recent study reported detection of HBV subpopulations accounting for 2% of the total viral population<sup>[50]</sup>.

The 454 Sequencing System supports analysis of samples from a wide variety of starting materials, including genomic DNA, PCR products, artificial bacterial chromosomes, and cDNA. The system relies on fixing nebulized and adapter-ligated DNA fragments to small DNA-capture beads in a water-in-oil emulsion. The emulsion is prepared to achieve oil drops with a diameter that

accommodates only one bead and a single DNA molecule. The oil drops act as microreactors for clonal PCR amplification. DNA-bound beads are placed on a PicoTiter-Plate (PTP), together with a fiber optic chip and a mix of enzymes such as DNA polymerase, ATP sulfurylase, and luciferase. The PTP is then placed in the GS FLX system for sequencing. The sequencing process is monitored by the light flashes released once a nucleotide is incorporated. However, the calibrated base calling cannot properly interpret long stretches (> 6) of the same nucleotide and for this reason, homopolymeric regions (defined as repeats of  $\geq 4$  identical bases) are prone to artifactual base insertions and deletions. In contrast, as each incorporation step is nucleotide-specific, substitution errors are rarely encountered in Roche/454 sequence reads.

The raw reads are processed by the 454 analytical software and then screened by various quality filters to remove poor-quality sequences. Fragments > 400 nucleotides can be sequenced (currently around 700 nucleotides), allowing haplotypic analysis that would theoretically be useful even for phylogenetic studies. UDPS is currently the only platform for quantitative analysis of long clonal sequences that allows detection of particular mutations as well as combinations of mutations in the same sequence. It is, therefore, highly useful as a high-throughput, massive sequencing method to describe quasispecies.

**Solexa (Illumina):** The single molecule amplification step of the Illumina Genome Analyzer starts with an Illumina-specific adapter library. This step automatically takes place on the oligo-derivatized surface of a flow cell, which is an eight-channel sealed glass microfabricated device that allows a special process, “bridge amplification”, of fragments on its surface. This process uses DNA polymerase to produce multiple DNA clusters, each representing the single molecule that initiated the cluster amplification. Bridge amplification generates multiple copies of a specific DNA molecule on an “oligo-decorated” solid support. Each cluster contains approximately 1 million copies of the original fragment.

The Illumina system utilizes a sequencing-by-synthesis approach in which all four nucleotides and the DNA polymerase are simultaneously added to flow cell channels for incorporation into the oligo-primed cluster fragments<sup>[46]</sup>. The nucleotides carry a unique base fluorescent label in which the 3'-OH group is chemically blocked. The DNA chains are then extended by one nucleotide, and an image is taken. Subsequently, the 3' blocking group is chemically removed to prepare each strand for the next incorporation by DNA polymerase. This series of steps continues for a specific number of cycles, permitting discrete read lengths of 50-250 nucleotides. However, this approach is not suitable for haplotypic analysis or quasispecies studies.

**SOLiD (Applied Biosystems/Life Technologies):** The SOLiD platform, which is similar to that of other NGS methods, uses an adapter-ligated fragment library

and an emulsion PCR (similar to 454 technology)<sup>[47]</sup>. However, it is based on the principle of “two-base encoding” and uses a DNA ligase as the enzyme. The system has random 8-mer fluorescent probes with all 16 combinations of di-bases at the 3' ends. Once one of these fluorescent probes hybridizes over the sequence, the probe is linked to the region of interest by ligase. Each ligation step is followed by fluorescence detection and removal of the ligated 8-mer. This method sequences short fragments of 35-50 nucleotides and does not allow haplotypic studies.

**Ion torrent: ion semiconductor sequencing (Life Technologies):** This platform is similar to the FLX 454 method, but has a semiconductor-based detection system<sup>[48]</sup>. The sequencing system is based on detection of hydrogen ions (protons) released during DNA polymerization, instead of the image capture used in other sequencing systems. A microwell containing a DNA template is flooded with a single type of nucleotide. If the nucleotide introduced is complementary to the leading template nucleotide, it will be incorporated into the growing complementary strand. This phenomenon releases a hydrogen ion that triggers a hypersensitive ion sensor, whose signal indicates that a reaction has occurred. If homopolymer repeats are present in the template sequence, multiple nucleotides will be incorporated in a single cycle. This leads to a corresponding number of released hydrogen molecules and a proportionately higher electronic signal. In this sense, this method can be considered more accurate than FLX-454 for homopolymeric sequences. Currently, fragments up 200 nt can be sequenced, with an approximation to haplotypic analysis. However, the sequence length is too short for phylogenetic studies, and the method is not useful to describe quasispecies.

### NGS applications and limitations

NGS techniques have been used to study some quasispecies, such as human immunodeficiency virus (HIV)<sup>[51,52]</sup>, HBV<sup>[53-59]</sup>, and hepatitis C virus (HCV)<sup>[46,60]</sup>. The 454 platform has been successful in analysis of HIV quasispecies, exhibiting high sensitivity for detecting treatment-resistant variants<sup>[51,61-63]</sup>, in describing HCV heterogeneity<sup>[46,60]</sup>, and more recently, in HBV quasispecies studies<sup>[53-59]</sup>. UDPS has been used in studies to quantify HBV minority variants carrying resistant mutations<sup>[55,58]</sup>, and to detect defective variants<sup>[53-58]</sup>. In addition, the UDPS NGS approach has enabled dynamic study of clonal evolution in cancer cells, detecting somatic mutations in rare sub-clones at a rate of 1 in 5000 copies<sup>[49]</sup>.

Several factors should be remembered in relation to NGS reads. First, the length of an NGS sequence read is shorter than that of Sanger sequencing, and second, each NGS platform has a unique error model that differs from the error model established for capillary sequence reads. In consequence, complex, accurate computational algorithms are required to process the raw data from massive parallel sequencing (background subtraction, base calling,

and quality assessment), and they should be specific for each platform. Bioinformatic filtering has been the main method developed to validate variants carrying substitutions, but it has not been completely optimized for variants with insertions and deletions, which are common in HBV, especially in the X ORF and basic Core promoter, mapped at the preCore region<sup>[64-68]</sup>. Therefore, study of insertion and deletion variants still requires classical cloning analysis, and that is why they have been little investigated.

In addition, UDPS is associated with sequencing errors in homopolymeric regions, although some of the variants identified have been true variants previously reported by cloning<sup>[69,70]</sup>. Currently, it is uncertain how natural insertion/deletion variants can be differentiated from variants resulting from errors in UDPS processing; therefore, the computational analysis of UDPS reads must be improved. One possible approach could include systematic analysis of forward and reverse reads in duplicate by NGS and in parallel with cloning. Another strategy could be targeted searching for sequences with known deletions and insertions, previously detected by classical clonal analysis<sup>[70]</sup>, or selection of insertions or deletions that cause relevant changes, such as *de novo* stop codons<sup>[69,70]</sup>.

The importance of investigating insertions and deletions in the HBV quasispecies is underscored with the G2091 deletion, described by Schories *et al.*<sup>[69]</sup> and recently detected by Ramírez *et al.*<sup>[70]</sup>. This deletion produces three *de novo* stop codons: (wild type, 2086TGG.GGG.GAA.TTG.ATG.ACT.CTA.GCT, and the deleted variant, 2086TGG.GGG.AAT.TGA.TGA.CTC.TAG.CT), resulting in an HBV “capsid-defective genome”. The presence of potentially defective genomes in the HBV quasispecies has been recently reported in the S ORF<sup>[56,58]</sup>, in the HBcAg start codon, and even in the HBxAg stop codon in studies using massive UDPS<sup>[56]</sup>.

Another factor that must be taken into account in NGS data analysis is the possibility of recombination events occurring during PCR amplification<sup>[71]</sup> by different mechanisms; for example, short incomplete amplicons can act as primers for different sequences present in the quasispecies, or crossing of amplified sequences can occur, similar to chromosome recombination events. These phenomena may be a potential limitation of UDPS, but they can also occur in classical cloning and any other technique that includes a PCR amplification step. This confounding factor questions the reliability of haplotype analysis of the quasispecies, which requires minimization of recombination events. Thus, the true impact of these events should be carefully evaluated in the future by UDPS analysis of *in vitro* mixtures from different clonal sequences. It would be particularly interesting to know the impact of recombination in order to derive conclusions from linkage analysis of different relevant nucleotide substitutions reported in the HBV polymerase and preCore region by classical cloning<sup>[72]</sup> and UDPS<sup>[56-58]</sup>.

Lastly, the limit of detection of the UDPS approach has not been established, and differences between studies



from different groups must be taken into account when viewing the results.

## QUASISPECIES NATURE OF HBV

The term quaspecies was coined to refer to RNA viruses that genetically evolve as complex distributions of variants that are closely related, but not identical<sup>[73]</sup>. In quaspecies infection, the genome is not precisely defined, and an average of different variants is used as the consensus genome. Study of quaspecies dynamics is important to understand the adaptability, pathogenic power, and persistence of viruses, and to design strategies to prevent and treat the diseases they cause. A viral quaspecies is seen as a swarm of variants in a host, among which variants carrying a biological advantage during replication are selected. When changes occur in the environmental conditions of the virus, the quaspecies structure responds by rebalancing its composition. The predominant sequence (master sequence) may even shift by selection of a variant that is better adapted to the new environment, in the classic Darwinian process of survival of the fittest<sup>[57]</sup>.

### *Variability only due to poor fidelity of the viral polymerase?*

The hepadnavirus family replicates by a reverse transcriptase that lacks proofreading activity, and this fact seems to justify their high mutation rate ( $3.2 \times 10^{-5}$  -  $7.9 \times 10^{-5}$  nucleotide substitutions/replicative cycle); 100 times higher than other DNA viruses<sup>[74]</sup>. The high mutation rate, intense replicative activity ( $10^{12}$  viral particles/d), and small genome size of HBV, results in approximately  $10^{10-11}$  point mutations being produced per day in individuals with active replication.

In addition, certain host factors are reported to be associated with hypermutational activity. One example is the innate antiviral defense mechanism that mediates host enzymes belonging to the APOBEC3 cytidine deaminase family, which cause extensive deamination of cytidine (C) to uridine (U) bases in negative-stranded DNA<sup>[75]</sup>. This activity results in a guanine (G) to adenine (A) hypermutation in positive-stranded DNA<sup>[76]</sup>. APOBEC-mediated G to A hypermutation was initially reported in HIV, but it has also been described in HBV<sup>[77-79]</sup>, other retroviruses, and retrotransposons<sup>[76]</sup>. In fact, several clinically relevant HBV variants result from G to A mutations, such as rtA181T (GCN to ACN) and rtM204I (ATG to ATA), interpreted as RT resistance mutations, and the G1896A and G1899A main preCore variants (see next sections).

Thus, viral and host factors yield an HBV quaspecies provided with plasticity and rapid adaptation to the changing environmental conditions caused by the immune response or antiviral treatments<sup>[80,81]</sup>. In this line, nascent HBV capsids can contain new mutations produced during reverse transcription or APOBEC activity; therefore, the genomes may differ from those that previously infected the nucleus. This reinfection process

generates a kind of intracellular quaspecies, in addition to the circulating one, thereby increasing the complexity of HBV. Another factor potentially responsible for causing variability is the fact that the same liver cell can be infected by multiple virions simultaneously or sequentially during its lifetime.

The versatility of the quaspecies structure may include mechanisms to tolerate variants with changes involving long fragments of the HBV genome, such as deletions reported by genome splicing<sup>[73,82,83]</sup>. This tolerance might yield defective genomes that can be maintained by transcomplementation mechanisms. For instance, HBsAg-defective variants might be enveloped by functional surface proteins encoded by other variants present in the quaspecies<sup>[58,84]</sup> (see next sections). For this reason, viral genomes that cannot self-replicate sufficiently may be detected in the viral quaspecies population<sup>[56,58,85]</sup>. Such defective genomes have been isolated from liver tissue of infected patients<sup>[86]</sup>, and the proteins they encode, which are known as hepatitis B spliced proteins, appear to contribute to persistent replication in patients with HCC<sup>[86]</sup>. This suggests that defective genomes may have modulatory roles and provides evidence of regulatory mechanisms directly associated with the HBV quaspecies structure.

These data seem to indicate that the quaspecies nature of HBV can further increase the encoding capacity of the virus, which is somewhat limited by the high overlapping of its genome. ORF overlapping represents a strategy to restrict the viral genome size and maximize its coding capacity<sup>[3,87-89]</sup>. An indication of gene overlapping may be the presence of unusually strong constraints at third codon positions<sup>[90-92]</sup>. Hepadnaviruses are representative of this situation, and overlapping must be systematically taken into account when HBV viral infection is investigated at the virological and clinical level.

As was discussed above, it is generally assumed that the main source of quaspecies variability is the viral polymerase lack of error correction. However, certain characteristics of the target sequences themselves, such as the presence of homopolymeric regions, may be associated with an increased risk of errors by polymerase “sliding”. This phenomenon would result in errors by deletion or insertion, giving rise to altered reading frame variants and resulting in potentially deficient genomes<sup>[69,70]</sup>. The presence of these variants in the circulating viral quaspecies would provide evidence of complementary mechanisms whose effect on infection is unknown.

In this line, it cannot be excluded that truncated proteins encoded by these variants may have some type of regulatory function. For example, lengthening the life of infected cells by changing cell apoptosis (carcinogenesis) has been reported in association with truncated HBx proteins<sup>[93-96]</sup>. To explain the existence of these truncated versions of HBx, it has been hypothesized that transcomplementation could be achieved by using functional protein encoded by competent variants. This process would potentially modulate viral encapsidation and replication, with direct effects on the clinical evolution of the infection.





**Table 1** Main amino acid variants described in the hepatitis B virus genome and its effect

ORF	Amino acid variant	Effect
P	A181T and M204 I/V (compensatory: L80I/V, T128N, R153Q, V173L, L180M, A200V <sup>1</sup> , V207I <sup>1</sup> ) N236T (compensatory: A181 T/V/S. Low sensitivity: V84M, S85A, L217R, I233V) I169T, T184S, S202C/I/G, M250I A194T <sup>1</sup>	LMV resistance ADV resistance ETV resistance <sup>2</sup> TDF resistance
S	G145R W156Stop, W163Stop, W172Stop, W196Stop D144A, P142S, K141E, Q129H, I/T126N, T131I, M133L	Immune therapy failure HBsAg structural alterations
X	I130M, V131I, F132Y	Contribution to HCC development
C		
preCore	A1762T and G1764A	HBeAg negative forms
Core	G1896A and G1899A P5H/L/T, D32N/H, C/E43K, cP50A/H/Y, E83D, I97F/L, L100I, A131G/N/P, S181H/P and C/Q182K/stop Regions 50-69 and 74-84	Disease progression HCC development Immune scape variants

<sup>1</sup>Antiviral treatment resistances not completely defined<sup>[58]</sup>; <sup>2</sup>Linked to lamivudine (LMV) signature (L180M + M204V). ORF: Open reading frames; ADV: Ad-efovir; ETV: Entecavir; TDF: Tenofovir; HBsAg: Hepatitis B virus (HBV) surface antigen; HBeAg: HBV e antigen; HCC: Hepatocellular carcinoma.

*et al.*<sup>[98]</sup> analyzed a group of entecavir-treated patients and found that responders presented a less complex quaspecies than the group of partial responders, whereas partial and non-responders showed similar patterns of complexity. A recent report based on a cloning method has shown that viral diversity after lamivudine treatment was higher in HBeAg-seroconverters than in non-seroconverters<sup>[72]</sup>. Nonetheless, these studies have the limitation of a low number of clones analyzed.

Nishijima *et al.*<sup>[59]</sup> applied UDPS to study quaspecies complexity in a group of chronic hepatitis B patients, comparing the viral genome sequences determined in liver tissue with those in serum. Although the results cannot be compared with those of the cloning studies, the authors found no significant differences in the viral population between liver and serum from the same individual. In addition, they found no significant differences in viral complexity at the HBV DNA level, or according to age or degree of fibrosis. Nishijima *et al.*<sup>[59]</sup> did not compare quaspecies diversity in relation to nucleos(t)ide analog (NUC) response or HBeAg seroconversion, but they observed similar complexity between naïve and NUC-treated cases. A preliminary study by our group using UDPS and Shannon entropy calculation indicated a decrease in quaspecies complexity at the HBV preCore/Core region after NUC treatment failure, likely due to selection of specific NUC-resistant variants. In contrast, diversity mainly increased during the natural evolution of the virus, probably because of immune system evolutive pressure. In this sense, it should be remembered that the main HBV epitopic regions are located in the preCore/Core region<sup>[99]</sup>.

## CLINICALLY SIGNIFICANT HBV GENOMIC VARIANTS: MASSIVE SEQUENCING FOR A DETAILED DEPICTION OF THE SITUATION

Several clinically and pathologically relevant variants have

been characterized along the viral genome, despite the restriction resulting from the high degree of HBV genome overlapping. This restriction is especially important in the P and S gene overlapping regions, but is less significant in the regions corresponding to the X and preCore/Core genes. Therefore, the X and preCore/Core genes may be the most suitable for studying quaspecies variability, even though they contain the major enhancer of the viral genome (ENH II). The following sections will discuss the major variants of each of the HBV genome regions (P, S, X, preCore and Core) and their clinical and pathological implications, which are summarized in Table 1. Most of the reported data were obtained by direct sequencing or short clonal studies, which means that the variants were present in significant percentages. However, recent results obtained by massive sequencing are also included to provide a more detailed picture.

### P gene variability and its implications in antiviral treatment

The polymerase gene (P ORF, nucleotides 2307-1623), the largest HBV gene, encodes the 90-kDa viral polymerase protein, a multifunctional enzyme involved in DNA synthesis and pgRNA retrotranscription (RT domain), with additional priming [TP domain and RNase (RH) domain] functions. The main genomic variants of this region have been reported in the proper viral polymerase RT domain, which has both DNA polymerase and retrotranscriptase activities (retrotranscribes pgRNA to the minus DNA strand and synthesizes the incomplete plus strand). RT activity is located between the non-functional spacer region (Spc) and the RH domain. The TP domain, which acts as a primer for synthesis of the negative DNA strand is located at the N-terminal of the P ORF. The Spc region, located next to TP, is dispensable for enzyme function and therefore, easily tolerates mutations. The Spc region overlaps the preS region of the envelope gene and accumulates important mutations, such as long deletions<sup>[3,100,101]</sup>. The crystal structure of

HBV polymerase has not yet been reported, but the 3D structure of HBV RT has been modeled, based on the crystal structure of HIV-1 RT<sup>[2,102]</sup>. The model shows the common right-handed configuration of both polymerases and identifies seven different domains (A-G). The catalytic center of RT activity (nucleotides 736-747) corresponds to the YMDD sequence, identical to what is observed in HIV-1<sup>[103]</sup>. The YMDD motif contains two of the three essential D (aspartate) residues of the polymerase. Attending to the specific nomenclature of the RT region, position 348 of the polymerase gene corresponds to the first amino acid of RT; hence, the catalytic motif is located at positions 203-206 (Y<sub>203</sub>M<sub>204</sub>D<sub>205</sub>D<sub>206</sub>)<sup>[32]</sup>. The RT region is highly conserved among retroviruses and hepadnaviruses<sup>[14]</sup>. In this putative structure, the YMDD main catalytic motif is identified in the C domain, located in the palm region of the right-handed RT structure<sup>[104,105]</sup>.

As in HIV, therapy for HBV infection is currently based on the use of NUCs [lamivudine (LMV), adefovir (ADV), emtricitabin (EMT), telbivudin (LdT), entecavir (ETV), and tenofovir (TDF)], which are HBV RT inhibitors. Among them ETV and TDF are usually recommended as the first-line treatment option because of their high potency and low resistance rates. NUCs and host nucleotides, the natural polymerase substrates, bind at the YMDD motif. NUCs act as competitive inhibitors by blocking elongation of new HBV DNA strand. The central role of HBV polymerase in the viral replication cycle seems to justify the stability of its AA sequence relative to the remaining HBV protein products. However, non-synonymous nucleotide changes that result in amino acid substitutions have been reported in relation to resistance to NUC therapies. The presence of these drugs induces selection of HBV variants carrying amino acid substitutions in the RT domain. These mutations may cause structural changes in the polymerase, resulting in a decrease of drug affinity and antiviral activity. Thus, NUCs efficiently inhibit wild-type HBV variants present in the viral quasiespecies, whereas variants carrying resistant mutations can maintain their replicative activity. Under NUC treatment, the percentage of resistant variants in the quasiespecies may increase and ultimately be selected as the major variant, thereby causing treatment failure, manifested as viral breakthrough (VBK).

Because they can confer resistance to oral antiviral treatment, mutated strains are of great interest clinically. The intensity of viral resistance is related to the type of drug and the viral variant. The most commonly reported variants, rtM204V and rtM204I, are changes in the YMDD motif to YVDD or YIDD (located in RT domain C and analogous to the M184V/I LMV resistance mutation of the HIV-1 RT) (Table 1). Both these variants show low affinity for LMV, making them highly resistant to the drug and easily selected during treatment (70% of patients show resistance after 5 years of LMV therapy). These variants are also resistant to other nucleoside therapies such ETV and LdT, but with lower resistance rates than

with LMV (*e.g.*, 17% after 2 years of LdT treatment)<sup>[106]</sup>.

The fitness of viruses with rtM204V and rtM204I variants is markedly reduced in comparison to wild-type<sup>[107]</sup>, but they alone can replicate under LMV therapy<sup>[108,109]</sup>. Long-term LMV therapy increases the probability of new variants emerging, which will restore the replication capacity of the mutant and worsen the outcome of infection<sup>[110]</sup>. The most common of these additional, potentially compensatory mutations is rtL180M<sup>[107]</sup>, which is often detected together with rtM204V and less often with rtM204I. Other mutations, such as rtV173L and rtL80I/V, do not alter the sensitivity of HBV to LMV, but instead, enhance its replication efficiency<sup>[111]</sup>. The rtL80I/V mutation (located in the RT A domain) is associated with severe disease in HBV genotype C patients<sup>[112]</sup>. rtV173L (combined with rtL180M and rtM204V) is the second most commonly detected compensatory mutant (19% of cases showing LMV resistance)<sup>[109]</sup>. Selection of compensatory mutants also occurs with the rtA200V/rtM204I combination<sup>[113]</sup> and with the variants rtT128N or rtR153Q in combination with the rtL180M/M204V polymerase mutations<sup>[114]</sup> (Table 1).

The compensatory effect of additional variants has been explained by molecular interactions between the various substitutions that provide HBV polymerase with a more efficient catalytic structure<sup>[102]</sup>. The rtA181T variant has been associated with LMV resistance in less than 1% of cases and has also shown resistance to ADV treatment<sup>[84]</sup>. The rare rtA181S mutation, which is similar to rtA181T, presents cross-resistance to LMV and ADV treatment in combination with M204I<sup>[115]</sup>. Emergence of resistance to ETV is uncommon in treatment-naïve patients (< 1% over 6 years), but in those with previous LMV failure, it increases dramatically to 40% after 4 years<sup>[106,116]</sup>. In fact, ETV resistance seems to be associated with the concomitant presence of LMV resistant variants and mutations in other RT codons (169, 184, 202 or 250, with at least four different substitutions in the same sequence), which confer decreased susceptibility to both LMV and ETV<sup>[117]</sup> *in vitro*, explaining the high genetic barrier of ETV.

The NUC ADV is active against HBV wild-type virus and variants resistant to LMV, EMT, and LdT. Emergence of ADV-resistant variants is less frequent than with LMV therapy (29% at 5 years)<sup>[118]</sup>. ADV resistance has been associated with the A181T/V and rtN236T variants, and less frequently, with rtI233V<sup>[119-121]</sup>. Interestingly, variant A181T has also shown LMV resistance according to *in vivo* and *in vitro* evidence; thus, rtA181T is cross resistant to LMV and ADV<sup>[84]</sup>. Other amino acid changes have been occasionally linked to resistance or low sensitivity to ADV, such as rtV84M and rtS85A in the A domain and rtL217R; a natural polymorphism in the D domain observed in subgenotype A2 HBV strains<sup>[2,120,122]</sup>. In addition to the main resistant variant, other minor variants are selected after LMV or ADV failure<sup>[123]</sup>, and some of them seem to be associated with the viral genotype (*e.g.*, rtS85F, rtL91I, and C2456G associated with LMV resistance



in HBV genotype D, or rtI53V, rtW153R and rtF221Y associated with ADV resistance in HBV genotype A). Of note, it seems that the main LMV resistant variants, M204V and M204I, are also associated with viral genotype (rtM204I is not often detected in genotype A)<sup>[123]</sup>.

TDF treatment, which is extensively used in HIV-1 infection, is also highly active against wild-type and LMV-resistant HBV polymerase variants<sup>[122,124,125]</sup>. TDF is associated with high sustained viral response (SVR) rates and a low rate of resistances: 0% after 6 years of therapy<sup>[106]</sup>. However, the rtA194T variant observed in some HIV/HBV coinfecting patients has been related with TDF resistance, and shows a reduced *in vitro* replication rate in combination with the rtL180M and rtM204V LMV variants<sup>[110]</sup>. Use of LMV, ETV, LdT, and ADV has been largely replaced by the new potent NUCs, ETV and TDF<sup>[106]</sup>. However, extensive application of LMV and the use of ADV as rescue therapy for LMV failure over many years has resulted in a considerable percentage of chronic HBV patients in whom resistance to these NUCs has developed, and a quaspecies enriched in LMV-resistant variants can be expected. These variants can limit the response to new-generation NUCs. This is the case of the rtL180M + rtM204V combination (known as the LMV signature) in ETV therapy and rtN236T in TDF treatment.

For all these reasons, early detection of RT resistant variants by highly sensitive methods, even minor components of the HBV quaspecies in treatment-naïve patients, could be highly useful for therapy purposes. Detection of minor variants can easily be performed by conventional techniques (reverse hybridization or clonal sequencing) or by NGS methods<sup>[53-58]</sup>. An essential consideration to bear in mind is the S and P gene overlapping, which can lead to reciprocal consequences when there is a nucleotide substitution in either ORF. For example, the rtA181T and rtM204I polymerase variants also produce a stop codon in the S gene (sW172stop and sW196stop, respectively) (Figure 1). Therefore, antiviral treatment pressure may cause selection of viral genomes that are potentially defective for envelope proteins<sup>[58,84]</sup>. Another relevant variant in this regard is rtW153Q, which leads to the sG145R variant in the S ORF, associated with failure of immunotherapy.

Complex variants with two or more amino acid changes, such as those conferring ETV resistance or causing a fitness increase during LMV treatment, cannot be properly studied by indirect methods (LiPa, RFLP, 5'-nuclease assays, melting points, mass spectrometry, DNA chip technology, or real-time PCR), because they do not allow simultaneous detection of different substitutions in the same viral genome. Identification of these substitutions requires clonal techniques, such as classical clonal sequencing or the recent NGS methods. Specifically, UDPS enables simultaneous analysis of thousand of clonally amplified long fragments (700 nucleotides), and deeper and more sensitive detection of minor populations in complex mixtures; therefore, it may be the most

suitable method for viral quaspecies studies.

As was mentioned above, the limit of detection of UDPS remains to be resolved. To date, different studies have achieved mixed results. Ijaz *et al.*<sup>[126]</sup> reported a lower quantitation limit of 2% for minor HBV populations. More recently, Mello *et al.*<sup>[50]</sup> reported values of 4%-17% for LMV-resistant variants<sup>[50]</sup>. Our group recently reported a cut-off value of 0.03% that enabled detection of extremely low percentages (0.04%-0.09%) of RT variants in treatment-naïve patients. In another recent UDPS study we detected RT variants in treatment-naïve samples at values of 0.1%-0.55%<sup>[56]</sup>. In the study by Nishijima *et al.*<sup>[59]</sup>, in which drug-resistant mutants were investigated in chronic-naïve cases by Illumina, frequencies of 0.3%-30% were reported. These three recent studies<sup>[56,58,59]</sup> as well as the previously reported ones<sup>[53,54]</sup> have all shown that resistant RT variants are present at baseline in treatment-naïve chronically HBV-infected individuals. This suggests that a reservoir of RT variants may exist, which would be prone to selection by the effect of antiviral therapies. Furthermore, LMV-resistant variants (LMV signature) linked to specific mutations responsible for ETV resistance have been simultaneously detected with 454 UDPS<sup>[58,127]</sup> (Figure 2). Moreover, Margeridon-Thermet *et al.*<sup>[55]</sup> reported low-level persistence of LMV-resistant variants even 1 year after LMV treatment discontinuation using UDPS with a sensitivity level of 0.5%.

Nevertheless, the clinical significance of these minor drug-resistant mutations remains uncertain. Nishijima *et al.*<sup>[59]</sup> concluded that pre-existing drug-resistant mutants (at naïve status), such as low-abundance mutant clones, may provide the opportunity to develop drug resistance against NUCs through selection of dominant mutations. However, a predictive cut-off value for baseline percentages to define preferential selection after NUC treatment has not been reported. Along this line, we found<sup>[56,58]</sup> that after VBK on LMV treatment, the variants selected were not the ones most frequently detected at baseline, suggesting that the low percentages observed at baseline ( $\leq 1\%$ ) do not determine the variant selected at VBK. Interestingly, we detected a small population of ETV-resistant variants<sup>[58]</sup> after LMV treatment in a patient who developed ETV resistance. See examples of UDPS haplotypic study in Figure 2. Additional sequential studies with a larger number of cases must be performed to define a cut-off value for the baseline percentage of resistant RT variants that can predict drug resistance. Hence, massive sequencing has opened the door to a more profound knowledge of the dynamic behavior of the HBV quaspecies that may clarify the role of minor variants in the HBV RT region on the outcome of infection<sup>[55,58]</sup>.

### ***S gene variability and its implications in immunoprophylaxis***

As was discussed in the HBV virology section, the surface ORF (nucleotides 2848-835) is completely overlapped by the P gene (Figure 1). S ORF has three in-frame start codons encoding the three types of surface antigens pres-



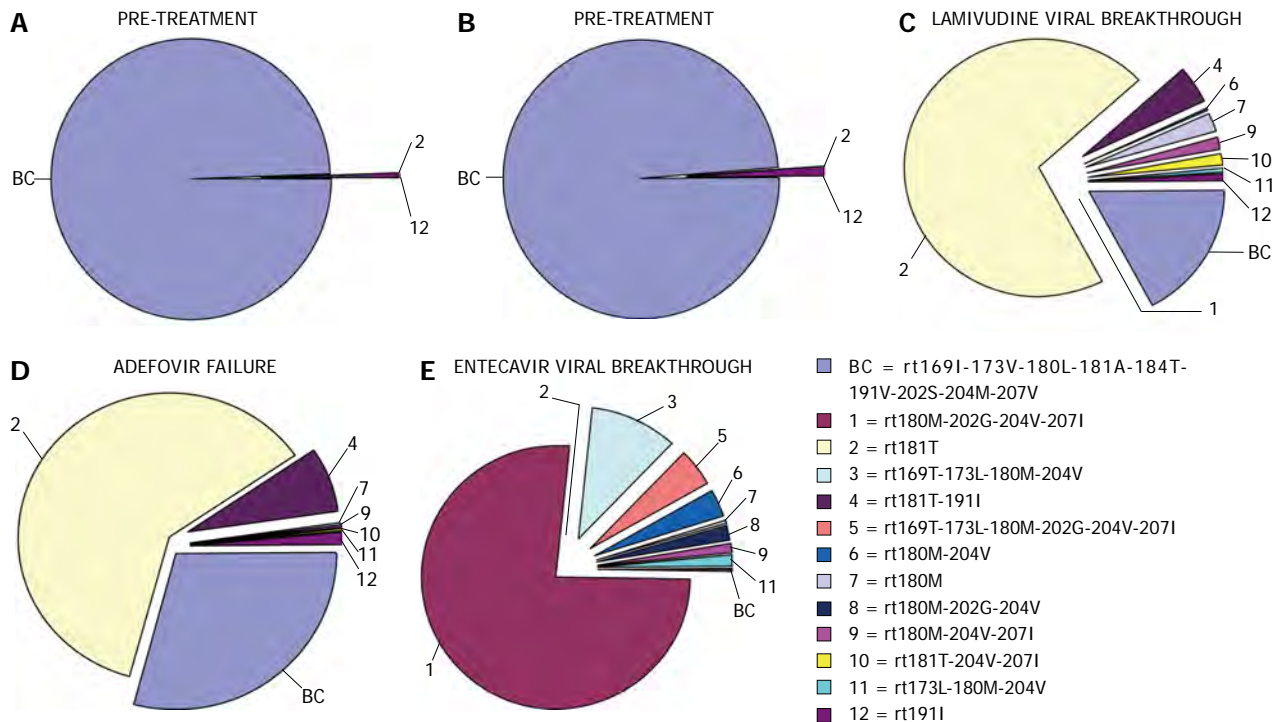


Figure 2 Changes in percentages of reverse transcriptase variants during follow-up of a patient included in one of our studies. Reproduced from Rodriguez-Frias *et al*<sup>[80]</sup>. BC: Baseline combination.

ent on the outer envelope: small (SHBS), middle (MHBS), and large (LHBs, 39 kDa). SHBS, which is common to all three, contains the main antigenic loop, also called the “a” determinant (codons 124-147 within the major hydrophilic region, which covers codons 100-170).

The antigenic loop activates the primary response of the neutralizing antibodies in HBV infection. In the so-called preS region, located in front of the SHBS start (positions 2950 and 3125 of the HBV genome), there is a highly variable sequence that is used to distinguish four major HBV serotypes (adr, adw, ayr, and ayw). HBsAg can self-assemble without containing the HBV genome, and adopt non-infective structural spheres or filamentous forms, which represent nearly the entire population of HBV-related particles (> 99.9%)<sup>[128]</sup>. The preS region overlaps the dispensable spacer domain of the P protein, which allows high heterogeneity. In contrast, the SHBS region (shared by all S-derived proteins) overlaps the essential RT domain of the P protein, which strongly restricts its variability. Deletions are the most relevant variants in the preS region<sup>[129]</sup>, being the main cause of the different genome lengths of the HBV genotypes and affecting the balance between the various types of HBsAg proteins<sup>[130]</sup>. Thus, it can be speculated that a partial or complete HBsAg defect may decrease virion assembly and secretion and lead to a parallel increase in the amount of cccDNA by enhancement of the capsid recycling pathway. An increase in preS deletions has been reported in HBeAg-positive chronic cases (35% in the sixth decade of infection), and has been related to a poor outcome (detected in 60% of HCC patients)<sup>[130,131]</sup>. The study of Kao *et al*<sup>[100]</sup> has reported a significantly higher rate of

preS deletions in HCC patients than in chronic carriers, mainly clustered (> 70%) in the C-terminal preS1 and N-terminal pre-S2 sequences. Interestingly, all these deletions encompassed T and B cell epitopes, and functional mapping showed that they affect the viral secretion site.

In addition, preS deletions can induce oxidative DNA damage and genomic instability; upregulation of certain genes, such as cyclooxygenase-2 and cyclin A, induces cell cycle progression and hepatocyte proliferation - a phenomenon linked to a high risk of developing HCC<sup>[130]</sup>. It must be kept in mind that the S region encoding HBsAg is completely overlapped with the HBV RT region. A mutation in RT codon 153, selected as compensatory after lengthy LMV therapy, causes an sG145R change in the S region (Figure 1), which is strongly associated with immunotherapy failure<sup>[111]</sup> (Table 1). Other relevant examples are the rtA181T or rtM204I RT variants, which result in stop codons in envelope proteins (sW172stop and sW196stop, Figure 1). In the shared S/P region, the main variants have been described in the antigenic determinant “a” (located the major hydrophilic region, amino acids 100-170). These variants (mainly sG145R) were first reported in an Italian boy, son of an HBsAg/HBeAg-positive mother, who had HBsAg and anti-HBs in serum despite receiving both active (vaccine) and passive [hepatitis B immune globulin (HBIG)] immunization<sup>[132]</sup>. The variant was not detected in his mother<sup>[129,132]</sup>, which suggests that sR145 presumably arose by immune selection pressure in the infant after HBV vaccination (vaccine escape variant), selected from a very small population in the mother’s HBV quasispecies. Most hepatitis B vaccines contain the major surface protein, SHBS, which induces

an immune response against the “a” determinant and constitutes an evolutive factor for variant selection.

Other minor substitutions in the S gene include sG145R (again, a G to A mutation in nucleotide 587), followed by sD144A, sP142S, sK141E, sQ129H, sI/T126N/A, sT131I, and sM133L (Table 1), all of which strongly affect the HBsAg structure<sup>[114,133,134]</sup>. The sR145 main variant seems to alter the projecting loop (aa 139-147) of the “a” determinant, inhibiting recognition of induced neutralizing antibody<sup>[135]</sup>. This variant can horizontally infect<sup>[136]</sup> and replicate for several years<sup>[137]</sup>, but at lower rate than the wild-type sG145 variant, probably because of a decrease in virion stability<sup>[138]</sup>. However, the presence of anti-HBs (hepatitis B immunoglobulin prophylaxis or vaccine-induced), which would block the sG145 strain, may allow selection of sR145 when it is present in the quaspecies as a minor variant. This mechanism would ultimately establish infection with the predominant presence of this variant in a clear manifestation of the adaptation capability of quaspecies structures. Such immune selection from the quaspecies would explain the strong association of this and other “a” determinant variants with HBV vaccination failure.

Variants observed in low percentages under immunoprophylaxis escape are detected in low prevalence in practically all the clinical stages of HBV infection<sup>[133,139]</sup>. Longitudinal studies have reported their accumulation during the course of chronic infection<sup>[140,141]</sup> as the major cause (70% of cases) of the paradoxical coexistence of HBsAg and anti-HBs<sup>[142]</sup>. Variant sG145R in conjunction with other S mutations located in the HLA I T cell epitope have been observed in fulminant HBV cases<sup>[143]</sup>. However, unlike sG145R, other variants appearing after vaccination often rapidly revert to the strain seen in the mother<sup>[144]</sup>.

Vaccination at birth is an ideal situation for escape variant selection, similar to administration of high-titer anti-HBs preparations to prevent graft infection in liver transplantation<sup>[145-147]</sup>. A screening program for school-age children in Taiwan found a 0.7% prevalence of “a” determinant mutants<sup>[148]</sup>. Interestingly, the percentage of HBsAg mutants increased from 8% to 25% over 10 years after introduction of a universal vaccination program from 1984 to 1994, but remained stable (23%) in 1999. This study clearly suggests a role for HBV vaccine in selecting HBsAg mutations. In a study in the United States, only 0.8% of vaccinated children born to HBsAg-positive mothers were infected with sG145R<sup>[149]</sup>. The prevalence of this variant in North Americans and in Europeans seems to be low<sup>[150]</sup>. However, some recent studies have reported relevant new data about this type of variant. Perinatal transmission of HBV has not been fully controlled despite adequate immunoprophylaxis in infants in Thailand, with escape mutants in the “a” determinant region (residues 144 and 145) being observed in 14% of infected infants<sup>[151]</sup>. Shahmoradi *et al.*<sup>[152]</sup> reported HBV-DNA activity in 28% of children born to HBsAg-positive mothers, and 62% of these cases carried enve-

lope variants, mainly (77%) the sG145R variant. In fact, these variants seem to be present as minor populations in 9% of HBV carriers who have not been exposed to HBV vaccination or HBIG prophylaxis<sup>[153]</sup>.

HBIG therapy is used to prevent recurrent HBV infection after liver transplantation (LT) for end-stage HBV liver disease. However, in some LT patients who become HBsAg<sup>+</sup> and HBV DNA-positive on HBIG therapy, emergence of mutations in putative neutralizing epitopes such as sG145R, similar to what occurs in vaccine failure, has been described<sup>[154-156]</sup>. A recent report found that 50% of reinfected LT recipients had mutations in the “a” determinant region and flanking sequences; a fact suggesting that quaspecies formation contributes to HBV reinfection following LT<sup>[156]</sup>. Therefore, HBIG-associated variants, like HBV vaccine-related variants, would arise from a pre-existing, but extremely minor population. Confirmation of this hypothesis would require application of ultrasensitive NGS massive sequencing methods, as has been used for other HBV regions<sup>[53-59]</sup>.

The longer the duration of HBIG therapy, the greater likelihood that “a” determinant variants will arise. Discontinuation of HBIG often leads to reversion to the pretransplant sequence<sup>[140,155]</sup>, thus providing further evidence of HBV quaspecies dynamics and reinforcing the idea that these variants are less replication-efficient than the wild-type strain. HBIG-treated liver transplant patients infected with “a” determinant escape variants in positions 144 or 145 showed a poorer clinical outcome than those infected with other variants or wild-type viruses<sup>[157]</sup>. In order to decrease selection of these variants, HBIG plus LMV combination therapy is used. This strategy has drastically reduced recurrence rates from 35% to less than 10%<sup>[158]</sup>. However, it should be remembered that there is a relationship between these variants and those in the overlapping polymerase region that arise during NUC therapy; especially the polymerase variant rtR153Q, which partially restores the replication efficiency of LMV-resistant variants such as rtM204V, and is associated with the main “a” determinant sG145R variant<sup>[114]</sup>. Therefore, patients with pre-LT polymerase variants may have a high risk of reinfection despite HBIG therapy<sup>[159]</sup>, and patients with “a” determinant variants due to HBIG therapy may develop polymerase variants, associated with high viral replication and a poor outcome<sup>[160]</sup>.

Currently, there are no available studies in which the S ORF is analyzed by NGS techniques. However, because of the complete overlapping between the S and P ORFs, some NGS studies mainly focussing on P ORF variability have reported interesting findings about the HBV quaspecies in S ORF<sup>[53,58]</sup>. Our group performed massive UDPS sequencing of samples from patients sequentially treated with LMV and ADV (Figure 2), and found a high frequency of rtA181T<sup>[58]</sup>, the substitution that causes the sW172stop stop codon in S ORF. The consequence of this change is that two-thirds of all HBV particles lacked 50 amino acids in HBsAg (in all types of envelope proteins) including several essential codons (hot spots

sS174 and sL175, sV177, sQ181, sW191, sL192, and sI195 placed in the Core-Surface interphase and residues sV184, sL186, sS187, and sW190 from the S-S interaction interphase<sup>[161]</sup>. Therefore, it is suggested that HBsAg carrying this stop codon may not be completely functional. Moreover, despite this drastic alteration, samples with a high prevalence of this defective variant showed considerable replication, which suggests that the quaspecies has some type of recourse to compensate for this theoretical handicap. That mechanism could be trans-complementation of the defective S protein genomes for enveloping with complete S proteins encoded by other quaspecies members. As has been suggested by Villet *et al.*<sup>[84]</sup>, existence of such a mechanism may be a requirement for emergence and maintenance of this incomplete variant. Hence, this may be an example of cooperation within the quaspecies<sup>[162]</sup>, as a relevant property that fits in with the idea that this population structure has remarkable plasticity. In our UDPS study<sup>[58]</sup>, other surface positions showed *de novo* stop codons at frequencies of 0.13% to 0.17% (sW156stop, sW163stop, sW165stop, and sW191stop); of note, sW156 is involved in HBV infectivity<sup>[163]</sup>. These *de novo* stop codons accounted for around 1.5% of the viral population in our study<sup>[58]</sup> and between 1% and 2.8% in the study of Solmone *et al.*<sup>[53]</sup>; both using UDPS. Therefore, UDPS has brought to light what seems to be a systematic phenomenon in the HBV quaspecies: the presence of defective genomes that participate in HBV virology. The pathological consequence of this phenomenon must be defined in further studies.

### ***X gene variability and its implications in HCC***

The X gene (nucleotides 1374-1838) encodes the HBV X protein (HBx). HBxAg is a 154-amino-acid protein with an N-terminal negative regulatory domain and a C-terminal transactivation/coactivation domain that plays a key role in control of cell proliferation, viability, and transformation<sup>[164-166]</sup>. This protein has been detected in both the cytoplasm and nuclei of infected hepatocytes<sup>[167-169]</sup>. HBx is a regulatory protein that is not packaged in virions during assembly, but is expressed in the new host cell to allow epigenetic control of HBV transcription from cccDNA<sup>[7,170]</sup>. In contrast to the other HBV genes, but similar to retroviral oncogenes, Miller *et al.*<sup>[14]</sup> reported that HBx shows codon usage preference (third nucleotide in degenerated codons), which is more related to the behavior of eukaryotic genes than viral genes and suggests that HBxAg is of eukaryotic origin. However, the X ORF lacks homology to host protein<sup>[167]</sup>.

The high conservation of X gene in mammalian hepadnavirus genomes strongly suggests that HBx is essential to the viral life cycle. It has been reported that HBx is required to initiate and maintain HBV replication, making HBx the key regulator of the natural infection process<sup>[170]</sup>. It is believed that HBx contributes to HBV oncogenicity<sup>[96,167,171-173]</sup> and it is reported to transform SV40-immortalized murine hepatocytes, induce cell cycle progression within the regenerating liver, and cause or

accelerate liver cancer in transgenic mouse models<sup>[174-177]</sup>. HBx expression affects several cellular functions in transfected cells, such as cytoplasmic calcium regulation, cell signaling, transcription, cell proliferation, DNA repair and apoptosis<sup>[169,177-180]</sup>.

To develop its functions, HBx interacts with many cellular partners, such as nuclear proteins involved in regulation of transcription and transcription factors<sup>[167,179]</sup>. Furthermore, HBx interacts and cooperates with cAMP response element binding (CREB)-binding protein/p300 to modify chromatin dynamics of target genes and to synergistically enhance CREB activity<sup>[181]</sup>.

HBx stimulates HBV replication 5-10-fold; likely by enhancing transcription of pgRNA<sup>[167]</sup> by activating the proteasome<sup>[182]</sup>. In this sense, HBx regions 61-69 and 105-140 seem to be essential for viral replication and expression. Paradoxically to its antiapoptotic capacity related to inhibition of tumor suppressor genes, the HBx 68-104 region is associated with mitochondrial membrane alterations that promote cell death<sup>[172]</sup>. Multiple evidence has related HBV infection with inhibition of the innate antiviral immune response, such as inhibition of the Toll-like receptor response<sup>[183]</sup>. HBx directly binds to interferon promoter stimulator-1 factor and inhibits activation of interferon  $\beta$ <sup>[184]</sup>, thereby inhibiting the innate antiviral immune response; a pathway in common with other viruses, and even inhibiting signals through the mitochondrial proteins<sup>[185]</sup>. These and other data clearly indicate that HBx protein has a key role in HBV infection.

In liver tumor tissue, the X gene is often integrated into the genome of infected hepatocytes while retaining its functionality, especially antiapoptotic capability, mediated by inhibition of p53 transcriptional activation<sup>[96,186]</sup> or adenosylmethionine<sup>[187]</sup>. HBx stimulates methyltransferases leading to hypermethylation, which is associated with chromosomal instability<sup>[96]</sup>. HBx also activates cell proliferation by repression of tumor suppressor genes, such as melanoma inhibitory activity 2<sup>[188]</sup>, or by increasing  $\beta$ -catenin expression<sup>[189]</sup>. HBx activity over nuclear factor  $\kappa$ B has been associated with antitumor therapy failure<sup>[187,190-192]</sup>. HBx does not directly bind to DNA; it acts through the activation process of various transduction signal cascades *in cis*<sup>[172]</sup>. It seems that HBx binds to the cccDNA HBV minichromosome histone-like transcriptional complex, thus modifying epigenetic regulation of this essential structure<sup>[7]</sup>.

The multiple functions of HBx may indicate that nucleotide substitutions in the X gene would have important consequences in HBV infection. Several specific substitutions have been reported in the region where X and ENH II overlap (T1485, C1479, A1613, T1653, T1689, A1753, T1766, A1768, and A1776)<sup>[82,193-195]</sup> and some of them seem to be related with the viral genotype<sup>[196]</sup>. These variants, which are found alone or in combination, can change the regulatory function of basal core promotor (BCP) motifs, thereby decreasing HBeAg expression and facilitating HBeAg seroconversion<sup>[197-199]</sup>. Furthermore, some of these variants, particularly the T1762/A1764 double muta-



tion, are strongly associated with cirrhosis and HCC<sup>[200,201]</sup>. Other variants, such as those detected in preCore region, may also show this relationship and there may even be a link with HBeAg status<sup>[202,203]</sup>. In one study, 40% of HCC patients presented six or more of these mutations, while they were present in only 2.7% of non-HCC patients, indicating a predictive value of these variants for HCC similar to that of  $\alpha$ -fetoprotein<sup>[201]</sup>. In another, more recent study performed in India, the A1753 mutation was detected in 35% of cases of HBV-related liver cirrhosis<sup>[204]</sup>.

Multiple X gene variants directly affect the HBx amino acid sequence; these include xI130M, xV131I, and xF132Y, which are associated with the main BCP variants (Table 1). Mutations in amino acids 5, 130, and 131 may contribute to HCC development and could be useful to predict clinical outcome in patients with chronic HBV infection<sup>[205]</sup>. Some HBx amino acid variants are typically located in the region between TATA boxes TA2 and TA3 of the BCP sequence (overlapping with the X gene). Our group observed that the most common variants are 8-nucleotide deletions-some of these (unpublished) deletions in the quasiespecies, obtained by UDPS are shown in Figure 3 - which result in frame shifting and often create a *de novo* stop codon at position 134, resulting in a 20-amino-acid truncation of the C terminus of the HBx protein<sup>[56]</sup>. This could cause a huge change in the proapoptotic functionality of this region<sup>[96]</sup> or in its transactivator role<sup>[206]</sup>. X gene deletions are commonly found in DNA integrated in liver tumor tissue<sup>[192]</sup> and are strongly associated with the development of HCC by multiple mechanisms, such as deregulation of the centromeric protein A<sup>[93]</sup>, regulation of miRNA<sup>[94]</sup>, and loss of the proapoptotic effect<sup>[96]</sup>.

It is likely that the highly complex scenario of the quasiespecies in this extremely relevant region of the HBV genome can be clarified by applying massive sequencing. However, no UDPS studies in this line have been reported to date, probably because of the technical difficulties associated with the presence of deletions. In a recent UDPS study by our group<sup>[56]</sup>, mainly focused on the preCore region but including the last eight HBx codons (positions 1814-1838), we found that around 3% of sequences carried the TAG amber stop codon instead of the major TAA codon. Even more interesting was the significant percentage of sequences (0.2%) in which a T1836C substitution changed the ochre stop codon TAA to CAA (Q). After this substitution, the HBx protein would be translated to the next in-frame stop codon TAG at position 1992, inducing expression of 51 additional amino acids at the C terminus, which could potentially alter HBx functionality. Thus, this preliminary UDPS study revealed a minor HBV population with highly relevant HBx changes (AA deletions or insertions) whose significance in the HBV quasiespecies and in the outcome of infection requires a more systematic and extensive UDPS study of this region.

### Variability in the preCore/Core regions

The Core gene (positions 1814-2548) has two in-frame

start codons encoding two proteins: the component of the viral capsid or Core (HBc, start codon at 1901), and the preCore protein, the precursor of HBeAg (start codon at position 1814). HBeAg protein is secreted by infected hepatocytes and seems to have an immunomodulatory function, establishing immune tolerance and predisposing infants of HBV-infected mothers to develop persistent infection. HBeAg promotes a Th2 response that leads to suppression of the host immune response against the virus, preventing clearance and allowing viral persistence<sup>[207]</sup>. HBeAg-mediated IL-1 activation may also increase hepatocyte proliferation, inducing anti-apoptotic genes and promoting hepatocarcinogenesis<sup>[207]</sup>.

In clinical practice, HBeAg expression differentiates between chronic HBV carriers as HBeAg-positive and HBeAg-negative; the latter are usually positive for the corresponding antibody (anti-HBe). The anti-HBe seroconversion event is a crucial turning point in the natural history of chronic hepatitis B. Anti-HBe seroconversion is considered a favorable prognostic factor in the disease, and sustained virological response in HBeAg-positive cases is a treatment endpoint<sup>[106]</sup>. The factors involved in HBeAg seroconversion have been extensively studied. However, seroconversion is not always followed by establishment of HBV inactive carrier status. Some patients show elevated transaminases and active viral replication after seroconversion to anti-HBe, indicating that the disease continues to be active in the absence of HBeAg<sup>[208]</sup>. In addition, significant differences in treatment response have been seen between HBeAg-positive and -negative status. In particular, HBeAg-negative patients present response rates 10%-20% higher than HBeAg-positive ones; even with the last generation antivirals ETV and TDF<sup>[106]</sup>. This phenomenon may be associated with different levels of viral replication between patients positive and negative to HBeAg<sup>[106]</sup>. However, other mechanisms associated with viral characteristics may also be implicated in response rates, such as baseline quasiespecies composition<sup>[72,98]</sup> and differing sensitivities to antivirals of certain HBV variants<sup>[57,59]</sup>. In fact, as described below, certain preCore and BCP variants that prevent or significantly decrease HBeAg expression (HBeAg-negative variants) may promote the process of HBeAg seroconversion. In many HBV cases from the Mediterranean area, the disease remains active after seroconversion, and HBeAg-negative preCore or BCP variants have been detected in these patients<sup>[209,210]</sup>. Once these variants become detectable, they perpetuate the infection beyond seroconversion<sup>[211]</sup>. Nonetheless, recent evidence seems to suggest that some HBeAg-negative variants may be more sensitive to antivirals<sup>[56,57,59]</sup>.

Variants in the preCore region (between start codons 1814 and 1901) are clinically important because they abolish HBeAg expression. The most common is G1896A (interestingly another G to A substitution), which leads to premature termination of the preCore protein (the HBeAg precursor) and creates a *de novo* in-frame stop codon at codon 28 (TGG to TAG) in the pre-



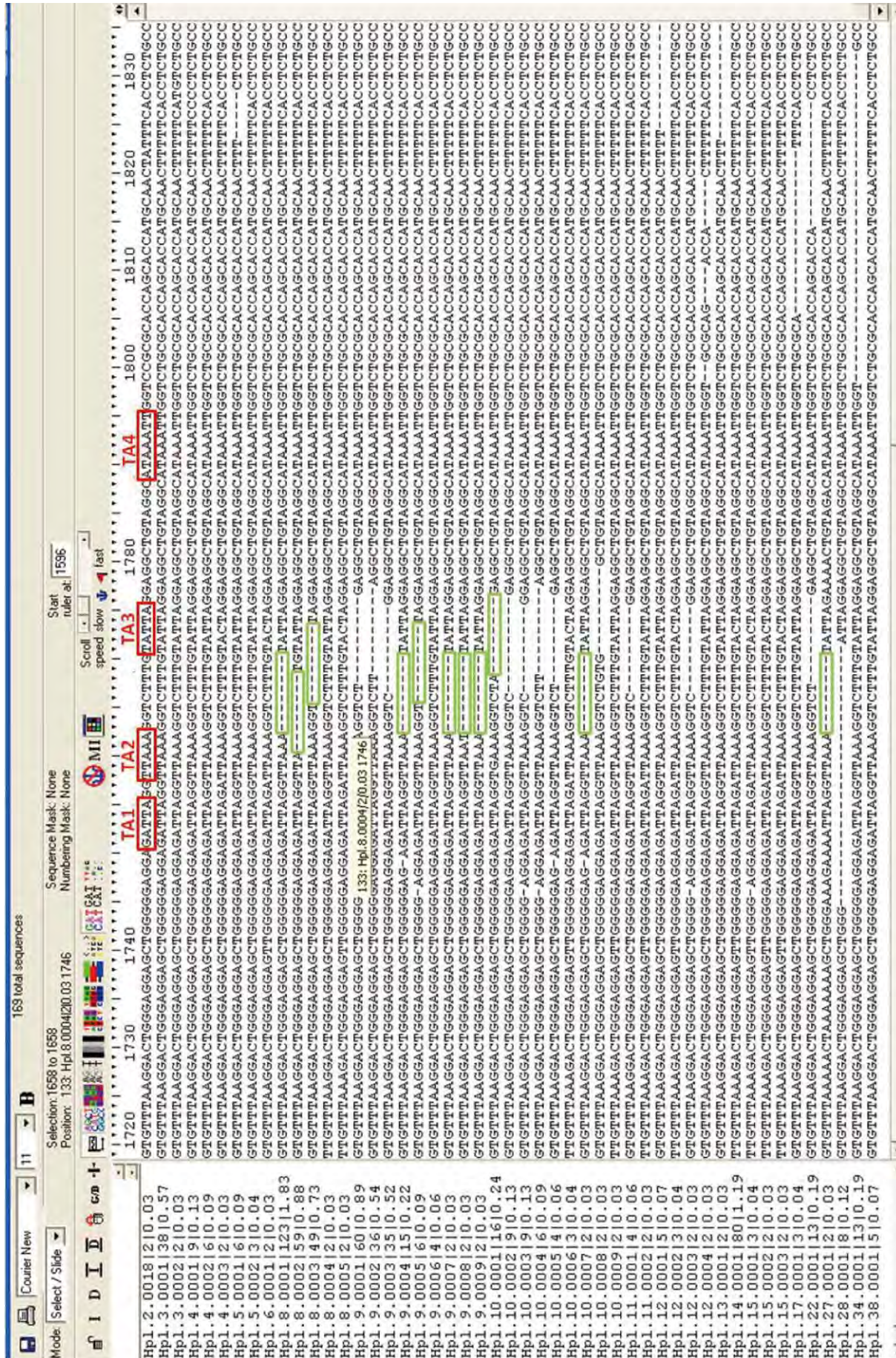


Figure 3. Quasispecies of the X gene obtained by unpublished result. The four TA-like boxes are highlighted in red and the eight nucleotides detected are indicated in green.



Core region (pcW28stop, Table 1). This change is located in a region of four G residues that are prone to G to A mutations<sup>[129]</sup>. G1896A abrogates HBeAg synthesis, does not disturb HBeAg production<sup>[212]</sup>, and has been detected in virtually all clinical stages of HBV infection, from extremely severe to the most benign forms<sup>[210-214]</sup>. Although G1896A-containing strains are independently transmissible<sup>[214]</sup>, HBeAg-negative HBV carriers do not develop chronic infection, but acute or fulminant hepatitis can occur<sup>[204,215,216]</sup>. This mutation shows a strong association with viral genotype, and is commonly found in Mediterranean and oriental anti-HBe-positive chronic HBV patients who show a high prevalence of genotypes D and B, respectively.

In contrast, the mutation is notably less prevalent in chronic HBV patients from Western Europe or North America, where genotype A is more prevalent<sup>[217]</sup>. This genotype association is clearly related to the overlapping of the preCore and the encapsidation signal sequences of  $\epsilon$ . In this essential pgRNA secondary structure, positions 1896 and 1858 are paired in the lower stem of the  $\epsilon$  stem loop secondary structure<sup>[218,219]</sup> (Figure 4). The G1896A substitution results in stabilization of the  $\epsilon$  structure in genotypes B, D, E, G and H, and in C strains in which there is a T in the paired position, 1858. In contrast, in genotype A and F strains, there is a C in position 1858, and the G1896A substitution would result in a loss of thermodynamic stability of  $\epsilon$ , producing a decrease in encapsidation and viral replication<sup>[209,220,221]</sup>. This observation explains the frequent detection of G1896A in genotype D strains, but rarely in genotype A strains<sup>[222]</sup>. The G1899A substitution (again, G to A) produces a pcG29D change, is frequently associated with liver cirrhosis, and is usually detected together with G1896A<sup>[222]</sup>.

A lack of HBeAg expression can also be the result of mutations in any of the three nucleotides of the preCore start codon. Although they are less common than G1896A, these mutations represent a large percentage of preCore defective variants<sup>[210]</sup>. Mutations in the preCore start codon do not disturb the encapsidation process, because they are located outside of the canonical  $\epsilon$  signal sequence<sup>[219]</sup> and are not restricted by genotype. preCore frameshift mutations due to insertions or deletions are uncommon events occurring outside essential regions, such as  $\epsilon$  or DR1 (*e.g.*, deletion in nt 1836-9)<sup>[129]</sup>. In contrast, the rare G1862T variant (pcV17F) affects the  $\epsilon$  structure bulge and disrupts viral replication, but not HBeAg expression.

The G1862T variant is found in the African A1 sub-genotype, is associated with lower viremia than European A1<sup>[223]</sup>, and is more common in HBeAg-positive than -negative patients (37% *vs* 11%)<sup>[224]</sup>. In addition to the G1896A change, three other preCore stop codons have been observed (positions 1817, 1874 and 1897), all of which disrupt the secondary pgRNA structure (as deduced by the secondary structure predicted by magnetic resonance study)<sup>[219]</sup>. The 1817, 1874 and 1897 changes need compensatory changes to stabilize the pgRNA structure, a

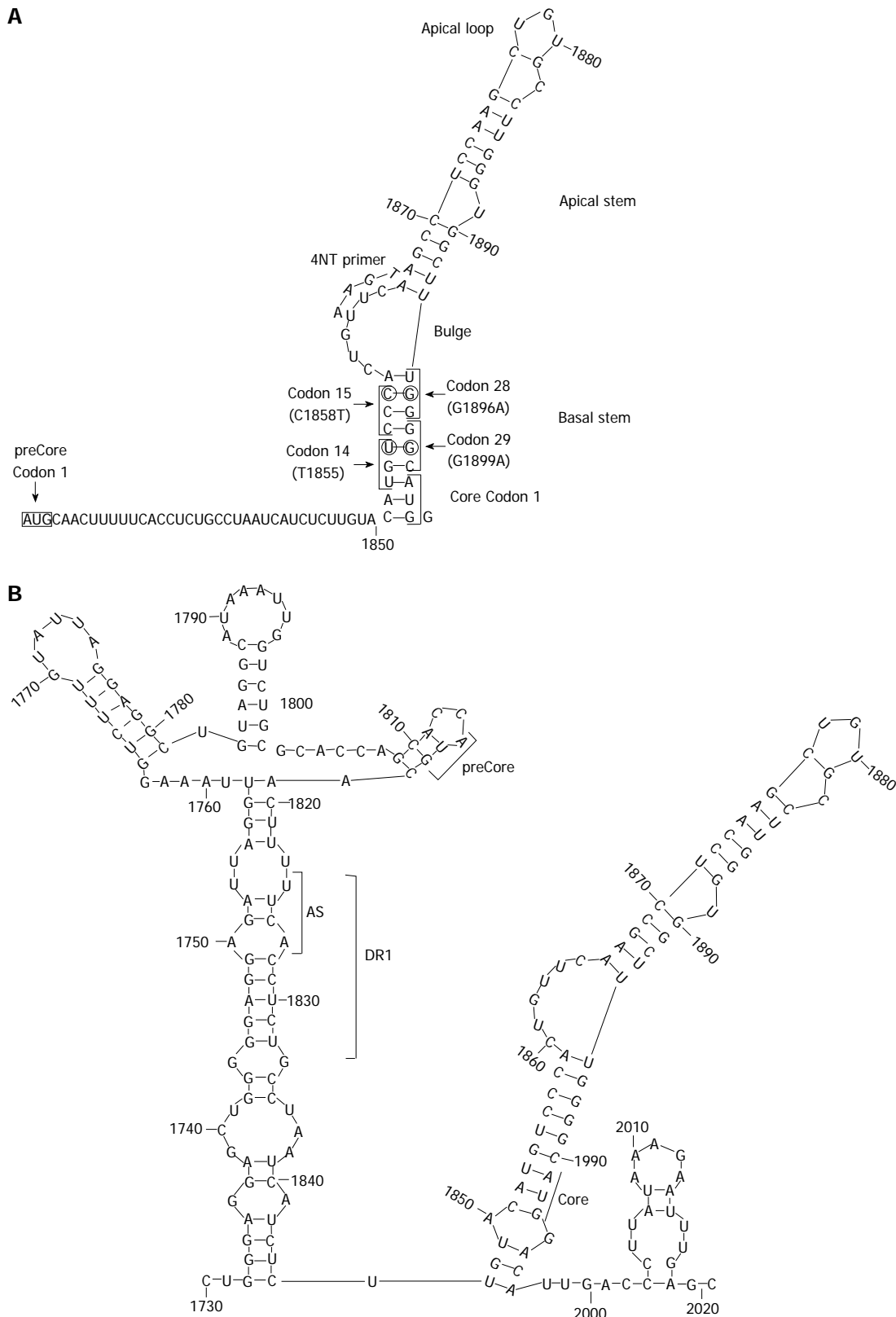
fact that seems justify their low-frequency detection.

In HBeAg-positive patients (immunotolerance phase), defective preCore variants are rarely found as minority populations by conventional direct Sanger sequencing or even by clonal analysis. However, these variants emerge following activation of hepatitis, often associated with anti-HBe seroconversion. In fact, the G1896A preCore mutation and the double A1762T/G1764A BCP mutation are associated with HBeAg seroconversion<sup>[197,225]</sup>. These variants accumulate along seroconversion and are the predominant viral population in anti-HBe-positive patients<sup>[226]</sup>. Whether HBeAg-defective preCore variants are selected from the viral quaspecies in persistent infection is still uncertain. However, the host immune system seems to be the main cause of emergence of preCore variants.

Hypermutations in the B and T epitopes of the HBcAg sequence have been observed in the presence of preCore mutations (HBeAg-negative)<sup>[209]</sup>, allowing escape of these variants. Some preCore variants, such as G1899A, have been clearly associated with HCC progression in HBeAg-positive patients, while G1896A plays a similar role in HBeAg-negative cases<sup>[202]</sup>. Therefore, early detection of preCore variants seems to have predictive value for spontaneous or interferon-induced seroconversion<sup>[220,227]</sup>.

Our group investigated the variability of the preCore region and its relationship with  $\epsilon$  structure functionality using UDPS at a sensitivity of 0.03%<sup>[56]</sup>. Minor populations of G1896A, G1899A variants, and preCore start codon variants were observed, but these mutations showed thermodynamic and structural restrictions in the encapsidation signal. Minor percentages of preCore variants were also seen in HBeAg-positive patients, but not in HBeAg-negative cases<sup>[56]</sup>. The study included a small number of patients, however, and did not explain the presence of a minor population of preCore variants in the HBV quaspecies. Hence, NGS studies including a large number of patients are needed to elucidate the significance of these findings.

The C gene also encodes HBcAg, which forms the HBV nucleocapsid. This protein is highly immunogenic, whereas the envelope proteins have low immunogenicity. Thus, HBV-infected individuals develop an early, intense anti-HBc humoral response that remains after clinical recovery. Multiple epitopic regions in nearly the entire HBc sequence have been described in relation with B cell and T helper epitope response<sup>[228,229]</sup>. Among them, the region flanked by codons 74 to 84 (B74-84) located at the tip of the spike structures of the capsid and included in the so-called major immunodominant region should be highlighted. This epitope is shared by HBc and HBeAg, hence it constitutes the major epitope for B cells<sup>[13,230]</sup>. Other regions stimulate CD4<sup>+</sup> Th cells, the best known being the immunodominant epitope flanked by amino acids 50 and 69 (TH50-69). Variability in these two regions has been related with immune response escape, disease persistence, and interferon therapy failure<sup>[57,231]</sup>. Furthermore, amino acid variants in the C gene, specifically cE83D and



and G1899A (pcG29D), and certain C variants (Table 1), such as cP5H/L/T, cD32N/H, cC/E43K, cP50A/H/Y, cE83D, cI97F/L, cL100I, cA131G/N/P, cS181H/P and cC/Q182K/stop. In a recent study performed in India, single nucleotide substitutions such as C1914G were found in 32% of HCC cases<sup>[204]</sup>.

In addition to amino acid substitutions, deletions leading to truncated HBc proteins have been described, such as the single G deletion in position 2090 in a group of six Gs<sup>[69,70]</sup>. It has been reported that these C-defective genomes are unable to replicate, but they are always present in the HBV quasiespecies, regardless of the region studied (S or C)<sup>[69,70]</sup>. Hence, their presence might indicate that there are active transcomplementation mechanisms in HBV infection that tolerate them. The natural tendency to maintain these defective particles may be because they confer a benefit for viral evolution<sup>[69,70]</sup>, although they seem to be poorly represented in the baseline HBV quasiespecies, making them difficult to detect by conventional sequencing technologies, even clonal analysis. In contrast to what was previously thought, UDPS study has brought to light the fact that these defective variants can replicate and form minor populations in the HBV quasiespecies<sup>[56,58]</sup>. Study of these variants by massive sequencing may help to establish whether their presence is only occasional and a mere side effect of the quasiespecies structure, or whether they are more prevalent and have functions affecting the outcome of infection.

Significant changes in the immunodominant epitopes of the viral capsid have been observed by direct Sanger sequencing, mainly under the effect of antiviral treatment with the immunomodulator interferon and even with NUCs<sup>[231]</sup>. Similar results were obtained in a preliminary study using massive sequencing<sup>[57]</sup>. However, it should be noted that HBeAg shares many epitopes of the HBc protein, and an association between the presence of preCore HBeAg-negative variants and mutations in Core epitopes has been reported<sup>[209]</sup>. This relationship shows that in the absence of HBeAg, immune pressure stimulates selection of some Core variants and provides further evidence that the immune system is an evolutionary factor acting on the HBV quasiespecies<sup>[57,209]</sup>.

The baseline presence of HBV RT variants resistant to antiviral therapy has been reported in multiple studies by conventional direct sequencing and cloning<sup>[233]</sup>, and recently by NGS methods<sup>[53-56,58,234]</sup>. However, study of the preCore region, which could help to understand the phenomenon of anti-HBe seroconversion and its association with SVR rates, has only been reported by our group<sup>[56,57]</sup>. Highly sensitive methods are required to detect minor variants in certain environmental situations, and evaluate the evolution of these variants within the quasiespecies. We found strong selection of a minor member of the quasiespecies (around 1%) in the absence of antiviral therapy in an HBeAg-negative case; possibly as a result of immune pressure. In this case, variants carrying amino acid substitutions were found in a motif commonly recognized by T helper cells (D<sub>64</sub>VTN<sub>67</sub> instead of E<sub>64</sub>LMT<sub>67</sub>,

located in the main TH 50-69 epitope). To date, only one preliminary study has analyzed epitope variability in the C region of the viral capsid by massive sequencing<sup>[99]</sup>. The results seem to confirm the dynamics of variant selection in the major epitopes of the C region in the absence of treatment, as well as some degree of simplification or decreased quasiespecies diversity after antiviral treatment, as evaluated by Shannon entropy<sup>[70]</sup>. Study of the variability of immunodominant epitopes might permit a redefinition of these epitopic motifs (*e.g.*, the B74-84 epitope could include positions 71, 72 and 87 or putative new immune-stimulating positions, such as 40, 41, 92 and 93).

The BCP region in preCore/Core controls expression of the preCore mRNA (precursor of HBeAg) and pre-genomic RNA. BCP is located between positions 1742 and 1842 and overlaps with the 3' end of the X gene and the 5' end of precore/Core. BCP contains 4 TATA boxes (TA1-TA4) and includes a fragment of the 3' terminal region of ENH II, which is involved in controlling S and X gene expression<sup>[210]</sup>. Despite its essential nature, clinically relevant BCP variants have been detected. The most common of these changes is the double mutation A1762T/G1764A associated with significant decreases in HBeAg levels and in viral activity<sup>[64,235-241]</sup>, and with severe liver disease<sup>[199-201,205]</sup>.

The liver-specific activity of ENH II is regulated by multiple liver-enriched transcription factors, specifically nuclear receptor hepatocyte nuclear factor (HNF) 4 or 1, which span the TATA box-like sequence of the pre-C promoter. A functional synergism between some nuclear receptors (including HNF4) upregulates the liver-specific activity of ENH II<sup>[242]</sup>. The effect of BCP variants at ENH II is associated with modification of specific sites recognized by hepatic factors. For example, the HNF1-binding site created by the A1762T/G1764A double mutation is imperfect for a response to HNF1, which results in suppression of preCore mRNA synthesis and increases in pregenomic RNA. Both these actions seem to explain the decrease in HBeAg (promoting seroconversion) and the increase in viral activity in the presence of the A1762T/G1764A mutation. In addition, this double mutation is associated with HBx substitutions in amino acids 130 and 131, which may contribute to HCC development<sup>[205]</sup>. Other minor mutations in the BCP region, such as C1740, C1753, and T/A1768, have been associated with severe liver damage<sup>[200,210,243-245]</sup> and even fulminant hepatitis<sup>[85,246]</sup>. The presence of BCP mutations in inactive carriers is significantly lower than in patients with active chronic infection and those with persistently elevated ALT levels<sup>[210]</sup>. Both BCP and preCore mutations (*e.g.*, A1762T/G1764A and G1896A stop codon) are present in most cases of HBV reactivation in immunosuppressed anti-HBc-positive individuals<sup>[247,248]</sup>. Early, sensitive detection of BCP and/or preCore mutant populations in the HBV quasiespecies could reveal potential predictors of seroconversion to anti-HBe or of evolution of the disease<sup>[82,190,211,232]</sup>. Fortunately, quantification of HBV variants is currently possible by massive sequencing



techniques<sup>[70]</sup>.

Due to the overlapping of BCP and X, BCP changes can also cause changes in the 3' region of the X gene<sup>[205]</sup>. Some such changes have been reported in the region immediately preceding the precore start codon (positions -5, -3 and -2 from 1814, the preCore start). This region corresponds to Kozak sequences, and associated nucleotide changes have been found with effects similar to those related to BCP mutations<sup>[249]</sup>. BCP variants between positions 1763 and 1779, which cause X gene deletions and decrease HBeAg expression have been associated with reactivation after chemotherapy and with disease severity<sup>[64,65]</sup>. However the effect of these variants on viral replication varies depending on the specific deletion (*e.g.*, deletion affecting TA3 1768-1775 causes a decrease in pregenomic and preCore RNA with a concomitant decrease in viral activity)<sup>[66-68]</sup>. Many BCP deletions produce a frame shift that results in a 20-amino-acid truncation of the HBx protein (amino acid 154 passes to 134) and partial loss of the C-terminal region, which contains the HBx transactivating activity. All these mutations have enormous clinical-pathological interest and have been related to interferon failure<sup>[250]</sup>.

## TREATMENT RESPONSE: VIRAL VARIANTS AND GENOTYPE

### BCP and precore variants

As was discussed above, HBeAg-negative variants result from changes in the preCore and/or BCP regions, and are observed in all clinical forms of HBV infection (from asymptomatic to severe) and in all the viral genotypes. However, their distribution and prevalence show important differences<sup>[210,246]</sup>. PreCore mutations are more common in genotype D<sup>[209,251]</sup> and BCP mutations are more frequent in genotype A<sup>[210]</sup>. In Asia, where HBV infection is widespread, genotype C is associated with a higher prevalence of BCP mutations, whereas genotype B shows more preCore mutations<sup>[252,253]</sup>. The poorer prognosis of genotype C is probably associated with the prevalence of BCP mutations<sup>[68,240,254]</sup>. During seroconversion in genotype C, BCP variants are selected before preCore variants, whereas in genotype B the opposite occurs<sup>[253]</sup>. BCP and preCore mutations are more prevalent in severe forms of infection<sup>[68,240,240,255]</sup>, in which BCP deletions are particularly important<sup>[254]</sup>. Although the impact of HBV genomic features during seroconversion is unknown, the viral quaspecies structure and its qualitative and quantitative composition is likely associated with this essential phenomenon in natural infection<sup>[28]</sup> and in the response to antiviral therapy<sup>[98]</sup>. In this sense, it has been reported that the evolutionary pattern of the viral reverse transcriptase region differs between responders and partial responders during the initial phase of ETV treatment<sup>[98]</sup>.

### Treatment of chronic HBV infection and its relationship with variability: is the RT region the only one involved?

Chronic HBV infection is now primarily treated with

NUCs, and these treatments are associated with a risk of selecting resistant RT variants that cause treatment failure. Currently, ETV or TDF are universally recommended as first-line therapy<sup>[106]</sup>. Both treatments present a small risk of resistance development (< 1% for ETV and 0% for TDF after 6 years of treatment). In contrast, LMV has been associated with a high risk of emerging resistance (> 80% at 5 years of treatment). LMV has been widely used, and a large number of LMV-treated patients have developed treatment-resistant variants. The percentage of RT variants increases after LMV relative to their baseline proportion. We looked into this situation by UDPS study and found the following: the LMV resistant variant L180M + M204V was undetectable in pre-LMV samples, was selected during LMV therapy, remained in significant percentages after ADV treatment, and was re-selected after ETV treatment<sup>[58]</sup>. A recent UDPS study by Margeridon-Thermet *et al*<sup>[55]</sup> also reported a high percentage of resistant RT variants after LMV treatment.

In general, HBeAg-negative patients (with preCore variants) showing viral activity after seroconversion present higher rates of SVR to NUCs than HBeAg-positive patients<sup>[106]</sup>. However, the response rates to interferon treatment differ: HBeAg-negative patients present lower response rates than HBeAg-positive ones<sup>[256,257]</sup>. These differences seem to be related to viral genotype, because significantly higher SVR rates have been found for genotype A, in which preCore variants are strongly restricted<sup>[258]</sup>, than for the remaining genotypes, especially genotype D. The reason for this genotype-dependent response is not clear, but the diversity of the viral quaspecies may play a role, as has been described for HBeAg seroconversion and treatment response<sup>[98]</sup>.

In chronic LMV-treated HBV patients, major defective precore mutations occasionally revert to wild-type forms, with reappearance of HBeAg<sup>[238,259]</sup>. This seems to suggest that HBeAg-negative variants are more sensitive to antiviral treatment than HBeAg-positive ones<sup>[238,260]</sup>. *In vitro* studies indicate that defective preCore mutations restore the replication efficacy of LMV-resistant mutants<sup>[261]</sup>. In one UDPS study, we found that the percentage of HBeAg-negative G1896A preCore variants in the quaspecies of HBeAg-positive cases (present as minor populations and undetectable by conventional sequencing) decreased after a period of treatment with LMV<sup>[56,57]</sup>. This was also seen in BCP variants associated with low HBeAg expression in a preliminary study<sup>[99]</sup>. These data suggest that these variants are more sensitive to LMV treatment than HBeAg-positive variants, and could explain the seroreversion phenomenon. In a recent study using quantitative real-time PCR, Nishijima *et al*<sup>[59]</sup> reported that liver tissue of NUC-treated HBeAg-negative cases showed low levels of the G1896A preCore mutant (0.0%-1.1%). The authors also reported a significant reduction in the percentage of this mutant following treatment in 13 of 14 cases (92.9%). These results reinforce the hypothesis that the G1896A preCore mutant is highly sensitive to NUCs. A decline in the percentage of HBeAg-negative variants (preCore and/or BCP muta-

tions) and selection of HBeAg-positive variants (without preCore or BCP mutations) after antiviral treatment<sup>[56,57,99]</sup> seems to agree with the lower SVR rates in the presence of HBeAg than in the absence of this antigen<sup>[106]</sup>.

Interestingly, preCore mutations reappear after prolonged therapy, with return to HBeAg-negative status (seroconversion)<sup>[238]</sup>. It has also been reported that HBeAg-negative preCore and BCP variants compensate for the decreased replicative capacity of resistant RT variants<sup>[261]</sup>. This observation, and the reappearance of HBeAg-negative variants after therapy continuation seem to be contradictory and suggest that additional factors such quasispecies complexity or adaptation to the host immune system would be involved. The application of NGS to analyze quasispecies dynamics in cases with seroreversion after anti-HBe loss might elucidate this phenomenon.

## CONCLUSION

In conclusion, HBV infection remains a major health problem worldwide, despite the continuing advances in treatment and development of effective vaccines. The high incidence of HBV infection is mainly due to the virological characteristics of this pathogen. HBV shows high variability that characterizes it as a quasispecies in a manner similar to other infectious agents such as HCV and HIV, but with a much higher prevalence than these. Detailed study of the viral population structure is essential to combat these infections successfully, and this type of study is now possible with NGS methods. These techniques have contributed important data over recent years, and they are expected to undergo spectacular development in the medium term. NGS promises to be a powerful resource that will help the scientific community in the management of HBV and other infections, as well as other key problems related to human health, such as genetic and oncologic disease.

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## Potential role of *Helicobacter pylori* infection in nonalcoholic fatty liver disease

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### Abstract

Accumulating evidence has implicated *Helicobacter pylori* (*H. pylori*) infection in extragastrointestinal diseases, including obesity, type 2 diabetes mellitus, cardiovascular disease, and liver disease. Recently, there has been a special focus on *H. pylori* infection as a risk factor for the development of nonalcoholic fatty liver disease (NAFLD). NAFLD is currently considered to be the most common liver disorder in western countries, and is rapidly becoming a serious threat to public health. The mechanisms of pathogenesis underlying NAFLD remain unclear at present and therapeutic options are limited. The growing awareness of the role of *H. pylori* in NAFLD is thus important to aid the development of novel intervention and prevention strategies, because the eradication of *H. pylori* is easy and much less expensive than long-term treatment of the other risk factors. *H. pylori* infection is involved in the pathogenesis of insulin resistance (IR), which is closely linked with NAFLD. It provides a new insight into the pathogenesis of NAFLD. This review probes the possible relationship between *H.*

*pylori* and NAFLD, from the perspective of the potential mechanism of how *H. pylori* infection brings about IR and other aspects concerning this correlation.

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**Key words:** *Helicobacter pylori*; Nonalcoholic fatty liver disease; Insulin resistance; Inflammation; Cytokines

**Core tip:** A growing body of evidence suggests that *Helicobacter pylori* (*H. pylori*) infection is linked with nonalcoholic fatty liver disease (NAFLD). There are some potential pathogenic mediators and mechanisms involved in this progress, including fetuin-A, tumor necrosis factor- $\alpha$  and adiponectin. Long-term *H. pylori* infection may cause insulin resistance and inflammation, contributing to NAFLD. *H. pylori* toxins in the portal circulation arising from the gastroduodenal area may be another intriguing point, which might be related to the increased intestinal permeability in patients with NAFLD. It is hoped that eradication of *H. pylori* will provide a new treatment strategy for NAFLD.

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### INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a Gram-negative and micro-aerophilic bacterium<sup>[1]</sup>. The incidence of *H. pylori* infection in adults is particularly high in developing countries compared with developed countries<sup>[2]</sup>.

*H. pylori* colonizes the stomach in childhood and persists throughout life, causing diseases mainly in adults,

including chronic gastritis, peptic ulcer disease, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer<sup>[3,4]</sup>. This persistent infection elicits a chronic inflammatory and immune response<sup>[5]</sup>, inducing both local and remote lesions. The interaction of the host with *H. pylori* can have profound systemic effects<sup>[6]</sup>. A growing body of evidence has implicated *H. pylori* infection in extragastrintestinal diseases such as cardiovascular, liver and biliary diseases<sup>[7-9]</sup>. The possible causative role of *H. pylori* infection in these diseases is intriguing. The contribution of *H. pylori* to the development of hepatic encephalopathy and hyperammonemia has been revealed<sup>[10]</sup>, and the possible correlation of *H. pylori* with other liver diseases has attracted a lot of attention<sup>[8,11]</sup>. Recent reports have emerged on the relationship between *H. pylori* infection and nonalcoholic fatty liver disease (NAFLD).

In recent years, there has been increased appreciation of the significance of NAFLD, which is currently considered to be the most common liver disorder in western countries, affecting up to 25%-30% of individuals<sup>[12-14]</sup>. NAFLD encompasses a range of related disorders<sup>[15]</sup>. The earliest stage is simple steatosis. It can progress to nonalcoholic steatohepatitis (NASH) with the cardinal features including hepatocyte injury, and inflammation with or without fibrosis<sup>[16-18]</sup>. NASH, in turn, may progress to cirrhosis and ultimately liver cancer in some patients<sup>[19]</sup>. Consequently, NAFLD is rapidly becoming a serious threat to public health. However, the mechanisms of the pathogenesis underlying NAFLD are complex and remain unknown at present<sup>[20,21]</sup>.

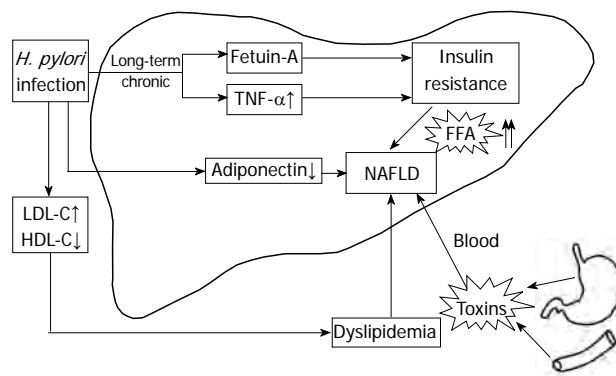
NAFLD is now regarded as the liver manifestation of the metabolic syndrome (MetS)<sup>[22]</sup>. It is strongly associated with obesity, diabetes, cardiovascular disease (CVD) and dyslipidemia<sup>[23]</sup>, because they spring from a "common soil", namely, insulin resistance (IR)<sup>[24]</sup>. A growing body of experimental evidence suggests that NAFLD and IR are closely related<sup>[25,26]</sup>.

*H. pylori* infection is implicated in the pathogenesis of IR<sup>[27]</sup>, which is important in the development of NAFLD, therefore, investigating the impact of *H. pylori* infection as a risk factor for IR might have implications in understanding its effect on NAFLD. It is hoped that the eradication of *H. pylori* can provide a novel strategy for the treatment of NAFLD. This review focuses on the possible relationship between *H. pylori* and NAFLD; mainly from the perspective of the potential mechanism of how *H. pylori* infection brings about IR and other aspects concerning this correlation (Figure 1).

## EXPERIMENTAL EVIDENCE LINKING

### *H. PYLORI* WITH NAFLD

A novel finding in the study of Cindoruk *et al.*<sup>[28]</sup> was the presence of 16S rDNA of *H. pylori* in the liver sample of a 44-year-old woman with NASH. They used polymerase chain reaction-based techniques to amplify 16S rDNA sequences of *H. pylori*. In that study, 27 of 75 patients with suspected liver disease were diagnosed with NASH. It



**Figure 1** Potential mechanism of how *Helicobacter pylori* contribute to nonalcoholic fatty liver disease. Insulin resistance (IR) may be an important link between *Helicobacter pylori* (*H. pylori*) infection and nonalcoholic fatty liver disease (NAFLD). IR favors accumulation of free fatty acids (FFAs) in the liver. *H. pylori*-induced IR may be mediated through fetuin-A. Tumor necrosis factor (TNF)- $\alpha$  plays a central role in the response to inflammation elicited by long-term *H. pylori* infection. The decrease in adiponectin is implicated in *H. pylori*-induced NAFLD. *H. pylori* toxins arising from the gastrointestinal area may cause liver damage. *H. pylori* may be associated with the altered lipid profile, leading to dyslipidemia, which is involved in the pathogenesis of NAFLD.

turned out in one sample that 16S rDNA of *H. pylori* was detected. This observation suggested that *H. pylori* play a role in NAFLD.

*H. pylori* is mainly identified by 16S rDNA sequencing<sup>[8]</sup>. In bacteria, there are three types of rDNA that are readily identifiable by size: the 120-nucleotide (nt) 5S rDNA, the 1600-nt 16S rDNA, and the 3000-nt 23S rDNA<sup>[29]</sup>. Researchers have recently tended to use genetic criteria including virulence gene and 16S rDNA sequencing for distinguishing it from other curved Gram-negative rods<sup>[30]</sup>.

In 2009 another study added credence to this finding, in which Pirouz *et al.*<sup>[31]</sup> noticed that patients with various chronic liver diseases (CLDs) had a greater probability of positive *H. pylori* 16S rDNA compared with the control group. In the 46 liver biopsies from patients with CLD, they detected *H. pylori* DNA in five of 11 samples from patients who were diagnosed with NAFLD.

## CLINICAL EVIDENCE LINKING *H. PYLORI* WITH NAFLD

Polyzos *et al.*<sup>[32]</sup> showed that NAFLD patients had significantly higher anti-*H. pylori* IgG, insulin, homeostatic model of assessment insulin resistance (HOMA-IR), and tumor necrosis factor (TNF)- $\alpha$ , but less total and high-molecular-weight adiponectin compared to the control group. However, there were no significant differences in steatosis grade, fibrosis stage, lobular or portal inflammation, or ballooning, when NAFLD patients were divided into subgroups according to *H. pylori* IgG seropositivity or <sup>13</sup>C-urea breath test positivity. This might be a clue that *H. pylori* infection is strongly linked to the pathogenesis of early-stage NAFLD, which is described as simple steatosis. Yet at the same time, it indicates that *H. pylori* infection may not contribute to the progression of NASH.

A randomized controlled single-blind study from



Doğan *et al*<sup>[33]</sup> showed that fatty liver was significantly more frequent in *H. pylori*-positive patients. The severity of the fatty appearance assessed by ultrasonography was also higher in the *H. pylori*-positive group. A study conducted in Japan demonstrated that *H. pylori* infection was one of the independent risk factors for the development of NAFLD<sup>[7]</sup>. These studies had some limitations and further research is warranted with larger, longer-term studies to confirm their findings. It is hard to determine if *H. pylori* is responsible for the natural course of NAFLD, or if it is merely an incidental finding. If this association is confirmed, eradicating *H. pylori* infection may have certain therapeutic perspectives in NAFLD.

## POTENTIAL PATHOGENETIC MEDIATORS AND MECHANISMS

### *IR: a possible bond linking H. pylori with NAFLD*

IR is a key pathogenic factor in NASH<sup>[34]</sup>. It leads to hyperinsulinemia and favors accumulation of free fatty acids (FFAs) in the liver because of decreased mitochondrial  $\beta$ -oxidation, on account that insulin inhibits hepatic mitochondrial  $\beta$ -oxidation of fatty acids<sup>[35]</sup>. Moreover, IR predisposes the liver to oxidative stress by stimulating microsomal lipid peroxidases<sup>[23]</sup>.

*H. pylori* infection is involved in diverse biological processes<sup>[36]</sup>, comprising inflammation, metabolism and oncogenic transformation<sup>[31,37]</sup>. In view of its effect on metabolic variables, *H. pylori* is associated with IR.

*H. pylori* infection is implicated in the pathogenesis of obesity<sup>[38]</sup> and type 2 diabetes mellitus (T2DM)<sup>[39-41]</sup>, which are closely related to MetS. IR is thought to be the underlying mechanism for MetS. However, so far, only limited clinical data directly suggest that *H. pylori* infection is involved in the development of NAFLD. Polyzos *et al*<sup>[32]</sup> showed that the contribution of *H. pylori* to NAFLD might be achieved indirectly through increasing IR, or directly, given that it can predict NAFLD independently from IR. Inspired by this hypothesis, studies investigating the relationship between *H. pylori* infection and IR would be of importance to infer the mechanism of how *H. pylori* induce NAFLD. A few studies have explored the possible link between *H. pylori* infection and IR. Besides, research on the influence of eradication therapy on IR and other metabolic parameters has been conducted. However, results are controversial, and whether *H. pylori* infection plays a role in IR remains to be determined. Assessed by HOMA-IR, existing data indicate a potential association between *H. pylori* infection and IR. HOMA-IR, which derives from fasting insulin and glucose levels<sup>[42]</sup>, is the most common method for assessment of IR in clinical practice and epidemiological studies<sup>[43]</sup>. Using this method, a high HOMA-IR score denotes low insulin sensitivity. In 2011, Polyzos *et al*<sup>[27]</sup> performed a systematic review<sup>[27]</sup>, summarizing the evidence for the association between *H. pylori* infection and quantitative indexes of IR. Summary data indicate a potential association between *H. pylori* infection and IR.

Eshraghian *et al*<sup>[44]</sup> showed that HOMA-IR score was significantly higher in the *H. pylori*-positive group compared with the negative group. They suggest that *H. pylori* infection is a risk factor for IR. However, Naja *et al*<sup>[45]</sup> have suggested no association of *H. pylori* infection with IR or MetS. They take the view that eradication of *H. pylori* infection to prevent IR or MetS is not warranted.

We must take into consideration that most of the studies that investigated the association between *H. pylori* and IR or MetS were small and did not adjust for potential confounders. Many of them did not control for the bacterial strain type or the host genetic factors. Due to the limited quantity and quality of these studies, more high-quality, multicenter, large-scale randomized controlled trials are required to clarify the association between *H. pylori* infection and IR development. A positive link between *H. pylori* infection and IR could have certain therapeutic prospects.

**A peculiar intermediary: fetuin-A:** *H. pylori* infection has been proposed in an attempt to elucidate the multifaceted aspects of the pathogenesis of IR. However, the pathogenetic link between *H. pylori* infection and IR is not fully understood as yet.

Among the various factors capable of inducing IR and subsequent IR syndrome, fetuin-A is peculiar because it is a glycoprotein that is produced exclusively in the liver and then secreted into the circulation in high concentrations<sup>[46]</sup>. Previous studies have shown that fetuin-A is closely related to IR<sup>[47-49]</sup>, and it has been linked with impaired insulin sensitivity, glucose metabolism, and the onset of diabetes mellitus<sup>[50,51]</sup>.

Recent studies have investigated whether the *H. pylori*-induced IR is mediated through fetuin-A. In the study of Kebapcilar *et al*<sup>[52]</sup>, fetuin-A level significantly decreases in *H. pylori*-infected patients when compared to control subjects. Moreover, *H. pylori* eradication reduces the levels of proinflammatory cytokines such as migration inhibitory factor and high-sensitivity C-reactive protein (CRP), with a significant increase in fetuin-A. They regarded fetuin-A as a potential anti-inflammatory cytokine<sup>[53]</sup>, on the basis of the theory that anti-inflammatory cytokines produced during inflammation tend to modulate the inflammatory reaction.

However, the findings of Manolakis *et al*<sup>[54]</sup> were the opposite. They noted that *H. pylori*-infected individuals showed higher levels of fetuin-A, insulin and HOMA-IR than controls. In addition, there was a positive correlation between fetuin-A and HOMA-IR. This has interesting therapeutic implications because it suggests that *H. pylori* eradication might decrease IR. This observation coincides with the results of Ou *et al*<sup>[55]</sup>, who showed that fetuin-A is considered to be a key proinflammatory mediator that plays a pivotal role in inflammatory and immune diseases. They suggest that elevated fetuin-A level has clinical implications in NAFLD and impaired glucose tolerance, which are features of IR<sup>[55]</sup>.

Fetuin-A is an endogenous inhibitor of insulin receptor tyrosine kinase in the liver and skeletal muscle<sup>[48]</sup>. Srin-

vas *et al*<sup>[48]</sup> have revealed that fetuin-A specifically inhibits insulin-stimulated insulin receptor autophosphorylation *in vitro* and *in vivo*, as well as exogenous substrate tyrosine phosphorylation. Moreover, they have demonstrated that fetuin-A influences insulin signaling by inhibiting insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1<sup>[56]</sup> and insulin-dependent mitogenesis. The glucoregulatory effects derived from insulin are predominantly exerted in three tissues consisting of liver, muscle and fat<sup>[57]</sup>. Thus, when IR occurs, the liver can be the point of attack. Based on the theory that fetuin-A inhibits insulin signaling in hepatocytes, it is reasonable to assume that the elevated fetuin-A in patients with NAFLD may contribute to the deteriorated hepatic IR.

Although the mechanism underlying fetuin-A-mediated IR remains elusive, it is a novel concept that fetuin-A may represent a promising index for assessing the *H. pylori*-related contributions to IR and MetS. If this particular association is confirmed, fetuin-A could be a potential target for therapy of IR and IR-related disorders, including T2DM, CVD and NAFLD.

### Chronic inflammation, cytokines and adipokines:

*Helicobacter* spp. are strong inducers of proinflammatory cytokines<sup>[58]</sup>. Long-standing *H. pylori* infection induces inflammation by stimulating excessive release of proinflammatory cytokines and vasoactive substances, such as interleukin (IL)-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$ <sup>[59-61]</sup>. *H. pylori*-positive individuals exhibit elevated levels of these proinflammatory cytokines<sup>[62]</sup>.

A growing body of evidence supports that inflammation is involved in the pathogenesis of IR and IR-related disorders<sup>[63]</sup>. Festa *et al*<sup>[64]</sup> have suggested that low-grade inflammation is a risk factor for the development of T2DM<sup>[64]</sup>. Several studies indicate that chronic subclinical inflammation is associated with CVD<sup>[65]</sup>.

Hotamisligil *et al*<sup>[66]</sup> and Feinstein *et al*<sup>[67]</sup> have demonstrated that TNF- $\alpha$  is able to induce IR. Therefore, we reckon that TNF- $\alpha$  may be a key mediator of both direct and indirect effects of *H. pylori* infection on NAFLD.

TNF- $\alpha$  interferes with insulin signaling, thereby favoring steatosis, and may play a proinflammatory role in the pathogenesis of NASH<sup>[68,69]</sup>. On the one hand, TNF- $\alpha$  promotes Ser phosphorylation of IRS-1<sup>[70]</sup>, resulting in a net decrease in insulin-receptor-mediated signaling. On the other hand, TNF- $\alpha$  can inhibit the autophosphorylation of insulin receptor or tyrosyl phosphorylation of IRS-1<sup>[67]</sup>. In addition, TNF- $\alpha$  downregulates the expression of key genes in adipose cells such as GLUT4<sup>[71]</sup>, resulting in decreased glucose transport<sup>[72]</sup>. Besides, TNF- $\alpha$  is capable of accelerating lipolysis, leading to an increase in FFAs, which can cause detrimental effects in hepatocytes, including oxidative stress<sup>[73]</sup>, induction of endoplasmic reticulum stress<sup>[74]</sup> and subsequent expression of proinflammatory cytokines. TNF- $\alpha$  promotes and is activated by IR *via* activation of IKK- $\beta$ <sup>[17]</sup>, which is a central coordinator of inflammatory responses through activation of nuclear factor (NF- $\kappa$ B)<sup>[75]</sup>. NF-

$\kappa$ B is a proinflammatory “master switch” that regulates inflammatory mediators including CRP, plasminogen activator inhibitor, TNF- $\alpha$ , IL-6 and IL-1 $\beta$ <sup>[76]</sup>.

Lower adiponectin level was observed in *H. pylori*-positive patients with NAFLD in the study of Polyzos *et al*<sup>[32]</sup>. This finding gives us a new clue that adiponectin may play a part in the process of *H. pylori*-induced NAFLD. Adiponectin, the adipocyte-derived hormone, is implicated in the pathogenesis of IR and NASH. Low serum adiponectin levels in NAFLD patients are suggestive of advanced hepatic fibrosis<sup>[77]</sup>. Adiponectin exerts several anti-inflammatory effects<sup>[78]</sup>, including inhibition of NF- $\kappa$ B activation<sup>[79]</sup> and suppression of macrophage function. Adiponectin and TNF- $\alpha$  are mutually antagonizing adipokines<sup>[80]</sup>. In contrast to TNF- $\alpha$ , adiponectin has an antilipogenic effect<sup>[81]</sup>. Thus, when adiponectin is decreased, its effects of controlling FFA entry and oxidation in the mitochondria are subsequently weakened, allowing FFAs to accumulate in the cytoplasm<sup>[82]</sup>.

### Lipid metabolism

Inflammation, IR and aberrant lipid metabolism may be interlinked components of the MetS<sup>[83,84]</sup>. Abnormalities of serum lipid concentrations are common in patients with NASH<sup>[23]</sup>. It is acknowledged that hypertriglyceridemia is involved in the pathogenesis of NASH. Each of the steps involved in hepatic lipid accumulation is altered in NAFLD, although to a different extent<sup>[85]</sup>. Satoh *et al*<sup>[86]</sup> have shown that *H. pylori* infection is a significant and independent risk factor for a modified lipid profile, including high low-density lipoprotein cholesterol (LDL-C) and low high-density lipoprotein cholesterol (HDL-C) in Japanese men, whereas these associations are not significant in women. Kebapcilar *et al*<sup>[52]</sup> demonstrated that *H. pylori* infection is significantly associated with lower HDL-C<sup>[52]</sup>, but they showed that eradication of *H. pylori* had no effect on the lipid profile. Akbas *et al*<sup>[87]</sup> reported that there was no significant difference in serum HDL-C, LDL-C, or total cholesterol between *H. pylori*-seropositive and *H. pylori*-seronegative individuals, whereas serum triglyceride level was higher in the *H. pylori*-positive group.

### Increased intestinal permeability, *H. pylori* toxins and cross-reactive antibody response

*H. pylori* is thought to have deleterious consequences on the hepatobiliary tract because the biliary epithelium can easily be colonized by bacteria from the duodenum<sup>[88]</sup>. The human gastrointestinal tract is an ecosystem integrated by microbiota. The mucosal epithelium of the small intestine is the barrier between the microbiota and gut lumen<sup>[89]</sup>. It is reported that increased intestinal permeability and small intestinal bacterial overgrowth (SIBO), may reflect qualitative and quantitative changes in the microbiota, leading to disruption of the intestinal barrier, subsequent bacterial translocation, and development of portal endotoxemia<sup>[90]</sup>. As a result, lipopolysaccharide, which is produced by Gram-negative bacteria, is increased in the portal circulation and accompanied by increased levels of

endotoxin-mediated cytokines in the liver. Bacterial translocation occurs due to impaired barrier function<sup>[91]</sup>, and bacterial constituents enhance hepatic inflammation and fibrosis<sup>[92]</sup>. Miele *et al.*<sup>[93]</sup> found that in NAFLD patients, increased gut permeability and the prevalence of SIBO correlated with the severity of steatosis but not with the presence of NASH.

In light of the above considerations, we speculate that the liver may be damaged by *H. pylori* toxins and constituents circulating in the blood coming out from the gastroduodenal area. And it is probably linked with the increased intestinal permeability in patients with NAFLD. Abenavoli *et al.*<sup>[94]</sup> reported a case of a 36-year-old woman with diagnosis of celiac disease (CD), primary biliary cirrhosis (PBC) and *H. pylori* infection. They found that strict adherence to a gluten-free diet, associated with ursodeoxycholic acid administration and eradication of *H. pylori* infection, led to a marked histological and serological improvement of PBC. *Helicobacter* spp. are implicated in the pathogenesis of PBC, because microbial DNA is found in liver tissue and bacterial antibodies in the serum of patients with PBC<sup>[95,96]</sup>, which is characterized by the presence of antimitochondrial antibodies directed predominantly against the E2 subunit of the pyruvate dehydrogenase complex<sup>[97]</sup>. They indicated that increased permeability to intraluminal antigens could induce an immune response against antigens sharing common epitopes to self-liver proteins and/or against cryptic antigens unmasked by the reaction with gliadin. Their study supports the pathogenetic role of increased intestinal permeability in the course of CD and *H. pylori* infection to induce PBC. However, this concept remains obscure. More studies are needed to clarify the reality of this association.

## CONCLUSION AND OUTLOOK

NAFLD affects both adults and children who present with particular risk factors, including obesity, sedentary lifestyle and/or a predisposing genetic background<sup>[98]</sup>. In some individuals it can progress to cirrhosis, or even hepatocellular carcinoma. Treatment strategies ranging from simple lifestyle modifications to pharmacological agents and even invasive surgical procedures<sup>[99]</sup> have been investigated as potential treatments for NAFLD. However, at present, it is regrettable that there is no one ideal therapy suitable for all patients<sup>[100]</sup>. Thus, new treatment strategies are important to halt the progression of NAFLD.

IR assumes importance in the pathogenesis of NAFLD progression<sup>[101]</sup>. Recent studies, largely on the possible contribution of *H. pylori* to IR, provide new insights into the link between *H. pylori* and NAFLD. Eradication of *H. pylori* is easy and relatively inexpensive, therefore, the interest in exploring its involvement in extragastric diseases is of importance for public health. Thus, understanding the pathogenetic role of *H. pylori* in NAFLD is important for devising new specific management strategies.

The clinical data regarding *H. pylori* infection in

NAFLD are limited, thus, it is premature to advocate intervention measures in patients with NAFLD. However, once this particular association is confirmed, it could drastically change our understanding of pathophysiology and treatment of NAFLD. Therefore, further studies are warranted to verify such associations before the strategy can be recommended in routine clinical practice.

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## Stages based molecular mechanisms for generating cholangiocytes from liver stem/progenitor cells

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### Abstract

Except for the most organized mature hepatocytes, liver stem/progenitor cells (LSPCs) can differentiate into many other types of cells in the liver including cholangiocytes. In addition, LSPCs are demonstrated to be able to give birth to other kinds of extra-hepatic cell types such as insulin-producing cells. Even more, under some bad conditions, these LSPCs could generate liver cancer stem like cells (LCSCs) through malignant transformation. In this review, we mainly concentrate on the molecular mechanisms for controlling cell fates of LSPCs, especially differentiation of cholangiocytes, insulin-producing cells and LCSCs. First of all, to certificate the cell fates of LSPCs, the following three features need to be taken into account to perform accurate phenotyping: (1) morphological properties; (2) specific markers; and (3) functional assessment including *in vivo* transplantation. Secondly, to promote LSPCs differentiation, systematical attention should be paid to inductive materials (such as growth factors and chemical stimulators), progressive materials including intracellular and extracellular signaling pathways, and implementary materials (such as liver enriched transcriptive factors). Accordingly, some recommendations were proposed to standardize, optimize, and enrich the

effective production of cholangiocyte-like cells out of LSPCs. At the end, the potential regulating mechanisms for generation of cholangiocytes by LSPCs were carefully analyzed. The differentiation of LSPCs is a gradually progressing process, which consists of three main steps: initiation, progression and accomplishment. It's the unbalanced distribution of affecting materials in each step decides the cell fates of LSPCs.

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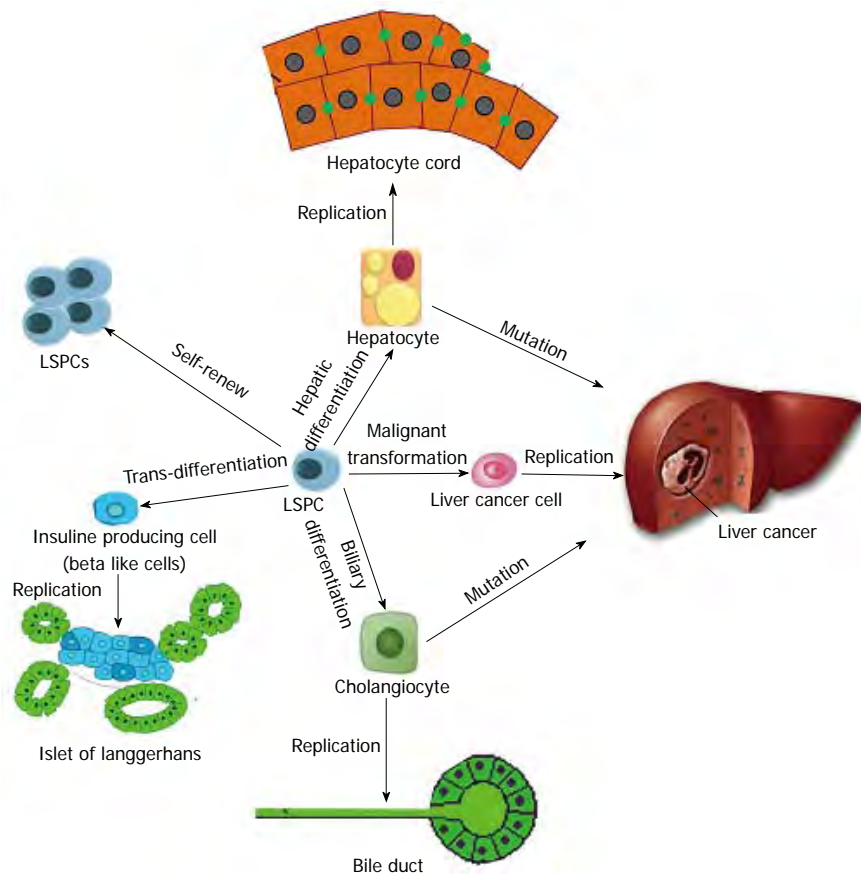
**Key words:** Liver stem/progenitor cells; Cholangiocytes; Biliary differentiation; Unbalanced distribution of materials; Cell therapy

**Core tip:** After liver stem/progenitor cells (LSPCs) are isolated by different groups from both fetal and adult livers, it is urgent to decide the cell fates of LSPCs. Especially, it is found that the core issue for LSPCs application lies in their accurate differentiation. Because there are lots of literatures concentrating on self-renewal and hepatic differentiation of LSPCs, in this review, we mainly summarize the molecular mechanisms for controlling other cell fates of LSPCs, especially differentiation into cholangiocytes. For biliary differentiation, we propose that it is a gradually progressing process consisting of three main steps: initiation, progression and accomplishment.

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### INTRODUCTION

Liver stem/progenitor cells (LSPCs) possess high pro-



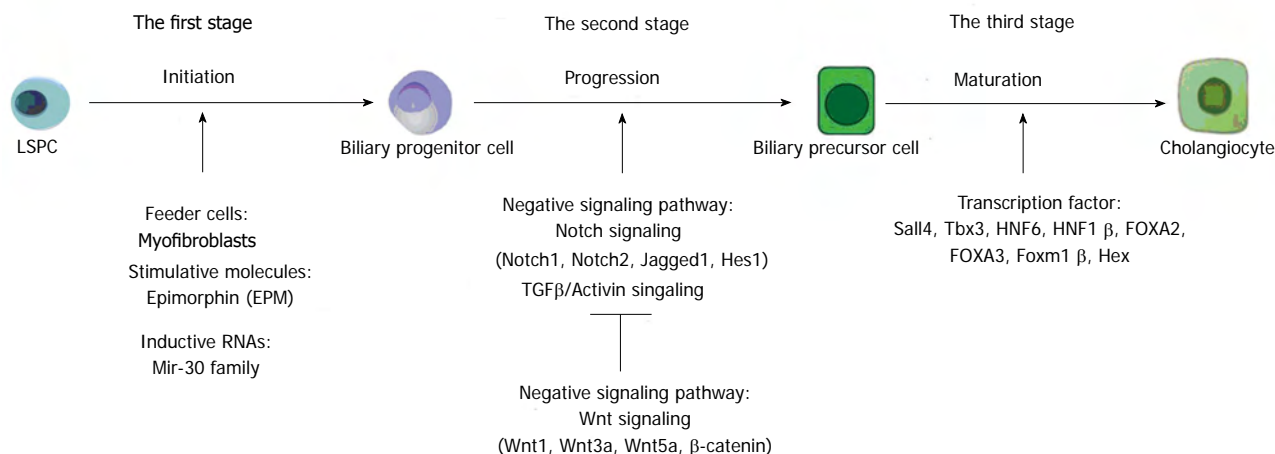
**Figure 1** The different cell fates of liver stem/progenitor cells under distinct situations. Under special stem microenvironment, LSPCs would probably self renew to keep stem properties. In the contrary, under differentiating stimuli, LSPCs could give birth to two kinds of fundamental mature cells in the liver, hepatocytes and cholangiocytes. This is very important for liver development and liver regeneration. Except for the traditional differentiation directions, LSPCs have the capacity to trans-differentiate into insulin producing cells, which is promising for treating diabetes. While in some bad situations, LSPCs may carry out malignant transformation to become liver cancer cells, even liver cancer stem cells, as a result, these cancer cells replicate themselves to cause liver cancer. This is a different way from mutation of hepatocytes for liver carcinogenesis. LSPC: Liver stem/progenitor cell.

liferative capacity and low immunogenicity and are robust in the face of cryopreservation or ischemic injury: properties that could enhance their engraftment within a recipient liver. Because of this, these LSPCs are very promising for the treatment of end-stage liver disease<sup>[1]</sup>. A series of animal models transplanted with LSPCs have been established, and several clinical trials have been reported. In one animal model, transplanted rat embryonic day (ED) 14 fetal liver stem/progenitor cells (FLSPCs) differentiated into the two mature epithelial cell phenotypes in the liver, *i.e.*, hepatocytes and cholangiocytes, and long-term, *in vivo* functional reconstitution of the liver tissue was achieved (Figure 1)<sup>[2,3]</sup>. The important recent progress is the use of human FLSPCs engrafted into naturally derived scaffolds to create a liver-like tissue *in vitro*<sup>[4]</sup>. However promising LSPCs are for cell therapy or tissue engineering, the fundamental purpose lies in generating mature, functional cells<sup>[5,6]</sup>.

LSPCs constitute approximately 0.5%-2.5% of liver parenchyma at all donor ages. The self-renewal capacity of LSPCs is demonstrated by their phenotypic stability after expansion for > 150 population doublings in a serum-free, defined medium, with a doubling time of approximately 36 h<sup>[7]</sup>. In fetal liver, LSPCs are commonly called hepatoblasts<sup>[2,3]</sup>. In some studies, some groups have used other terms than hepatoblasts to represent the cell populations with stem properties in fetal liver, such as embryonic hepatic stem cells or fetal liver stem-like epithelia. Thus, it would be more appropriate to denote these

cells as the “FLSPCs”, and we will adopt this description in this review. In adult liver, LSPCs are generally referred to as oval cells (OCs), with scant, lightly basophilic cytoplasm and pale blue-staining nuclei<sup>[8]</sup>. The appearance of OCs has been reported in rat livers treated with hepatotoxins, such as 2-acetylaminofluorene, combined with partial hepatectomy (PHx) and D-galactosamine<sup>[9,10]</sup>. However, in addition to OCs, small hepatocytes (SHs) are also well known in adult liver, and they are better suited to the appellation “progenitor” cells. As it is not an easy task to distinguish stem cells from progenitors because of the difficulty of proving the unlimited self-renewal activity of stem cells in many situations, we use the term “adult liver stem/progenitor cells (ALSPCs)” to describe such cells, including both OCs and SHs in this review article. In the field of liver biology, the definitions of “LSPCs” include the following: (1) cells responsible for normal tissue turnover; (2) cells that regenerate liver after PHx; (3) cells responsible for progenitor-dependent regeneration; (4) transplantable liver repopulating cells; and (5) cells that adopt hepatocyte and bile duct phenotypes *in vitro*.

Currently, researchers are working hard to characterize, localize and isolate LSPCs, though this has been difficult because of the lack of specific markers<sup>[11]</sup>. To avoid the restriction of lacking specific markers, Liu *et al.*<sup>[12,13]</sup> have tried other strategies for isolating LSPCs. Based on the concept that stem cells have specific physical and morphological properties, Liu *et al.*<sup>[12]</sup> isolated FLSPCs by a percoll continuous gradient centrifugation-centered



**Figure 2** The molecular mechanisms in each step of biliary differentiation of liver stem/progenitor cells. The biliary differentiation of LSPCs can be divided into three main stages comprising of initiation, progression and maturation. Some important jacent or feeder cells and specific molecules are proposed to be responsible for initiating the first stage of biliary differentiation. When the biliary differentiation goes on, several key signaling pathways including Notch and TGFβ have essential impacts on guarantee of the second stage. After some transcription factors activated, the third stage of biliary differentiation could be accomplished and LSPCs could be matured into cholangiocytes. LSPCs: Liver stem/progenitor cells; TGFβ: Transforming growth factor β.

three-step method. Because stem cells have specific functional characteristics, such as excluding biological vital dyes such as Hoechst 33342, Liu *et al.*<sup>[13]</sup> obtained ALSPCs by side population (SP) enrichment. Although many groups have isolated LSPCs using various strategies, LSPCs for disease application remain far off. The core issue is how to manipulate LSPC differentiation, which is essential for both cell therapy and liver regeneration<sup>[14,15]</sup>. Thus, to guarantee the efficiency and security of LSPC-based therapy for liver diseases, it is important to clarify the strategies and related mechanisms for proper differentiation of LSPCs.

In recent years, many studies have shed light on the tangle of regulatory mechanisms that govern the complex process of LSPC differentiation, but an overall understanding remains a challenge. Here, we review the current understanding of the exact mechanisms related to the differentiation of LSPCs, especially toward cholangiocyte differentiation. We divided the process of LSPC differentiation into three stages (Figure 2). The first stage is the onset of differentiation, when LSPCs are induced to mature into certain cell types. In this stage, the lineage-specific cytokines/growth factors (GFs), their (relative) doses and order of application are crucial for directing the lineage specification of the LSPCs<sup>[16]</sup>. The second stage is the acceleration of differentiation, when LSPCs are quickly progressing through the process. Many developmental regulatory signaling pathways, including the Wnt, Notch, bone morphogenetic protein and fibroblast growth factor pathways, may play a role in directing the cell fates of LSPCs<sup>[17]</sup>. The third stage is to guarantee the accomplishment of differentiation. In this stage, transcription factors are vital to make cells express lineage-specific markers<sup>[5]</sup>.

## IDENTIFICATION OF CHOLANGIOCYTES GENERATED FROM LSPCs

LSPCs can differentiate into a wide range of cell types, including hepatocytes, cholangiocytes, pancreatic cells and intestinal epithelial cells (Figure 1)<sup>[18]</sup>. However, in this review, we focus on cholangiocyte specification from LSPCs. To ascertain the cell fates of LSPCs, the following three features inherent to LSPC transitions must be taken into account for accurate phenotyping: (1) the differentiation of LSPCs toward a specific lineage often involves uncontrolled processes, resulting in a heterogeneous cell population; (2) the differentiation into mature cells is a steady process; and (3) the ultimate proof of functional cell behavior is *in vivo* transplantation of *ex vivo* generated LSPC-based mature cells into immunodeficient animal models with liver injury<sup>[19,20]</sup>.

As LSPCs differentiate into cholangiocytes, the cells grow in size to > 12 μm and display a keystone morphology with cholangiocyte-type epithelial polarity. These cells are concentrically layered to form a cyclic structure or arranged in lines to form ductal plates. Under the electron microscope, these cells acquired the classic cholangiocyte features of small numbers of organelles and many primary cilia on their surface.

Aside from morphological identification, the analytical work is limited to the elucidation of (1) cholangiocyte RNA transcripts *via* (quantitative) reverse transcriptase polymerase chain reaction and (2) cholangiocyte proteins by immunofluorescence. During the process of LSPC differentiation into cholangiocytes, cells transition from the expression of early biliary markers (such as Sox9, which is a representative transcriptional factor expressed in biliary precursor cells), to the expression of mid-stage



biliary markers (such as the cytokine CK19 and E-cadherin), and then mature biliary markers (such as CK7)<sup>[21]</sup>. In addition, gamma-glutamyl transpeptidase (GGT), a major enzyme of glutathione homeostasis, is often used as a biliary marker to follow the differentiation of LSPCs<sup>[22]</sup>. Furthermore, multidrug resistance-associated protein 3<sup>[23]</sup> and secretin receptors<sup>[24]</sup> are also found to be expressed in cholangiocytes.

Although the induced differentiation of cholangiocytes has been performed, the functional examination of LSPC-derived cholangiocytes is very scarce. Thus, the *in vivo* identification of induced cholangiocytes is essential, and to some extent it can be considered the “gold standard” of certifying the cell fates of LSPCs<sup>[25]</sup>. LSPC-derived cholangiocytes *in vivo* should be able to replace injured cholangiocytes or lost bile duct cells.

## STRATEGIES FOR CHOLANGIOCYTIC DIFFERENTIATION OF LSPCs

The components of the stem-cell microenvironment regulating differentiation include distinct cell-cell interactions and paracrine signals, which comprise both soluble and extracellular matrix factors, as well as the three-dimensional architecture, which shapes and dictates the delivery of these cues. It is reported that mature stellate cells and/or myofibroblasts resulted in differentiation of LSPCs into cholangiocytes<sup>[26]</sup>. These feeder cells control the cell fates of LSPCs through either paracrine signaling pathways or cell-cell interaction<sup>[27-30]</sup>. Thus, if the paracrine signals produced by the feeders are replaced with similar components, the same induced differentiation of LSPCs could be achieved. There are feeder-free conditions that yield equivalent results, consisting of the embedding of LSPCs into hydrogels containing type I collagen (60%) and Matrigel (40%) with modified Kubota's medium for cholangiocytes. It is also demonstrated that the murine FLSPC cell line, hepatoblast cell line-3, can be induced to differentiate toward cholangiocyte by plating in Matrigel<sup>[31]</sup>. Furthermore, Matrigel-coated films are also widely used for manipulating LSPCs. Although PLL-terminal t-(poly-L-lysine/poly-L-glutamic acid) (PLL/PLGA) films are less favorable for stem cell cultures than PLGA-terminal t-(PLL/PLGA) films, the cell fates of LSPCs are correlated with the film thickness on both types of film, with differentiation favored on the thinner films<sup>[32]</sup>.

Recent evidence has shown that expression of miRNAs can regulate the divergent differentiation pathways of stem cells<sup>[33]</sup>. Therefore, Liu *et al.*<sup>[33]</sup> reasoned that miRNAs could be responsible for regulating cell fate decisions in LSPCs by regulating the cells' responses to ubiquitous GFs. It was found that the miR-23b cluster, including miR-23b, miR-27b, and miR-24-1 and miR-10a, miR-26a and miR-30a, was highly expressed in LSPCs<sup>[34]</sup>. MiR-23b cluster repressed bile duct gene expression in LSPCs while promoting their growth; low levels of the miR-23b miRNAs were needed in cholangiocytic differentiation and bile duct formation<sup>[34]</sup>.

## MOLECULAR MECHANISMS OF CHOLANGIOCYTIC DIFFERENTIATION FROM LSPCs

The process of intrahepatic bile duct (IHBD) formation from LSPCs involves cholangiocyte differentiation (lineage specification) and morphogenesis of ductal structures<sup>[21]</sup>. Understanding how LSPCs can generate differentiated bile ducts is crucial for studies on epithelial morphogenesis and for development of cell therapies for hepatobiliary diseases. Many groups<sup>[35-37]</sup> have demonstrated that, during *in vivo* liver development and *in vitro* differentiation, LSPCs located around the portal vein first develop as biliary precursor cells and then generate cholangiocytes. Nevertheless, the molecular mechanisms behind these events have yet to be fully elucidated. It is shown that Wnt and Notch signaling are active in the adult human liver to drive proliferation and differentiation of LSPCs into the hepatocyte or cholangiocyte lineages<sup>[38]</sup>. The Notch pathway is triggered by expression of the Notch ligand Jagged1 by myofibroblasts, thereby promoting biliary differentiation of LSPCs, and the enhancement of Wnt3a expression in macrophages after uptake of hepatocyte debris and paracrine activation of Wnt signaling in neighboring LSPCs specifies hepatocytic differentiation<sup>[39,40]</sup>. The opposing roles of Wnt and Notch signals in cholangiocyte fate determination in the LSPCs are described below. The molecules responsible for differentiation of LSPCs into cholangiocytes are also discussed in this section (Figure 2).

## INITIATION OF CHOLANGIOCYTIC DIFFERENTIATION

When the LSPCs are cultured in Matrigel, they are likely to differentiate into cholangiocytes. Recently, the key stimulator has been found. Epimorphin/syntaxin 2 (EPM) is a highly conserved and very abundant protein involved in epithelial morphogenesis in various epithelial organs<sup>[41]</sup>, and in the liver, it is exclusively expressed on the surface of hepatic stellate cells and myofibroblasts<sup>[42]</sup>. Biliary differentiation markers elevated by EPM include Yp, Cx43, aquaporin-1, CK19 and GGT<sup>[41]</sup>. Moreover, the signaling pathway of EPM was analyzed by focal adhesion kinase (FAK), extracellular regulated kinase 1/2 (ERK1/2) and RhoA. Most importantly, RhoA was found to be necessary for EPM-induced activation of FAK and ERK1/2 and bile duct formation. In addition, EPM regulated GGT IV and GGT V expression differentially, and this was possibly mediated by C/EBP $\beta$ . Taken together, these data demonstrated that EPM regulates biliary differentiation of LSPCs through effects on RhoA and C/EBP $\beta$ , implicating a dual aspect of this morphoregulator in bile duct epithelial morphogenesis. In another study, it was reported that EPM selectively induced bile duct formation through upregulation of CK19 expression and suppression of hepatocyte nuclear factor

(HNF) 3 $\alpha$  and HNF6<sup>[43]</sup>. These results demonstrate a new biophysical action of EPM in bile duct formation, during which the determination of LSPCs play a crucial role. MiRNAs could also initiate biliary differentiation of LSPCs. In the previous section, we described the requirement for miR-23b miRNAs in growing hepatocytes to repress bile duct genes and repress tumor growth factor (TGF)  $\beta$  signaling. There has also been another report providing evidence that miR-30 family miRNAs were required for complete bile duct formation to repress hepatocyte genes<sup>[44]</sup>.

## PROGRESSION OF CHOLANGIOCYTIC DIFFERENTIATION

Bile ducts are formed only around the portal side, suggesting that region-specific signals induce cholangiocytes from LSPCs. Two signaling pathways, TGF $\beta$ /Activin<sup>[21,45]</sup> and Notch<sup>[46,47]</sup>, are specifically activated in LSPCs near the portal vein. Although differentiation of LSPCs to cholangiocytes by TGF $\beta$  and Notch signaling occurs in mid-gestation, surprisingly, LSPCs can be induced to differentiate into cholangiocytes and form ectopic duct structures in the parenchyma upon Notch activation after birth<sup>[48]</sup>. That is, the Notch pathway plays an essential role in the morphogenesis of bile duct structures<sup>[49]</sup>. Indeed, conditional knockout of Recombination signal binding protein J $\kappa$ , an essential downstream signal component of the Notch receptor, results in a reduced number of cholangiocytes at ED 16.5, confirming a role for this signaling pathway in cholangiocyte cell fate specification<sup>[48]</sup>. In general, Notch signaling is likely to play the most important role in controlling biliary differentiation of LSPCs.

A study using an *in vitro* culture of FLSPCs has shown that activation of the Notch signaling pathway promotes LSPC differentiation into the cholangiocyte lineage by coordinating a network of LETFs including HNF1 $\alpha$ / $\beta$ , HNF4 $\alpha$  and C/EBP $\alpha$ <sup>[46]</sup>. Among multiple Notch signaling components, Notch1, Notch2 and Jagged1, Hes1 are widely accepted as essential for promoting bile duct differentiation<sup>[49-51]</sup>, while Notch3 and Jagged2 play key roles in hepatic differentiation<sup>[52]</sup>. Lacking Hes1, a target of the Notch signaling, ductal plate formation occurs normally, but the subsequent remodeling and tubular structure formation is completely blocked<sup>[53]</sup>. In humans, mutations in Jagged1, a ligand for the Notch receptors, are associated with Alagille syndrome, an autosomal dominant disorder characterized by multiple developmental defects including neonatal cholestasis caused by a paucity of IHBD<sup>[54-56]</sup>. In addition, another form of Alagille syndrome has been found to be caused by mutations in the *Notch2* gene<sup>[57]</sup>.

TGF $\beta$  is necessary for the formation of bile ducts<sup>[58]</sup>. The inhibition of TGF $\beta$  signaling allows LSPCs to undergo normal hepatocyte differentiation<sup>[45]</sup>. Wnt signaling is also involved in regulating biliary epithelial cell fate. The addition of Wnt3a in *ex vivo* culture experiments supports biliary epithelial cell differentiation of FLSPCs<sup>[59]</sup>. However, as to Wnt5a, a non-canonical Wnt ligand, *in*

*vitro* differentiation assays showed that Wnt5a-mediated signaling in FLSPCs suppresses biliary differentiation through the activation of phosphorylated Calcium/calmodulin-dependent protein kinase II<sup>[60]</sup>. Similarly, in the absence of Wnt1 signaling, LSPCs failed to differentiate into hepatocytes and underwent atypical ductular hyperplasia, exhibiting epithelial metaplasia and mucin production<sup>[61,62]</sup>. Furthermore, the inhibition of  $\beta$ -catenin, a core component of canonical Wnt signaling, prevents LSPCs from expressing biliary markers<sup>[63]</sup>.

In brief, Notch signaling promotes LSPCs differentiation into the biliary epithelial lineage and concurrently inhibits hepatic differentiation by reducing the expression of hepatic genes. In contrast, Wnt signaling is more likely to aid in promoting hepatic differentiation and repressing biliary differentiation. The unbalanced activation of Wnt and Notch signaling pathways influences the cell fates of LSPCs.

## ACCOMPLISHMENT OF CHOLANGIOCYTIC DIFFERENTIATION

With regard to the molecular mechanisms involved in cholangiocyte differentiation, several transcription factors have been implicated, including Sal-like 4 (Sall4), T-box transcription factor 3 (Tbx3), the Onecut transcription factor HNF6 and HNF1 $\beta$ , HES1, FOXA2, FOXA3, forkhead Box (Fox) m1 $\beta$  (Foxm1 $\beta$ ), and Hex<sup>[36,37,64-68]</sup>. Sall4 is expressed in LSPCs but not in mature liver cells. The expression level of Sall4 gradually falls during liver development. Sall4 has been shown to play a role in regulating the lineage commitment of LSPCs by inhibiting their differentiation into hepatocytes while driving differentiation toward cholangiocytes<sup>[36]</sup>. When bile duct-like structures were induced by collagen gel-embedded culture conditions, overexpression of Sall4 markedly augmented the size and number of CK19<sup>+</sup> branching structures. These results suggest that Sall4 plays a crucial role in controlling the lineage commitment of LSPCs not only by inhibiting their differentiation into hepatocytes but also by driving their differentiation toward cholangiocytes<sup>[36]</sup>. Tbx3 also contributes to the hepato-biliary lineage decision<sup>[37,69]</sup>. Tbx3 functions to maintain expression of the hepatocyte transcription factors HNF4 $\alpha$  and C/EBP $\alpha$  while suppressing expression of the cholangiocyte transcription factors HNF6 and HNF1 $\beta$ <sup>[69]</sup>. In addition, as a direct and critical target of HNF6, HNF1 $\beta$  shows a decisive effect in bile duct development<sup>[65]</sup>.

## DIFFERENTIATION OF LSPCs INTO INSULIN-POSITIVE CELLS

Although organ-specific stem cells possess plasticity that permits differentiation along new lineages, production of endocrine pancreas and insulin-secreting beta cells from stem cells has not been fully demonstrated. The liver and pancreas share a common developmental origin, and a

bipotential precursor cell population for these organs has been identified within the embryonic endoderm<sup>[70]</sup>. Consistent with these facts, many studies have demonstrated that LSPCs can be converted to insulin-producing cells by stable expression of pancreatic duodenal homeobox 1 (Pdx1) or its super-active form (Pdx1-VP16) or to functional pancreatic beta-cell-like cells, and/or islet-like cell clusters containing other pancreatic lineages under certain other conditions<sup>[71-74]</sup>. The most common condition under which LSPCs are induced to differentiate into insulin-producing cells is a high-glucose environment<sup>[75]</sup>. In addition, there are studies indicating an efficient chemical protocol for differentiating LSPCs into functional insulin-producing cells using small molecules, and they represent a promising LSPC-based treatment for diabetes mellitus. When ALSPCs were incubated with a combination of 5 mmol/L sodium butyrate and 1 nmol/L betacellulin, most of the cells were converted into morphologically beta cell-like cells. An immunoreactive pancreatic polypeptide, somatostatin, and insulin were detected in sodium butyrate and betacellulin-treated ALSPCs<sup>[72]</sup>. Based on induction by a combination of 5-aza-2'-deoxycytidine, trichostatin A, retinoic acid and a mix of insulin, transferrin and selenite, LSPCs could also trans-differentiate into beta-like cells<sup>[76]</sup>. Furthermore, transduction of pancreatic transcription factors, such as Pdx1, Neurogenin3, NeuroD and MafA, can induce the formation of ectopic islet-like cells and the production of insulin in ALSPCs<sup>[77,78]</sup>. Stepwise differentiation from LSPCs into functional insulin-secreting cells will identify key steps in beta-cell development and may yet prove useful for transplantation therapy for diabetic patients<sup>[79]</sup>.

## MALIGNANT TRANSFORMATION OF LSPCs INTO LIVER CANCER STEM LIKE CELLS

Stem cells have potential for therapy of liver diseases, but they may also be involved in the formation of cancer<sup>[80]</sup>. At present, it is widely accepted that cancer arises from the malignant transformation of stem cells<sup>[81,82]</sup> because these are the only cells that persist sufficiently long to acquire the required number of genetic changes. Specifically, LSPCs are hypothesized to be the precursors for a subset of liver cancer<sup>[83,84]</sup>. Presently, accumulating evidence supports the above notion as follows<sup>[85]</sup>: (1) similar signaling pathways may regulate self-renewal in LSPCs and liver cancer cells; and (2) liver cancer contains rare cells with stem cell-like properties, which may derive from malignant transformation of LSPCs. Herein, we propose that liver cancer stem like cells (LCSCs) might arise from LSPCs it would facilitate our understanding of stem-cell origin of liver cancer. It has been demonstrated that deletion of p53 from LSPCs is sufficient to induce tumor formation<sup>[86]</sup>. Recently, through loss expression of Tg737, You *et al.*<sup>[87]</sup> successfully induced FLSPCs to malignantly transform into LCSCs. These LCSCs from LSPCs could

generate liver cancer after transplantation into immunodeficient mice. In addition to gene manipulation, dysregulated miRNAs may also initiate malignant transformation. To find the possible target miRNAs, Liu *et al.*<sup>[13]</sup> compared the miRNA profiles between LSPCs and LCSCs. As a result, Liu *et al.*<sup>[13]</sup> found 78 miRNAs were dysregulated, including miR-200a (the most down-regulated miRNA in LCSCs) and miR-181 (the most greatly upregulated miRNA in LCSCs)<sup>[13]</sup>. After inhibition of miR-200a in LSPCs, Liu *et al.*<sup>[13]</sup> found that cells displayed malignant properties such as unlimited proliferation and strong metastasis. A novel regulatory link between miR-181 and LCSCs was proven by a study from another group<sup>[88]</sup>. They found that miR-181 could induce LSPCs' malignant transformation by directly targeting hepatic transcriptional regulators of differentiation (for example, caudal type homeobox transcription factor 2 and GATA binding protein 6) and an inhibitor of Wnt/beta-catenin signaling (nemo-like kinase).

## CONCLUSIONS AND FUTURE DIRECTIONS

Despite uncertainty surrounding the mechanism underlying the role of LSPCs in liver regeneration<sup>[89]</sup>, there is great hope for the use of these cells in liver-based therapies<sup>[90]</sup>. First, LSPCs can be used for the treatment of inherited end-stage liver disease. Second, they can also serve as a source of cells for cell transplantation in acquired liver diseases such as acute failure due to toxic or viral injury. Third, because LSPCs can be expanded *in vitro* to a desired extent, they can be used to populate liver assist devices or artificial livers based on bioengineered matrices. Lastly, they can be used as targets for gene therapies in primary liver diseases or diseases where extra-hepatic manifestations arise from abnormal gene expression or defective protein production in the liver. Considering the strong proliferative potential and amenability for *in vitro* manipulation, LSPCs may be attractive candidates for liver disease treatment. In addition, LSPCs may be useful for cell therapy to treat diabetic patients, given their potential to be effectively reprogrammed toward pancreatic lineages<sup>[91]</sup>. Furthermore, the development of such protocols would reduce the likelihood of malignant transformation upon transplantation.

Although LSPCs are promising for the future use in many fields, the accurate control of cell fates of LSPCs is far from accomplishment. Thus, it is necessary to clear the mechanisms for LSPCs differentiation and build standardisation of the production of functional cholangiocytes from LSPCs. Here, we want to list several directions that may help to guide future research of LSPCs differentiation: (1) The knowledge of biliary development and liver regeneration can best provide detailed information for *in vitro* cholangiocyte differentiation of LSPCs. It is a good choice to thoroughly investigate the molecular basis of biliary development during the period from fetal liver to adult liver; (2) LSPCs react differently to stimulative



materials at different stages. The dosage, timing, and combinations of materials should thus be fine-tuned according to the differentiated stage LSPCs located. Hence, it is important to figure out what state LSPCs are presented; (3) The molecular mechanism in each step of cholangiocytic differentiation from LSPCs is essential for cell-based therapies. Both positive and negative factors responsible for the initiation, progression and maturation of cholangiocytic differentiation should be specially considered; and (4) Although we divide the process of cholangiocytic differentiation into three stages, it is actually a continuous evolving process. That is to say, it should be kept in mind that many key factors may not only take effect in some stage of cholangiocytic differentiation from LSPCs.

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## Presence of phthalates in gastrointestinal medications: Is there a hidden danger?

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### Abstract

Pharmaceutical companies that produce gastrointestinal (GI) medications often utilize phthalates for their ability to localize medication release. Commonly prescribed GI medications that may utilize phthalates are 5-Aminosalicylates, proton pump inhibitors, and pancreatic enzymes. Our understanding of the cumulative health effects of phthalates from medications remains unclear, and there is increasing evidence that phthalates are not harmless. Experimental studies in animals have shown that phthalates, specifically dibutyl phthalate and Di-(2-ethyl-hexyl) phthalate, have the potential to alter and/or inhibit reproductive biology and in utero development. Despite the lack of definitive human data, many cohort and cross-sectional studies demonstrate concerning associations between phthalates and poor health status, specifically developmental problems. Longitudinal studies and studies with larger sample sizes are required to determine whether phthalates actually cause negative health consequences. It is also important that physicians regularly review and discuss with patients the medicinal ingredients in their medications and supplements, specifically in pregnant woman with

inflammatory bowel disease.

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**Key words:** Phthalates; Dibutyl phthalate; 5-Aminosalicylates; Medications; Development; Pregnancy

**Core tip:** Phthalates are widely used as excipients in medications used to treat gastrointestinal disease. Research into the adverse effects associated with certain phthalates continues to produce uncertainty regarding the safety of their use in medications. Gastroenterologists should be aware of the potential harm of specific phthalates so that they can make informed decisions of whether the benefits of the medication outweigh the potential risks. Additional studies using human populations will help elucidate if regulatory bodies should mandate the use of alternative excipients.

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### INTRODUCTION

Phthalates are plasticizers with widespread industrial use. Their unique chemical structure allows them to make plastic more flexible and durable<sup>[1]</sup>. Phthalates are commonly used as softeners, solvents and additives, and are employed as excipients in gastrointestinal (GI) medications<sup>[2-4]</sup>. Pharmaceutical companies that produce GI medications often utilize phthalates for their ability to localize medication release. More specifically, low molecular weight (LMW) phthalates are found in oral medications that require both controlled time release and location

sensitive release at certain points along the GI tract<sup>[5]</sup>. Our understanding of the cumulative health effects of phthalates from medications remains unclear, and there is increasing evidence that phthalates are not harmless. This paper will review phthalate utilization in GI medications and summarize the evidence for the possible hidden danger of these common additives.

## CHEMICAL STRUCTURE

Phthalates are diesters of 1,2-benzendicarboxylic acid (phthalic acid) and are present in both industrial and commercial synthetic products<sup>[5]</sup>. Phthalate esters are prepared by the esterification of two moles of monohydric alcohol with one mole of phthalic anhydride<sup>[6]</sup>. When used as an additive to industrial products, phthalates are often combined with polyvinyl chloride (PVC) because they are cheap and are able to provide important properties to plastics such as flexibility and durability. As a result, phthalates are found in more than 80% of the global plasticizer market<sup>[6]</sup>. LMW phthalate subgroups have fewer than eight carbon atoms and include diethyl phthalate (DEP) and dibutyl phthalate (DBP), while high molecular weight (HMW) phthalates have eight or more carbon atoms in an alkyl chain<sup>[7]</sup>. The commonly used HMW phthalate, Di-(2-ethyl-hexyl) phthalate (DEHP), is found in many products containing PVC<sup>[7]</sup>. Most phthalates used as plasticizers have between 4 and 13 carbon atoms. These specific carbon lengths are used since fewer than four carbons can make compounds too volatile and more than 13 carbons are less effective at combining with PVC molecules<sup>[6]</sup>.

## BIOABSORPTION

Since phthalates are the most widely used additives in plastics, their absorption in the body has been extensively studied. Phthalates do not bio-accumulate in the body. However, their widespread use translates into a large exposure in the general population<sup>[8]</sup>. Phthalates are quickly metabolized to mono-alkyl metabolites and glucuronides and are excreted in both urine and feces<sup>[9-12]</sup>. The urine content of phthalates and their metabolites have been shown to be sensitive biomarkers of phthalate intake. Therefore, urine screening has been used in many studies to assess phthalates levels in the population<sup>[2,13,14]</sup>. Specifically, United States and German population data have shown widespread exposure to phthalates in urine samples<sup>[5,14,15]</sup>. A United States study using data from the National Health and Nutrition Examination Survey found that over 75% of urinary samples contained some form of a phthalate metabolite, and it has been speculated that urine studies may underestimate phthalate levels in humans, as metabolites may be metabolized into undetectable byproducts<sup>[2,14]</sup>.

## GI MEDICATIONS AND PHTHALATES

Scientists utilize various techniques to permit the release

of medication at specific parts of the luminal GI tract. For instance, using the prodrug technique, an inert drug is transformed into its active form at various pH levels. As an alternative method, the pharmaceutical industry has relied heavily on phthalates to assist with delivery of GI medications to precise areas of the luminal GI tract.

Compared to HMLW phthalates, LMW phthalates are more commonly used in pharmaceutical products. Phthalates used as excipients include cellulose acetate phthalate, DBP, DEP, dimethyl phthalate, hypromellose phthalate, and PVC<sup>[16]</sup>. Excipients are defined as inactive ingredients found in medications that aid in the manufacturing, administration or absorption of the drug<sup>[17]</sup>. They usually possess no active pharmacological ingredients and are regarded as inert. For example, LMW excipients such as DBP and DEP are listed in the FDA Inactive Ingredients Database for use in oral capsules, delayed action, enteric coated and controlled release tablets<sup>[18]</sup>. Phthalates can also be combined with different polymers to maintain medication flexibility<sup>[19]</sup>. This can assist with the localization of active ingredients through the delayed release of the inner components of solid drugs<sup>[19,20]</sup>.

An extensive review of pharmaceutical literature revealed that many GI medications contain phthalates as both excipients and inactive ingredients<sup>[17]</sup>. For instance, this review found that mesalamine, pancreatic lipase, sulfasalazine, ranitidine and omeprazole are prescription drugs marketed in either Canada or the United States with labels that identified an ortho-phthalate as an inactive ingredient. The phthalate DBP, which has been shown to have potentially harmful adverse effects, is found in nonprescription medications such as bisacodyl and many probiotic supplements used frequently by gastroenterologists<sup>[17]</sup>. Omeprazole and ranitidine contain the phthalate DEP, of which there is no evidence of potential harm.

The extensive use of phthalates in GI medications has prompted research into the cumulative effects of phthalates on those taking these drugs for prolonged periods of time. GI medications utilize phthalates more than most medications and are, therefore, more likely to result in high exposure to phthalates. Studies have shown that among patients prescribed, some of the aforementioned GI medications, specifically mesalamine and omeprazole, urine concentrations of phthalates have been documented at levels 100 times higher than the general population<sup>[5]</sup>. It has also been shown that DBP and DEP, commonly used as excipients, can be found at concentrations of 9000 micrograms per capsule in some GI medications<sup>[11]</sup>. These concentrations are concerning, as it has been shown that only 3600 micrograms per capsule can result in DBP metabolites in urine that are above the recommended tolerable daily intake<sup>[11]</sup>. Well-designed retrospective studies are needed to determine the long-term effects of using GI medications with high levels of phthalates.

## HARMFUL EFFECTS OF PHTHALATES

Experimental studies in animals have shown that phthal-



ates, specifically DBP and DEHP, have the potential to alter and/or inhibit reproductive biology and in utero development<sup>[5]</sup>. One study demonstrated that mice exposed to 190 times the recommended amount of Asacol, a 5-ASA drug that contains DBP, were at risk for developing skeletal malformations and reproductive adverse effects<sup>[21]</sup>. These concerns prompted additional studies which revealed that phthalates can act as anti-androgens and subsequently have toxic interactions with androgen receptors<sup>[22,23]</sup>. Nonetheless, little data exists to help determine whether phthalates act as endocrine hormones at high levels in humans. Whether phthalates have meaningful interactions with proteins at the cellular level also remains unclear<sup>[24,25]</sup>.

Despite the lack of definitive human data, many cohort and cross-sectional studies demonstrate concerning associations between phthalates and poor health status, specifically developmental problems. For instance, a study in the United States found positive associations between LMW phthalate metabolites and several developmental indicators, including gestational age and head circumference. These results demonstrate that phthalates may potentially alter childhood development from birth<sup>[26]</sup>. Research from Denmark showed a potentially detrimental correlation between phthalate monoesters and hormones essential for normal in utero development<sup>[26,27]</sup>. Multi-center cohort studies from the United States and Mexico studying male children demonstrated that prenatal urinary phthalate concentration is negatively correlated with genital development, including anogenital distance, an index of demasculinization of the male reproductive tract, and penile width<sup>[23,28,29]</sup>. Cross-sectional data from the United States, China, and Sweden comparing phthalates levels with semen concentration and semen quality have raised concern about deleterious interactions<sup>[30-34]</sup>. By measuring phthalate metabolites in urine, dose-response relations have been found between some phthalate metabolites and sperm concentration, motility, and morphology<sup>[30,32]</sup>. Despite the associations between phthalates and semen indices, this data has not been reproduced in the general population.

Additionally, phthalates have been associated with stunted neurodevelopment<sup>[35,36]</sup>. A cross-sectional study from South Korea displayed a negative relationship between urinary concentration of phthalate metabolites and performance on various IQ tests<sup>[35]</sup>. Moreover, United States cohort data indicated a positive association between maternal urine concentration of certain phthalates and increased negative behavior on validated behavior reporting tools<sup>[36]</sup>. One cohort study from Denmark showed a negative association between phthalate metabolites in urine and normal serum levels of thyroid hormone<sup>[37]</sup>. Interestingly, a cohort study from South Korea showed an association between phthalate metabolites in the urine, specifically DEHP, and increased attention deficit hyperactivity disorder symptoms<sup>[38]</sup>. Recent research has provided conflicting data on the association of phthalates with the early onset of puberty and its associ-

ated symptoms<sup>[22,39]</sup>. A case-control study from Turkey demonstrated an association between plasma levels of certain phthalates and gynecomastia, while a multicenter cohort study performed in the United States showed no association between phthalates concentration in the urine and precocious puberty<sup>[22,39]</sup>. Finally, cross-sectional and cohort studies out of Sweden, Russia and Finland have implicated respiratory complications such as rhinitis and asthma with phthalates<sup>[40-43]</sup>. However, the evidence for the association between phthalates and these clinical manifestations remains weak as most of these studies used PVC exposure as a proxy to phthalate exposure.

## 5-AMINOSALICYLATES

5-Aminosalicylates (5-ASAs) are used as first line therapy in treatment for mild to moderate ulcerative colitis (UC). Initial research in phthalate exposure and GI medications has focused on 5-ASAs users. Specifically, absorption data shows concerning levels of phthalates in the urine of chronic users of mesalamine, a 5-ASA drug. United States data demonstrated that six individuals taking mesalamine had metabolites of DBP 50-fold higher than those not using mesalamine<sup>[10]</sup>. Similarly, one third of patients taking mesalamine had urine levels of phthalates that exceeded FDA recommended levels<sup>[5,10]</sup>. While no equivocal evidence exists, gastroenterologists treating UC should consider prescribing 5-ASAs without DBP. This consideration should be especially taken in women of child-bearing age, as DBP may have deleterious effects during pregnancy based on animal studies.

Studies of pregnant and lactating women have shown that phthalates appear in maternal and umbilical blood, amniotic fluid and breast milk<sup>[27,44-46]</sup>. As a result, women taking 5-ASA formulations have been evaluated for potential adverse effects during pregnancy<sup>[47-50]</sup>. While no randomized control studies exist, a meta-analysis using 7 cohort studies did not indicate that woman taking 5-ASA during pregnancy have significantly higher rates of congenital abnormalities compared to control groups using no medication<sup>[51]</sup>. Pooled odd ratios from these studies demonstrated 1.16, 2.38, 1.14, 1.35 and 0.93 fold increase in congenital malformations, still births, spontaneous abortions, preterm deliver and low birth weight, respectively<sup>[51]</sup>. Based on this data, the 5-ASA formulation under the brand name of Asacol has been classified by the FDA as a pregnancy class C, which reflects adverse effects in animal but not human studies<sup>[21]</sup>. As such, it is important that women taking 5-ASA drugs are informed about the potential risk of drugs containing DBP, especially when there are alternative 5-ASA formulations that do not contain DBP. Nonetheless, it must be emphasized that the risks of not taking 5-ASA while in remission far outweigh the benefits of avoiding phthalates. In addition, clinicians should consider 5-ASA formulations that release predominantly into the colon and do not contain phthalates. For example, Mezavant is a 5-ASA drug that uses an Multi Matrix system delayed release mechanism,

which allows release to be primarily in the colon where it can be most effective at treating ulcerative colitis. It has been shown to be equally efficacious at achieving IBD remission and does not contain phthalates in its coating<sup>[52]</sup>. Salofalk is another alternative 5-ASA formulation available in Canada and utilizes pH-dependent release. Its Eudagrit-L coating, contains the DEP rather than DBP. Unlike the latter, DEP has not been shown to be harmful in animal studies.

## REGULATION OF PHTHALATES

Throughout the previous decade, much of the media attention covering phthalates has targeted the presence of these plasticizers in children toys. Multiple agencies throughout the world have regulated phthalates in non-medical products including toys, cosmetics, environmental chemicals and health related products<sup>[53-57]</sup>.

Only recently has more attention been focused on phthalates in medications. In December 2012, the Center for Drug Evaluation and Research, a group affiliated with the FDA, recommended against the use of DBP and DEHP as excipients in prescription and nonprescription medications, and encouraged the use of alternative phthalates when possible<sup>[20]</sup>.

The FDA has likely limited their advice to recommendations since clinical and nonclinical research has only demonstrated an association between exposure to these phthalates and developmental problems, and there remains no evidence that medications with phthalates cause phenotypic physiologic abnormalities. These studies have been strictly correlational in nature, and thus a cause-effect relationship cannot be proven.

Accumulating pressure on pharmaceutical companies has encouraged the development of alternatives to phthalates. Pharmaceutical companies have developed excipients that do not contain phthalates. As mentioned, Salofalk and Mezavant are alternative 5-ASA formulations that contain alternative phthalates other than DBP or DEHP or a delayed release mechanism that does not incorporate phthalates<sup>[52,58]</sup>.

## FUTURE DIRECTIONS FOR PHTHALATES

It is currently challenging to identify which medications contain phthalates, along with the specific dosage of phthalates included. Levels of phthalates for many medications are not openly displayed, due to proprietary formulations<sup>[17]</sup>. Current standards do not require that inactive components are included on the package labeling of dietary supplements<sup>[59]</sup>. It is the authors' opinion that government regulators should continue to advocate for the display of all components on drug packaging. It is also important that physicians regularly review and discuss with patients the medicinal ingredients in their medications and supplements. Patients should also be encouraged to use their pharmacists as a resource. Specifically, pregnant woman should review their medications

with pharmacists and discuss the potential presence of phthalates and possible alternatives. Of course, all these decisions should be made in conjunction with the advice of a physician.

Based on the empirical evidence available to date, government regulators and physicians must take caution against phthalates. Recommendations from government regulators should be followed if feasible and will hopefully facilitate the development and utilization of alternatives to phthalates. In order to further explore preliminary concerns, additional research with robust methodology should be conducted. Longitudinal studies capable of demonstrating causation are required to determine whether phthalates actually cause negative health consequences. Studies with larger sample sizes will also help quantify how much DBP and DEHP is being absorbed through specific medications. These studies might help with comparative quantification of bioabsorption between medication and environment (non-medical) exposures, which will help direct policy. Such research will permit government regulatory bodies, drug companies and doctors to respond appropriately.

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## Neuromodulation for fecal incontinence: An effective surgical intervention

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mechanism. Neuromodulation is a minimally invasive procedure with a low rate of adverse events and apparently favorable cost-efficacy profile. This review is intended to expand knowledge about this effective intervention among the non-surgically skilled community who deals with this disabled group of patients.

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**Key words:** Fecal incontinence; Neuromodulation; Sacral nerve stimulation; Biofeedback; Anal sphincter

**Core tip:** This review summarizes the evidence for neuromodulation of fecal incontinence. Neuromodulation is effective for some patients with fecal incontinence of different etiology unknown mechanism; when analyzed by intent to treat analysis, the median responder rate is 59%. The most common serious adverse event is infection at the site of implant which occurs in about 3% of patients. Cost of treatment is high relative to conservative treatment and biofeedback but seems to be cost-effective when offset by gains in quality-adjusted years. Randomized controlled trials comparing neuromodulation with biofeedback therapy in fecal incontinence are advisable to tailor patients' management.

### Abstract

Fecal incontinence is a disabling symptom with medical and social implications, including fear, embarrassment, isolation and even depression. Most patients live in seclusion and have to plan their life around the symptom, with secondary impairment of their quality of life. Conservative management and biofeedback therapy are reported to benefit a good percentage of those affected. However, surgery must be considered in the non-responder population. Recently, sacral nerve electrostimulation, lately named neuromodulation, has been reported to benefit patients with fecal incontinence in randomized controlled trials more than placebo stimulation and conservative management, by some unknown

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### INTRODUCTION

Fecal incontinence (FI) is defined as the accidental loss of solid or liquid stools and is a common disabling condition that is often under-reported at medical consultation

because of fear and embarrassment. In a recent study of > 1500 primary care patients, FI was self-reported by 36.2% of patients, but only 2.7% of them had a medical diagnosis of FI<sup>[1]</sup>. FI has a significant impact on quality of life (QOL) and health expenditure and may facilitate the placement of older patients in nursing home facilities<sup>[2]</sup>. Therefore, increased medical screening of FI is needed because both conservative and interventional treatments are available. Biofeedback therapy to increase rectal awareness of stools and ameliorate anal sphincter response improved continence in about two-thirds of patients in open and randomized controlled trials (RCTs)<sup>[3,4]</sup>. However, patients with severe impairment of rectal sensation and/or previous anal trauma do poorly with biofeedback, and alternative options are desirable in selected patients<sup>[5]</sup>. In the past a number of surgical procedures has been proposed to treat FI. Major drawbacks were the small sample sizes and potential worsening of incontinence<sup>[6]</sup>. Sacral nerve electrostimulation, later also called neuromodulation (NRM), was first applied in 1995 by Matzel *et al*<sup>[7]</sup> with encouraging results in a small group of patients with FI without evidence of anal sphincter defects. The technique was attractive because of its limited side effects and for being minimally invasive. Since then, the effectiveness of NRM in improving FI has been proven in a number of studies, although its mechanism of action remains ill defined<sup>[8]</sup>. However, physicians involved in the treatment of disordered anal continence should consider NRM among potential treatment options, and this review is intended to be a primer for the non-surgical community.

### Search methods

Search terms were fecal incontinence OR anal incontinence and sacral nerve stimulation OR neuromodulation. These searches were limited to human subjects, adults, and studies published in full in the English language between January 1995 and December 2012. Case reports, preliminary studies, and small sample series investigating < 15 patients were not considered. Databases searched were PubMed, Web of Science, Cochrane Reviews, and Embase. The bibliographies of identified studies were also searched for additional references. To address NRM effectiveness, special consideration was given to RCTs and adequately powered prospective trials.

## TECHNIQUE, SAFETY AND MECHANISM OF ACTION OF NRM

### Technique

NRM is a minimally invasive surgical intervention consisting of: (1) a testing evaluation interval; and (2) a second stage with permanent stimulator implantation, provided the testing interval results are clinically successful. The first stage, also termed percutaneous nerve evaluation (PNE), is of most relevance to determine the feasibility of electrode implantation into the sacral foramina, and to demonstrate clinical benefits worth pursuing

with permanent NRM<sup>[9]</sup>. Two technical options are available for PNE: a temporary, percutaneously placed, unipolar stimulation lead to be later removed, or the surgical placement of a quadripolar lead next to a target nerve<sup>[9]</sup>. Both types of leads are then connected to an external pulse generator to be substituted by a permanent pulse generator implanted subcutaneously in case of positive outcome. The permanent implant sized a quarter dollar or 2 euro coin (diameter 24.26 mm, thickness 1.75 mm) is commonly placed in the gluteal area and can be managed by a small handheld device<sup>[9]</sup>. A small retrospective study evaluated outcome and complications of the two PNE techniques<sup>[10]</sup>. No difference in outcome was shown, but the infection rate was slightly higher in patients undergoing surgical placement.

### Safety

The commonest adverse events are implant site pain and paresthesia, which is seen in up to 28% of patients in some large series with careful reporting about safety<sup>[11,12]</sup>. Pain is usually managed conservatively and explant of the device is rarely needed. However, a recent meta-analysis concluded that incidence of implant site pain may be as low as 6%<sup>[8]</sup>. The most serious complication is infection at the implant site, which was seen in up to 10.8% in the largest series of > 100 patients<sup>[11,12]</sup>. The control of site infection may require device explant in approximately half of those affected<sup>[12]</sup>. The meta-analysis by Tan *et al*<sup>[8]</sup> supports diverse evidence indicating that the typical infection rate is 3%, with the proportion requiring the device to be removed for refractory infection being about 3% of those infected. Additional side effects reported in < 8% of patients are urinary incontinence, diarrhea, and extremity pain, which always resolve spontaneously or are effectively managed by medication<sup>[8]</sup>. In older series, broken or displaced electrodes occurred in about 4% of cases<sup>[8]</sup>, and sometimes required device explantation. However, this problem is becoming less frequent since the electrodes were redesigned. Battery replacement is usually required after a median of 7 years<sup>[13]</sup>.

### Mechanism of action

In 1999, Vaizey *et al*<sup>[14]</sup> first reported the effect of NRM on anorectal physiology measured by 24-h solid state catheter manometry in a small group of 10 patients with FI. Resting anal pressure did not change significantly and some evidence of modification of rectal sensitivity and tone was observed. The authors speculated that NRM worked via complex neuromodulation of sacral reflexes to regulate rectal sensitivity and anorectal motility<sup>[14]</sup>. Since then, several studies focused on modifications of anorectal physiology associated with NRM in FI, with conflicting results. In their meta-analysis, Tan *et al*<sup>[8]</sup> concluded that NRM is associated with improvement in anal canal pressure at rest and with voluntary squeezing, and a decrease in the maximum tolerable rectal volume. However, subsequent studies had inconsistent results, with RCTs and long-term studies failing to show a rel-



evant influence of NRM on anal pressure<sup>[15,16]</sup>. When there are significant improvements in anal canal pressure, the size of the effect is small and the final resting and squeeze pressures appear to be below the normal range for healthy controls<sup>[8]</sup>. This is not commensurate with the large clinical effects seen for FI and suggests that the mechanism by which NRM improves continence is not primarily an improvement in anal canal pressure. This issue was addressed in a recent review by Gourcerol *et al*<sup>[17]</sup>, which specifically focused on defining the potential mechanism of action of NRM. The authors speculated on three potential mechanisms: (1) somatovisceral reflex; (2) modulation of the perception of afferent information; and (3) increase in external anal sphincter activity<sup>[17]</sup>. However, no definitive evidence could be found to support any of these and a multifactorial component was further speculated to justify the efficacy of NRM. The authors concluded that NRM is effective almost certainly *via* modulation of spinal and/or supraspinal afferent inputs, but many gaps remain in the understanding of the mechanism of action of NRM<sup>[17]</sup>.

## EFFECTIVENESS

### RCTs

After the early report of Matzel *et al*<sup>[7]</sup>, a number of trials were developed to evaluate the efficacy of NRM in FI<sup>[7,8]</sup>. A major drawback to assessing this literature is the huge variance in inclusion and outcome criteria and follow-up intervals<sup>[8]</sup>. Additional limitations are small sample size (often < 20 patients) and lack of adequate control groups<sup>[8]</sup>. However, the majority of uncontrolled trials reported a favorable outcome in more than two-thirds of patients with limited side-effects. Researchers were unable to identify any clinical and/or functional variable that could predict outcome<sup>[8]</sup>. In earlier reports, patients were selected on findings of either no or marginal evidence of anal sphincter defects. However, this limitation was later dropped because of the unclear definition of the mechanism of action of the treatment<sup>[8]</sup>. In 2005, Leroi *et al*<sup>[18]</sup> reported on the first randomized, controlled, double blind, multicenter study testing the efficacy of NRM in FI and/or severe urgency of any etiology. Patients with an ultrasound diagnosis of sphincter defect were included, provided the defect was not considered to be the main determinant of incontinence<sup>[18]</sup>. After implantation, 27 of 34 patients with FI were randomized in a double-blind crossover manner to NRM active treatment (electrostimulator ON) *vs* placebo (electrostimulator OFF) for a 1-mo period with the device *in situ*. A final interval of 3 mo was also included in the evaluation with patients still blinded, potentially choosing either the ON or the OFF modality<sup>[18]</sup>. Twenty-four patients completed the trial, making the sample underpowered. However, patients reported a significant improvement in both symptoms and QOL scores, and anal physiology when in active treatment compared to placebo, providing evidence that a placebo effect was not the main determi-

nant of NRM outcome<sup>[18]</sup>. However, until recently, NRM was not compared to conservative management (diet, lifestyle modification, constipating drugs, and biofeedback), which is cheaper, commonly available, and often associated with benefits in at least 50% of patients with FI<sup>[3]</sup>. Tjandra *et al*<sup>[15]</sup> randomized 120 patients with FI to either supervised optimal medical therapy or NRM. Conservative treatment included bulking agents, pelvic floor exercises, or lifestyle and dietary manipulations, but it did not include biofeedback<sup>[15]</sup>. NRM was significantly more effective in improving frequency of incontinence with 25 patients regaining perfect continence<sup>[15]</sup>. Cleveland Clinic Continence Score and QOL score were both significantly improved as well<sup>[15]</sup>.

### Open label trials and meta-analysis

In a recent prospective, open label, multicenter trial, Wexner *et al*<sup>[11]</sup> confirmed the effectiveness of NRM in improving FI in a large sample of 120 patients with 112 of them undergoing permanent implantation. The vast majority of patients (83%) reported significant improvement of FI according to the outcome measurement selected, including 41% gaining complete anal continence, after a mean follow-up of 28 mo<sup>[11]</sup>. FI is a chronic disorder, therefore, Mellgren *et al*<sup>[12]</sup> reported on the same cohort after a mean follow-up of 3.1 years (range: 0.2-6.1 years) with at least a partial data set available in 64% of the patients. A significant decrease in episodes of incontinence was still reported by 86% of available patients with 41% regaining continence. A stable improvement in QOL score was also reported by patients<sup>[12]</sup>. To deepen the analysis, a carried forward observation at 3 years was performed showing a 78% success rate. However, the success rate dropped to 59% at 3 years when considering all missing data as failures<sup>[12]</sup>. Historically, anal sphincter disruption has been considered a contraindication to perform NRM, which was not even considered in the presence of a relevant morphological alteration<sup>[7-9]</sup>. However, Chan *et al*<sup>[19]</sup> provided sound evidence against this assumption in a comparative cohort study. The effectiveness of NRM in improving FI and QOL at 1 year was not significantly different in 21 patients with a disrupted external anal sphincter (81% persisting after previous sphincter repair) when compared to the outcome of 32 patients with FI and an intact anal sphincter<sup>[19]</sup>. These data were confirmed by an RCT comparing NRM with conservative treatment in which many patients with defects in both the internal and external sphincters were included, showing that NRM was equally effective in those with or without sphincter disruption<sup>[15]</sup>. The therapeutic potential of NRM compared to conservative treatment in FI has also been reported in several, mostly small studies including patients with distinct pathological conditions, including rectal resection and pelvic irradiation<sup>[8,20,21]</sup>. In these distinct conditions dealt with in the following section, FI response rates may be lower, with about 50% of patients responding to temporary stimulation<sup>[20,21]</sup>. A recent meta-analysis by Tan *et al*<sup>[8]</sup> reported on a total of

Table 1 Effectiveness of neuromodulation in fecal incontinence

Ref.	Sample	Study design	Major findings	Adverse events	Comments
Tjandra <i>et al</i> <sup>[9]</sup>	120 patients with severe FI (solid or liquid FI > 1/ wk) were randomized to 2 groups. Of 60 randomized to test stimulation, 53 received permanent implants. Average age 63 yr; > 90% female. Sphincter defect or scar in 47% of both groups	Single site RCT comparing NRM to optimal medical management	71% of permanently implanted patients (63% of randomized patients - ITT analysis) reported > 50% reduction in FI episodes/wk at 12 mo. FI episodes/wk decreased from 9.5 to 3.1 in SNS group, and not at all in controls. All 4 QOL domains significantly improved in SNS. Anal squeeze pressure was unchanged. SNS significantly different from control on all outcomes	Pain in 6%, seroma in 2%, and excessive tingling in vaginal region in 9%, but no septic complications	Low complication rate and excellent outcomes may be related to this being a single-site study
Wexner <i>et al</i> <sup>[11]</sup>	Multicenter study. 133 received test stimulation; 120 (90.2%) qualified for permanent implant. Average age 60.5 yr; 92% females. Inclusion required > 2 solid or liquid accidents/wk for > 6 mo and > 12 mo postpartum	Multicenter cohort study in United States, Canada, and Australia. Hypothesis was that > 50% would report > 50% reduction in FI frequency at 12 mo compared to baseline. QOL and safety were secondary endpoints	73% of permanently implanted patients (66% of all undergoing test stimulation - ITT analysis) showed > 50% reduction in FI episodes/wk at 12 mo. FI episodes/wk decreased from 9.4 at baseline to 1.9 at 12 mo. All 4 domains of the FI QOL improved significantly. An IAS defect predicted poorer outcome	Pain in 25.8%, paresthesia in 12.5%, infection in 10.8%	
Mellgren <i>et al</i> <sup>[12]</sup>	See Wexner (2010). 77 patients completed the 36 mo FU assessment	This reports the 36 mo outcomes for the Wexner (2010) study	At 36 mo, 86% of 77 patients available for assessment, but only 55% of 120 enrolled patients, reported > 50% reduction in FI	Pain in 28%, paresthesia in 15%, infection in 10%. 5/120 required device explant and 2 required device replacement	ITT analysis under-estimates efficacy because some patients were lost to FU for reasons unrelated to efficacy
Michelsen <i>et al</i> <sup>[16]</sup>	177 patients at single Danish hospital. Average age 60. 142 (80%) had positive PNE and 126 received NRM	Uncontrolled case series	In 107 of 111 who still had stimulator in place at 12 mo, Wexner score decreased in 87 (median decrease of 7) and was unchanged or worse in 20. No significant change in anorectal manometry	15 of 126 with permanent implant had device explanted. There were 2 infections requiring explant	ITT analysis was not possible. Many patients were lost to FU
Hollingshead <i>et al</i> <sup>[13]</sup>	118 patients received PNE, 91 (77%) qualified for NRM; and 86 received NRM	Uncontrolled case series	For all 86, median FI episodes/wk decreased from 8.5 to 1.3 and Wexner score decreased from 15 to 9. In 16% of patients reporting 50% reduction initially, efficacy was lost at median of 11.5 mo	Broken leads in 2. Battery replacement in 7 at mean of 81 mo. No other AEs reported	ITT analysis not possible
Altomare <i>et al</i> <sup>[23]</sup>	94 patients from 6 hospitals underwent PNE, and 60 qualified for and underwent NRM. Average age 58 yr, 83% female	Uncontrolled case series	Of 60 implanted, 2 died (unrelated) and 6 had devices explanted, leaving 52 for 5 year FU. At 5 yr, 37 (39% by ITT) had > 50% decrease in FI frequency. Squeeze and resting pressures increased, maximum tolerated volume decreased	AEs in 8 patients: electrode displacement in 8; pain in 3, allergic reaction in 1; myocardial infarct in 1; unrelated death in 2	ITT success rate at 5 yr was 40% after adjustment for 2 unrelated deaths
Muñoz-Duyos <i>et al</i> <sup>[24]</sup>	Spanish study of 47 patients who received PNE, of whom 29 (62%) received NRM. PNE was ineffective in 16 and 3 had technical failures	Uncontrolled case series with median 3 yr FU. Cost analysis was primary focus	At last FU, 14 were continent and 11 had > 50% reductions in FI frequency. QOL significantly improved. Total direct costs for NRM were €371 434, estimated to be €16 181 per quality adjusted life year. No improvement in anal canal pressures	8 patients experienced pain but none required explantation	ITT response rate was 53.2%
Dudding <i>et al</i> <sup>[22]</sup>	British study of 70 patients who received PNE, of whom 61 had > 50% reduction. At analysis, 51 had received permanent implants, and FU was available for 48. These patients may also be included in the Hollingshead (2011) report	Uncontrolled case series with median 24 mo FU. Primary focus was cost-effectiveness. Direct and indirect costs were estimated by theoretical model of services required rather than on actual costs	At 2 yr FU, 41 of 48 with long-term FU (85.4%) had >50% reduction. Direct costs were estimated at £9795 for SNS compared to £2529 for conservative treatment. The estimated incremental cost-effectiveness ratio was £25 070, which is convenient, being within £30 000 recommended by United Kingdom national guidelines	10/48 had complications including 2 wound infections, 1 lead migration, 5 pain, 2 device failures	ITT response rate was 58.6%. Cost analysis was based on theoretical/ imputed data rather than real costs

Chan <i>et al</i> <sup>[19]</sup>	60 consecutive patients underwent PNE and 53 received NRM. These were separated into 21 with EAS defect vs 32 with intact EAS. One surgeon did all surgeries	Prospective cohort study comparing those with EAS defect to those with intact sphincter	There was a trend for patients with EAS defect to have worse incontinence and poorer squeeze pressures at baseline and FU, but not significant. Outcomes were similar: At 12 mo FU 68.8% with sphincter disruption vs 72.0% with intact sphincter had > 50% reduction in FI. No differences in anal manometry or QOL outcomes	Seroma in 1/53; pain in 3/53. No AEs required explant	Strong support for hypothesis that NRM is equally effective in patients with EAS defects. ITT responder rate for combined group was 63%
Michelsen <i>et al</i> <sup>[28]</sup>	20 patients randomized; 19 had complete data	Randomized prospective crossover comparing NRM continuously for 3 wk to NRM on only during waking hours for 3 wk	Wexner and St Mark's incontinence scores and frequency of soiling were significantly worse during device off period. However, FI frequency was not significantly different between conditions	AEs were not reported	Not directly relevant to efficacy of NRM
Leroi <i>et al</i> <sup>[8]</sup>	34 consecutive FI patients (31 females) considered, 27 eventually studied, 24 completed the trial	Randomized, double-blind, crossover, controlled trial. All 27 patients underwent NRM then randomized in a double-blind crossover design to stimulator ON vs stimulator OFF for 1-mo interval. Patients while blinded choose to meet the final period of 3 mo ON or OFF	Cleveland Clinic Continence score, frequency of FI and urgency, delay in postponing defecation, subjective feeling of improvement, anal physiology, QOL score all significantly improved in the ON interval compared to the OFF interval	10 out of initial 34 reported AE, 4 device explantations; 3 for pain and 1 for infection	First RCT to show effectiveness of NRM compared to placebo; underpowered sample

AE: Adverse event; EAS: External anal sphincter; FU: Follow-up; IAS: Internal anal sphincter.

994 patients undergoing NRM with 665 permanently implanted, confirming significant improvement in symptoms and QOL in patients with FI. A disrupted anus was not the main determinant of outcome<sup>[8]</sup>. However, some criticisms of NRM outcome reports should be considered (Table 1). Outcomes for NRM are often expressed as the proportion of patients receiving a permanent implant who continue to have at follow-up assessment a > 50% reduction in FI relative to baseline<sup>[8,11,12]</sup>. However, this likely overestimates the efficacy of NRM compared to other treatments (e.g., biofeedback therapy), where it is conventional to report effectiveness in terms of an intention-to-treat (ITT) analysis<sup>[13]</sup>. To calculate the ITT response rate, one must include in the denominator all patients who received test stimulation (PNE). When this is done (Table 1), the responder rate ranges from 40% to 66%, with a median of 59%, compared to a median of 85% when only those who received a permanent implant were included in the denominator<sup>[11,22,23]</sup>. In addition, some large studies did not provide the data to calculate the ITT responder rate<sup>[13,14]</sup>. The response to PNE ranges from 64% to 96.7%, with a median of 87%, and 10 reporting at least a 50% reduction in FI from baseline<sup>[8,22-24]</sup>. In a recent Danish study, a questionnaire was mailed to 127 patients with FI and ongoing NRM, to assess subjective patient satisfaction and frequency of incontinence, and 85% responded<sup>[25]</sup>. A total of 57.3% of the responders reported positively about NRM treatment<sup>[25]</sup>, a percentage close to the calculated ITT response rate. In addition, satisfaction with treatment was closely related to pretreatment frequency of incontinence; namely, the more incontinent the patient, the more likely to report treatment dissatisfaction<sup>[25]</sup>. Finally, the effects of NRM are well sustained with 75% of those treated still reporting > 50% symptom reduction at about 7 years<sup>[26]</sup>. In a separate study, Uludağ *et al*<sup>[27]</sup> reported that 84% were still reporting > 50% reduction in FI at 7 years. Switching off the sacral nerve stimulator at night might reduce the device-associated cost, but it is likely associated with a poor long-term outcome<sup>[28]</sup>.

Distinct and rare conditions

The efficacy of NRM has also been investigated under several distinct and rare conditions associated with FI, including: (1) double incontinence; (2) rectal resection; (3) pelvic radiotherapy; (4) anal sphincter atrophy; and (5) spinal lesions. In these conditions, FI is commonly deemed unresponsive to conservative treatment and poorly amenable to surgical intervention<sup>[2-3]</sup>. These conditions have mostly been studied in case reports and small case series of NRM of FI with encouraging results, but no adequately powered RCTs<sup>[9]</sup>. Notwithstanding that NRM to treat FI was developed by sporadic observations of symptom benefit in urinary urge incontinence, few studies have addressed the bene-



fit of NRM in double incontinence<sup>[9]</sup>. Caremel *et al*<sup>[29]</sup> first reported on clinical questionnaires sent to 57 patients with double incontinence treated by permanent implantation, with FI as the main indication for NRM in 60% of them. About two-thirds of patients responded, with 49% reporting an improvement in both fecal and urinary incontinence. Patients implanted for urinary incontinence as the main indication were more likely to report full amelioration of both types of incontinence<sup>[29]</sup>. Recently, Faucheron *et al*<sup>[30]</sup> reported a single-center study of 57 patients (54 women) who underwent PNE and permanent implantation for double incontinence of multiple etiology, with a median follow-up of 62.8 mo. Improvement in both fecal and urinary incontinence was evaluated by dedicated scores, with about 50% of patients reporting amelioration of both symptoms<sup>[30]</sup>. Surprisingly, bladder-related clinical improvement scored slightly lower than bowel-related improvement. Re-intervention rate (29%) and complication rate (12%) were both relatively high<sup>[30]</sup>. Rectal resection for cancer and pelvic radiotherapy are conditions commonly associated with secondary severe alterations in bowel compliance<sup>[3]</sup>. Incontinence is predominant at night and mostly deemed incurable<sup>[2,3]</sup>. Two European groups investigated the efficacy of NRM in these hard-to-treat conditions in small samples. Both studies reported PNE to be effective in improving continence in approximately half of those treated, but the efficacy of permanent implantation was not reported<sup>[20,21]</sup>. Atrophy of the anal sphincter is an additional hard-to-treat FI disease for which NRM has been associated with clinical benefit in open trials. Santoro *et al*<sup>[31]</sup> have reported a single-center study of 28 patients with magnetic-resonance-imaging-documented external anal sphincter atrophy of different severity undergoing permanent implantation for FI. A significant improvement in both FI and QOL scores was reported regardless of severity of sphincter atrophy<sup>[31]</sup>. This study provided indirect evidence of improvement in anal sphincter function as the mechanism of action of NRM<sup>[31]</sup>. Finally, a few studies have evaluated the efficacy of NRM for loss of normal bowel function due to nerve injury, neurological disease, or congenital defects of the nervous system - so-called neurogenic bowel. Holzer *et al*<sup>[32]</sup> assessed clinical outcome in a cohort of 29 patients undergoing permanent implantation for FI of mixed neurological etiology, including diabetes. The authors claimed that most patients were symptomatically improved, but outcome parameters were ill defined<sup>[32]</sup>. Recently, an Italian group reported on the efficacy of NRM in improving symptoms of pelvic floor dysfunction in 23 patients with incomplete spinal cord damage<sup>[33]</sup>. A significant improvement in FI was found in the majority of patients, but the grouping of patients with both constipation and FI made it hard to interpret the results<sup>[33]</sup>.

## COST

The cost of NRM is high when compared to conserva-

tive medical management, pelvic floor exercises and biofeedback therapy. Actual cost of NRM varies widely among countries as well as health insurance conditions. However, studies from three different countries have concluded that NRM is cost-effective when offset by the quality-adjusted life-years gained, and that it is likely to be reimbursed by government health programs<sup>[22,24,34,35]</sup>.

## CONCLUSION

In conclusion, NRM is effective for FI of diverse etiology. Encouraging results have also been reported for FI therapy in distinct and rare conditions, but RCTs are lacking and no firm conclusion can be actually drawn. NRM is reported to have long-term benefit in more than two-thirds of patients with FI undergoing permanent implantation, by some as-yet-unknown mechanism. However, when analyzed by ITT analysis, the median responder rate drops to 59% of those treated. NRM is a minimally invasive procedure. The most common serious adverse event is infection at the site of implantation, which occurs in about 3% of cases and requires device explantation in about 3% of all patients receiving permanent implants. Cost of treatment is high relative to that of conservative treatment and biofeedback but there are studies from different countries suggesting that NRM is cost-effective when offset by gains in quality-adjusted life years. However, RCTs comparing NRM to biofeedback therapy for FI are required to resolve this issue.

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## Interaction of IFNL3 with insulin resistance, steatosis and lipid metabolism in chronic hepatitis C virus infection

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### Abstract

Metabolic changes are inextricably linked to chronic hepatitis C (CHC). Recently polymorphisms in the IFNL3 (IL28B) region have been shown to be strongly associated with spontaneous and treatment induced recovery from hepatitis C virus (HCV) infection. Further, circumstantial evidence suggests a link between IFNL3 single nucleotide polymorphisms and lipid metabolism, steatosis and insulin resistance in CHC. The emerging picture suggests that the responder genotypes of IFNL3 polymorphisms are associated with a higher serum lipid profile, and less frequent steatosis and insulin resistance. This review analyzes the current data regarding this interaction and its meaning for HCV pathogenesis and disease progression.

reserved.

**Key words:** IFNL3; Chronic hepatitis C; Insulin resistance; Lipids

**Core tip:** Metabolic changes are inextricably linked to chronic hepatitis C (CHC). Recently polymorphisms in the IFNL3 region have been shown to be strongly associated with spontaneous and treatment induced recovery from hepatitis C virus (HCV) infection. Further, circumstantial evidence suggests a link between IFNL3 single nucleotide polymorphisms and lipid metabolism, steatosis and insulin resistance in CHC. The emerging picture suggests that the responder genotypes of IFNL3 polymorphisms are associated with a higher serum lipid profile, and less frequent steatosis and insulin resistance. This review analyzes the current data regarding this interaction and its meaning for HCV pathogenesis and disease progression.

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### INTRODUCTION

Hepatitis C virus (HCV) infection affects about 170 million people worldwide. It leads to slow but progressive hepatic inflammation and fibrosis in as many as 70% of infected individuals. Over time, 20% will develop cirrhosis and its related complications, and about 1%-2% of subjects may develop hepatocellular carcinoma after 2-3 decades of infection<sup>[1]</sup>. The natural history of HCV infection in terms of chronicity and disease progression seems to be largely determined by the host immune response to



virus-infected hepatocytes. The interplay between many viral, host, genetic and environmental factors modifies the course of HCV infection and the degree of hepatic inflammation. In this context, the discovery of the association between IFNL3 polymorphisms and spontaneous or treatment induced clearance of HCV presented a major milestone in the study of chronic hepatitis C (CHC)<sup>[2-5]</sup>.

Metabolic syndrome is a constellation of problems that includes obesity, dyslipidemia, diabetes, and insulin resistance<sup>[6]</sup>. The prevalence of metabolic syndrome is increasing, paralleling the obesity epidemic worldwide and in the United States and European countries especially<sup>[7]</sup>. Multiple levels of interaction between HCV, metabolic syndrome and genetics have been recently postulated, including a molecular interaction between IFNL3 polymorphisms and HCV associated glucose and lipid metabolism. In this review we summarize the current clinical evidence for an interaction between IFNL3 polymorphisms and metabolic syndrome, and its clinical implications.

## IFNS LAMBDA AND HCV

The type III interferon (IFN) or IFN- $\lambda$  (IFNL) family consists of three members: IFN- $\lambda$ 1, IFN- $\lambda$ 2 and IFN- $\lambda$ 3 (formerly known as IL-29, IL-28A and IL-28B), which were discovered in 2003 by computational prediction and are genetically distinct from type I IFNs<sup>[8,9]</sup>, and a fourth, IFN- $\lambda$ 4, recently described from primary human hepatocytes<sup>[10]</sup>. Another recent study identified a novel TT/-G polymorphism in the CpG region upstream of IL28B, which is a better predictor of HCV clearance than rs12979860<sup>[11]</sup>. Whereas IFN- $\alpha$  binds to the constitutively expressed type I IFN receptor in almost all nucleated cells, IFN- $\lambda$  cytokines bind to a heterodimer of part of the IL-10 receptor and the IFNL receptor (IL10RA and IL28R, respectively)<sup>[12]</sup>, the latter of which is only expressed in restricted cell types, including epithelial cells, plasmacytoid dendritic cells and hepatocytes<sup>[13]</sup>. IFNL stimulation results in the upregulation of interferon stimulated genes (ISGs) *via* the Jak-STAT (Janus kinase-signal transducer and activator of transcription) pathway similar to type I IFNs<sup>[12,14]</sup>.

In an attempt to identify host genetic markers for IFN responsiveness to predict treatment outcome in CHC, genome-wide association studies identified single nucleotide polymorphisms (SNPs) in the IFNL3 (IL28B) region to be strongly associated not only with response to treatment with pegylated IFN- $\alpha$  (Peg-IFN- $\alpha$ ) with ribavirin (RBV) in HCV genotype 1 infection<sup>[2-5]</sup>, but also spontaneous recovery<sup>[15]</sup> (Table 1). This association has been validated in different ethnic populations and for various HCV genotypes<sup>[16,17]</sup>. In a recent meta-analysis, IFNL3 polymorphisms seem to be clinically useful even in the era of new direct acting antiviral drugs<sup>[18]</sup>. Moreover, the responder genotypes of the IFNL3 polymorphisms have been reported to be associated with increased hepatic inflammation in CHC patients<sup>[2,19]</sup>.

**Table 1 Summary of the IFNL3 polymorphisms identified by genome-wide association studies**

GWAS	Total population	IFNL3 SNP	Wild (responder) type/non-responder allele
Suppiah <i>et al</i> <sup>[3]</sup>	848	rs8099917 <sup>2</sup>	T/G
Tanaka <i>et al</i> <sup>[4]</sup>	314	rs8099917	T/G
		rs12980275	A/G
Rauch <i>et al</i> <sup>[5]</sup>	914	rs8099917	T/G
Ga <i>et al</i> <sup>[6]</sup>	1137	rs12979860 <sup>1</sup>	C/T

<sup>1</sup>rs12979860 and <sup>2</sup>rs8099917 single nucleotide polymorphisms (SNPs) are strongly associated with clearance and commonly used in clinical practice. GWAS: Genome-wide association studies.

## HCV-ASSOCIATED METABOLIC CHANGES

CHC can be considered not only a viral disease, but also a metabolic disease. HCV interacts with lipid metabolism leading to steatosis, it impairs glucose metabolism leading to insulin resistance (IR) and diabetes mellitus type II and is associated with an increased risk of carotid atherosclerosis<sup>[20,21]</sup>.

The prevalence of steatosis in patients with CHC is reported to be between 40% and 80% depending on the features of the population studied in terms of alcohol consumption, prevalence of overweight/obesity, diabetes and other risk factors for fatty liver<sup>[22,23]</sup>. However, when all common contributing factors to steatosis have been excluded, the prevalence of steatosis in CHC still remains about 40%. This figure represents an approximately 2-fold increase compared to the prevalence of steatosis in other common chronic liver diseases such as chronic hepatitis B virus infection (20%)<sup>[24]</sup>.

Various studies have shown that both host and viral factors may contribute to the development of steatosis, with the relative importance of each varying with HCV genotype. In particular, in patients infected with HCV genotype 3, steatosis seems to be mostly virus-induced and often severe<sup>[2,25]</sup>. In contrast, in patients infected with non-genotype 3, steatosis seems to be mainly associated with host metabolic factors and correlates with body mass index (BMI) and central adiposity<sup>[3,26,27]</sup>.

Steatosis is known to have deleterious clinical consequences in CHC, as it is associated with accelerated progression of liver fibrosis<sup>[28]</sup> and probably HCC<sup>[29]</sup>. It has also been shown in large clinical trials that steatosis impairs the response to antiviral therapy<sup>[30]</sup>. However, the effect is more prominent in patients with non-3a genotype<sup>[30]</sup>, likely due to IR as the underlying mechanism affecting the response to standard dual therapy with Peg-IFN- $\alpha$ /RBV, and suggesting that the viral steatosis does not impair response to treatment<sup>[25]</sup>. Increasing levels of IR are associated with reduced rates of rapid virological response (RVR) as well as sustained virological response (SVR) in patients with HCV genotype 1, 2, 3 and 4 infections when treated with dual therapy comprising Peg-IFN- $\alpha$  and RBV<sup>[31-33]</sup>. This observation has been confirmed in two meta-anal-

yses<sup>[34,35]</sup>. A direct correlation between lipid profiles and the virologic response to Peg-IFN- $\alpha$  and RBV was also reported in some recent studies<sup>[36,37]</sup>.

## IFNL3 POLYMORPHISM AND LIPID METABOLISM

Since the discovery of the correlation of IFNL3 polymorphisms with HCV clearance, there is an accumulating body of evidence about an association between these polymorphisms and metabolic changes in CHC.

The IFNL3 responder genotype is associated with less pronounced disturbances of lipid metabolism in CHC, as reflected by higher serum cholesterol and lipoprotein levels: CHC genotype 1 individuals with IFNL3 rs12979860 CC genotype have significantly higher apolipoprotein B and low-density lipoprotein cholesterol (LDL-C) levels<sup>[38]</sup>, and lower serum apolipoprotein E levels<sup>[39]</sup>, compared to those with the non-responder CT and TT genotypes. In accordance with these findings, another study from Japan showed that the responder genotype of rs8099917 TT was associated with high LDL-C levels and high SVR<sup>[40]</sup>. This association may result from suppression of hepatic lipase and lipoprotein lipase by endogenous interferon, which in turn decreases serum LDL-C, implying a stronger endogenous interferon response to HCV in those subjects<sup>[36]</sup>.

In a small cohort (55 patients), Sheridan *et al.*<sup>[39]</sup> showed that although HCV RNA was significantly higher in those with rs12979860 CC genotype, this difference was mainly accounted for by a higher non-lipoviral particles (LVP) fraction. It is known that LVPs are low-density HCV particles that have high infectivity<sup>[41]</sup>. This may partially explain the paradox that the IFNL3 responder genotypes have higher HCVRNA total viral load, despite high viral load being a negative predictor of SVR<sup>[42]</sup>. However, further confirmation of this data in larger cohorts is required before any final conclusion can be extracted.

Chiba-Falek *et al.*<sup>[43]</sup> investigated the genetic basis for the variance of LDL-C and apolipoprotein B levels in CHC patients and their potential interaction with IFNL3 genotype: Their data show that two of the APOE genomic region polymorphisms, rs7412 and rs429358 (which defines the  $\epsilon$ 4 isoform), appear to be associated with serum lipoprotein levels in HCV patients. Polymorphisms in  $\epsilon$ 4, however, were not associated with apolipoprotein E levels in HCV-infected Caucasians patients (in contrast to a healthy control cohort), and the overall amount of variance in serum apolipoprotein E levels explained by APOE genotype was much lower in the HCV cohort (7% *vs* 20%). A recent genome-wide association studies in non-HCV-infected cohorts suggested associations of polymorphisms at the TOMM40-APOE genomic region with multiple lipid traits (LDL-C, triglycerides)<sup>[44]</sup>. In particular, two nonsynonymous SNPs in exon 4 of the APOE gene, rs429358 and rs7412 have been associated with lipid levels<sup>[44]</sup>. Further this study also refers

to a potential interaction between TOMM40-APOE and IFNL3 polymorphisms, as rs429358 was associated with apolipoprotein B levels in a IFNL3 genotype dependent manner, *i.e.*, the effect was more profound in patients carrying rs12979860 CC, than those carrying rs12979860 CT/TT. These results together suggest that HCV-associated dyslipidemia may not be controlled to the same extent by the same genes that affect lipids/lipoproteins in healthy (non-HCV infected) cohorts<sup>[43]</sup>.

There is increasing evidence that the life cycle of HCV is directly linked to host lipoproteins: (1) HCV circulates in plasma with lipoprotein as an infectious complex; (2) Hepatocyte lipoprotein receptors are involved in HCV entry; (3) Replication of HCV RNA in hepatic cells is inhibited by inhibitors of lipid metabolism; (4) HCV particles released from hepatocytes are attached to lipoproteins; and (5) Serum lipid profiles (LDL-C, HDL-C and triglycerides) are associated with higher rates of spontaneous or treatment-induced HCV clearance<sup>[45]</sup>. At least, the latter is also affected by IFNL3 polymorphisms, opening up the possibility of interaction in mediating this effect. Thus, better understanding of the interaction between lipids, IFNL3 polymorphisms and the HCV life cycle will improve our understanding of HCV pathogenesis and open new avenues in treating HCV infection.

## IFNL3 POLYMORPHISM AND STEATOSIS

The relationship between steatosis and IFNL3 genotype is still subject to debate, as the current literature demonstrates conflicting results (see Table 2). A retrospective analysis of 1604 patients enrolled in the IDEAL trial (Individualized Dosing Efficacy Versus Flat Dosing to Assess Optimal Pegylated Interferon Therapy) of HCV genotype 1 patients showed that the IFNL3 rs12979860 CC responder genotype was significantly associated with higher pretreatment LDL-C levels and less frequent hepatic steatosis<sup>[46]</sup>. In keeping with this, other recent studies show the same association between the IFNL3 rs12979860 CC genotype and less frequent steatosis in CHC genotype 1<sup>[47-49]</sup>. This observation also extends to two other IFNL3 polymorphisms, rs8099917<sup>[50]</sup> and rs12980275, which were associated with steatosis in genotypes non-3<sup>[51]</sup>. In contrast, a study from Japan failed to find a significant association between rs8099917 and hepatic steatosis in 122 Japanese Mongolian patients infected with HCV genotype 1b<sup>[52]</sup>, and another study from Spain failed to confirm an association between rs12979860 and steatosis in 445 Caucasian patients<sup>[53]</sup>. These conflicting results may be owing to the relatively small sample size of these cohorts, differences in ethnicity, population characteristics and local other risk factors for steatosis, different IFNL3 SNPs being investigated, which may exhibit different features, and the respective assessments of individual pathologists.

In the setting of liver transplantation for CHC, a recent abstract presented at the European Association for the Study of the Liver (EASL) meeting 2013 suggested

**Table 2** *IFNL3* polymorphisms and steatosis in chronic hepatitis C

No. of patients	Study design	HCV genotype	<i>IFNL3</i> SNP	Results	Ethnicity	Ref.
1604	Retrospective	HCV-1	<i>rs12979860</i>	CC genotype associated with higher pretreatment LDL-C levels and less frequent hepatic steatosis	Caucasians	46
145 180	Retrospective analysis of two Independent cohorts: (1) (antifibrotic) Study cohort; (2) Duke cohort	HCV-1	<i>rs12979860</i>	CC genotype associated with less hepatic steatosis	122 (84.1%) Caucasians 130 (72.2%) Caucasians	47
434	multi-center, Retrospective	HCV-1	<i>rs12979860</i>	CC genotype associated with less frequent hepatic steatosis	Caucasians	48
202	Prospective	HCV-1: 181 (89.6%) HCV-4: 21 (10.4%)	<i>rs12979860</i>	CC genotype associated with less frequent hepatic steatosis	Caucasians	49
153	Retrospective	HCV-1b	<i>rs8099917</i>	TT genotype associated with less hepatic steatosis (vesicular and clear cell changes)	Japanese	50
626	Retrospective analysis of the Swiss Hepatitis C Cohort Study	Non-HCV-3	<i>rs12980275</i>	G associated with less hepatic steatosis only in non-HCV-3	Caucasians	51
122	Retrospective	HCV-1b	<i>rs8099917</i>	No association with hepatic steatosis	Japanese Mongolian	52
445	Retrospective	HCV-1: 303 (68.1%) HCV-2: 13 (2.9%) HCV-3: 82 (18.4%) HCV-4: 47 (10.6%)	<i>rs12979860</i>	No association with hepatic steatosis	Caucasian	53

HCV: Hepatitis C virus; LDL-C: Low-density lipoprotein cholesterol.

**Table 3** *IFNL3* polymorphisms and insulin resistance in chronic hepatitis C

Cohorts size	Study design	HCV genotype	<i>IFNL3</i> SNP	% IR	HOMA-IR	Results	Ethnicity	Ref.
434	Multi-center, retrospective	HCV-1 ( <i>n</i> = 434)	<i>rs12979860</i>	50%	> 3	CC genotype associated with reduced IR	Caucasians	48
202	Prospective	HCV-1 ( <i>n</i> = 181) HCV-4 ( <i>n</i> = 21)	<i>rs12979860</i>	32.20% (65/202)	≥ 3	CC genotype associated with reduced IR	Caucasians	49
328	Retrospective	HCV-1 ( <i>n</i> = 328)	<i>rs8099917</i>	50% (84/168) in TT genotype <i>vs</i> 69.7% (53/76) in TG/GG genotype	≥ 2.45 in TT genotype <i>vs</i> ≥ 1.55 in TG/GG genotype	No differences in <i>IFNL3</i> genotype distribution according to HOMA-IR	Japanese	57
240	Retrospective	HCV-1 ( <i>n</i> = 188) HCV-2 ( <i>n</i> = 3) HCV- (n = 30) HCV-4 ( <i>n</i> = 19)	<i>rs12979860</i>	46% (89/193)	≥ 2	No differences in HOMA-IR levels according to <i>IFNL3</i> genotypes	Caucasians	59

HCV: Hepatitis C virus; IR: Insulin resistance.

that *IFNL3* rs12979860 TT nonresponder genotypes had an increased incidence of graft steatosis over time, while recipient *IFNL3* was not associated with steatosis<sup>[54]</sup>.

In an attempt to better understand the interaction of *IFNL3* polymorphisms with other polymorphisms in influencing steatosis, a recent study investigated the interaction between *IFNL3* rs12979860 and the Patatin like phospholipase domain-containing 3 (PNPLA3) rs738409 polymorphism, a strong determinant of hepatic fat accumulation and steatohepatitis<sup>[55]</sup>. Albeit, the association between rs12979860 genotype and steatosis was independent of PNPLA3 GG genotype, the rs12979860 CC genotype protected from steatosis only in patients positive for the PNPLA3 G variant, a genetic risk factor for severe steatosis<sup>[56]</sup>. In another study, the PNPLA3 G variant showed a close association with steatosis in patients with

rs12979860 CT/TT, but not rs12979860 CC genotype<sup>[53]</sup>. These findings suggest a potential interaction between *IFNL3* and PNPLA3 polymorphisms on the risk for steatosis in non-genotype 3 CHC patients, though further analysis is required to better understand the nature of this interaction.

Finally, an interaction between *IFNL3* genotype and an amino acid substitution at residue 70 (aa70) of the HCV core region has been suggested<sup>[52]</sup>. Although these authors failed to find a direct association between rs8099917 and hepatic steatosis, they found significant associations between rs8099917 and aa70 and between aa70 and hepatic steatosis<sup>[52]</sup>. This suggests that the amino acid at residue 70 of the HCV core region should be considered as a parameter for adjustment in any future studies of the correlation between *IFNL3* genotype and steatosis.



## IFNL3 POLYMORPHISM AND INSULIN RESISTANCE

The relationship between IR measured by HOMA and IFNL3 genotype is still subject to debate (see Table 3). Two recent reports showed that the responder IFNL3 rs12979860 CC genotype was associated with reduced IR in HCV genotype 1 patients<sup>[48,49]</sup>, while other reports failed to find this association<sup>[57-59]</sup> with either rs8099917<sup>[57]</sup> or rs12979860<sup>[57-59]</sup>. Interestingly, a recent study from Spain shows that IR can predict SVR in CHC patients independently of the IFNL3 rs12979860 polymorphism<sup>[59]</sup>. This is quite intriguing as it sheds new light on the clinical observations linking higher LDL, less steatosis and lower insulin resistance with SVR.

## CONCLUSION

In conclusion, the discovery of IFNL3 polymorphisms and their impact on CHC presents a major breakthrough in HCV research. The association of IFNL3 responder genotypes with higher LDL, less steatosis, less insulin resistance and SVR suggests a mechanistic link between IFNL3 and the metabolic syndrome in CHC. This sheds new light on the pathogenesis of CHC and opens exciting avenues to explore. Further work is needed to better understand the mechanistic explanation of these inter-related associations, and its potential implications in improving the current management of CHC patients.

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## Gastrointestinal complications of systemic sclerosis

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**Key words:** Systemic sclerosis; Gastrointestinal tract; Manometry; Endoscopy; Diagnosis; Treatment

**Core tip:** Although often overlooked by clinicians, the gastrointestinal tract is the most commonly damaged system in patients with progressive systemic sclerosis. Virtually all parts of the gastrointestinal tract can be involved, although the esophagus is the most frequently reported. The mechanisms of gastrointestinal tract involvement have not been clarified; however, vascular damage, excessive accumulation of collagen, and immunological abnormalities may play a role because they are the most frequent histological findings in biopsies and autopsies. Non-specific symptoms, including dyspepsia, nausea, vomiting, and abdominal distension are common complaints. Although supportive and symptomatic treatment is the main therapeutic strategy for systemic sclerosis, early diagnosis is critical for improving patient prognosis.

### Abstract

Systemic sclerosis is an autoimmune disease characterized by progressive skin thickening and tightness. Pulmonary interstitial fibrosis and kidney damage are the most important indicators for mortality; however, the gastrointestinal tract is the most commonly damaged system. Virtually all parts of the gastrointestinal (GI) tract can be involved, although the esophagus is the most frequently reported. The mechanisms that cause such extensive damage are generally unclear, but vascular changes, immunological abnormalities, excessive accumulation of collagen in the submucosa, smooth muscle atrophy and neuropathy may participate because these are the most common histological findings in biopsies and autopsies. Most patients with GI tract involvement complain about dyspepsia, nausea, vomiting, abdominal bloating/distension, and fecal incontinence. These symptoms are generally mild during the early stage of the disease and are likely ignored by physicians. As the disease becomes more advanced, however, patient quality of life is markedly influenced, whereby malnutrition and shortened survival are the usual consequences. The diagnosis for systemic sclerosis is based on manometry measurements and an endoscopy examination. Supportive and symptomatic treatment is the main therapeutic strategy; however, an early diagnosis is critical for successful management.

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### INTRODUCTION

Systemic sclerosis (SSc) is a chronic autoimmune disease with an unknown etiology. The most common clinical presentations include: Raynaud's phenomenon, skin thickening and tightness caused by widespread vasculopathy and excessive fibrosis. The gastrointestinal (GI) tract is the most commonly involved internal organ in SSc. It is estimated that GI involvement occurs in approximately 70%-90% of SSc patients<sup>[1-3]</sup>; however, a recent study by Schmeiser *et al*<sup>[4]</sup> has shown that 98.9% of SSc patients

suffered from GI symptoms. Additionally, the symptoms of GI manifestation can be mild to severe, including pain, dysphagia, vomiting, diarrhea, constipation and fecal incontinence<sup>[5]</sup>. Anatomically, GI involvement can affect the whole length of the GI, starting from the mouth to the anus. In this review, we have highlighted the clinical features of each anatomical GI region believed to be involved in SSc, as well as the possible treatment approaches.

## **PATHOLOGY AND PATHOGENESIS OF THE GI INVOLVEMENT IN SSc**

Although the entire GI tract can be affected, the underlying pathological changes and symptoms are similar in all parts of the GI, from the esophagus to the rectum. Additionally, vascular changes, immunological abnormalities, excessive accumulation of collagen in the submucosa and smooth muscle atrophy are histological hallmarks of SSc found in the digestive tract walls from patient biopsies and autopsies<sup>[1]</sup>. The progression of the GI involvement in SSc patients ranges from a grade of 0-2. These scores were based on the following parameters: (1) Vascular damage to the vasa nervorum (grade 0), which is a part of the characteristic SSc vasculopathy that can lead to ischemia; (2) Neurogenic impairment (grade 1), which is secondary to ischemia but causes damage to neurons of the intestinal wall; and (3) Myogenic dysfunction (grade 2), whereby normal smooth muscle is replaced by collagenous fibrosis and may cause atrophy<sup>[2,4]</sup>. This progression may explain the characteristic pathological changes observed in SSc patients.

The pathogenesis of the GI complications that occur during SSc is generally unknown. Vascular and auto-immune hypotheses have been proposed to explain the GI histopathological changes observed in SSc<sup>[5]</sup>. The vascular change hypothesis suggests that the initial GI lesions occur because of a neural dysfunction caused by arteriolar changes in the vasa nervorum or by increased collagen deposition. Moreover, studies have shown that mucosal blood flow to the stomach and duodenum are reduced and that vascular insufficiency occurs before smooth muscle atrophy develops. Additionally, increased proliferation and fibrosis of the adventitia may also occur. All these vascular changes can lead to ischemia, which in turn may cause neuron damage and collagen tissue compression of nerves. Vascular ectasia with focal intra-vascular thrombi and antrum fibromuscular hyperplasia also can occur with SSc, providing additional evidence for the vascular change hypothesis.

It is generally accepted that the immune system participates in SSc pathogenesis. One study has shown that damaged stomach endothelial cells express high levels of the cell adhesion molecules including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and can attract activated lymphocytes that move into the damaged sites. The increased CD4<sup>+</sup>/CD8<sup>+</sup> ratio in the gastric mucosa T cell infiltrate suggests that the acquired immune response is a trigger of GI damage during the

early stage of SSc<sup>[1]</sup>. Additionally, weak expression of vascular endothelial growth factor in the gastric submucosa suggests angiogenesis impairment, which then causes nerve plexuses dysfunction and smooth muscle atrophy. Antibodies to the M3 muscarinic receptor, which can block binding of the enteric cholinergic neurotransmission, have also been detected in SSc patients<sup>[6]</sup>. As GI damage advances during SSc, severe fibrosis, with abundant type I and III collagen deposition in the lamina propria and muscularis mucosa, can be observed, and these changes are associated with smooth muscle atrophy and fibrosis. Furthermore, a prominent T-cell infiltration, with a significantly elevated CD4<sup>+</sup>/CD8<sup>+</sup> ratio, has been detected in SSc patients with GI involvement. Moreover, over-expression of fibrogenic cytokines, such as transformation growth factor  $\beta$ , connective tissue growth factor, endothelin-1 and  $\alpha$ -smooth muscle actin have been observed around intestinal glands and blood vessels<sup>[7,8]</sup>.

The association between autoantibodies and SSc GI damage has also attracted great interest. Howe *et al.*<sup>[9]</sup> reported the presence of anti-myenteric neuron antibodies in some SSc patients, suggesting that this autoantibody may be associated with the GI symptoms that occur in some SSc patients. Nishimagi *et al.* had found that the presence of anti-centromere antibodies (ACA) or Scl-70 antibodies were less frequent in patients with severe GI damage; however, there was an increased frequency of anti-U3RNP (ribonucleoprotein) and anti-U1RNP antibodies as well as an increased ratio of Th/To cells<sup>[10,11]</sup>. Additionally, there was a higher incidence of severe diarrhea in patients with anti-U3RNP antibodies but not in patients with anti-U1RNP antibodies. Thoma *et al.* found that there was a negative association between diarrhea and pulmonary fibrosis, although this association was not statistically significant. In general, however, there is no reported SSc GI involvement between localized and diffuse SSc subtypes<sup>[12]</sup>.

## **CLINICAL FEATURES**

The symptoms of GI damage in SSc patients, including pain, dysphagia, vomiting, diarrhea, constipation, fecal incontinence, and weight loss vary in severity. Even in patients without GI symptoms, up to 77% of them had reflux-esophagitis, 85% had distal esophagus dysmotility, and 92% had gastritis when evaluated by oesophago-gastro-duodenoscopy<sup>[13]</sup>. In fact, the whole GI tract may be involved and contribute to the symptoms listed above; however, different GI regions also have their own specific symptom presentations.

### **Oral cavity**

Facial skin tightness and thickening limits the opening of the mouth and interferes with oral intake and mastication. Approximately 20% of patients develop secondary Sjögren's syndrome, which may cause dysphagia, difficulty in swallowing, and periodontal disease and may further impair a patient's ability to maintain a good quality of life

and nutritional status<sup>[9]</sup>.

### Esophagus

The esophagus is the most commonly involved and most intensively studied GI complication in SSc patients. Up to 96% of SSc patients have esophageal complications, including esophageal motility abnormalities, lower esophageal sphincter (LES) abnormalities, gastroesophageal reflux disease (GERD) and Barrett's esophagus. A recent study has shown that esophageal involvement is more pronounced in SSc patients with positive anti-centromere antibodies compared with patients with increased levels of anti-topoisomerase I (Scl-70) or anti-nuclear antibodies<sup>[14]</sup>.

**Esophageal dysmotility and GERD:** Esophageal dysmotility is the most common GI manifestation in SSc patients. Damage to the distal two-thirds of the esophagus smooth muscle during SSc causes decreased or even complete loss of peristalsis in the smooth muscle and distal portion of the esophagus, delaying food bolus transportation and clearance of refluxed materials from the stomach<sup>[15]</sup>. Dysphagia and difficulty in swallowing are common complaints.

**GERD:** The damage to the lower two-thirds of the esophagus caused by SSc often leads to a weakened LES. LES abnormalities, which presents as a low baseline pressure, results in the sphincter neither opening normally when swallowing nor closing completely afterwards. Such abnormalities allow for a pathological gastric acid reflux to the esophagus, which causes further damage to the LES. This condition is known as GERD. Initially esophageal damage caused by GERD manifests as simple peptic esophagitis, but it can progress to erosive esophagitis, bleeding and frank ulceration. If left untreated, esophageal stricture, fistulas and achalasia-like syndrome may occur<sup>[2]</sup>. Patients with GERD usually have heartburn, dysphagia, substernal chest pain, nausea and vomiting after eating; however, the intensity of the symptoms is not related to the severity of GERD. It is reported that GERD severity is associated with pulmonary interstitial fibrosis<sup>[1]</sup>.

**Barrett's esophagus:** Chronic GERD can lead to Barrett's esophagus. The estimated prevalence of Barrett's esophagus in SSc patients is 6.8%-12.7%<sup>[16]</sup>. Additionally, Barrett's esophagus in SSc patients is associated with an increased risk of esophageal carcinoma<sup>[1,2,5]</sup>.

### Stomach

It is reported that stomach involvement occurs in 10%-75% of SSc patients<sup>[17]</sup>. The gastric manifestations of SSc include gastric antral vascular ectasia (GAVE), which is typically presented as "watermelon stomach" during gastro-endoscopy examinations. Additionally, GAVE can cause gastric dysmotility, which leads to delayed gastric emptying or gastroparesis. Moreover, these SSc patients may have GI bleeding, early satiety, bloating,

dyspepsia, nausea and vomiting.

**GAVE:** The appearance of GAVE under gastroendoscopy observation is unique and is characterized by multiple, parallel longitudinal columns of red vessels within the gastric antrum radiating to the pylorus, resembling the stripes on a watermelon. This condition, therefore, is also known as "watermelon stomach". GAVE can precede an SSc diagnosis<sup>[18]</sup>. It is estimated that the prevalence of GAVE ranges from 5.7% to 14% of SSc patients<sup>[1,5]</sup>. GAVE can sometimes manifest itself as severe GI bleeding, although achlorhydria, with or without pernicious anemia, is more common. Furthermore, the pernicious anemia is usually microcytic.

**Gastroparesis:** This condition is the result of chronic gastric motility alternations. These patients may experience delayed gastric emptying or complete gastric paralysis. Delayed emptying can result in early satiety, bloating, dyspepsia, nausea and vomiting. Succussion splashing during examinations often suggests gastroparesis. Delayed emptying occurs equally with solid and liquid meals and can make GERD even worse.

### Small bowel

The small intestine is the second most commonly involved portion of GI tract during SSc, following the esophagus. It is suspected that the small intestine function is compromised in 40% of SSc patients<sup>[19]</sup>. Although most mild cases have no symptoms, bloating, vomiting, abdominal pain, diarrhea, pseudo-obstruction, malabsorption and weight loss may occur in severely affected patients. Small intestine hypomotility is the primary abnormality and may lead to pseudo-obstruction and bacterial overgrowth, which is the major cause of malnutrition in SSc patients. Additionally, pneumatosis cystoides intestinalis (PCI) may occur but is a rare condition.

### Intestinal hypomotility and secondary bacterial overgrowth

Intestinal dysmotility has been reported in 40%-88% of SSc patients<sup>[5]</sup>. Manometric and electrophysiological studies have revealed neuropathy of the enteric nervous system in SSc patients with intestinal dysmotility. Additionally, the autoantibody that inhibits M3-muscarinic receptor-mediated enteric cholinergic neurotransmission was also detected in these patients<sup>[20,21]</sup>. Intestinal hypomotility can result in nausea, vomiting, bloating, distension, anorexia and abdominal pain. Because decreased motility of the small intestine can result in intestine contents stasis, it is believed that stasis of intestinal contents can cause small intestinal bacterial overgrowth (SIBO). SIBO is defined as the presence of more than  $1 \times 10^5$  organisms per millimeter of duodenal aspirate fluid. It is not a rare disorder and has been detected in up to 55.5% of SSc patients<sup>[22,23]</sup>. Additionally, it has been observed that SIBO is more prevalent in patients with limited SSc. Bacteria overgrowth competes with the host for nutrition and causes malabsorption of fat, proteins, carbohy-



drates and vitamins<sup>[5]</sup>. SSc patients with SIBO, therefore, have lower levels of serum albumin and total protein, as well as vitamin B<sub>12</sub> and ferritin. The symptoms caused by SIBO are similar to those caused by small intestinal hypomotility; however, steatorrhea, multiple nutritional deficiencies and weight loss can occur when the flora overgrowth is severe enough to cause prominent malabsorption.

**Small intestine pseudo-obstruction:** This complication is secondary to small intestinal hypomotility because decreased peristalsis, or even aperistalsis, may provoke luminal dilatation and overt pseudo-obstructions. There is no difference in the clinical and radiographic features of the pseudo-obstructions caused by SSc or by other reasons. Abnormal collagen deposition in the small intestinal wall, which can occur during SSc, is irreversible, resulting in recurrence of pseudo-obstructions.

**PCI:** PCI is characterized by the presence of intramural gas in the gastrointestinal tract<sup>[24]</sup>. It is a rare SSc GI complication. Like small intestinal pseudo-obstructions, it is secondary to small intestine dysmotility. It is basically a radiological diagnosis and usually has no consequences. Rarely, however, intestinal ischemia can occur and surgical intervention is needed. Occasionally, the air-filled cysts in the bowel may rupture, leading to benign pneumoperitoneums<sup>[5,25]</sup>. Generally, the prognosis of PCI is good.

### Colon

Colon involvement is observed in 10%-50% of SSc patients<sup>[1,5]</sup>. Colon hypomotility is the most common colonic complication during SSc and can cause delayed colon transit. As a result, constipation and evacuation difficulty may occur. Constipation, however, does not often persist for long because of intestinal bacterial overgrowth-induced diarrhea. Therefore, constipation and diarrhea are the most common clinical symptoms of SSc patients. Although wide-mouth diverticula in the colon may occur in SSc patients, it is rarely symptomatic. Colonic telangiectasias are common during SSc and may cause overt bleeding, which can result in anemia.

### Anorectal SSc

The reported anorectal involvement in SSc is 50%-70%<sup>[5]</sup>. Patients may present with chronic diarrhea, fecal incontinence and rectal prolapses. Fecal incontinence is the most frustrating symptom and seriously impairs patient's quality of life. It is reported that 37.1% to 70% of SSc patients develop incontinence<sup>[2,5]</sup>; however, the prevalence of fecal incontinence is likely under-estimated because most patients are reluctant to report the symptoms. Neuropathy plays a key role in the development of SSc fecal incontinence.

Defecation requires the collaboration of the internal and external sphincter as well as intact rectoanal inhibitory reflex (RAIR). RAIR consists of relaxation of the smooth muscle internal anal sphincter (IAS) and contrac-

tion of the striated muscle external anal sphincter (EAS), which makes it possible to maintain anal continence. The IAS is primarily responsible for the anal resting tone and the EAS is primarily responsible for the voluntary contraction of the anal sphincter. IAS weakness leads to passive fecal incontinence, while EAS weakness leads to urge fecal incontinence. As smooth muscle is more likely to be damaged because of SSc, the IAS is more likely to be affected in the anorectum. IAS atrophy may be secondary to vascular or neurological dysfunction. Heyt *et al*<sup>[26]</sup> demonstrated that SSc patients had a thinned IAS. Additionally, the circular and longitudinal IAS smooth muscle layers were replaced with fibrous tissue. A lower IAS resting pressure is also common in SSc patients with anorectal involvement; however, the squeeze pressure is usually normal, as the EAS is generally not affected. Because there is a decrease in the rectal resting tone in these patients (due to smooth muscle cell atrophy that results from ischemia); rarefaction of innervations and neurogenic dysfunction often occur as well, consequently impairing the RAIR. Thoua *et al*<sup>[27]</sup> demonstrated that the RAIR was compromised in 46% of SSc patients with incontinence and provided evidence that neuropathy played a key role in the development of fecal incontinence in these observed patients. Furthermore, Malandrini *et al*<sup>[28]</sup> observed nerve degeneration in the rectal mucosa of SSc patients with fecal incontinence. Most studies have shown that the resting anal pressure is also reduced in fecal-incontinent SSc patients, resulting in an absent or impaired RAIR; however, their maximal squeeze pressures are normal. Additionally, inappropriate collagen and connective tissue deposition often occurs in SSc patients, which disrupts neural fiber connections and insults neural tissue, usually resulting in neuropathy. Interestingly, however, although the IAS response is diminished or absent and the EAS response is normal or increased in rectal-incontinent SSc patients, no correlation between disease duration, ACA status or SSc subtype has been observed<sup>[25]</sup>.

### Liver and biliary tract

Liver and biliary involvement in SSc is relatively rare; however, primary biliary cirrhosis (PBC) is the most common hepatobiliary manifestation in SSc patients, with an estimated prevalence of 2.5%<sup>[29]</sup>. Eight percent of SSc patients have positive anti-mitochondrial antibodies, while anti-glycoprotein and anti-sp100 antibodies have been detected in up to 15% of SSc patients<sup>[29-31]</sup>. The onset of PBC may precede, occur concomitantly with, or more commonly, follow SSc onset. Patients with a concomitant SSc and PBC disease occurrence have a higher prevalence of calcinosis and telangiectasia than patient with only SSc<sup>[32]</sup>.

## DIAGNOSIS

The diagnosis of SSc related GI disorders generally depends on the location of the involvement. Oral cavity

problems can be diagnosed by routine oral examination. Esophageal motility disorders, such as GERD, can be diagnosed by the combination of upper GI endoscopy and esophageal manometry procedures, together with ambulatory PH studies. Esophageal biopsies can confirm the diagnosis of Barrett's esophagus. Electrogastrographic recordings and scintigraphic evaluations following a radiolabeled meal are both useful for the diagnosis of delayed stomach emptying. A typical endoscopic procedure, whereby "watermelon stomach" is obvious, is diagnostic for GAVE. Small bowel manometry is helpful not only in the screening for SSc patients who have small intestine involvement but also in identifying symptomatic patients with intestinal pseudo-obstructions who may benefit from octreotide. Additionally, low resting anal canal pressure and impaired or absent RAIP observations are helpful in diagnosing anorectal disorders.

## TREATMENT

Treatments for SSc-induced GI impairment are generally symptomatic and supportive. Nutrition status assessment should be a routine component of clinical care for SSc patients<sup>[33]</sup>. Moreover, a multi-disciplinary approach is important for the optimal care of SSc patients with GI involvement.

### Oral cavity

For patients with decreased oral aperture, techniques such as facial grimacing exercises, mouth stretching exercises and oral opening augmentations with tongue depressors are recommended. Bilateral commissurotomy may also be performed to increase mouth opening. For patients with dry mouth, attention should be paid to oral hygiene to prevent caries.

### Esophagus

For patients with esophageal dysmotility, modified lifestyle measures should be initiated and have proven to be helpful. These measures include frequent small meals, sitting up during and after meals, elevating the heads of patients' beds, and avoiding known irritants and bedtime meals or snacks. Patients with endoscopically documented GERD require chronic treatment with proton-pump inhibitors. Use of prokinetic drugs that increase gastric emptying, such as metoclopramide, may help reduce reflux. Additionally, esophageal strictures can also be dilated under endoscopic guidance. Patients not responding to medication therapy can be treated with anti-reflux surgery. The outcomes of anti-reflux surgeries, however, are variable, and careful pre-operative evaluations are warranted. Partial funduplications (Toupet procedure) may also be helpful in some patients<sup>[32]</sup>.

### Stomach

Bipolar cautery, heat probe, sclerotherapy and laser ablation are available for the treatment of GAVE. Prokinetic drugs, such as metoclopramide, domperidone, pruc-

lopride and tegaserod, are helpful in patients with early stage stomach disease, but become less effective as the disease progresses. Macrolide antibiotics are believed to have motilin agonist properties and have been evaluated in delayed stomach emptying patients. Erythromycin, however, is the most widely studied drug. It has been shown to stimulate intestinal motility even with low dosages; however, its effectiveness may decrease with time<sup>[34]</sup>.

### Small intestine

Bacterial overgrowth is the major cause of symptoms in patients with small intestinal involvement. Antibiotics, such as metronidazole (500 mg BID) and ciprofloxacin (500 mg BID), administered for 14 to 28 d can be helpful for these patients. An alternative antibiotic regimen includes oral intake of chloramphenicol and the third generation cephalosporins. Rotating antibiotics monthly is suggested to circumvent bacterial resistance. Probiotics have been proven to be effective and safe for patients with bloating caused by bacterial overgrowth<sup>[35]</sup>. Lactobacillus can be used to treat this condition because it can competitively inhibit the attachment and growth of pathogenic organisms and restore the microbial balance in the GI tract. Additionally, lactobacillus may also enhance the immune-modulating effects in patients by increasing the IgA response or by modifying mucosal IL-10 and Th1/Th2 lymphocyte levels. Studies have shown that lactobacillus can also produce proteinaceous factors that alter epithelial permeability, inhibit bacterial translocation, and influence the level of gut mucin glycoprotein<sup>[5]</sup>.

Small intestine pseudo-obstructions are common in SSc patients. The initial treatment for this condition should include bowel rest, intravenous fluid infusion and electrolyte correction. Octreotide has also been shown to be effective<sup>[36]</sup>. The starting dosage is usually 50 µg *bid* (given subcutaneously) during acute onsets; however, the dosage can be increased up to 200 µg if a satisfactory response is not observed. For patients with recurrent pseudo-obstruction episodes, 50 µg of octreotide at bedtime is usually effective and depot octreotide can be prescribed on a monthly basis. Neostigmine can lead to prompt colon decompression; therefore, it can be used for this condition. If octreotide and neostigmine treatments are not effective, however, colonoscopic decompression is normally the treatment of choice. Surgery procedures are reserved for cases of peritonitis and perforation.

### Colon and anorectal disorders

Constipation, diarrhea and fecal incontinence are the major symptoms in patients with colon and anorectal involvement. High-fiber diets and bulk-forming laxatives should be avoided in constipated patients because these can worsen constipation. Fluid ingestion and osmotic laxatives, such as senna, lactulose, bisacodyl and polyethylene glycol, are recommended because these medications can alter intestinal mucosa electrolyte transportation and also increase intestinal motor activity. Antibiotics can be given to patients with diarrhea caused by bacteria

overgrowth syndrome. For patients with incontinence, sacral nerve stimulation has been shown to be successful in most patients and may also abolish incontinence in some patients<sup>[5]</sup>. Posterior anal repair may be considered when sacral nerve stimulation fails. Rectal and vaginal prolapses should be detected and surgically repaired, as these two conditions can contribute to incontinence. Additionally, biofeedback may be helpful in improving rectal continence. Surgical procedures such as dynamic graciloplasties or the installation of artificial bowel sphincters should be considered in patients with resistant and severe incontinence.

### Liver and biliary disorders

PBC in SSc patients can be treated with ursodeoxycholic acid, which delays the histological progression rate. Patients with severe liver disorders, however, may need liver transplantation.

In summary, GI involvement in SSc patients is common and sometimes troublesome. An early diagnosis is crucial for improving patient prognosis due to the insidious progressive nature of the disease. Symptomatic and supportive treatments, as well as modified life style measures are the management mainstays for this disease.

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## Advances in the management of acute liver failure

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### Abstract

Acute liver failure (ALF) is an uncommon but dramatic clinical syndrome characterized by hepatic encephalopathy and a bleeding tendency due to abrupt loss of liver function caused by massive or submassive liver necrosis in a patient with a previously healthy liver. The causes of ALF encompass a wide variety of toxic, viral, metabolic, vascular and autoimmune insults to the liver, and identifying the correct cause can be difficult or even impossible. Many patients with ALF develop a cascade of serious complications involving almost every organ system, and death is mostly due to multi-organ failure, hemorrhage, infection, and intracranial hypertension. Fortunately, the outcome of ALF has been improved in the last 3 decades through the specific treatment for the disease of certain etiology, and the advanced intensive care management. For most severely affected patients who fail to recover after treatment, rapid evaluation for transfer to a transplantation center and consideration for liver transplantation is mandatory so that transplantation can be applied before contraindications

develop. This review focuses on the recent advances in the understanding of various contributing etiologies, the administration of etiology-specific treatment to alleviate the liver injury, and the management of complications (*e.g.*, encephalopathy, coagulopathy, cardiovascular instability, respiratory failure, renal failure, sepsis and metabolic disturbance) in patients with ALF. Assessment of the need for liver transplantation is also presented.

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**Key words:** Acute liver failure; Cerebral edema; Liver transplantation

**Core tip:** Acute liver failure (ALF) is a dramatic clinical syndrome of abrupt loss of liver function in a patient with a previously healthy liver. The causes of ALF encompass a wide variety of toxic, viral, metabolic, vascular and autoimmune insults to the liver. ALF patients develop serious complications, and death is mostly due to sepsis, followed by multiple organ failure, and intracranial hypertension. However, disease outcome has been improved through etiological treatment and advanced intensive care management. For most severely affected patients who fail to recover after treatment, liver transplantation may be life-saving.

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### INTRODUCTION

Acute liver failure (ALF) is an uncommon but dramatic clinical syndrome characterized by sudden and massive hepatic necrosis that results in jaundice, coagulopathy [international normalized ratio (INR)  $\geq 1.5$ ], and hepatic

encephalopathy (any degree of altered mentation) in the absence of pre-existing liver disease<sup>[1]</sup>. A timely diagnosis of ALF is critical because of its feature of rapid deterioration, and delayed diagnosis can be disastrous. The most prominent causes include drug-induced liver injury, viral hepatitis, autoimmune liver disease and shock or hypoperfusion; many cases have no discernible cause<sup>[2]</sup>. The mortality rate of ALF is as high as 40%-50%, depending on the cause and improper therapeutic management. The immediate cause of death in 35% of ALF patients is brain herniation due to elevated intracranial pressure (ICP), and most other deaths are the result of severe refractory hypotension resulting from supervening sepsis culminating in multiorgan failure<sup>[3]</sup>. Orthotopic liver transplantation (OLT) has emerged as the only therapeutic intervention with proven benefit for patients with advanced ALF<sup>[4]</sup>.

## ETIOLOGY AND SPECIFIC THERAPIES

The causes of ALF encompass a wide variety of toxic, viral, metabolic, and vascular insults to the liver, and the etiology of ALF varies with geography. In Africa and Asia, viral hepatitis dominates the causes of ALF<sup>[5]</sup>. By contrast, in Europe and North America, toxic etiologies predominate<sup>[6]</sup>. In many cases, the cause of ALF cannot be established and remains indeterminate. Determination of etiology is important because specific therapies can be given once the diagnosis is established. In addition, knowing the cause could provide a reasonably valid guide to predicting outcome<sup>[7]</sup>.

### Toxins

Acetaminophen (APAP) overdose accounts for 46% of ALF cases in some areas, and APAP-induced ALF is currently more commonly seen after unintentional than intentional overdose<sup>[8]</sup>. The development of liver failure from APAP is dose dependent; hepatic failure is more likely with ingested dosages > 150 mg/kg. Various risk factors increase the probability of acute liver damage even at therapeutic doses of APAP. The factors include alcoholic addiction, malnutrition (resulting in glutathione depletion), and concurrent use of narcotic analgesics compounded with APAP. The liver damage leads to a characteristic pattern of pericentral necrosis due to cytochrome P450-mediated oxidative metabolism of APAP to the highly reactive, intermediate metabolite, *N*-acetyl-p-benzoquinone imine (NAPQI)<sup>[9]</sup>. Accumulation of NAPQI leads to cell death and hence hepatocellular necrosis. *N*-Acetylcysteine (NAC) is established as a proven beneficial agent for APAP-induced hepatotoxicity<sup>[10]</sup>. It acts by replenishing glutathione that detoxifies NAPQI. In addition, excessive NAC also provides substrates for hepatic ATP synthesis, thus supporting mitochondrial energy metabolism. The latter pathway may be particularly important in delayed administration of NAC.

Mushroom poisoning, most commonly from *Amanita* genus mushrooms, should be suspected in patients with

a history of severe gastrointestinal symptoms (nausea, vomiting, diarrhea and abdominal cramping), which occur within hours to a day after mushroom ingestion<sup>[11]</sup>. Amanitin toxin, recycled *via* the enterohepatic circulation, interrupts hepatocyte mRNA synthesis resulting in dose-dependent hepatotoxicity. The mortality of ALF secondary to mushroom poisoning approaches 10%-30%. Penicillin G and silybinin may ameliorate the hepatic injury, however, patients should be listed for hepatic transplantation, because this procedure is often the only life-saving option<sup>[12]</sup>.

Drugs other than APAP rarely cause dose-related liver injury<sup>[13]</sup>. Many of these injuries are idiosyncratic, and they often occur within the first 6 mo after intake of the drug. In this setting, it is necessary to discontinue all but the most essential medications. Medications commonly associated with acute liver injury include antimicrobials, neurological and psychiatric drugs, and nonsteroidal anti-inflammatory drugs (Table 1)<sup>[14]</sup>.

### Virus

Patients with viral hepatitis that develop hepatic failure are largely suffering from hepatitis B, and less frequently hepatitis A. Hepatitis B carriers undergoing immunosuppressive or cancer chemotherapy may experience reactivation of hepatitis B virus (HBV) replication, which may lead to ALF. Prophylactic antiviral therapy is recommended for HBV carriers at the onset of cancer chemotherapy or of a finite course of immunosuppressive therapy<sup>[15-17]</sup>. High viral load at baseline is the most important risk factor for HBV reactivation. Patients with baseline HBV DNA < 2000 IU/mL level should continue treatment for 6 mo after completion of chemotherapy or immunosuppressive therapy. Patients with a high baseline HBV DNA (> 2000 IU/mL) level should continue treatment until they reach treatment endpoints as in immunocompetent patients. Lamivudine or telbivudine can be used if the anticipated duration of treatment is short (< 12 mo) and baseline serum HBV DNA is not detectable. Tenofovir or entecavir is preferred if longer duration of treatment is anticipated<sup>[17]</sup>. In addition, all transplant recipients positive for hepatitis B surface antigen should receive antiviral therapy, preferably using tenofovir or entecavir<sup>[15]</sup>.

In an endemic area such as Russia, Pakistan, Mexico, or India, hepatitis E remains an important cause of hepatic failure, particularly in the context of pregnancy, and it carries a high mortality in this setting<sup>[18]</sup>. Moreover, vertical transmission of hepatitis E from women with acute infection results in ALF in more than half of neonates. So far, ALF due to acute hepatitis C infection is uncommon and occurs in < 1% of patients. Few data suggest that hepatitis G virus plays a major pathogenic role in ALF<sup>[19]</sup>. Herpes viruses occasionally cause ALF, usually among immunosuppressed and pregnant patients<sup>[20]</sup>. Epstein-Barr virus, adenoviruses, cytomegalovirus, varicella zoster virus, parvovirus B19, yellow fever virus and hemorrhagic fever virus are also implicated as causes of ALF. For ALF caused by herpes viruses or varicella zoster,



**Table 1** Drugs which may cause idiosyncratic liver injury leading to acute liver failure

Classification	Drugs
Anti-infective agents	Amoxicillin/clavulanate erythromycin, roxithromycin, telithromycin, doxycycline, minocycline nitrofurantoin, ciprofloxacin, levofloxacin, moxifloxacin trimethoprim-sulfamethoxazole, sulfasalazine, isoniazid, rifampin, pyrazinamide, ethambutol, dapsone, fluconazole, itraconazole, terbinafine, ketoconazole, chloroquine Didanosine, fialuridine, efavirenz, abacavir, nevirapine-lamivudine
Cardiovascular agents	Amiodarone, labetalol, diltiazem, methyldopa, valsartan, lisinopril, angiotensin converting enzyme inhibitor Asparaginase, flutamide
Hypolipidemic agents	Atorvastatin, cerivastatin, simvastatin, pravastatin, fluvastatin, ezetimibe
Hypoglycemic Agents	Metformin, troglitazone
Anti-allergic agents	Zafirlukast, loratadine, diphenhydramine
Herbal products/dietary supplements	Kava, Herbalife, Comfrey, Senecio, Greater Celandine, Polygonum multiflorum, ginseng, Teucrium polium, usnic acid, ma huang, Chaso, Onshido, Hydroxycut, LipoKinetix
Neurological and psychiatric drugs	Halothane, isoflurane, butorphanol opiates, amphetamines, marijuana, cocaine Phenytoin, valproic acid, carbamazepine, felbamate, lamotrigine, vigabatrin amitriptyline, imipramine, sertraline, paroxetine, venlafaxine, pemoline, bupropion Chlorpromazine, quetiapine, clonazepam tolcapone
Nonsteroidal anti-inflammatory drugs	Diclofenac, bromfenac, etodolac, naproxen, ibuprofen, indometacin
Miscellaneous	Propylthiouracil, retinol, infliximab, allopurinol, cyclosporine, disulfiram, iron sulfate, anabolic steroids, carbon tetrachloride, phenprocoumon, nicotinic acid
Antineoplastic agents	Methotrexate, cytoxan, etoposide, dactinomycin, azathioprine, tamoxifen

acyclovir (5-10 mg/kg *iv* every 8 h) is the recommended treatment<sup>[21]</sup>.

### Metabolic causes

Metabolic disorders like Wilson disease (WD), HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome, acute fatty liver of pregnancy, Reye's syndrome, galactosemia, hereditary fructose intolerance, hemochromatosis,  $\alpha$ 1-antitrypsin deficiency and tyrosinemia may also cause ALF.

WD accounts for 6%-12% of all patients with ALF who are referred for emergency liver transplantation. ALF due to WD occurs predominantly in young women (female:male ratio 4:1)<sup>[22]</sup>. Diagnostic tests for WD should include ceruloplasmin, serum and urinary copper levels, total bilirubin/alkaline phosphatase ratio, slit lamp examination for Kayser-Fleischer rings, and hepatic copper levels when liver biopsy is feasible<sup>[23]</sup>. High bilirubin (> 20 mg/dL) and low alkaline phosphatase levels (including

undetectable levels) due to profound hemolytic anemia help with its rapid recognition. Liver transplantation is the only effective option for those with WD who present with ALF. One-year survival following liver transplantation ranges from 79% to 87%, and those who survive this early period continue to survive in the long term<sup>[23]</sup>.

The hepatic damage of HELLP syndrome is proposed to result from disordered placentation, leading to either the circulation of antiangiogenic factors and endothelial dysfunction, or cytokine production causing the characteristic periportal hemorrhage and fibrin deposition observed by Sánchez-Bueno *et al.*<sup>[24]</sup>. Acute fatty liver degeneration of pregnancy is a sudden catastrophic illness occurring most frequently in the third trimester, when mitochondrial dysfunction due to maternal and fetal fatty acid  $\beta$ -oxidation defects resulting in microvesicular fatty acid accumulation in hepatocytes<sup>[25]</sup>. There is an overlap of these two clinical syndromes, and they play a major role in the pathogenesis of pre-eclampsia, with hypertension, and proteinuria. Early recognition of these syndromes and prompt delivery are critical in achieving good outcomes. Failure to recover from the illness should be promptly listed for liver transplantation<sup>[26]</sup>.

### Vascular causes

The Budd-Chiari syndrome (acute hepatic vein thrombosis) is an uncommon cause of ALF<sup>[27]</sup>. Right upper quadrant pain, striking hepatomegaly, and fluid retention characterize the initial clinical picture and may help distinguish this syndrome from other forms of ALF in which the liver is small and not tender. Therapeutic strategies have included anticoagulation, use of transjugular intrahepatic portocaval shunting, or transplantation. It is important to rule out underlying cancer prior to transplantation for these patients. Liver ischemic injury can also cause ALF and could be seen in the setting of sepsis, cardiac arrest, heart failure, or hypotension induced by long-acting niacin or cocaine<sup>[28]</sup>. Aminotransferase levels will be markedly elevated and respond rapidly to stabilization of the circulatory problem. Cardiovascular support is the treatment of choice in this setting.

### Miscellaneous causes

Some rare causes of ALF include heat shock, protracted seizures, autoimmune hepatitis, and malignant infiltration<sup>[29,30]</sup>. ALF occurs in a small fraction of autoimmune hepatitis patients-probably < 20%. These cases are usually recognized prior to hospitalization as having autoimmune disease that proceeds to rapid deterioration. The clinical picture is in the form of a subacute presentation, with intermediate elevation of enzyme levels and high bilirubin concentrations. Presence of autoantibodies and a compatible picture on biopsy help to confirm the diagnosis, but they may not be notable. Some autoimmune hepatitis patients may respond well to steroid therapy, and others may still require transplantation. The most common forms of malignant infiltration implicated in ALF are lymphoma, breast cancer, and melanoma<sup>[31]</sup>.

Others include small cell lung cancer and prostate cancer. Diagnosis should be made by imaging and biopsy, and treatment appropriate for the underlying malignant condition is indicated.

### Indeterminate causes

About 15%-20% of ALF occurs with an indeterminate cause, which includes unrecognized idiosyncratic drug toxicity, non-A-E viral hepatitis, and possibly unrecognized metabolic and genetic diseases. The reasons for this misdiagnosis may include failure to obtain an adequate history as mentioned, failure to perform the definitive diagnostic tests, or simply due to some other elusive diagnoses. As has been noted, about 20% of ALF of indeterminate cause is related to obscure APAP toxicity through detection of APAP-protein adducts, the byproducts of the toxic reaction<sup>[32]</sup>. However, the adduct assay is not available for routine use at this time.

## CLINICAL MANIFESTATIONS

The clinical presentation of ALF is multifaceted, ranging from slightly altered conscious level with profound coagulopathy to a catastrophic failure of multiple organs. The initial clinical features of ALF may be nonspecific and may include anorexia, fatigue, abdominal pain and fever. As the metabolic and detoxification function of liver becomes impaired, the signs of ALF emerge, including jaundice, encephalopathy, coagulopathy, haemodynamic instability, acute lung injury/acute respiratory distress syndrome (ARDS), renal failure, sepsis, and metabolic disturbance.

Depending on the interval between development of jaundice and onset of encephalopathy, clinical manifestation could be stratified into three groups such as hyperacute (< 7 d), acute (7-28 d) and subacute (4-26 wk)<sup>[33]</sup>. This classification is popular but not particularly helpful because it does not have prognostic significance that is distinct for identifying the cause of the illness. Hyperacute failure, most commonly caused by APAP hepatotoxicity, is characterized by high aminotransferase level and low bilirubin level<sup>[34]</sup>. Hepatic encephalopathy develops rapidly in this setting, sometimes preceding jaundice. Subacute liver failure due to idiosyncratic drug toxicity presents as minimal encephalopathy with no cerebral edema<sup>[35]</sup>. This condition is usually associated with severe jaundice, renal dysfunction, and moderate coagulopathy.

## ICU TREATMENT

NAC is a proven effective therapy for APAP hepatotoxicity. It is also beneficial in non-APAP ALF patients showing early (grades I / II) hepatic encephalopathy<sup>[36]</sup>. NAC can increase non-transplant survival among these patients. Thus, administration of NAC should be initiated immediately when ALF is established. Except for NAC, there is no other proven therapy for ALF. Management consisting of intensive care support should be initiated to address

the various organ dysfunctions associated with ALF.

### Cerebral edema and intracranial hypertension

Cerebral edema leading to intracranial hypertension (ICH) is one of the major causes of morbidity and mortality in patients with ALF. The pathogenesis of cerebral edema and ICH in ALF appears to be multifactorial. Ammonia is converted in the astrocytes to osmotically active glutamine, producing osmotic cerebral edema<sup>[37]</sup>. Other factors such as impaired cerebral blood flow (CBF) autoregulation, systemic inflammatory response and ischemic injury have also been proposed as the cause of ICH. Some risk factors for the development of cerebral edema in patients with ALF include high-grade encephalopathy (grade III or IV), high serum ammonia concentrations (> 200  $\mu\text{mol/L}$ ), and requirement for vasopressor support or renal replacement therapy.

Cerebral edema presents clinically as hepatic encephalopathy due to ICH. Basic interventions for the management of cerebral edema should be applied universally in patients with high-grade hepatic encephalopathy. These interventions include elevation of the head of the bed to 30°, maintenance of a neutral neck position, endotracheal intubation, minimizing painful stimuli, and control of arterial hypertension<sup>[38]</sup>. Propofol is a reasonable choice for adequate sedation because it may protect from ICH. For treatment of pain, fentanyl is preferred as the first-line agent. Factors that increase ICP need to be avoided and include hypercapnia, hyponatremia, frequent movements, neck vein compression, fluid overload, fever, hypoxia, coughing, sneezing, seizures, and endotracheal suctioning.

ICP monitoring is recommended in ALF patients with high-grade hepatic encephalopathy, in centers with expertise in ICP monitoring as well as in patients awaiting and undergoing liver transplantation<sup>[39]</sup>. ICP monitoring can detect elevations in ICP to direct interventions, which may preserve brain perfusion and prevent cranial herniation. Generally, the goal of therapy in ALF is to maintain ICP < 20 mmHg and cerebral perfusion pressure (CPP) > 60 mmHg. Prolonged ICP > 40 mmHg and CPP < 50 mmHg are associated with a poor outcome. CPP < 40 mmHg for > 2 h indicates reduced neurological blood flow to maintain intact brain function and should contraindicate liver transplantation because of poor post-transplantation prognosis. However, patients with refractory ICP elevation > 35 mmHg and CPP < 50 mmHg who made a full neurological recovery contradicted previous findings.

In patients with persistently elevated ICP, osmotic therapy can be considered. Mannitol reduces ICP by osmotically drawing water from the brain parenchyma into the intravascular space<sup>[40]</sup>. ICP > 20 mmHg necessitates intravenous administration of mannitol (0.5-1 g/kg) provided serum osmolality is < 320 mOsm/L. However, mannitol fails to normalize ICP once a level > 60 mmHg is reached. Thus, its best use is for mild to moderate ICH. Alternately, hypertonic saline mitigates ICH through both osmotic and nonosmotic effects<sup>[41]</sup>. Hypertonic saline to

target serum sodium levels 145-155 mmol/L are suggested to avoid complications associated with extreme hyponatremia, such as seizure and changes in mentation.

Hypothermia also has some benefit in reducing ICP, because it lowers brain energy metabolism, reduces arterial ammonia concentration and extraction of ammonia by the brain, normalizes CBF autoregulation, and reverses systemic inflammatory reactions<sup>[42]</sup>. In addition to its neurological effect, hypothermia results in significant improvement of cardiovascular hemodynamics, as manifested by increased mean arterial pressure (MAP) and systemic vascular resistance, and reduction in noradrenaline requirements. Therapeutic hypothermia (cooling to a core temperature of 34 °C-35 °C) is probably well tolerated and effective, but randomized, controlled trials are needed to confirm the benefits of hypothermia before it is applied routinely.

Barbiturates are centrally acting hypnotics that reduce brain oxygen utilization and are effective in lowering ICP<sup>[43]</sup>. However, untoward side effects such as arterial hypotension, negative inotropic effects, and immunosuppressant effects make barbiturates a poor first-choice treatment for ICH. Hyperventilation can induce hypocapnia that causes cerebral vasoconstriction, which in turn reduces CBF, thus leading to a decrease in ICP<sup>[44]</sup>. Although hyperventilation effectively reduces ICP, there is a concern that the resultant vasoconstriction could exacerbate cerebral ischemia and even cause hypoxia. It is believed that hyperventilation to maintain PaCO<sub>2</sub> between 30 and 35 mmHg may reduce ICP acutely, but it should not be used over a prolonged period.

The use of hepatectomy in patients awaiting liver transplantation is based upon the concept that the necrotic liver is the source of unknown humoral substances that contribute to increased ICP<sup>[45]</sup>. Removal of the liver in an ALF patient resulted in improved ICP possibly through a reduction in CBF, nitric oxide (NO) and liver-derived proinflammatory cytokines.

### **Hemodynamic failure**

ALF is characterized by a hyperdynamic circulation with high cardiac output, low MAP, and low systemic vascular resistance<sup>[46]</sup>. Increased NO production and cyclic GMP may be involved in these hemodynamic disturbances. Because the patients have such markedly deranged circulation, it is important to use monitoring devices that are able to provide information about changes in MAP, filling status, cardiac output, and oxygenation status. Due to poor oral intake, transudation of fluid into the extravascular space, and possibly gastrointestinal bleeding, most patients are volume depleted and require initial fluid resuscitation. The initial treatment of hypotension should involve intravenous infusion of normal saline and a volume challenge is recommended<sup>[47]</sup>.

After adequate fluid replacement and treatment of infection and sepsis, vasopressors may also be required to maintain adequate MAP and CPP. ALF patients have lost CBF autoregulation and an increase in MAP results in an

increase in CPP. The MAP should be maintained in a narrow range to achieve a CPP of 60-80 mmHg to prevent cerebral hypoperfusion on the one hand and further cerebral hyperemia on the other hand. Noradrenaline, with fewer  $\beta$ -adrenergic side effects, could increase hepatic blood flow in parallel with minimizing tachycardia and is often the preferred vasopressor<sup>[48]</sup>. In patients who do not respond to a volume challenge and norepinephrine, vasopressin or terlipressin may potentiate the effects of norepinephrine. Patients with uncorrectable hypotension after volume repletion and vasopressor administration should be evaluated for adrenal insufficiency, which occurs frequently in this setting<sup>[49]</sup>. Adrenal insufficiency could be corrected with a stress dose of hydrocortisone 200-300 mg/d in divided doses.

### **Respiratory failure**

Acute lung injury/ARDS is not uncommon in patients who have ALF and severe multiple organ dysfunction; particularly a requirement for vasopressors and concurrent ICH<sup>[50]</sup>. The hypoxemia caused by acute lung injury and ARDS should be managed with low tidal volume ventilation to minimize risks of pulmonary volume trauma and barotrauma. Upregulation of respiratory rate is needed to ensure adequate minute ventilation, avoiding marked hypercapnia. It is desirable to maintain the lowest level of positive end-expiratory pressure that achieves adequate oxygenation because high levels may exacerbate cerebral edema and hepatic congestion. Recruitment, a transient increase in mean airway pressure to expand the lungs, is also beneficial in improving oxygenation.

### **Acute renal failure**

The incidence of acute renal failure in ALF is as high as 50%-80%. Acute renal failure resembling hepatorenal syndrome is multifactorial in the setting of ALF<sup>[51]</sup>. Direct drug nephrotoxicity and acute tubular necrosis due to ischemia from hypotension are among the most important associated disease entities. In addition, development of abdominal compartment syndrome, due to ascites, intra-abdominal hemorrhage or severe abdominal and gut wall edema, is a common cause of renal impairment in ALF. Management includes avoidance of nephrotoxic agents, treatment of infection, maintenance of adequate renal perfusion, and renal replacement therapy. Early targeted volume replacement and vasoactive agent administration are essential to avoid arterial hypotension and ensure adequate renal perfusion. Worsening renal failure needs to be addressed with renal replacement therapy. Continuous renal replacement therapy is recommended, because most patients with ALF tolerate intermittent hemodialysis poorly because of circulatory instability, precipitous fluid shifts, and a rise in ICP<sup>[52]</sup>.

### **Infection**

ALF patients have enhanced susceptibility to infection because of the presence of indwelling lines and catheters, dysfunction of monocytes, and impaired complement



system and neutrophil and Kupffer cell function<sup>[53]</sup>. Bacterial infections have been documented in 80% of cases; most commonly pneumonia, urinary tract infections, intravenous catheter-induced bacteremia, and spontaneous bacteremia. Infectious organisms are mainly Gram-negative enteric bacilli, Gram-positive cocci and *Candida* species. Infection inhibits hepatic regeneration, and it is associated with progression of hepatic encephalopathy and renal failure, reduces successful rate of transplantation, and increases mortality in ALF. Thus, close surveillance for infection should be maintained in all ALF patients, with frequent chest radiographs and cultures of blood, urine and sputum. Empirical antibiotics should be administered when surveillance cultures are positive. To patients who develop progression to grade 3 or 4 hepatic encephalopathy and elements of systemic inflammatory response syndrome, antibiotic treatment is also recommended<sup>[54]</sup>.

### Bleeding

Deficiencies of fibrinolytic proteins, anticoagulant proteins (protein C/S or antithrombin III) and procoagulation factors (II, V, VII, IX and X) are often present in ALF; in part due to failure of synthesis as well as consumption of these factors. Data have also shown quantitative and qualitative platelet dysfunction in ALF. Hemostatic changes thus incorporate coagulopathy [confirmed with prolonged prothrombin time (PT) and partial thromboplastin time] as well as a tendency to develop thrombotic events such as disseminated intravascular coagulation. However, there are abnormalities in both the coagulation and the fibrinolytic pathways, and data suggest that the defects are balanced; that is, there is a relative preservation of hemostasis<sup>[55]</sup>. Clinically significant bleeding occurs rarely (about 5% of cases) and the perceived bleeding risk based upon INR may be overstated.

Bleeding generally occurs from superficial mucosal lesions, especially gastric erosions. Administration of histamine-2 receptor antagonists or proton pump inhibitors has been shown to decrease the risk of gastric mucosal bleeding in patients with ALF. In general, infusion of fresh frozen plasma is indicated only for control of active bleeding or during invasive procedures such as insertion of ICP monitor, to maintain an INR  $< 1.5$ <sup>[56]</sup>. When fresh frozen plasma fails to normalize PT/INR adequately, the use of recombinant factor VIIa can be considered. Cryoprecipitate is recommended in patients who have significant hypofibrinogenemia ( $< 1$  g/L). Platelet transfusion is indicated only to aid in controlling active bleeding or during invasive procedures if the count is  $< 50 \times 10^9$ /L or prophylactically if  $< 15 \times 10^9$ /L<sup>[57]</sup>. Finally, vitamin K (5-10 mg subcutaneously) should be considered in all patients with ALF, because its deficiency can occur in  $> 25\%$  of patients.

### Metabolic concerns

Patients are prone to develop hypoglycemia because hepatocyte necrosis causes glycogen depletion and defective

glycogenolysis and gluconeogenesis. Rapid development of hypoglycemia, which can confound the hepatic encephalopathy, should be managed with continuous intravenous glucose infusion<sup>[58]</sup>. Hyperglycemia should also be avoided because it may contribute to poor ICP control. Low systemic blood pressure and poor systemic microcirculation result in a build-up of lactate; a complication that may be accentuated by the lack of the lactate metabolism in the failing liver. Correction of hyperlactatemia is important because it can affect circulatory function and aggravate cerebral hyperemia. Serum phosphate, potassium and magnesium are frequently low, requiring repeated supplementation. Severe restrictions of protein should be avoided; normal protein intake of about 1 g/kg per day is reasonable in most cases<sup>[59]</sup>. Owing to the hypercatabolic state of ALF, nutrition is vital and enteral feedings should be initiated early. If enteral feeding is contraindicated, parenteral nutrition is a reasonable alternative.

## LIVER SUPPORT DEVICES

Extracorporeal supportive devices have been advocated to replace the liver function in ALF patients; however, the complexity of liver metabolic, synthetic, detoxifying, and excretory functions makes the extracorporeal hepatic support extremely difficult. Currently available liver support systems comprise nonbiological systems and bioartificial systems. As the most common techniques of nonbiological systems, molecular adsorbent recirculatory system and Prometheus therapy are useful methods of detoxification for patients with ALF<sup>[60]</sup>. Unfortunately, no survival benefit could be demonstrated compared with standard medical therapy.

Bioartificial liver (BAL) systems rely on the use of liver cells (human or nonhuman) to perform detoxification and secretion of hepatocyte-derived factors. The selection of the ideal cell source and the design of more sophisticated bioreactors are the main issues in this field of research. Preliminary data on the use of BAL devices suggest some improvement in encephalopathy, but no real improvement could be demonstrated in overall survival.

## LIVER TRANSPLANTATION

OLT remains the only definitive treatment for patients with ALF proven to have irreversible liver injury. Rapid evaluation for transfer to a transplantation center and consideration for liver transplantation is mandatory so that transplantation can be applied before contraindications develop. Towards this end, multiple prognostic indicators and scoring systems have been devised to predict outcome in ALF. The King's College criteria are widely used to assess of the severity of ALF and the potential variability of the prognosis, with a sensitivity of 68%-69% and a specificity of 82%-92% (Table 2). Recently, the addition of arterial lactate levels in patients with APAP-induced ALF has been proposed to improve sensitivity of the criteria and identifies patients in need

**Table 2 King's College criteria for selecting recipients of emergency liver transplants**

Acetaminophen-induced ALF	Non-acetaminophen-induced ALF
Strongly recommended list for OLT if: Arterial lactate > 3.5 mmol/L after early fluid resuscitation	List for transplantation if: INR > 6.5 and encephalopathy present irrespective of grade
List for transplantation if: Arterial pH < 7.3 or arterial lactate > 3.0 mmol/L after adequate fluid resuscitation	Or if any 3 of the following features (encephalopathy irrespective of grade) are present: Age < 10 yr or > 40 yr <sup>1</sup> Interval from jaundice to encephalopathy > 7 d <sup>1</sup>
List for transplantation if all 3 of the following occur within a 24-h period: Grade 3 or 4 hepatic encephalopathy INR > 6.5 Creatinine > 300 µmol/L	INR ≥ 3.5 Serum bilirubin ≥ 300 µmol/L Unfavorable etiology, such as seronegative hepatitis, idiosyncratic drug reaction or Wilson disease

<sup>1</sup>These criteria have not been found to be predictive of outcome in recent analyses. ALF: Acute liver failure; OLT: Orthotopic liver transplantation; INR: International normalized ratio.

for OLT earlier<sup>[61]</sup>. The Clichy/Villejuif criteria are widely used in Northern Europe for ALF patients with severe encephalopathy, and assess outlook with consideration of coagulation factor V concentrations and patient age. The criteria include grade 3 and 4 hepatic encephalopathy and factor V levels < 20% in patients < 30 years of age or < 30% in patients aged > 30 years. Other systems such as Acute Physiology and Chronic Health Evaluation (APACHE) II score and the Model for End-Stage Liver Disease (MELD) score have also been used to determine the prognosis in ALF. The MELD score is calculated by the formula: MELD = 3.8 [Ln serum bilirubin (mg/dL)] + 11.2 (Ln INR) + 9.6 [Ln serum creatinine (mg/dL) + 6.4]. The sensitivity of APACHE II score and MELD score is too low to determine outcome, but the specificity is acceptable. This means that they are more applicable for predicting death rather than spontaneous survival.

In general, key factors involved in determining outcome of ALF are the etiology, degree of encephalopathy, degree of hepatocyte damage, and risk of extrahepatic complications. First, the etiologic diagnosis *per se* appears to be the strongest driver of outcome. ALF cases due to APAP toxicity, hepatitis A, ischemia, and pregnancy may have a better prognosis<sup>[62]</sup>. Approximately 90% of APAP-induced ALF cases recover with supportive measures, whereas ALF cases due to idiosyncratic drug injury, acute hepatitis B, autoimmune hepatitis, mushroom poisoning, WD, Budd-Chiari syndrome and indeterminate causes carry a much poorer prognosis in the absence of OLT. Up to 80% of patients who develop liver failure due to idiosyncratic drug injury might die without transplantation. Second, grade 3 or 4 encephalopathy is considered to show irreversible liver damage; spontaneous recovery is rare, and in most cases the patient is transferred to a transplantation centre and undergoes OLT as soon as possible<sup>[63]</sup>. Moreover, the degree of hepatocyte damage,

reflected as coagulopathy or jaundice, is viewed as inverse correlation with survival. Finally, extrahepatic complications, such as comorbid cardiovascular, respiratory and systemic conditions have a negative affect on patient outcomes. In addition, studies have also identified serum phosphate, blood NH<sub>3</sub> levels, high body mass index, genetic polymorphism, and surrogate markers of cell death as additional predictive or diagnostic factors<sup>[64]</sup>. Hypophosphatemia is an indication of increased hepatic ATP production during liver regeneration and serve as a good prognostic indicator especially in APAP-induced ALF. Genetic polymorphisms in keratins 8 and 18, the sole keratins expressed by hepatocytes, confer susceptibility to ALF and are also prognostic.

Before OLT, contraindications to transplantation such as substance abuse, suicidal predilection, psychiatric disorders, uncontrollable sepsis and other organ system involvement (irreversible brain damage, extrahepatic malignancy, cardiovascular failure requiring > 1 µg/kg per minute norepinephrine infusion, and ARDS requiring FiO<sub>2</sub> > 60% and PEEP > 12 cm H<sub>2</sub>O) must be excluded. Once listed for OLT, patients waited an average of 3.5 d. However, 66% of patients were transplanted, and of the remainder, 22% died prior to transplantation and 12% recovered spontaneously. The 1-year survival of cadaveric liver transplant in ALF patients is lower than that in chronic liver failure patients; in part because of the extreme emergency conditions often encountered. After the first year, this trend has reversed and ALF patients have a better long-term survival. In addition to whole-organ deceased donor liver transplantation, live donor and auxiliary liver transplantation have been attempted but still remain controversial<sup>[65]</sup>.

## CONCLUSION

The management of ALF challenges our best skills because of its rapid progression and frequently poor outcomes. Early identification of ALF and the administration of etiology-specific treatment are crucial to improve the outcome. Extrahepatic organ failure should be well managed with advanced intensive care management. Better-targeted use of OLT techniques becomes important to save the patients who fail to recover spontaneously. A better understanding of the pathophysiology of ALF will probably lead to further improvement in survival rates.

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## Overexpression of miR-196b and HOXA10 characterize a poor-prognosis gastric cancer subtype

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### Abstract

**AIM:** To identify molecular biologic differences between two gastric adenocarcinoma subgroups presenting different prognoses through the analysis of microRNA and protein expression.

**METHODS:** Array technologies were used to generate 1146 microRNAs and 124 proteins expression profiles of samples from 60 patients with gastric cancer. For the

integrative analysis, we used established mRNA expression data published in our previous study. Whole mRNA expression levels were acquired from microarray data for 60 identical gastric cancer patients. Two gastric adenocarcinoma subgroups with distinct mRNA expression profiles presented distinctly different prognoses. MicroRNA and protein expression patterns were compared between gastric cancer tissue and normal gastric tissue and between two different prognostic groups. Aberrantly expressed microRNA, associated mRNA, and protein in patients with poor-prognosis gastric cancer were validated by quantitative reverse transcription polymerase chain reaction and immunochemistry in independent patients.

**RESULTS:** We obtained the expression data of 1146 microRNAs and 124 cancer-related proteins. Four microRNAs were aberrantly expressed in the two prognostic groups and in cancer *vs* non-cancer tissues ( $P < 0.05$ ). In the poor-prognosis group, miR-196b, miR-135b, and miR-93 were up-regulated and miR-29c\* was down-regulated. miR-196b expression positively correlated with Homeobox A10 (HOXA10) expression ( $r = 0.726$ ,  $P < 0.001$ ), which was significantly increased in poor-prognosis patients ( $P < 0.001$ ). Comparing gastric cancer with non-cancer tissues, 46/124 proteins showed differential expression ( $P < 0.05$ ); COX2 ( $P < 0.001$ ) and cyclin B1 ( $P = 0.017$ ) were clearly over-expressed in the poor-prognosis group.

**CONCLUSION:** Co-activation of miR-196b and HOXA10 characterized a poor-prognosis subgroup of patients with gastric cancer. Elucidation of the biologic function of miR-196b and HOXA10 is warranted.

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**Key words:** Gastric cancer; Gene expression; Microarray; MicroRNA; miR-196b; Homeobox A10

**Core tip:** Using an integrative analysis of the tumor transcriptome and protein expression profiles obtained by microarray techniques, we have elucidated the molecular biologic characteristics of a poor prognosis gastric cancer subgroup. Overexpression of miR196b and Homeobox A10 are implicated in gastric cancer, particularly in tumors with poor prognosis features. We anticipate this integrative approach will contribute to the characterization of cancer heterogeneity and the development of personalized therapy through the identification of cancer targets.

Lim JY, Yoon SO, Seol SY, Hong SW, Kim JW, Choi SH, Lee JS, Cho JY. Overexpression of miR-196b and HOXA10 characterize a poor-prognosis gastric cancer subtype. *World J Gastroenterol* 2013; 19(41): 7078-7088 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i41/7078.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7078>

## INTRODUCTION

Gastric cancer is the second leading cause of cancer-related deaths worldwide<sup>[1]</sup>. Surgery is the only curative treatment strategy and conventional chemotherapy has shown limited efficacy for the treatment of patients with advanced disease. Understanding the molecular mechanisms governing carcinogenesis, progression, and prognosis in gastric cancer is a prerequisite for the construction of a convincing management strategy. It is widely acknowledged that there are favorable and poor-prognosis subtypes of cancer, although the treatment strategy is generally determined by clinical and histopathologic stage rather than disease type. Patients with gastric cancer are also clinically heterogeneous, which suggests an underlying molecular diversity<sup>[2,3]</sup>. Molecular subtypes of gastric cancer have been suggested through analysis of gene or protein expression profiles and oncogenic signaling pathways<sup>[4-9]</sup>. In a previous study, we identified distinct gastric cancer subclasses by analyzing gene expression profiles and we described two gastric cancer subtypes that were strongly associated with prognosis<sup>[10]</sup>.

MicroRNAs play a role in the pathogenesis of various human cancers<sup>[11]</sup>. Some microRNAs function as oncogenes and others as tumor suppressors, although their mechanisms remain to be elucidated<sup>[12,13]</sup>. The relationship between microRNA expression profile and gastric cancer prognosis has been actively explored<sup>[14,15]</sup>, as has the pathogenesis of gastric cancer<sup>[16,17]</sup>. Overexpression of miR-196b has been linked to leukemia and several solid cancers including gastric cancer and may represent a useful gastric cancer marker<sup>[18,19]</sup>. A role for miR-196b as an oncogene or tumor suppressor has not yet been confirmed<sup>[20]</sup>, nor has its role in gastric carcinogenesis and progression.

Homeobox A10 (HOXA10) is transcription factor involved in the proliferation of hematopoietic stem cells

and progenitor cells. Its over-expression is associated with cancer development and poor prognosis in patients with acute myeloid leukemia and solid cancers<sup>[21-23]</sup>. HOXA10 is a neighboring gene of miR-196b but these genes have no known function in gastric cancer pathogenesis.

In this study, we generated microRNA and protein expression profiles using samples from 60 patients with gastric cancer to identify molecular biologic differences between previously identified good- and poor-prognosis patient subgroups.

## MATERIALS AND METHODS

### Patients and samples

Tumor specimens and clinical data were obtained from patients with primary gastric adenocarcinoma who underwent curative gastrectomy as the primary treatment between 1999 and 2007 at Severance Hospital and Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Three independent patient cohorts were used for each experiment: Group 1 (frozen tissue samples from 60 patients, each of whom provided a cancer sample and 8 of whom provided a non-cancer sample) for microRNA, and protein microarray analysis (Table 1); Group 2 (12 paired cancer and non-cancer frozen tissue samples from 12 patients) for validation tests using quantitative reverse transcription polymerase chain reaction (qRT-PCR); and Group 3 (paraffin-embedded samples from 368 patients with gastric cancer) for tissue microarray immunohistochemistry (IHC). Frozen tissue samples were examined by pathologists at the time of collection and were stored below -80 °C at a tissue bank until the analysis. Samples were collected after obtaining written informed consent from the patients. The study was approved by the institutional review board of Yonsei University Health System.

### MicroRNA microarray

Total RNA was extracted from fresh frozen tissues using a mirVana<sup>TM</sup> miRNA isolation kit (Ambion, Austin, TX, United States). Microarray experiments were performed according to the manufacturer's protocols (Illumina microRNA Expression Profiling Assay; Illumina, San Diego, CA, United States). The panel contained 1146 miRNA assays described in the Sanger Institute miRBase Release 12.0 (from miR-1 to miR-1827)<sup>[24]</sup>; 200 ng RNA was used for labeling and hybridization. Chips were scanned with an Illumina BeadArray Reader (Illumina, San Diego, CA, United States) and intensity values were analyzed using BeadStudio version 3.1.3. A cut-off detection *P* value of 0.01 was used to determine whether a microRNA probe was significantly detected. Microarray data were normalized using the quantile normalization method. MicroRNA microarray data is available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) public database (accession number GSE26595).



**Table 1** Clinicopathologic factors of patients with gastric cancer participating in the microarray analysis *n* (%)

Characteristic	Group 1 ( <i>n</i> = 60)	miR-196b high ( <i>n</i> = 28)	miR-196b low ( <i>n</i> = 29)	<i>P</i> value	HOXA10 high ( <i>n</i> = 29)	HOXA10 low ( <i>n</i> = 28)	<i>P</i> value
Age, yr							
Median (range)	63 (32-83)	66 (34-78)	58 (32-83)	0.134	64 (34-83)	60 (32-78)	0.134
> 65		15 (52)	9 (32)		15 (52)	9 (32)	
Sex							
Male	41 (68)	19 (68)	20 (69)	0.928	18 (62)	21 (75)	0.294
Histologic type							
Diffuse	27 (45)	9	17	0.066	8	18	0.007
Intestinal	23 (38)	14	9		16	7	
Mixed	10 (17)	5	3		5	3	
T stage							
T1/T2	26 (43)	10 (36)	14 (48)	0.337	11 (38)	13 (46)	0.516
T3/T4	34 (57)	18 (64)	15 (52)		18 (62)	15 (54)	
N stage							
N0/N1	36 (60)	15 (54)	19 (66)	0.358	16 (55)	18 (64)	0.483
N2/N3	24 (40)	13 (46)	10 (34)		13 (45)	10 (36)	
AJCC stage <sup>1</sup>							
I / II	23 (38)	11 (39)	12 (41)	0.872	11 (38)	12 (43)	0.705
III / IV	37 (62)	17 (61)	17 (59)		18 (62)	16 (57)	

<sup>1</sup>Based on the American Joint Committee on Cancer staging manual 6<sup>th</sup> edition. HOXA10: Homeobox A10; AJCC: American Joint Committee on Cancer.

### Protein expression assay

Reverse-phase protein array (RPPA) was performed according to the manufacturer's protocol and the data were analyzed as previously described<sup>[25]</sup>. Frozen tissues were lysed in RPPA lysis buffer and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein-containing samples were diluted to a uniform protein concentration and transferred into 384-well plates. The lysate was printed onto nitrocellulose-coated glass slides and probed using 124 antibodies. Signal intensity was measured by scanning the slides: slides were scanned, analyzed, and quantified using the customized Microvigene software (VigeneTech, Inc., Carlisle, MA, United States) to measure spot intensity.

### Analysis of microarray data

The microarray expression level was transformed into a log 2 base before further analysis. Cluster analysis was performed after median centering using the Cluster 3.0 program and visualized in Treeview (Eisen Lab. CA, United States). BRB-ArrayTools (version 3.6; Biometrics Research Branch, National Cancer Institute, MD, United States) were used for microarray data analysis<sup>[26]</sup>. The previously generated gene expression data from the poor-prognosis group<sup>[10]</sup> were obtained from the NCBI GEO public database (accession number GSE13861).

### Quantitative RT-PCR and analysis

Quantitative RT-PCR was carried out according to the manufacturer's protocols (miRURY LNA microRNA PCR System, Exiqon, Vedbaek, Denmark) and was performed in duplicate. The RT reactions were incubated for 30 min at 50 °C followed by heat inactivation at 85 °C for 5-10 min. cDNA templates were diluted and added to the PCR master mix. Cycling conditions were as follows: 37 °C for 10 min, 95 °C for 10 min, followed by 40

cycles of 95 °C for 20 s and 60 °C for 60 s. RT-PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) with a 96-well block module. Relative amounts of microRNA were calculated from the threshold cycle (C<sub>T</sub>) number using the expression of U6 snRNA for normalization.

### Tissue microarray construction and IHC staining

Paraffin-embedded tissue microarray blocks were created using gastric cancer tissue specimens obtained from 368 patients. A tissue microarray set contained 14 slides with 30 tissue cores per slide. Sections 4 µm thick were deparaffinized and treated to block endogenous peroxidase activity. After antigen retrieval, the primary antibody - anti-HOXA10 antibody (polyclonal, dilution 1:75; Santa Cruz Biotechnology, Santa Cruz, CA, United States) - was applied to the sections. The sections were incubated with a secondary antibody (horse radish peroxidase-rabbit/mouse), developed using a NovaRED substrate kit (VECTOR Laboratory, Burlingame, CA, United States) and counterstained with Harris hematoxylin. Stained slides were examined with a Zeiss Axio Imager M2 microscope with an AxioCam HRc camera and photographed using AxioVision 4.8.2 software (Zeiss, Jena, Germany). Strong cytoplasmic staining was considered as positive expression. Positive expression was defined as staining stronger compared to smooth muscle. Negative expression was defined as staining positivity lower than or similar to smooth muscle.

### Statistical analysis

Statistical analysis was performed with PASW Statistics 18 (SPSS Inc., San Diego, United States) and graphical interpretations were generated with GraphPad Prism 5 (GraphPad Software, San Diego, CA, United States). The Kaplan-Meier method was used to estimate patient

prognosis; differences between genotypes were compared using the log-rank test. Differences between groups were analyzed using the two-tailed Student's *t* test and the  $\chi^2$  test. Associations between the expression levels of the two targets were analyzed using the Pearson correlation coefficient. Generally, differences were considered statistically significant at  $P < 0.05$ .

## RESULTS

### Gene expression microarray-established gastric cancer subgroups C1 and C2

In our previous study<sup>[10]</sup>, two gastric cancer subgroups [a poor-prognosis group C1 ( $n = 32$ ) and a good-prognosis group C2 ( $n = 28$ )] were established through unsupervised hierarchical clustering of gene expression data from the 60 patients with gastric cancer in Group 1. Genes with expression levels that were at least 2-fold different in at least 15 tissues, relative to the median value across tissues, were selected for hierarchical clustering analysis (2077 gene features) (Figure 1A). Following comparison of gene expression profiles of samples from C1, C2, and non-cancer patients, unique gene sets were identified for the C1 and C2 groups that contained 2755 and 1437 genes, respectively ( $P < 0.001$ ). C1 patients had significantly poorer relapse-free survival than C2 patients ( $P = 0.0019$ ; log-rank test). This was independent of disease stage, indicating that the molecular features reflected in gene expression patterns might be strong independent predictors of clinical outcome (Figure 1B). Gene expression data for 5922 transcription factor (TF) genes were selected and C1-specific TF genes were extracted (Figure 1C). A total of 413 up-regulated and 326 down-regulated TF genes are shown in Figure 1D; the top 20 up- and down-regulated TF genes are listed in Figure 1E.

### microRNA expression profile of gastric cancer

A total of 410 out of 1146 probes in the microRNA expression assay were significantly detected ( $P < 0.01$ ) and further processed (Figure 2A). Though unsupervised clustering analysis revealed two major subtypes, these did not appear to have any prognostic or clinico-pathologic value. Subsequently, 164 microRNAs significantly ( $P < 0.05$  in the *t* test) aberrantly expressed in gastric cancer tissue were identified (Figure 2A). Non-cancer tissue showed relatively homogenous microRNA expression patterns, in contrast to the highly heterogeneous expression patterns observed in cancer tissues.

### Aberrant microRNA expression in C1 and C2 patients

MicroRNA expression data were compared between non-cancer and cancer tissues and between C1 and C2 samples. Four microRNAs were uniquely dysregulated ( $P < 0.05$ ) among non-cancer, C1, and C2 samples (Figure 2A). miR-196b, miR-135b, and miR-93 were up-regulated and miR-29c\* was down-regulated in the C1, poor-prognosis group (Figure 2B). Log-transformed expression lev-

els are shown in Figure 3A. miR-196b showed the most significantly different expression patterns between cancer and non-cancer samples ( $P < 0.001$ ), and between the C1 and C2 groups ( $P < 0.001$ ).

### Validation of microRNA expression by quantitative RT-PCR

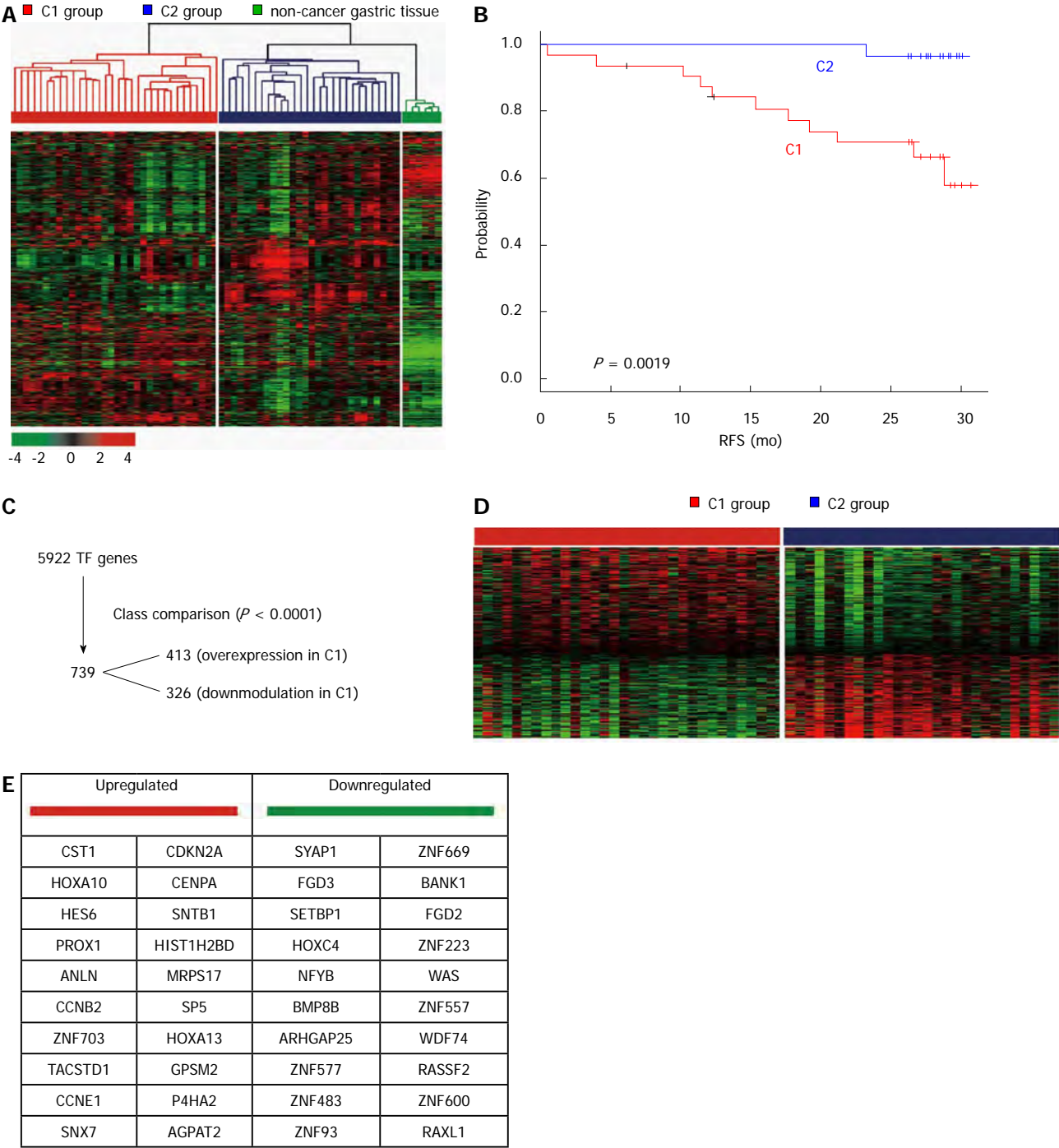
To validate the microRNA expression microarray data, qRT-PCR was performed using RNA from the 12 patients in Group 2. Using Ct numbers, miR-196b was determined to be up-regulated in gastric cancer tissue compared with non-cancer tissue ( $P = 0.007$ ; two-sample paired *t* test; Figure 3B). The up-regulation of miR-135b in gastric cancer samples was not statistically significant ( $P = 0.118$ ).

### Correlation between HOXA10 and miR-196b expression

HOXA9 and HOXA10 are neighboring genes to miR-196b (Figure 4A) and are regarded as miR-196b-associated genes. Therefore, the expression level of HOXA9 and HOXA10 and their correlation with miR-196b expression were evaluated. Analysis of microRNA and mRNA expression data from gastric cancer tissue samples obtained from patients in Group 1 showed that HOXA10 mRNA expression was positively correlated with miR-196b expression (Figure 4B;  $r = 0.726$ ,  $P < 0.001$ ). HOXA10 expression was up-regulated in gastric cancer compared with non-cancer tissue and was higher in C1 compared with C2 patients (Figure 4C). Overexpression of miR-196b was significantly associated with shorter overall survival (OS;  $P = 0.022$ ; Figure 4D). Overexpression of HOXA10 exhibited a tendency to poor OS ( $P = 0.202$ ; Figure 4D) but HOXA9 did not exhibit any significant association with miR-196b or prognosis (Figure 4B and D). HOXA10 protein-positive expression was identified in 56 of 368 gastric cancer tissue samples (15.2%) from patients in Group 3 (Figure 5A and B). In non-cancer gastric tissue, body glands were stained variably. In contrast to well and moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma and signet ring cell carcinoma showed less positive staining for HOXA10 (29.4% vs 8.4%,  $P < 0.001$ ).

### Protein expression profile of gastric cancer

The unsupervised clustering analysis revealed that protein expression was highly variable compared with mRNA and microRNA expression in non-cancer and cancer tissues. Two-group two-sample *t* tests comparisons were performed using cancer and non-cancer samples to identify gastric cancer-specific proteins. Univariate analyses revealed 48 out of 124 proteins with significantly different levels ( $P < 0.05$ ) between cancer and non-cancer tissues (Figure 5B). Among these 48 proteins, the expression of COX2 and cyclin B1 was significantly increased in gastric cancer vs non-cancer tissue (both  $P < 0.001$ ), and up-regulated in the C1 compared with C2 groups ( $P < 0.001$  for COX2 and  $P = 0.016$  for cyclin B1).



**Figure 1** Two prognostic groups of patients with gastric cancer, C1 and C2, have distinct mRNA expression patterns. A: mRNA expression patterns of gastric cancer and non-cancer gastric tissue are depicted in a heat map. Hierarchical clustering revealed two subtypes of gastric cancer, C1 and C2. The data are presented as a matrix in which rows represent individual mRNAs and columns represent each tissue. The color red represents relatively high expression levels and green reflects lower expression levels; B: Kaplan-Meier plot of relapse-free survival (RFS) revealed C1 and C2 represented poor- and good-prognosis gastric cancer tissues, respectively; C: Workflow of C1-specific 739 transcription factors (TFs) selected from a total 5,922 TFs; D: Hierarchical clustering of gene expression for 739 TF genes; E: List of top 20 up- and down-regulated TF genes in the C1 group.

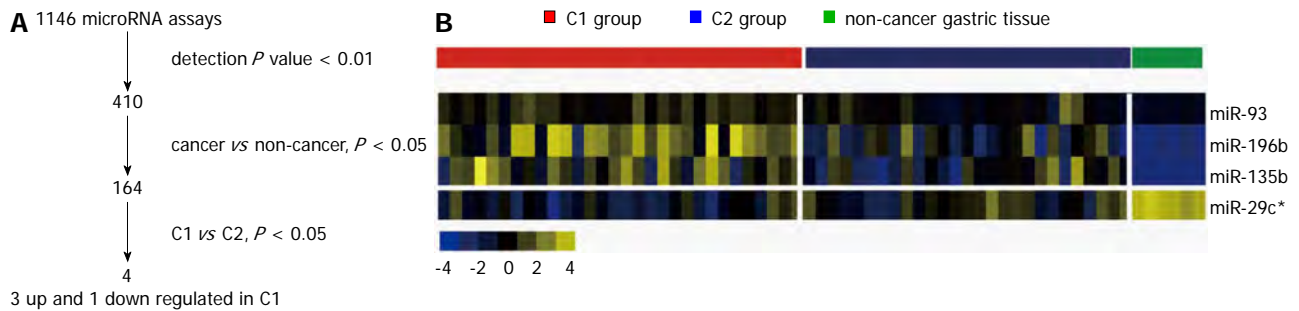
## DISCUSSION

We previously identified two prognostic subgroups of patients with gastric cancer through gene expression microarray technology<sup>[10]</sup>. Our findings suggested that their gene expression signatures reflected clinical differences between good- and poor-prognosis patients independent

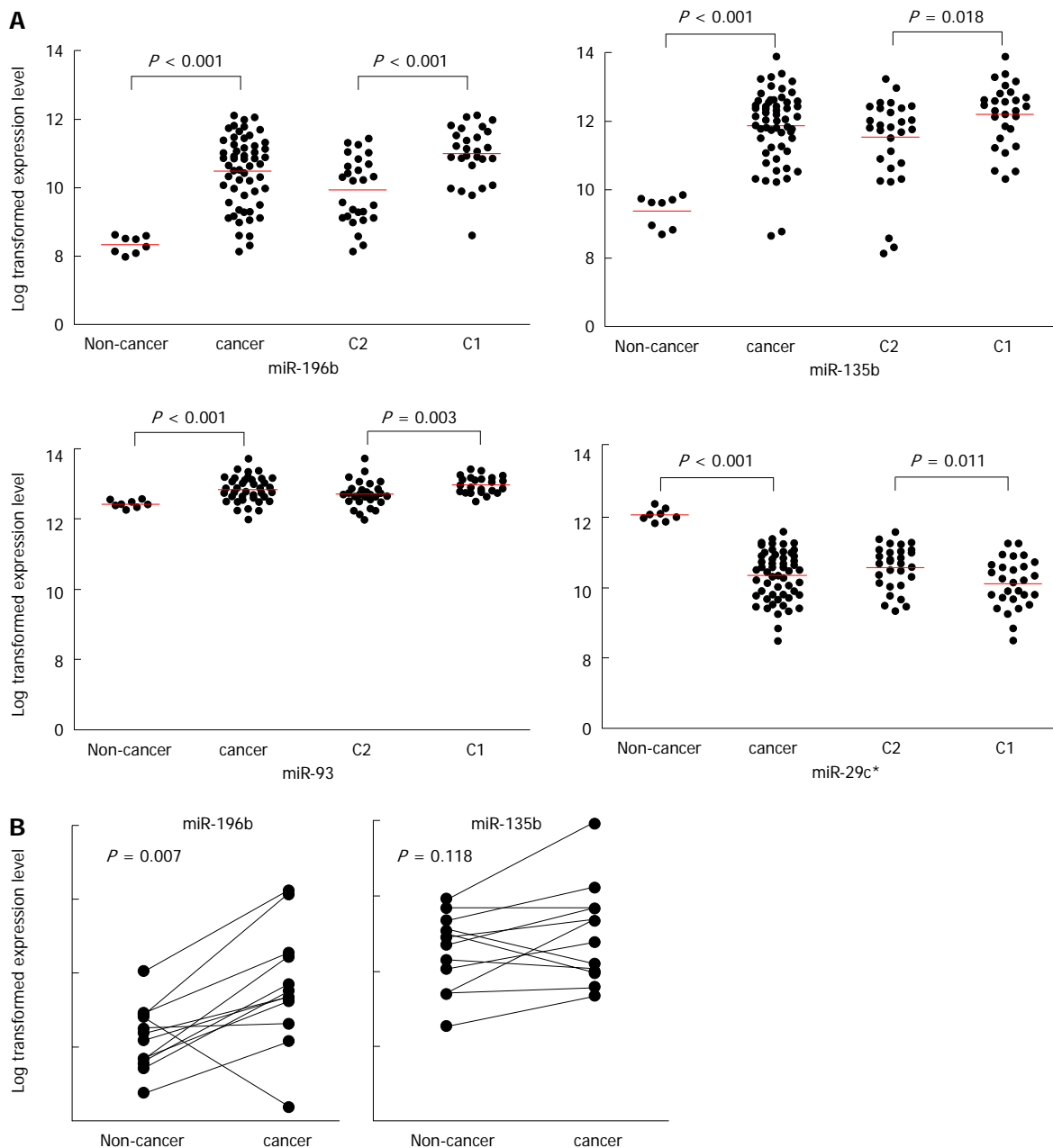
of tumor-node-metastasis stage and histology. The molecular biologic mechanisms responsible for these clinical differences were unknown. In an effort to understand these molecular mechanisms, we obtained microRNA and protein expression data from tumor tissue samples using high-throughput microarray techniques.

First, two-group comparison analysis revealed 164 mi-

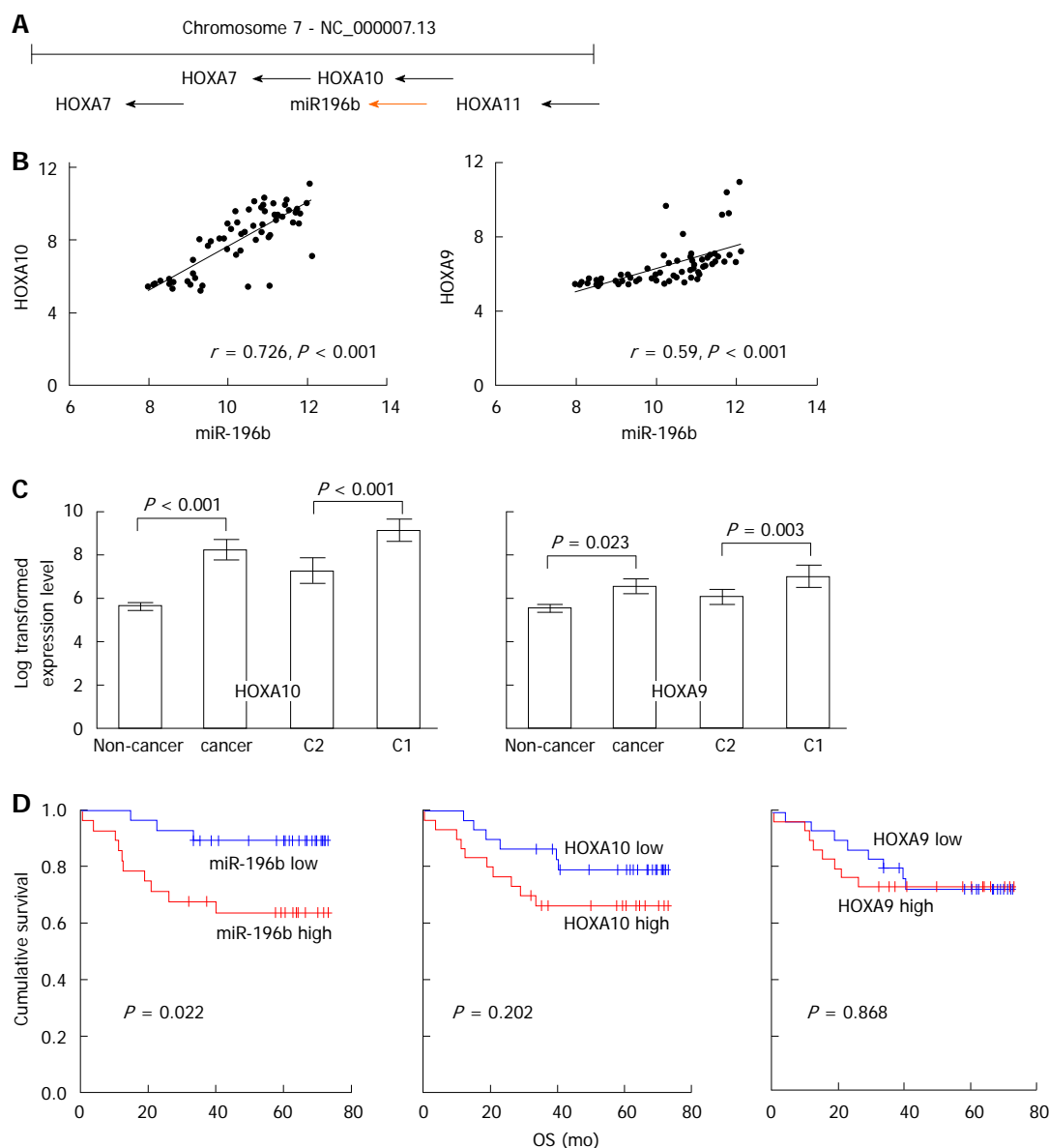




**Figure 2** Aberrant microRNA expression in gastric cancer. A: Flowchart of the data analysis to identify poor-prognosis C1-specific microRNAs. Four microRNAs (miR-196b, miR-135b, miR-93, and miR-29c\*) were selected; B: Expression patterns of the four microRNAs are shown in a heat map for C1, C2, and non-cancer gastric tissues. The color yellow represents relatively high expression levels and blue reflects relatively low levels.



**Figure 3** The expression levels of four microRNAs. A: Four microRNAs expressed differentially in gastric tissues. Scatter plots of gene expression levels with a bar indicating the mean; B: The expression level of miR-196b and miR-135b were validated in a quantitative reverse transcription polymerase chain reaction study of 12 patients samples.



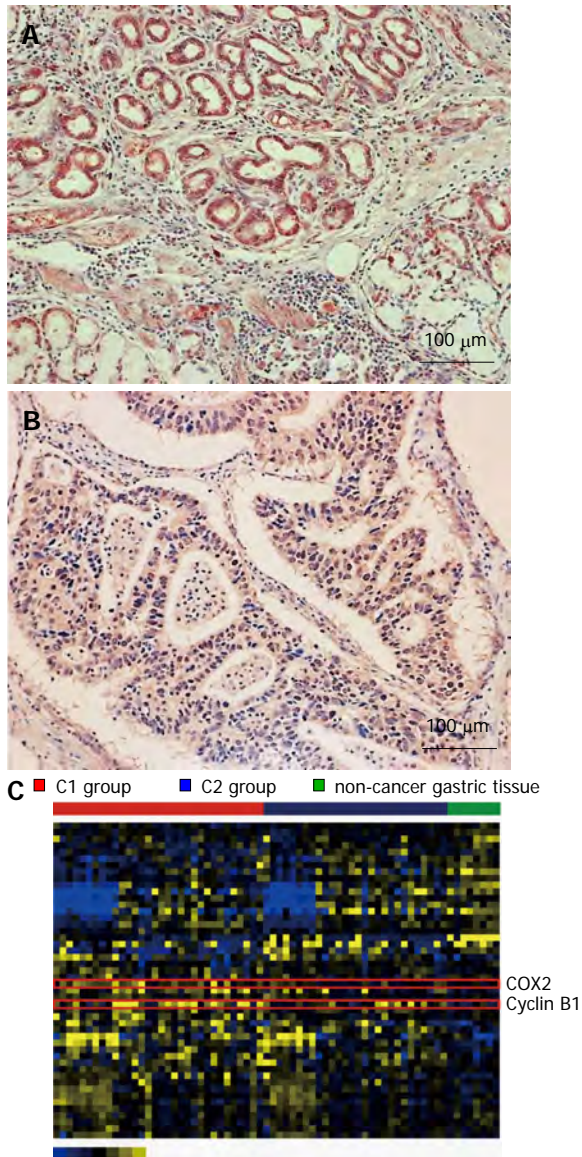
**Figure 4** The correlation between miR-196b and homeobox A10 expression in gastric cancer. **A:** miR-196b, homeobox A10 (HOXA10), and HOXA9 are neighbor genes and located on chromosome 7p15.2; **B:** Expression of miR-196b and HOXA10 were positively correlated ( $r = 0.726$ ;  $P < 0.001$ ). The correlation between miR-196b and HOXA9 expression was less significant; **C:** HOXA10 and HOXA9 expression level in gastric cancer vs non-cancer tissues and in the C1 vs the C2 group. Results are presented as means with 95%CI; **D:** Kaplan–Meier plot of overall survival according to the expression level of miR-196b, HOXA10, and HOXA9.

croRNAs that showed different expression profiles in the cancer and non-cancer tissue samples. Supervised clustering (C1 vs C2 vs non-cancer) using these 164 microRNAs revealed that non-cancer tissues manifested relatively homogenous microRNA expression. A comparison of the microRNA expression revealed that three microRNAs (miR-196b, miR-135b, and miR-93) were up-regulated in C1 vs C2 and in cancer vs non-cancer tissues. Conversely, miR-29c\* was down-regulated in C1 samples. We suggest, therefore, that dysregulation of those four microRNAs might characterize C1, our previously identified poor-prognosis gastric cancer subgroup. The accumulated knowledge about microRNA-associated genes and cancers is summarized in Table 2.

The overexpression of miR-196b has previously been linked to leukemia and several solid cancers<sup>[18,19]</sup>. The

increased expression of miR-196b contributes to the development of leukemia and the relationship between differential miR-196b expression and clinical and biologic features has been studied in leukemia<sup>[18,39–41]</sup>. miR-196b expression is also increased in pancreatic ductal adenocarcinoma and bronchial squamous cell carcinoma, although its role in these cancers has not been established<sup>[27,28]</sup>. Tsai *et al*<sup>[19]</sup> reported that a lack of promoter methylation in miR-196b resulted in its overexpression in gastric cancer cell lines and patient samples, and they proposed that miR-196b overexpression might provide a useful tumor marker. However, it has not yet been determined whether miR-196b functions as an oncogene or a tumor suppressor<sup>[20]</sup>, and its role in gastric carcinogenesis and progression has not yet been identified.

In recent *in vitro* study, a leukemia oncogene was found



**Figure 5** Aberrant protein expression in gastric cancer. A: Representative image from the homeobox A10 (HOXA10) immunohistochemical assay ( $\times 200$ ) of gastric cancer tissue. The moderately differentiated adenocarcinoma shows positive for HOXA10 expression. A strong cytoplasmic immunoreaction was noted; B: Negative for HOXA10 expression; C: The expression pattern of 48 proteins from Reverse-phase protein array (RPPA) was depicted using a heat map. The color yellow represents relatively high expression levels and blue reflects relatively low levels.

to promote the expression of miR-135b, with elevated miR-135b expression decreasing chemosensitivity, suggesting the contribution of miR-135b to the oncogenic activity of the leukemia oncogene<sup>[29]</sup>. It has been suggested that oncogenic kinase-linked miR-135b contributes to tumorigenesis through modulation of the tumor-immune phenotype and microenvironment<sup>[29]</sup>. Others have identified miR-135b as a novel biomarker for pancreatic ductal adenocarcinoma<sup>[30]</sup>. In chronically inflamed or genetic models of colon cancer, miR-135b was suggested along with other microRNAs, to regulate signaling pathways related to mitogen-activated protein kinase, phosphoinositide 3-kinase, WNT, and transforming growth factor

(TGF)- $\beta$ , all of which are known to be involved in cell transformation<sup>[31]</sup>. However, there are as of yet no data relating to the pathogenesis of gastric cancer.

miR-93 is a well-known constituent of the miR-106b approximately 25 cluster, paralogs of which have oncogenic activity in several cancers, including gastric cancer. Up-regulated miR-93 in gastric cancer leads to cell cycle activation and impaired apoptosis<sup>[17,32]</sup>. miR-29c\* is, however, poorly understood with no known target and it has only been shown to be an independent favorable prognostic factor in malignant pleural mesothelioma<sup>[38]</sup>.

Information about each of these microRNAs provides support for the proposal that our poor-prognosis microRNA signature - up-regulation of miR-196b, miR-135b, and miR-93 and down-regulation of miR-29c\* - is relevant. As miR-93 has largely been investigated in gastric cancer and miR-29c\* is poorly understood, we focused our attention on miR-196b and miR-135b. The qRT-PCR assay demonstrated that miR-196b was significantly overexpressed in cancer *vs* non-cancer tissue while miR-135b was not (Figure 3B). Therefore, the role of miR-196b in gastric cancer was further investigated.

Using correlation analysis, we identified that HOXA10 levels were also elevated in gastric cancer. The expression of miR-196b appears to be most positively associated with HOXA10 expression. Our previous gene expression microarray data indicated that HOXA10 was ranked as the second most up-regulated TF in gastric cancer (Figure 1E), as it was significantly elevated in gastric cancer *vs* non-cancer and in poor-prognosis C1 *vs* good-prognosis C2. Evidence is accumulating that HOXA10 is involved in the proliferation of hematopoietic stem cells and progenitor cells, leading to cancer development through activating the target genes coding for TGF $\beta$ 2, dual-specificity protein phosphatase 4, and integrin- $\beta$ 3<sup>[21,42,43]</sup>. Up-regulation of HOX genes is associated with a tumor stem-like cell phenotype of glioblastoma, and high HOXA10 protein expression has been associated with resistance to chemotherapy<sup>[44]</sup>. HOXA10 is also over-expressed in a poor-prognosis subset of patients with acute myeloid leukemia and in several solid cancers<sup>[21-23]</sup>. On the basis of these findings, we postulate that HOXA10 may induce gastric carcinogenesis and function as a marker of poor prognosis. Recently, it was reported that upregulation of HOXA10 was correlated with favorable prognosis and inversely correlated with the depth of invasion in gastric cancer<sup>[45]</sup>. Different patient populations and detection methods for HOXA10 seems to be responsible for inconsistent results. More reliable evidences should be accumulated to elucidate the role of HOXA10 in gastric cancer.

miR-196b is located in the HOXA cluster on chromosome 9 and expression levels of pri-miR-196b are highly correlated with its neighboring gene HOXA10<sup>[18]</sup>. A previous study showed that expression patterns of miR-196b were inversely correlated with the methylation status of promoter CpG-rich regions in gastric cancer cell lines and primary gastric cancer samples<sup>[19]</sup>. Suzuki



**Table 2** microRNAs differentially expressed in gastric cancer tissues; non-cancer tissue and C1 and C2 groups ( $P < 0.05$ )

Name	Chromosome location	Function	Related gene	Related cancer	References
Expression level: C1 > C2 > non-cancer					
hsa-miR-196b	7p15.2	Tumor suppressor, progression	HOXA, HOXC8	T-cell ALL, MLL-rearranged leukemia, ALL, stomach, lung, pancreas	18,19,27,28
hsa-miR-135b	1q32.1	Tumorigenesis	FOXO1, APC	Leukemia, anaplastic large cell lymphoma, pancreas, colon, prostate, osteosarcoma	29-31
hsa-miR-93	7q22.1	Tumorigenesis, progression, prognosis	E2F1, ZBTB4, FUS1, TP53INP1, ITGB8, VEGFA, CDKN1A	Kidney, breast, colon, liver, stomach, lung, osteosarcoma, adult T-cell leukemia	17,32-37
Expression level: C1 < C2 < non-cancer					
hsa-miR-29c*	1q32.2	Prognosis		Malignant pleural mesothelioma	38

ALL: Acute lymphoblastic leukemia; MLL: Mixed lineage leukemia gene; HOXA10: Homeobox A10.

*et al.*<sup>[46]</sup> analyzed genome-wide profiling of chromatin signatures combining microRNA expression and suggested that DNA demethylation can alter the chromatin signatures of numerous microRNAs in cancer. Elevated expression of miR-196b in gastric cancer cells may result from the preferential unmethylated status of CpG islands and from other TFs. We suggest that HOXA10 expression might also be governed by miR-196b modulation; however, the exact mechanism of miR-196b promoter activity modulation by TFs remains to be elucidated.

In the present study, miR-196b and HOXA10 were co-activated in gastric cancer samples, especially in the poor-prognosis group. miR-196b and HOXA10 are also closely related to the development and progression of hematopoietic stem cells. This suggests that gastric cancer co-activated by miR-196b and HOXA10 might share the same pathogenesis as leukemia. Chronic inflammation caused by *Helicobacter pylori* (*H. pylori*) is associated with gastric carcinogenesis and there is recent evidence that *H. pylori* infection recruits bone marrow-derived cells (BMDCs) in the gastric epithelial mucosa that participate in gastric preneoplasia<sup>[47,48]</sup>. Over-expression of miR-196b and HOXA10 may mediate the development of hematopoietic progenitor cells and gastric cancer; thus, it is necessary to elucidate the mechanism and function of the co-activation of miR-196b and HOXA10 in gastric cancer.

RPPA is a quantitative assay that analyzes large samples for known proteins and their active forms. We evaluated the expression of 124 proteins to understand the physiology and pathogenesis of gastric cancer at the protein level. The limitations of this platform, measuring only small numbers of known antibodies, were taken into consideration when interpreting the data. As shown in Figure 5B, protein expression patterns are highly heterogeneous, even between gastric cancers, indicating that a few tumor targets will apply to almost all patients with gastric cancer and that most markers are likely to only apply to selected patients. In the present study, COX2 and cyclin B1 were significantly elevated in the C1 group compared with the C2 group. In combination with gene expression array data, the HOXA10 promoter has a nuclear factor- $\kappa$ B (NF $\kappa$ B) binding site, and NF $\kappa$ B controls COX2 expression, leading to inflammation and

carcinogenesis. The elucidation of this relationship might explain the mechanism of HOXA10 over-expression in gastric cancer.

In conclusion, using an integrative analysis of tumor transcriptome and protein expression data by microarray techniques, we have elucidated the molecular biologic characteristics of a poor-prognosis gastric cancer subgroup. We postulate that the overexpression of miR-196b and HOXA10 are implicated in gastric cancer, particularly in tumors with poor-prognosis features. The personalized cancer therapy targets aberrations that drive tumor growth and survival. Administering the right drug for the right person consequently improves clinical outcomes and decreases toxicity. We anticipate that this approach will contribute to the characterization of cancer heterogeneity and the development of personalized therapy through the identification of cancer targets.

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## COMMENTS

### Background

Gastric cancer is clinically heterogeneous, which suggest underlying molecular diversity. Advances in high-throughput technologies such as microarray for gene or protein expression profiles have reinforced the identification of molecular biologic characteristics of cancer. It is of great clinical value to perform integrative analysis of data from diverse high-throughput technologies simultaneously, to elucidate gastric cancer subtype harboring poor prognosis.

### Research frontiers

MicroRNAs play a role in the pathogenesis of various human cancers by regulating the expression of target gene post transcriptionally. Microarray and bioinformatic technologies enable the investigation of the trans-correlation of hundreds of microRNAs and tens of thousands of genes.

### Innovations and breakthroughs

MiR-196b was the most significantly up-regulated microRNA in gastric cancer and was significantly correlated with poor prognosis. The expression of miR-196b appeared to be most positively associated with homeobox A10 (HOXA10) expression. The expression of miR-196b and HOXA10 seems to be regulated by identical biologic mechanism and characterize a poor-prognosis gastric cancer subtype.

## Applications

An integrative approach that simultaneously evaluates the expression profiles of mRNA, microRNA, and protein in cancer tissue will contribute to characterizing cancer heterogeneity and developing personalized therapy through the identification of cancer targets.

## Terminology

A microarray is a multiplex lab-on-a-chip and assays large amounts of biological material using high-throughput screening methods. The types of microarrays include DNA microarray, microRNA microarray, protein microarray, and tissue microarray. Bioinformatics is the analysis of biological data including gene and protein expression and regulation data.

## Peer review

This study generated and analyzed gene, microRNA, and protein expression profiles from 60 patients with gastric cancer and identified the overexpression of miR-196b and HOXA10 as a characteristic of a poor-prognosis gastric cancer subtype. Further investigation to elucidate the molecular biologic function of miR-196b and HOXA10 in gastric cancer is necessary.

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## Magnifying endoscopy for the diagnosis of specialized intestinal metaplasia in short-segment Barrett's esophagus

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III (long oval), IV (tubular), V (villous)] by Endo's classification. Then, a 0.5% solution of methylene blue (MB) was sprayed over columnar mucosa. The patterns of the magnified image and MB staining were analyzed. Biopsies were obtained from the regions previously observed by magnifying endoscopy and MB chromoendoscopy.

**RESULTS:** Three of five patients with a type V (villous) epithelial pattern had SIM, whereas 21 patients with a non-type V epithelial patterns did not have SIM. The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of pit-patterns in detecting SIM were 100%, 91.3%, 92.3%, 60% and 100%, respectively ( $P = 0.004$ ). Three of the 12 patients with positive MB staining had SIM, whereas 14 patients with negative MB staining did not have SIM. The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of MB staining in detecting SIM were 100%, 60.9%, 65.4%, 25% and 100%, respectively ( $P = 0.085$ ). The specificity and accuracy of pit-pattern evaluation were significantly superior compared with MB staining for detecting SIM by comparison with the exact McNemar's test ( $P = 0.0391$ ).

**CONCLUSION:** The magnified observation of a short-segment BE according to the mucosal pattern and its classification can be predictive of SIM.

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**Key words:** Short-segment; Barrett's esophagus; Magnifying endoscopy; Methylene blue chromoendoscopy; Specialized intestinal metaplasia; Dysplasia; Esophageal adenocarcinoma; Diagnosis

**Core tip:** Various endoscopic approaches and advancements have shown great promise. However, careful endoscopic observation and stepwise four quadrant bi-

### Abstract

**AIM:** To determine whether magnified observation of short-segment Barrett's esophagus (BE) is useful for the detection of specialized intestinal metaplasia (SIM).

**METHODS:** Thirty patients with suspected short-segment BE underwent magnifying endoscopy up to  $\times 80$ . The magnified images were analyzed with respect to their pit-patterns, which were simultaneously classified into five epithelial types [I (small round), II (straight),

opsy still represent the standard for the surveillance of Barrett's esophagus (BE). In our study, we investigated the usefulness of magnifying endoscopy for the diagnosis of specialized intestinal metaplasia (SIM) in patients with short-segment BE compared with methylene blue chromoendoscopy. We found that the magnified observation of a short-segment BE according to its mucosal pattern and classification can be predictive of SIM.

Ham NS, Jang JY, Ryu SW, Kim JH, Park EJ, Lee WC, Shim KY, Jeong SW, Kim HG, Lee TH, Jeon SR, Cho JH, Cho JY, Jin SY, Lee JS. Magnifying endoscopy for the diagnosis of specialized intestinal metaplasia in short-segment Barrett's esophagus. *World J Gastroenterol* 2013; 19(41): 7089-7096 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7089.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7089>

## INTRODUCTION

Barrett's esophagus (BE) is important clinically as the link between one of the most common gastrointestinal diseases, gastroesophageal reflux disease (GERD), and the most rapidly increasing cancer of the gastrointestinal (GI) tract, esophageal adenocarcinoma (EAC). For an adenocarcinoma to develop in the esophagus, the squamous epithelium must transition to columnar epithelium and subsequently become dysplastic. This metaplasia-dysplasia-carcinoma sequence is attributed to the repeated injury of the esophagus by gastroesophageal reflux<sup>[1-3]</sup>. According to the Montreal consensus from 2006, BE is characterized by the replacement of the squamous epithelia in the distal esophagus by columnar epithelia (gastric metaplasia), irrespective of the presence of specialized intestinal metaplasia (SIM)<sup>[4]</sup>. Controversy exists regarding the absolute requirement of intestinal metaplasia to define BE, primarily because long-term follow-up studies are not available to assess the risk of progression for each histologic subtype. However, cross-sectional and descriptive studies suggest that SIM either coexists with or precedes a significant majority of EAC cases and is likely the precursor lesion<sup>[5,6]</sup>. Therefore, histologic confirmation of SIM in BE is required. Because of the latent period of transition to high grade dysplasia, EAC is significantly shorter for patients with low grade dysplasia (median of 2.75 years) than for patients without low grade dysplasia (median of 9.88 years)<sup>[1]</sup>.

Patients with SIM are currently recommended to undergo periodic endoscopic surveillance to determine the progression to dysplasia at an early, potentially curable stage<sup>[5,7]</sup>.

Discerning SIM and obtaining satisfactory target biopsies at the region of interest by standard endoscopic observation is difficult<sup>[8,9]</sup>. Thus, to identify the presence of SIM and dysplasia according to the Seattle protocol, specimens are obtained using a predefined four-quadrant sampling technique<sup>[10]</sup>. The major disadvantages of this method are the need for multiple biopsies, random choice

of biopsy places, and the high cost.

Chromoendoscopy and magnifying endoscopy have been improving mucosal visualization to allow for better differentiation of the SIM and dysplasia from the columnar epithelium during endoscopy<sup>[11,12]</sup>. These techniques provide more accurate biopsies as well as reduce the number of biopsies<sup>[13,14]</sup>. Chromoendoscopy involves the use of dyes sprayed over the mucosa. Methylene blue (MB) stains actively absorbing cells, such as the intestinal epithelium and intestinal metaplasia<sup>[11]</sup>. The sensitivity and specificity of MB staining for SIM detection in BE is still under discussion<sup>[15,16]</sup>. Magnifying endoscopy, which provides images of fine mucosal detail that correspond to histologic structure, is now widely accepted for the study of GI disorders. After magnification, a characteristic relief called a pit-pattern is visible on the surface of the esophageal epithelium. The most widely known classification of esophageal pit-patterns in relation to histology were described by Endo *et al*<sup>[17]</sup>. The usefulness of this classification is its ability to predict the presence of SIM based on the structure of the mucosal surfaces.

BE can be subdivided into long-segment BE ( $\geq 3$  cm) and short-segment BE ( $< 3$  cm)<sup>[18]</sup>. Just as for long-segment BE, histologic confirmation of SIM in short-segment BE is also needed; not only long-segment BE but also short-segment BE, have been known as major risk factors for the development of EAC<sup>[19,20]</sup>. Furthermore, small areas of dysplasia can be difficult to diagnose.

The aim of this study was to determine whether the magnified observation of short-segment BE is useful for the detection of SIM and for the prediction of histological diagnosis compared with MB chromoendoscopy.

## MATERIALS AND METHODS

Patients with short-segment BE were prospectively enrolled into this study at Soonchunhyang University Hospital in South Korea between March 2002 and June 2002 (Figure 1). Patients underwent magnifying endoscopy, which could enhance the image up to  $\times 80$  (Olympus GIF-Q240Z, Japan) (Figure 2). Mucus was removed by a 10% solution of acetylcysteine instillation. The magnified images were analyzed with respect to pit-patterns, which were simultaneously classified into five epithelial types [I (small round), II (straight), III (long oval), IV (tubular), V (villous)] by Endo's classification (Figure 3). Then, a 0.5% solution of methylene blue was sprayed over the columnar mucosa. The excess of dye was flushed away with 50 mL of water after 2 min. The patterns of the magnified image and MB staining were analyzed. Biopsies were obtained from the regions previously observed by magnifying endoscopy and MB chromoendoscopy (Figure 4). If the biopsies were unsatisfactory or inaccurately targeted, other biopsies were performed. Every biopsy was classified into three types of epithelium by a pathologist: the fundic type, cardiac type and SIM (Figure 5). The study was performed after receiving approval from the Institutional Review Board of the Soonchunhyang University in Seoul, South Korea.

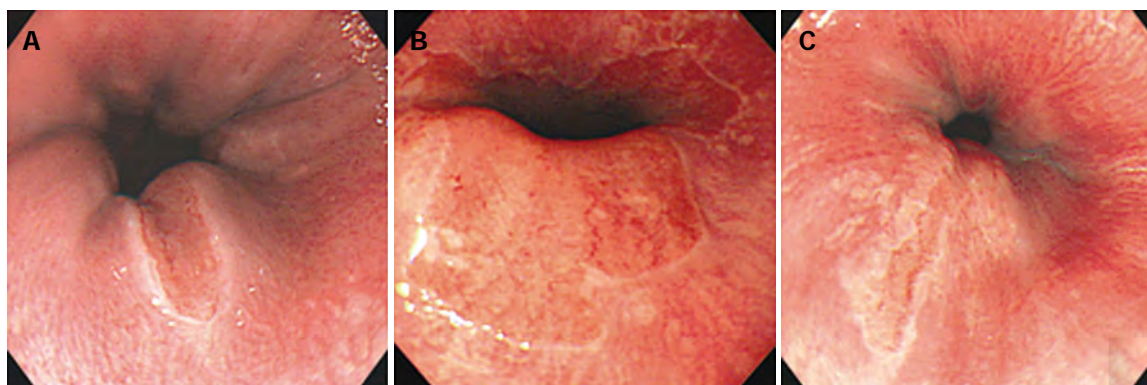


Figure 1 Screening endoscopy. A-C: Endoscopically suspected short-segment Barrett's esophagus (< 3 cm).

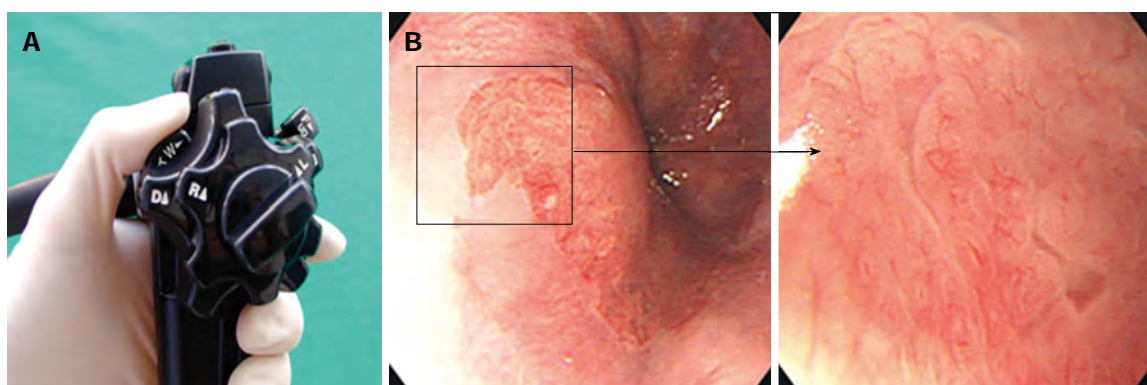


Figure 2 Magnifying endoscopy. A: Magnifying endoscopy up to  $\times 80$  (Olympus GIF-Q240Z,  $\times 80$ ); B: Magnified image of the short-segment Barrett's esophagus.



Figure 3 Classification of pit-pattern of Barrett's esophagus by Magnifying endoscopy (Endo's classification). A: I (small round); B: II (straight); C: III (long oval); D: IV (tubular); E: V (villous).

### Statistical analysis

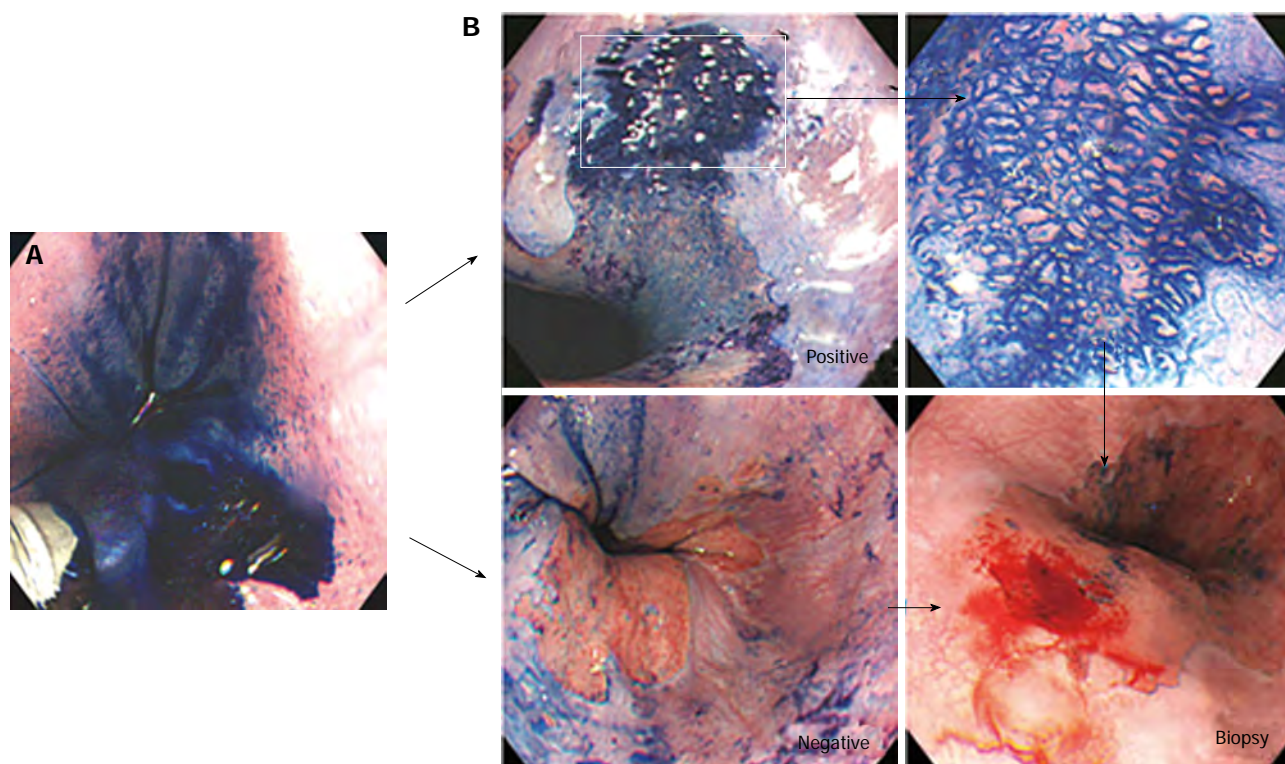
To analyze the relationships among the variables, Fisher's exact test was used. We performed an exact McNemar's test to compare the diagnostic value of MB chromoendoscopy and magnifying endoscopy for detection of SIM. Data analysis was performed using SPSS 14.0. All statistical hypotheses were verified at a significance level of  $P < 0.05$ .

## RESULTS

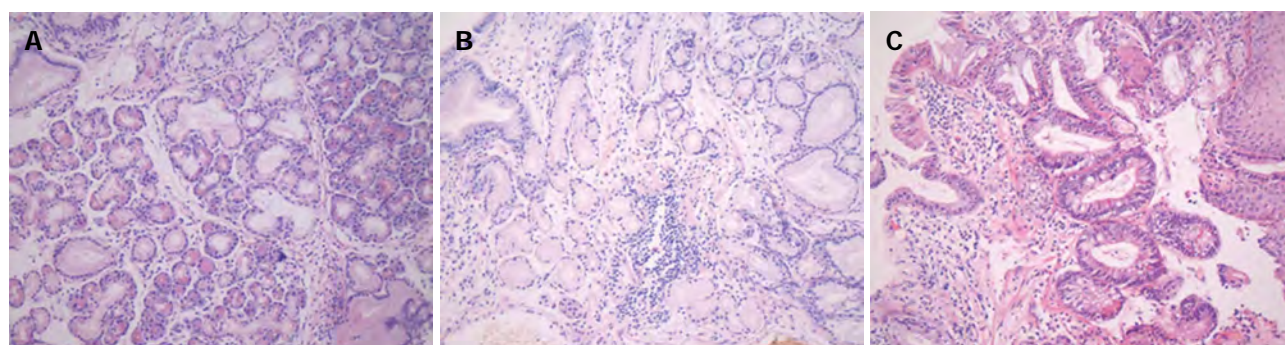
### Patient characteristics

Thirty patients, 16 men and 14 women, with an average age of 44.8 years (range 17-75 years), were enrolled into this study. All of the patients had tongue-like columnar epithelium in the tubular esophagus within 3 cm from the

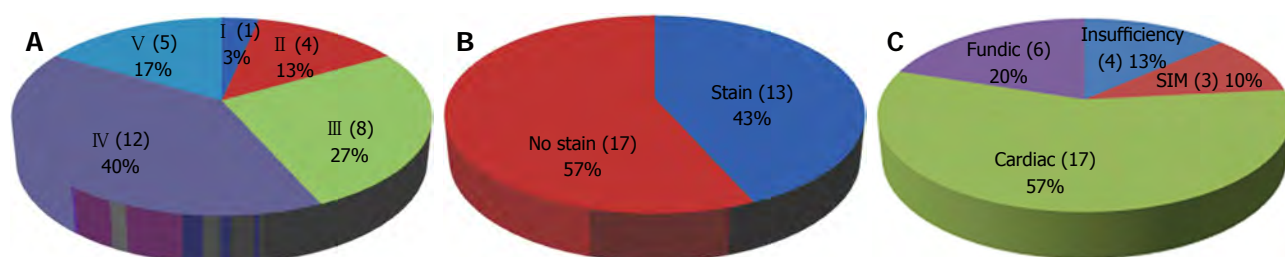




**Figure 4 Methylene blue chromoendoscopy.** A: 0.5% solution of methylene blue (MB) was sprayed over the columnar mucosa; B: Biopsies were obtained from the regions previously observed by magnifying endoscopy and MB chromoendoscopy.



**Figure 5 Histological diagnosis.** A: Fundic type (HE stain, × 200); B: Cardiac type (HE stain, × 200); C: Specialized intestinal metaplasia (HE stain, × 400).



**Figure 6 Distributions of pit-pattern, methylene blue staining, histologic diagnosis.** A: Pit-pattern; B: Methylene blue staining; C: Histologic diagnosis. SIM: Specialized intestinal metaplasia.

Esophagogastric junction, as identified by previous standard endoscopy. No patient had previous histologically proven SIM in the columnar lined epithelium.

The results for individual patients, including the pit-

pattern, MB staining, histologic diagnosis and reflux esophagitis, are listed in Table 1. Distributions of the types of pit-pattern, MB staining, and histologic diagnosis are shown in Figure 6.

**Table 1 Individual results**

Patient	Type	Stain	Histology	Reflux	Patient	Type	Stain	Histology	Reflux
1	Villous	Yes	SIM	Yes	16	Tubular	No	Insufficiency	Yes
2	Oval	No	Cardiac	No	17	Villous	Yes	SIM	Yes
3	Oval	No	Cardiac	No	18	Oval	No	Fundic	Yes
4	Straight	No	Fundic	No	19	Tubular	No	Insufficiency	No
5	Straight	Yes	Fundic	No	20	Oval	Yes	Fundic	Yes
6	Tubular	No	Cardiac	No	21	Villous	Yes	SIM	No
7	Oval	No	Cardiac	No	22	Tubular	No	Insufficiency	No
8	Tubular	Yes	Cardiac	No	23	Tubular	Yes	Cardiac	Yes
9	Tubular	No	Cardiac	No	24	Villous	Yes	Cardiac	Yes
10	Small round	Yes	Fundic	No	25	Oval	No	Cardiac	Yes
11	Tubular	No	Cardiac	Yes	26	Villous	No	Cardiac	No
12	Tubular	No	Cardiac	No	27	Straight	No	Cardiac	No
13	Tubular	No	Fundic	No	28	Tubular	No	Cardiac	No
14	Tubular	Yes	Insufficiency	No	29	Oval	Yes	Cardiac	Yes
15	Tubular	Yes	Cardiac	No	30	Oval	yes	Cardiac	Yes

SIM: Specialized intestinal metaplasia.

**Table 2 Relationship between specialized intestinal metaplasia and variables**

Variables	SIM (+)	SIM (-)	Total
Reflux esophagitis	2	9	11
No-Reflux esophagitis	1	14	15
Total <sup>1</sup>	3	23	26
Villous	3	2	5
Non-villous	0	21	21
Total <sup>2</sup>	3	23	26
MB stain	3	9	12
Non-MB stain	0	14	14
Total <sup>3</sup>	3	23	26

<sup>1</sup>Fisher's exact test:  $P = 0.538$ ; <sup>2</sup>Sensitivity = 100%, specificity = 91.3%, accuracy = 92.3%, PPV = 60%, NPV = 100%, Fisher's exact test:  $P = 0.004$ ; <sup>3</sup>Sensitivity = 100%, specificity = 60.9%, accuracy = 65.4%, PPV = 25%, NPV = 100%, Fisher's exact test:  $P = 0.085$ . SIM: Specialized intestinal metaplasia; PPV: Positive predictive value; NPV: Negative predictive value; MB: Methylene blue.

Histologic examination revealed SIM in 3 of 26 patients (11.5%). The remaining four patients could not be diagnosed due to the insufficiency of the specimens for histologic examination. Reflux esophagitis was diagnosed by histologic examination in 11 of 26 patients (42.3%). The patients without RE did not have a history of GERD. SIM in BE was not more common in patients with reflux esophagitis (2 patients, 18.1%) than in those without it (1 patient, 5.2%;  $P = 0.538$ , Table 2).

#### Relationship between type of pit-pattern and SIM

The fine mucosal patterns (pit-pattern) of 30 patients were recorded and classified according to Endo's classification. The specimens obtained previously from the regions observed by magnification without MB staining underwent histologic examinations to determine the relationship between the type of pit-pattern and SIM by magnifying endoscopy.

Of the 30 patients, one case was type I (small round);

four cases were type II (straight); eight cases were type III (long oval); 12 cases were type IV (tubular); and five cases were type V (villous). Type IV (tubular) was the most common epithelial type. As shown in Table 1 and Figure 6, three of five patients with a type V (villous) epithelial pattern had SIM. Twenty-one patients without type V epithelial patterns did not have SIM ( $P = 0.004$ ). These results suggest that a type V (villous) epithelial pattern is compatible with SIM, and the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of type V pit-pattern in detecting SIM were 100%, 91.3%, 92.3%, 60% and 100%, respectively (Table 2).

#### Relationship between MB staining and SIM

Out of 30 patients, 13 patients (43.3%) had positive MB staining, and 17 patients (56.7%) had negative MB staining. One of the 13 patients with positive MB staining and three of the 17 patients with negative MB staining did not receive a histological diagnosis due to insufficient specimens. As shown in Table 1 and Figure 6, three of 12 patients with positive MB staining had SIM, whereas 14 patients with negative MB staining did not have SIM ( $P = 0.085$ ). The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of MB staining in detecting SIM were 100%, 60.9%, 65.4%, 25% and 100%, respectively (Table 2).

#### Diagnostic value of pit-pattern evaluation and MB staining for detecting SIM

In comparison with MB staining, pit-pattern evaluation according to Endo's classification had much higher specificity (91.3% *vs* 60.9%), accuracy (92.3% *vs* 65.4%), and positive predictive value (60% *vs* 20%) for the detection of SIM in BE; however, it had a similar sensitivity (both 100%) and negative predictive value (both 100%). The exact McNemar's test revealed that the specificity and accuracy of pit-pattern evaluation was significantly superior to that of MB staining for detecting SIM by ( $P = 0.0391$ ;



**Table 3** Diagnostic value of pit-pattern evaluation and metaplasia staining for detection of specialized intestinal metaplasia ( $n = 3$ )

	TP	TN	P value
Pit-pattern	3	21	
MB stain	3	14	
	Pit-pattern	MB stain	
Sensitivity	100.00%	100.00%	
Specificity	91.30%	60.90%	0.0391 <sup>1</sup>
Accuracy	92.30%	65.40%	0.0391 <sup>1</sup>
PPV	60.00%	25.00%	0.1643 <sup>1</sup>
NPV	100.00%	100.00%	

<sup>1</sup>P value by exact McNemar's test. TP: True positive; TN: True negative; PPV: Positive predictive value; NPV: Negative predictive value; MB: Methylene blue.

Table 3).

## DISCUSSION

SIM in BE is a risk factor for EAC. A strong relationship has been established between the presence of SIM and the subsequent development of adenocarcinoma<sup>[5,6]</sup>.

Detecting esophageal neoplasias at an earlier stages will allow for the possibility of intervening more quickly and lowering the mortality from EAC. However, the effectiveness of the screening and surveillance of BE has not been studied in randomized, controlled trials. For example, various endoscopic approaches and advancements have shown great promise, yet the confirmation of their utility in high-quality clinical trials has yet to occur<sup>[21,22]</sup>.

Canto *et al.*<sup>[11]</sup> found that the overall accuracy of MB staining for detecting SIM was 95%. However, the same level of accuracy was not achieved in other studies. Dave *et al.*<sup>[16]</sup> reported that MB staining was associated with prolonged endoscopy, increased patient discomfort, and potentially serious adverse events; furthermore, it was neither very sensitive nor specific for SIM. According to Horwhat *et al.*<sup>[13]</sup>, chromoendoscopy might decrease the number of biopsies without an improving the overall detection rate of dysplasia compared with a conventional four-quadrant biopsy. Wasielica-Berger *et al.*<sup>[14]</sup> and Ferguson *et al.*<sup>[23]</sup> found no convincing data indicating that pit-pattern evaluations may replace multiple biopsies, according to the Seattle recommendations for the detection of SIM in BE. Therefore, the aim of this study was to determine whether the magnified observation of short-segment BE is useful for the detection of SIM or for the prediction of histological diagnosis, compared with MB chromoendoscopy.

Oberg *et al.*<sup>[3]</sup> showed that a long duration of reflux symptoms (RR = 1.3; 95%CI: 1.2-1.7) were independently associated with an increased risk of developing high-grade dysplasia or esophageal adenocarcinoma. However, SIM in BE was not more common in patients with reflux esophagitis who had a history of GERD compared with those without such a history ( $P = 0.538$ ).

Endo's study found that the type IV (tubular) and type V (villous) classifications were characteristic of

SIM. Similarly, we found a significant correlation between pit-patterns evaluated according to Endo's classifications and histology. The differences in the frequency of SIM were related to the particular mucosal pit-pattern types. We frequently found SIM in places with a type V (villous) epithelial pattern (3 of 5 patients). SIM did not coexist in any case with a non-type V epithelial pattern. Therefore, the surface structure of type V (villous) epithelial pattern is compatible with SIM ( $P = 0.004$ ).

MB is a vital stain that is taken up by actively absorbing tissues, such as the small intestinal and colonic epithelium. In BE, areas of intestinal metaplasia are positively stained, whereas non-absorptive epithelia, such as those found in squamous or gastric mucosa, remain unstained. We found SIM in places with MB-positive stained epithelium (3 of 12 patients). No case of SIM was associated with MB-negative stained epithelium. However, MB-positive staining cannot be considered characteristic of SIM, as the difference was not significant ( $P = 0.085$ ).

Compared with MB staining, the pit-pattern evaluation by magnifying endoscopy according to Endo's classification had much higher specificity (91.3% *vs* 60.9%) and positive predictive value (60% *vs* 20%) for the detection of SIM in BE, despite similar sensitivity (100% *vs* 100%) and negative predictive values (100% *vs* 100%). The specificity and accuracy of pit-pattern evaluations were significantly superior, according to McNemar's exact test, to those of MB staining for the detection of SIM ( $P = 0.0391$ ).

There were some limitations to our study. First, we found no sites with dysplasia or cancer cells, which may be attributed to the relatively small number of patients. In addition, the present study enrolled too few patients (3 out of 5 patients with type V pit-pattern). However, this study was very difficult regarding the recruitment of patients due to the refusal of many of the patients and the quite rare prevalence of this condition in Korea<sup>[24,25]</sup>. Second, long-segment BEs were excluded in our study. The risk of progression to malignancy appears to increase significantly with increasing lengths of BE<sup>[26,27]</sup>. It would be worth knowing about pit-patterns in long-segment, salmon-colored mucosa and also pit-pattern correlation with histological diagnosis of BE. However, there is conflicting evidence in the literature<sup>[28]</sup>. Short-segment and long-segment BE are biologically identical and have significant if not equivalent malignant potential. In addition, Kim *et al.*<sup>[29]</sup> showed that patients with long-segment BE are very rare in South Korea. So, we focused on short-segment BE in this study. Third, we did not address whether the simultaneous use of magnifying endoscopy and MB staining might improve the diagnostic yield. Sharma *et al.*<sup>[12]</sup> reported that high magnification chromoendoscopy might be a useful clinical tool for the increased detection of patients with intestinal metaplasia. Statistically, there is no doubt that the results are improved when magnifying endoscopy is performed with MB staining simultaneously, if both are characteristics of SIM. In our study, MB-positive staining could not be considered a characteristic of SIM. Therefore, we did not



try to demonstrate that the simultaneous performance of magnifying endoscopy and MB staining could improve the results. Fourth, we did not count the total number of biopsies. Thus, we could not show that the magnifying endoscopy might decrease the number of biopsies, generating an overall improvement in the detection rate of dysplasia compared with a conventional, four-quadrant biopsy.

In summary, we identified the usefulness of magnifying endoscopy for the diagnosis of SIM in patients with short-segment BE from preceding studies. However, we were still unable to demonstrate the usefulness of MB chromoendoscopy. Because we did not count the total number of biopsies, we could not confirm that both of the endoscopic examinations decreased the number of biopsies, costs and inspection time. We found that both methods were time-consuming and caused patient discomfort. These are among the disadvantages of the other studies.

Various endoscopic approaches and advancements have shown great promise. Still, careful endoscopic observation and stepwise four quadrant biopsy still represent the standard for the surveillance of BE<sup>[21,30]</sup>. In our study, the evaluation of mucosal surfaces under magnification has potential to allow the selection of the biopsy site according to the pit-pattern. In conclusion, the magnified observation of short-segment BE according to the mucosal pattern and its classification can be predictive for SIM.

## COMMENTS

### Background

Crosssectional and descriptive studies suggest that specialized intestinal metaplasia (SIM) either coexists with or precedes a significant majority of esophageal adenocarcinoma (EAC) cases and is the likely precursor lesion.

### Research frontiers

Detecting esophageal neoplasia at an earlier stage will allow for the possibility of intervening more quickly and the lowering mortality due to EAC. However, the effectiveness of screening and surveillance of Barrett's esophagus (BE) has not been studied in randomized controlled trials. In addition, discerning SIM and obtaining satisfactory target biopsies at the region of interest by standard endoscopic observation is difficult.

### Innovations and breakthroughs

The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of pit pattern in detecting SIM were 100%, 91.3%, 92.3%, 60% and 100%, respectively ( $P = 0.004$ ). The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of methylene blue (MB) staining in detecting SIM were 100%, 60.9%, 65.4%, 25% and 100%, respectively ( $P = 0.085$ ). The specificity and accuracy of the pit-pattern evaluation were significantly superior compared with MB staining for detecting SIM by comparison of exact McNemar's test ( $P = 0.0391$ ).

### Applications

The study results suggests that the magnified observation of short-segment BE according to the mucosal pattern and its classification can be predictive for SIM.

### Terminology

BE is characterized by the replacement of the squamous epithelia in the distal esophagus by columnar epithelia (gastric metaplasia), irrespective of the presence of specialized intestinal metaplasia.

### Peer review

The paper found that the magnified observation of a short-segment BE according to its mucosal pattern and classification can be predictive of SIM. It's an

informative manuscript, nicely written.

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## C/EBP homologous protein deficiency aggravates acute pancreatitis and associated lung injury

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induced with 6 injections of cerulein (Cn, 50  $\mu$ g/kg) at 1-h intervals, then intraperitoneal injection of lipopolysaccharide (LPS, 7.5 mg/kg) in CHOP-deficient (*Chop*<sup>-/-</sup>) mice and wild-type (WT) mice. Animals were sacrificed under anesthesia, 3 h or 18 h after LPS injection. Serum amylase, lipase, and cytokines [interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$ ], pathological changes, acute lung injury, and apoptosis in the pancreas were evaluated. Serum amylase and lipase activities were detected using a medical automatic chemical analyzer. Enzyme-linked immunosorbent assay kits were used to evaluate TNF- $\alpha$  and IL-6 levels in mouse serum and lung tissue homogenates. Apoptotic cells in sections of pancreatic tissues were determined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis. The mouse carotid arteries were cannulated and arterial blood samples were collected for PaO<sub>2</sub> analysis. The oxygenation index was expressed as PaO<sub>2</sub>/FiO<sub>2</sub>.

**RESULTS:** Administration of Cn and LPS for 9 and 24 h induced severe acute pancreatitis in *Chop*<sup>-/-</sup> and WT mice. When comparing *Chop*<sup>-/-</sup> mice and WT mice, we observed that CHOP-deficient mice had greater increases in serum TNF- $\alpha$  (214.40  $\pm$  19.52 pg/mL vs 150.40  $\pm$  16.70 pg/mL;  $P$  = 0.037), amylase (4236.40  $\pm$  646.32 U/L vs 2535.30  $\pm$  81.83 U/L;  $P$  = 0.041), lipase (1678.20  $\pm$  170.57 U/L vs 1046.21  $\pm$  35.37 U/L;  $P$  = 0.008), and IL-6 (2054.44  $\pm$  293.81 pg/mL vs 1316.10  $\pm$  108.74 pg/mL;  $P$  = 0.046) than WT mice. The histopathological changes in the pancreases and lungs, decreased PaO<sub>2</sub>/FiO<sub>2</sub> ratio, and increased TNF- $\alpha$  and IL-6 levels in the lungs were greater in *Chop*<sup>-/-</sup> mice than in WT mice (pancreas: *Chop*<sup>-/-</sup> vs WT mice, hemorrhage,  $P$  = 0.005; edema,  $P$  = 0.005; inflammatory cells infiltration,  $P$  = 0.005; total scores,  $P$  = 0.006; lung: hemorrhage,  $P$  = 0.017; edema,  $P$  = 0.017; congestion,  $P$  = 0.017; neutrophil infiltration,  $P$  = 0.005, total scores,  $P$  = 0.001; PaO<sub>2</sub>/FiO<sub>2</sub> ratio: 393  $\pm$  17.65 vs 453.8,  $P$  = 0.041; TNF- $\alpha$ :  $P$  = 0.043; IL-6,  $P$  = 0.040). Results from TUNEL analysis indicated increased acinar cell apoptosis in

### Abstract

**AIM:** To investigate the pathophysiological role of C/EBP homologous protein (CHOP) in severe acute pancreatitis and associated lung injury.

**METHODS:** A severe acute pancreatitis model was



mice following the induction of acute pancreatitis. However, *Chop*<sup>-/-</sup> mice displayed significantly reduced pancreatic apoptosis compared with the WT mice (201.50 ± 31.43 vs 367.00 ± 47.88, *P* = 0.016).

**CONCLUSION:** These results suggest that CHOP can exert protective effects against acute pancreatitis and limit the spread of inflammatory damage to the lungs.

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**Key words:** C/EBP homologous protein; Acute pancreatitis; Lung injury; Cytokines; Apoptosis

**Core tip:** We found that mice lacking C/EBP homologous protein (CHOP) had aggravated acute pancreatitis-induced increases in the severity of pancreatic pathology, pancreatitis-associated lung injury, and cytokines interleukin-6 and tumor necrosis factor- $\alpha$  levels compared with wild-type (WT) mice. Pancreatic apoptosis was also lower in *Chop*<sup>-/-</sup> mice than in WT mice during acute pancreatitis. These results suggest that CHOP exerts protective effects against acute pancreatitis and limits the spread of inflammatory damage to the lungs.

Weng TI, Wu HY, Chen BL, Jhuang JY, Huang KH, Chiang CK, Liu SH. C/EBP homologous protein deficiency aggravates acute pancreatitis and associated lung injury. *World J Gastroenterol* 2013; 19(41): 7097-7105 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i41/7097.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7097>

## INTRODUCTION

Acute pancreatitis is an acute inflammatory process of sudden onset, and occurs in the peripheral and internal areas of the pancreas. At an early stage of the disease, acute pancreatitis induces multiple organ system dysfunction syndromes in the lung, kidney, liver, and other organs. These are major contributory factors to the high mortality rate of severe acute pancreatitis<sup>[1,2]</sup>. Acute pancreatitis can spread systemically through systemic inflammatory responses<sup>[3]</sup>. Acute lung injury occurs frequently in acute pancreatitis and is a major component of acute pancreatitis-associated multiple organ dysfunction<sup>[4]</sup>. The overproduction of several cytokines and non-cytokine inflammatory mediators may account for the systemic manifestations of acute pancreatitis<sup>[5-7]</sup>.

Acinar cell damage initiates acute pancreatitis, resulting in local activation of the immune system and local inflammation of the pancreas<sup>[5]</sup>. The local pro-inflammatory response to acinar cell damage is counteracted by an anti-inflammatory response; however, uncontrolled local inflammation can lead to generalized inflammation and systemic inflammatory response syndrome<sup>[8]</sup>. Pancreatic acinar cell damage during acute pancreatitis generally oc-

curs through a combination of apoptosis and necrosis<sup>[9]</sup>. Recent studies have shown that mild acute pancreatitis is associated with extensive apoptotic acinar cell death, whereas severe acute pancreatitis involves acinar cell necrosis with minimal apoptosis<sup>[10,11]</sup>. Acinar necrosis-induced inflammation can progress systemically, causing multiple organ failure and death. Studies have proposed that acinar apoptosis might protect the pancreas from local and systemic cytokine release<sup>[10,11]</sup>.

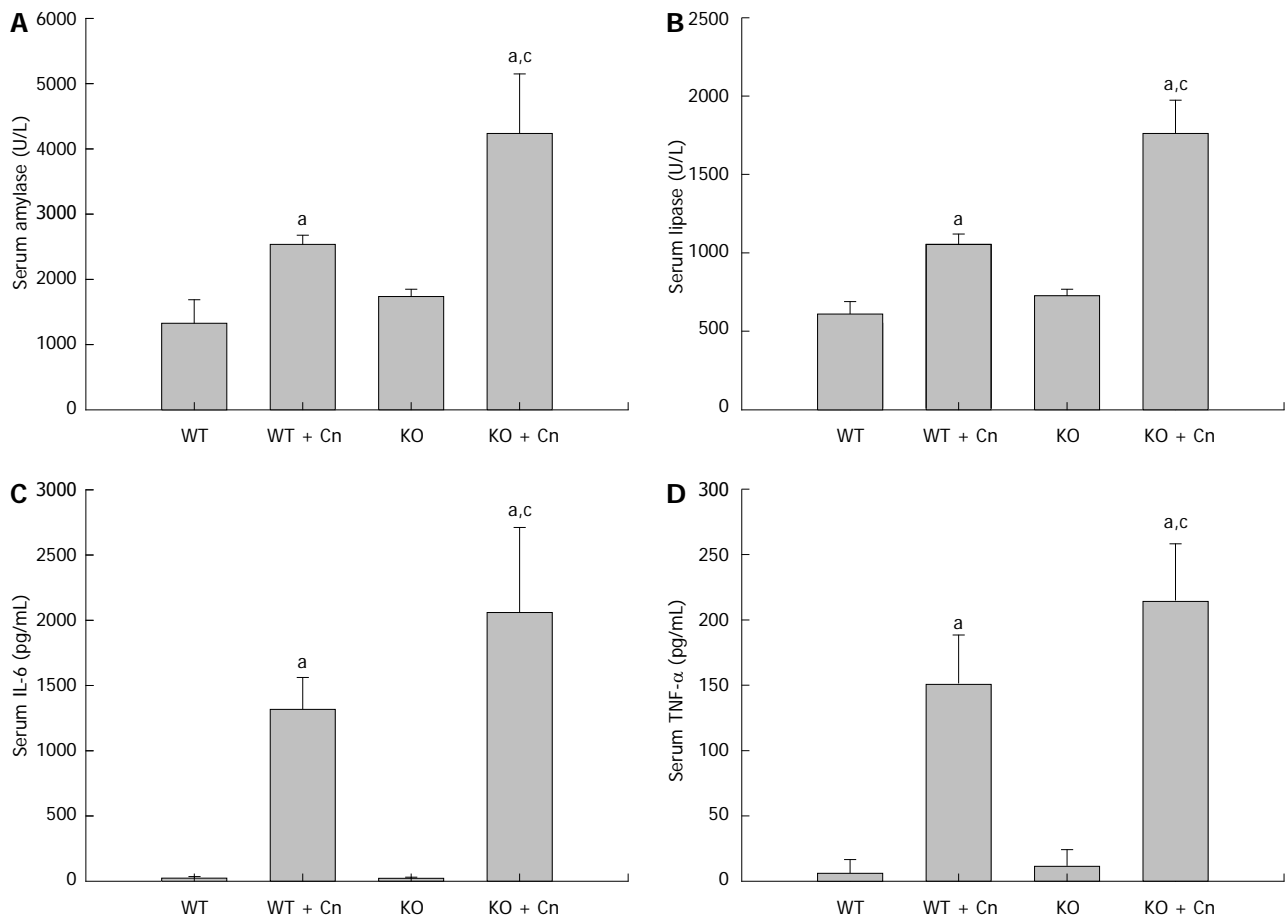
C/EBP homologous protein (CHOP), also known as G1 arrest and DNA damage 153 (Gadd153), is the 19.2-kDa protein product of DNA damage-induced transcript 3 (Ddit3) and a key regulator of stress responses. CHOP is known to play an important role in the induction of apoptosis. Overexpression of CHOP promotes apoptosis in several cell lines, whereas CHOP-deficient (*Chop*<sup>-/-</sup>) cells are resistant to endoplasmic reticulum (ER) stress-induced apoptosis<sup>[12]</sup>. Experiments using *Chop*<sup>-/-</sup> mice revealed that CHOP-mediated apoptosis contributes to the pathogenesis of a number of ER stress-related diseases<sup>[13]</sup>. ER stress is the most potent inducer of CHOP expression and CHOP is known as a pro-apoptotic factor<sup>[14,15]</sup>. It is also involved in several physiological adaptive processes<sup>[15,16]</sup>, including mitochondrial<sup>[17]</sup> and oxidative stress<sup>[18]</sup>, amino acid starvation<sup>[19]</sup>, and differentiation of keratinocytes<sup>[20]</sup> and osteoblasts<sup>[21]</sup>. Studies have reported CHOP upregulation in a murine model of acute pancreatitis<sup>[8,22]</sup>; however, the mechanism(s) and functional consequences of acute pancreatitis-induced CHOP expression are not well-understood. We hypothesized that CHOP plays an important role in acute pancreatitis and influences acute pancreatitis-induced systemic inflammation and acute lung injury. In this study, we used *Chop*<sup>-/-</sup> mice to investigate the pathogenesis of acute pancreatitis.

## MATERIALS AND METHODS

### Animal experiments

All animal care and experimental procedures were approved by the Animal Care Committee of the College of Medicine, National Taiwan University. Mice deficient in CHOP (*Chop*<sup>-/-</sup>) on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, United States). Adult male *Chop*<sup>-/-</sup> mice and wild-type (WT) C57BL/6 mice weighing 18-25 g were used in this study. Mice were housed at a constant temperature of 20 °C-22 °C with a 12 h:12 h light-dark cycle.

A mouse model of severe acute pancreatitis induced by cerulein (Cn) and lipopolysaccharide (LPS) has been well-established<sup>[20,21]</sup>. The *Chop*<sup>-/-</sup> and WT mice were injected with 6 doses of Cn (50  $\mu$ g/kg; Sigma, St Louis, MI, United States) at 1-h intervals, and then intraperitoneal injection with LPS (7.5 mg/kg; derived from *Escherichia coli* 0111:B4, Sigma), to induce severe acute pancreatitis. Animals were sacrificed under anesthesia (tribromoethanol, 250 mg/kg, dissolved in 2-methyl-2-butanol) by intraperitoneal injection at 3 h or 18 h after LPS injection, and their pancreases and lungs were dis-



**Figure 1** Mice deficient in C/EBP homologous protein displayed increased serum amylase, lipase, interleukin-6, and tumor necrosis factor- $\alpha$ . Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide (LPS) in Chop<sup>-/-</sup> (KO) and wild-type (WT) mice. Serum levels of amylase (A), lipase (B), interleukin (IL)-6 (C), and tumor necrosis factor (TNF)- $\alpha$  (D), were detected 24 h (A-C) and 9 h (D) after induction of acute pancreatitis. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). The data are normally distributed. <sup>a</sup> $P < 0.05$  compared with WT mice without pancreatitis; <sup>c</sup> $P < 0.05$  vs WT mice with pancreatitis.

sected immediately<sup>[23,24]</sup>. Blood samples were collected for amylase, lipase, and cytokine assays. After rinsing with saline and blotting on paper, segments of the tissues were fixed and embedded in paraffin wax for histological analysis. Other tissue parts were fully homogenized. The lung tissue homogenates were stored in liquid nitrogen before use to evaluate tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) levels.

#### Histological examination

To evaluate the morphological severity of acute pancreatitis, the pancreas was fixed in 10% formaldehyde for 24 h, embedded in paraffin, and stained with hematoxylin and eosin. A pathologist who was blinded to the treatment protocol scored the tissues for edema, inflammatory infiltration, and hemorrhage in 10 fields, each on a scale of 0-3. Grades of edema were 0, absent or rare; 1, edema in the interlobular space; 2, edema in the intralobular space; 3, isolated island shape of pancreatic acinus. Inflammation was graded as 0, absent; 1, mild; 2, moderate; 3, severe. Parenchymal hemorrhage was graded as 0, absent; 1, mild; 2, moderate; 3, severe. To evaluate the morphological severity of acute pancreatitis-associated lung injury, lung tissue was rapidly removed and

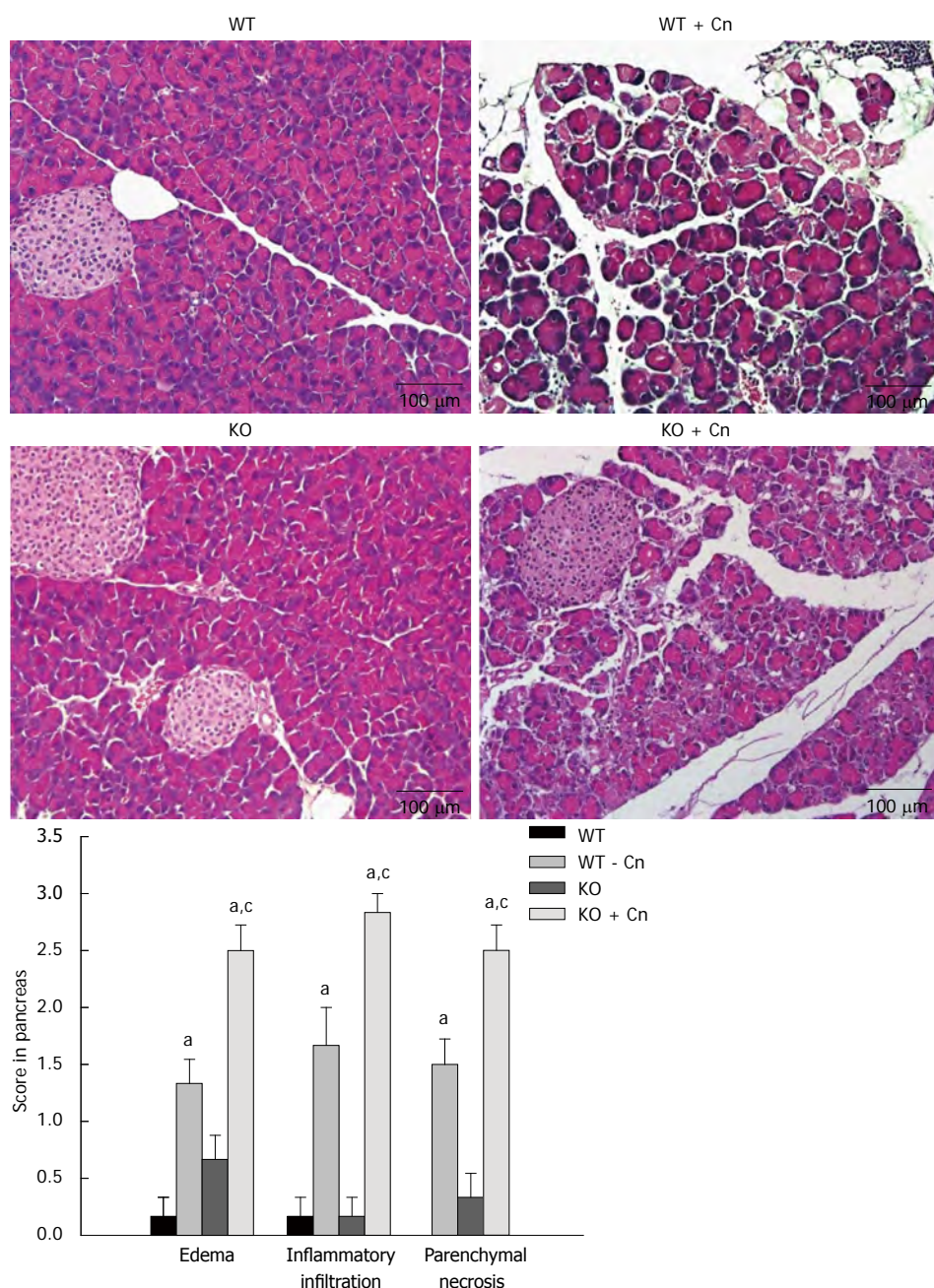
immersed in 10% formalin. Two areas of the lung, one central and one peripheral, were embedded in paraffin. Histological sections were stained with hematoxylin and eosin. Pulmonary alterations were scored by an experienced pathologist in a blind manner. Polymorphonuclear cellularity, pulmonary edema, congestion, necrosis, and hemorrhage were graded, each on a scale of 0-3.

#### Measurement of PaO<sub>2</sub>/FiO<sub>2</sub> ratio

Twenty-four hours after LPS injection, mice were anesthetized with tribromoethanol (250 mg/kg) dissolved in 2-methyl-2-butanol by intraperitoneal injection. The mouse carotid arteries were cannulated and arterial blood samples were collected for PaO<sub>2</sub> analysis. The oxygenation index was expressed as PaO<sub>2</sub>/FiO<sub>2</sub>.

#### Analysis of cell apoptosis

Apoptotic cells in sections of pancreatic tissues were determined using a TdT-Frag ELTM DNA fragmentation detection kit (Oncogene Research Products, Boston, MA, United States) according to the manufacturer's instruction. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis was conducted to detect cells containing labeled DNA frag-



**Figure 2** Experimental acute pancreatitis in *Chop*<sup>-/-</sup> mice. Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide (LPS) in *Chop*<sup>-/-</sup> (KO) and wild-type (WT) mice. Histological examination was performed 24 h after induction of acute pancreatitis. Representative histological changes in pancreatic sections stained with hematoxylin and eosin are shown. Scale bar = 100 μm. Pathological changes in the pancreas were scored. Data are presented as mean ± SEM (*n* = 4). The data are normally distributed. <sup>a</sup>*P* < 0.05 vs WT mice without pancreatitis; <sup>c</sup>*P* < 0.050 vs WT mice with pancreatitis.

ments. These were revealed as green staining in cell nuclei, indicating the internucleosomal cleavage of DNA.

#### Measurements of serum amylase, lipase, and cytokines

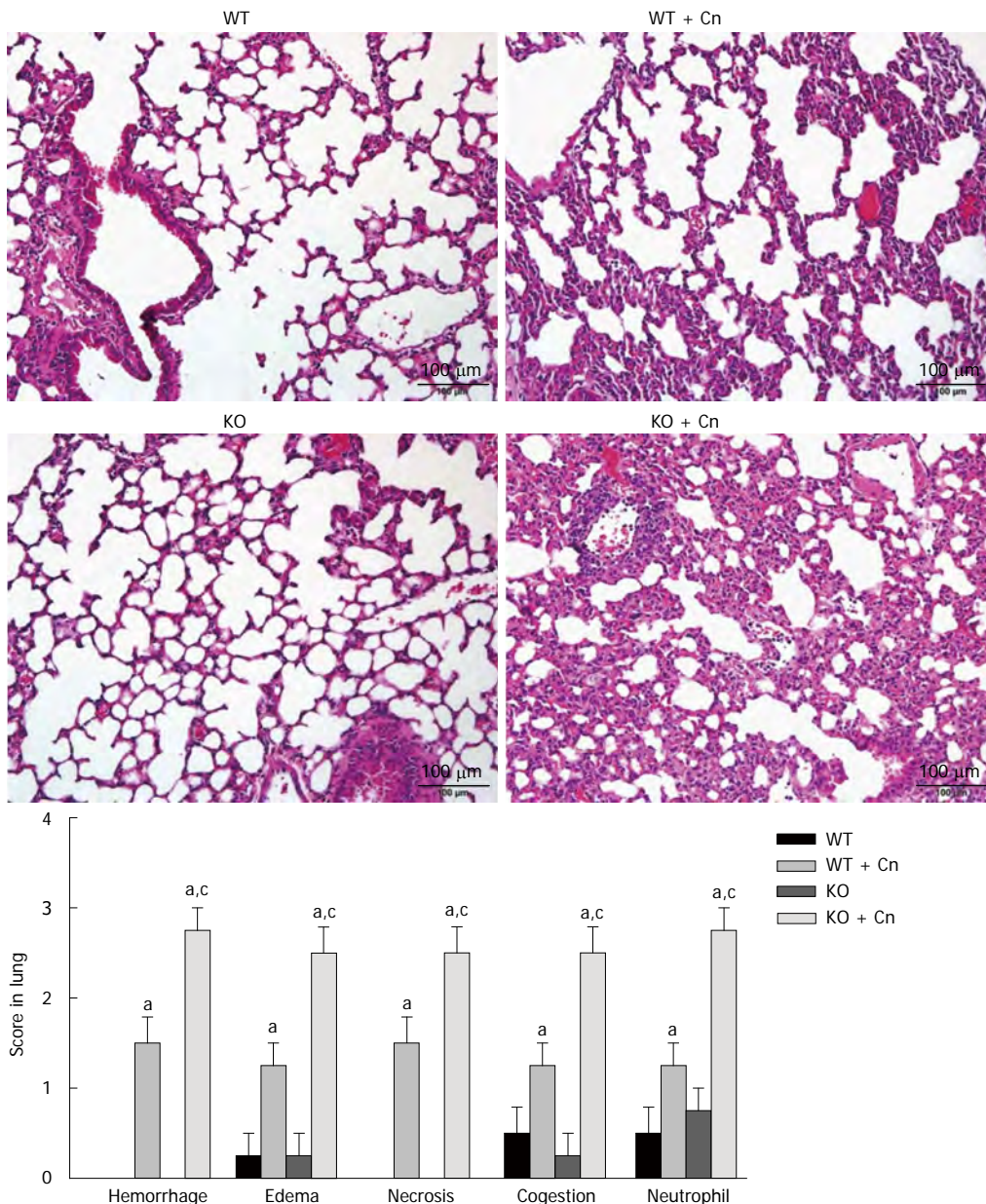
Serum amylase and lipase activities were detected using a medical automatic chemical analyzer. Enzyme-linked immunosorbent assay kits were used to evaluate the levels of TNF-α (R and D Systems) and IL-6 (Assaypro) in

mouse serum and lung tissue homogenates following the induction of acute pancreatitis.

#### Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons between experimental groups were performed using one-way analysis of variance test followed by the two-tailed Student *t* test. A *P* value < 0.05 was considered significant.





**Figure 3** Experimental acute pancreatitis-associated lung injury in *Chop*<sup>-/-</sup> mice. Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide in *Chop*<sup>-/-</sup> (KO) and wild-type (WT) mice. Histological examination was performed 24 h after induction of acute pancreatitis. Representative histological changes in lung sections stained with hematoxylin and eosin are shown. Scale bar = 100 μm. Pathological changes in lungs were scored. Data are presented as mean ± SEM (*n* = 4). The data are normally distributed. <sup>a</sup>*P* < 0.050 vs WT mice without pancreatitis; <sup>c</sup>*P* < 0.05 vs WT mice with pancreatitis.

## RESULTS

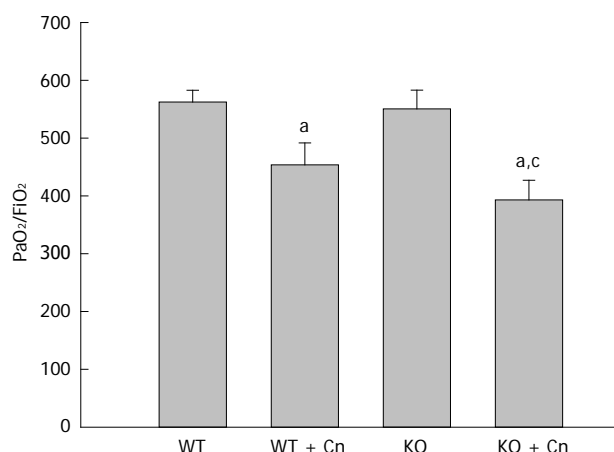
### Mice deficient in CHOP displayed acute pancreatitis-induced increases in serum amylase, lipase, IL-6, and TNF-α

Administration of Cn and LPS for 9 and 24 h induced severe acute pancreatitis in *Chop*<sup>-/-</sup> and WT mice. Following induction of acute pancreatitis, mice displayed increased serum amylase and lipase activities and TNF-α and IL-6 levels (Figure 1). When comparing *Chop*<sup>-/-</sup> mice and WT mice, we observed that CHOP-deficient mice demonstrated significantly greater increases in serum TNF-α ( $214.40 \pm 19.52$  pg/mL *vs*  $150.40 \pm 16.70$  pg/mL; *P* = 0.037), amylase ( $4236.40 \pm 646.32$  Units/L *vs*

$2535.30 \pm 81.83$  Units/L; *P* = 0.041), lipase ( $1678.20 \pm 170.57$  Units/L *vs*  $1046.21 \pm 35.37$  Units/L; *P* = 0.008), and IL-6 ( $2054.44 \pm 293.81$  pg/mL *vs*  $1316.10 \pm 108.74$  pg/mL; *P* = 0.046) than WT mice (Figure 1).

### Mice deficient in CHOP displayed increased acute pancreatitis-induced changes in lung histopathology and TNF-α and IL-6 levels

After administration of Cn and LPS for 24 h, mice showed features of typical acute pancreatitis in the pancreas, including the expansion of interlobular and intralobular spaces by moderate to severe interstitial edema, extensive infiltration of inflammatory cells, and pancreatic hemorrhage (Figure 2). They also displayed



**Figure 4** The PaO<sub>2</sub>/FiO<sub>2</sub> ratio in *Chop*<sup>-/-</sup> mice with acute pancreatitis. Acute pancreatitis was induced by using cerulein (Cn) and lipopolysaccharide in *Chop*<sup>-/-</sup> (KO) mice and wild-type (WT) mice. The PaO<sub>2</sub>/FiO<sub>2</sub> ratio was detected 24 h after induction of acute pancreatitis in mice. Data are presented as mean ± SEM (*n* = 6). The data are normally distributed. <sup>a</sup>*P* < 0.05 vs wild type mice without pancreatitis; <sup>c</sup>*P* < 0.05 vs wild type mice with pancreatitis.

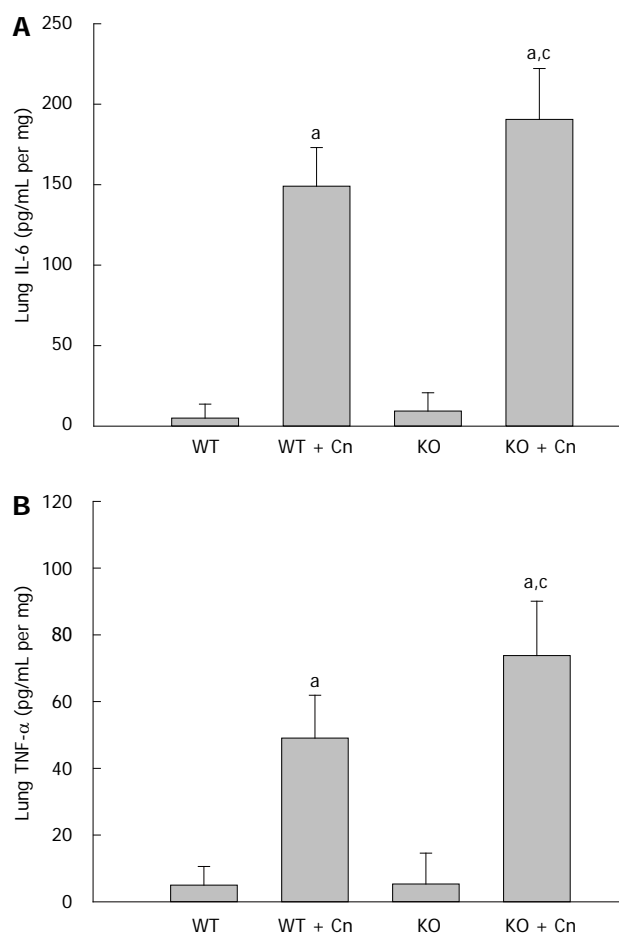
pulmonary changes such as marked pulmonary edema, inflammatory infiltration, and alveolar collapse (Figure 3), and decreased PaO<sub>2</sub>/FiO<sub>2</sub> ratio (an oxygenation index) (Figure 4). The levels of TNF-α and IL-6 in the lungs were markedly increased in mice with acute pancreatitis (Figure 5). Histopathological changes in the pancreases and lungs, decreased PaO<sub>2</sub>/FiO<sub>2</sub> ratio, and the increases of levels of TNF-α and IL-6 in the lungs were greater in *Chop*<sup>-/-</sup> mice than in WT mice (pancreas: *Chop*<sup>-/-</sup> vs WT mice, hemorrhage, *P* = 0.005, edema, *P* = 0.005, inflammatory cells infiltration, *P* = 0.005, total scores, *P* = 0.006; lung: hemorrhage, *P* = 0.017, edema, *P* = 0.017, congestion, *P* = 0.017, neutrophils infiltration, *P* = 0.005, total scores, *P* = 0.001; PaO<sub>2</sub>/FiO<sub>2</sub> ratio: 393 ± 17.65 vs 453.8, *P* = 0.041; TNF-α: *P* = 0.043; IL-6, *P* = 0.040).

#### Mice deficient in CHOP showed reduced acute pancreatic-induced apoptosis in the pancreas

Results from TUNEL analysis indicated increased acinar cell apoptosis (Figure 6) in mice following the induction of acute pancreatitis. However, *Chop*<sup>-/-</sup> mice displayed significantly reduced pancreatic apoptosis vs the WT mice (201.50 ± 31.43 vs 367.00 ± 47.88, *P* = 0.016; Figure 6).

## DISCUSSION

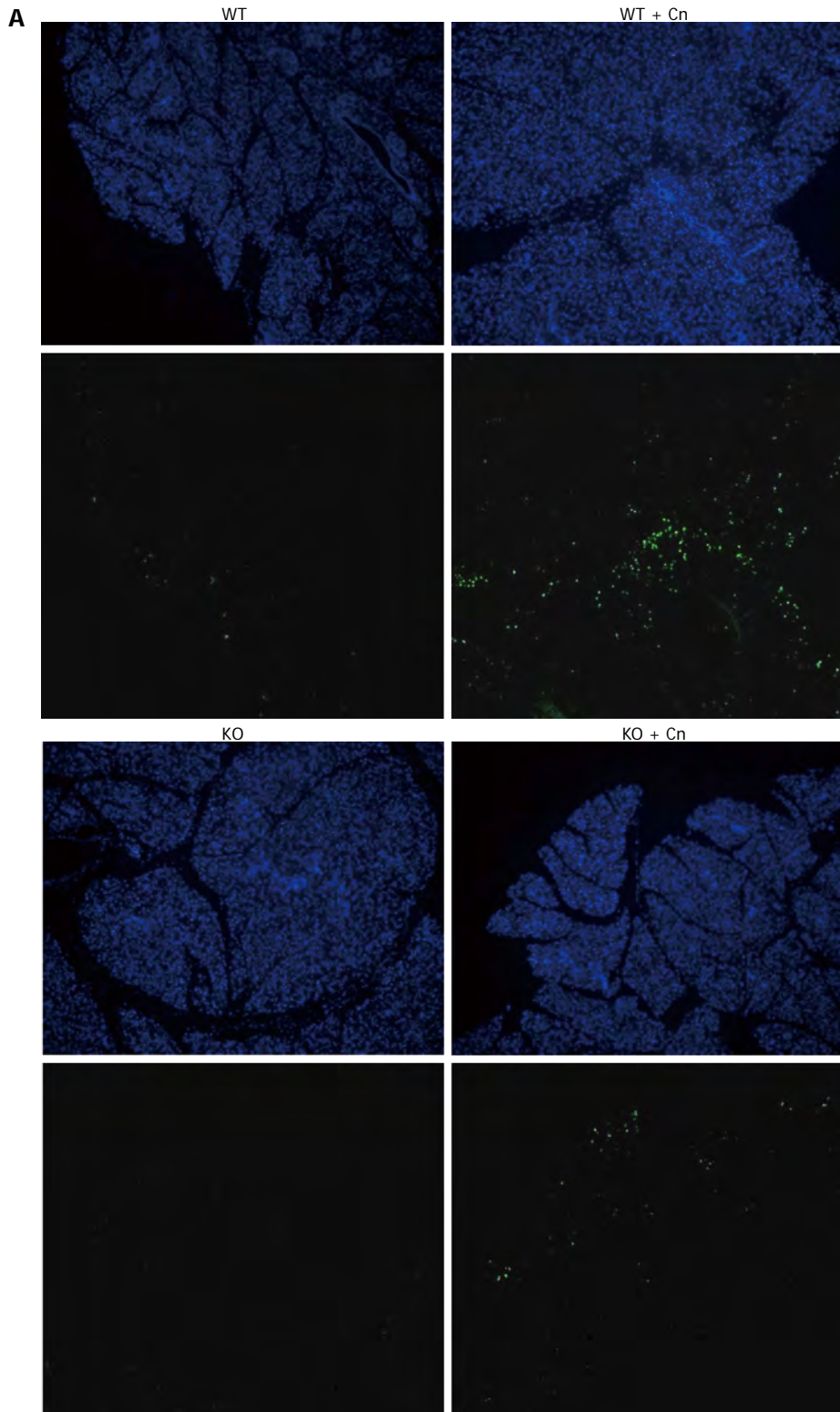
In a murine model, acute pancreatitis and its associated lung injury induced by Cn/LPS injection are compatible with the clinical manifestations of severe acute pancreatitis with lung damage<sup>[25,26]</sup>. In our previous study, we demonstrated the upregulation of pancreatic CHOP expression following Cn/LPS-induced acute pancreatitis. In the present study, we evaluated the pathophysiological role of CHOP in acute pancreatitis and its associated acute lung injury. Mice deficient in CHOP displayed increases in the severity of pancreatic pathology, increased activities of serum amylase and lipase, increased levels of pancreatitis-



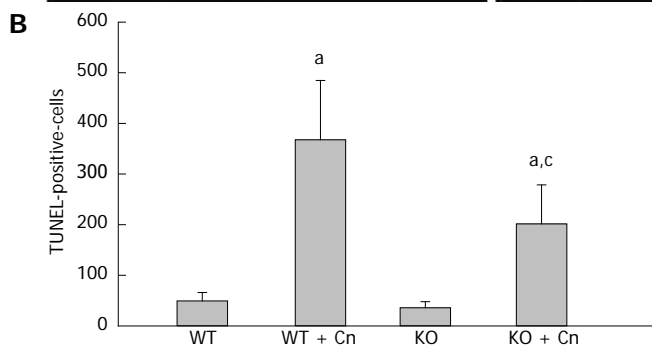
**Figure 5** Mice deficient in C/EBP homologous protein displayed increased interleukin-6, and tumor necrosis factor-α in the lungs. Levels of tumor necrosis factor (TNF)-α (A), and interleukin (IL)-6 (B) were detected 9 h (A) and 24 h (B) after induction of acute pancreatitis in *Chop*<sup>-/-</sup> and wild-type (WT) mice. Data are presented as mean ± SEM (*n* = 6). The data are normally distributed. <sup>a</sup>*P* < 0.050 vs WT mice without pancreatitis; <sup>c</sup>*P* < 0.05 vs WT mice with pancreatitis.

induced systemic inflammatory cytokines such as TNF-α and IL-6, and increased acute lung injury compared with WT mice. Moreover, mice with Cn-induced acute pancreatitis displayed low levels of apoptosis and high levels of necrosis<sup>[27]</sup>. It is known that severe acute pancreatitis is primarily associated with necrosis and, to a lesser extent, with apoptosis in acinar cells. Mild acute pancreatitis, however, is primarily associated with apoptotic acinar cell death<sup>[10]</sup>. In this study, we observed higher numbers of TUNEL-positive pancreatic cells in WT mice than in *Chop*<sup>-/-</sup> mice. These results suggest that CHOP mediates the increases in pancreatic cell apoptosis induced by Cn and LPS.

CHOP has been found to be upregulated during ER stress, which is typically associated with cellular death and associated organ dysfunction<sup>[28]</sup>. Recent evidence has shown that CHOP exerts diverse functional effects in addition to regulation of apoptosis. CHOP contributes to inflammation, the generation of reactive oxygen species, and altered cellular interaction within the extracellular matrix<sup>[29]</sup>. Lozon *et al*<sup>[30]</sup> found that *Chop*<sup>-/-</sup> mice displayed



**Figure 6 Mice deficient in C/EBP homologous protein displayed decreased apoptosis in the pancreas after induction of acute pancreatitis.** Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide in *Chop*<sup>-/-</sup> (KO) and wild-type (WT) mice. We performed transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis 9 h after induction of acute pancreatitis. Results indicated the presence of TUNEL-positive cells (A, B) in the pancreas. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). The data are normally distributed. <sup>a</sup> $P < 0.050$  vs WT mice without pancreatitis; <sup>c</sup> $P = 0.016$  vs WT mice with pancreatitis.





increased lung edema and permeability, indicating that CHOP plays a protective role after prolonged hyperoxia. Here we observed that CHOP exerts protective effects in a murine model of Cn/LPS-induced acute pancreatitis. This finding is in contrast to those of Suyama *et al.*<sup>[31]</sup>, who reported the role of the ER stress-CHOP pathway in the acceleration of pancreatitis. We applied a different protocol from the study by Suyama *et al.*<sup>[31]</sup> to induce severe acute pancreatitis, observing that experimental acute pancreatitis and acute pancreatitis-associated lung injury induced by 6 episodes of Cn injection and one subsequent LPS injection are compatible with the clinical manifestations of severe acute pancreatitis with lung damage<sup>[25,32-34]</sup>. In the study of Suyama *et al.*<sup>[31]</sup>, 12 doses of Cn and 3 doses of LPS were used to induce acute pancreatitis; they did not, however, evaluate the systemic inflammatory cytokines and the severity of lung injury. Acute lung injury is a major component of acute pancreatitis-associated morbidity and mortality<sup>[34]</sup>. Moreover, Kubisch and Logsdon have examined the effects of secretagogues on the function of acinar cells and the unfolded protein response components including CHOP<sup>[35]</sup>. They found that the cholecystokinin analog CCK8 significantly increased amylase secretion and mRNA expression of CHOP; however, the pathophysiological role of CHOP was still unclear. In our study, we observed that pancreatic and systemic inflammatory responses and acute lung injury during Cn/LPS-induced acute pancreatitis were greater in CHOP-deficient mice than in their WT counterparts. Taken together, these findings suggest that endogenous CHOP may play a systemic anti-inflammatory role and reduce the severity of acute lung injury during acute pancreatitis.

In conclusion, the present findings demonstrate for the first time that CHOP plays a significant role in the induction of apoptosis and prevention of systemic inflammation and acute lung injury during severe acute pancreatitis.

## COMMENTS

### Background

Severe acute pancreatitis is a life-threatening disease. Studies have reported C/EBP homologous protein (CHOP) upregulation in a murine model of acute pancreatitis. However, the mechanism(s) and functional consequences of acute pancreatitis-induced CHOP expression are not well-understood.

### Research frontiers

Overexpression of CHOP promotes apoptosis in several cell lines. The authors hypothesized that CHOP plays an important role in acute pancreatitis and influences acute pancreatitis-induced systemic inflammation and acute lung injury.

### Innovations and breakthroughs

The authors found for the first time that mice lacking CHOP have aggravated acute pancreatitis-induced increases in the severity of pancreatic pathology, pancreatitis-associated lung injury, and cytokine interleukin-6 and tumor necrosis factor- $\alpha$  levels compared with wild-type mice. Pancreatic apoptosis was also lower in *Chop*<sup>-/-</sup> mice than in wild-type mice during acute pancreatitis.

### Applications

CHOP exerts protective effects against acute pancreatitis and limits the spread of inflammatory damage to the lungs.

### Terminology

CHOP, C/EBP homologous protein, also known as G1 arrest and DNA damage

153 (Gadd153), is the 19.2-kDa protein product of DNA damage-induced transcript 3 (Ddit3) and a key regulator of stress responses. CHOP is known to play an important role in the induction of apoptosis.

### Peer review

The authors aimed to investigate the pathophysiological role of CHOP in severe acute pancreatitis and associated lung injury. This is a unique study and the acquired data support that CHOP has a protective effect against acute pancreatitis.

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## Photodynamic therapy vs radiofrequency ablation for Barrett's dysplasia: Efficacy, safety and cost-comparison

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Author contributions: Ertan A performed all the procedures and designed the study; Ertan A, Blackmon S and Zaheer I collected the data; Correa A and Thosani N analyzed the data; Ertan A, Blackmon S and Thosani N wrote the manuscript.

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### Abstract

**AIM:** To compare effectiveness, safety, and cost of photodynamic therapy (PDT) and radiofrequency ablation (RFA) in treatment of Barrett's dysplasia (BD).

**METHODS:** Consecutive case series of patients undergoing either PDT or RFA treatment at single center by a single investigator were compared. Thirty-three patients with high-grade dysplasia (HGD) had treatment with porfimer sodium photosensitizer and 630 nm laser (130 J/cm), with maximum of 3 treatment sessions. Fifty-three patients with BD (47 with low-grade dysplasia -LGD, 6 with HGD) had step-wise circumferential and focal ablation using the HALO system with maximum

of 4 treatment sessions. Both groups received proton pump inhibitors twice daily. Endoscopic biopsies were acquired at 2 and 12 mo after enrollment, with 4-quadrant biopsies every 1 cm of the original BE extent. A complete histological resolution response of BD (CR-D) was defined as all biopsies at the last endoscopy session negative for BD. Fisher's exact test was used to assess differences between the two study groups for primary outcomes. For all outcomes, a two-sided *P* value of less than 0.05 was considered to indicate statistical significance.

**RESULTS:** Thirty (91%) PDT patients and 39 (74%) RFA were men (*P* = 0.05). The mean age was  $70.7 \pm 12.2$  and  $65.4 \pm 12.7$  (*P* = 0.10) year and mean length of BE was  $5.4 \pm 3.2$  cm and  $5.7 \pm 3.2$  cm (*P* = 0.53) for PDT and RFA patients, respectively. The CR-D was (18/33) 54.5% with PDT vs (47/53) 88.7% with RFA (*P* = 0.001). One patient with PDT had an esophageal perforation and was managed with non-surgical measures and no perforation was seen with RFA. PDT was five times more costly than RFA at our institution. The two groups were not randomized and had different BD grading are the limitations of the study.

**CONCLUSION:** In our experience, RFA had higher rate of CR-D without any serious adverse events and was less costly than PDT for endoscopic treatment of BD.

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**Key words:** Barrett's esophagus; Dysplasia; Photodynamic therapy; Radiofrequency ablation; Cost comparison

**Core tip:** Barrett's esophagus containing dysplasia confers an elevated risk for developing esophageal adenocarcinoma. Photodynamic therapy (PDT) and radiofrequency ablation (RFA) have both been shown in randomized controlled trials to eradicate Barrett's dysplasia (BD) and reduce the risk for disease progres-



sion. We compared the effectiveness, safety, and cost of PDT and RFA in managing BD in consecutive case series performed at single center by single endoscopist. We found that RFA had significantly higher rate of complete histological resolution of Barrett's dysplasia and it was five times less costly than PDT at our institute compared to PDT.

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## INTRODUCTION

Adenocarcinoma of the esophagus and gastroesophageal junction (GEJ) is a devastating disease with 5-year survival rate of less than 15%<sup>[1]</sup>. Since 1970s, the incidence of this cancer has increased by more than 500% and it is considered one of the most rapidly increasing cancers in western society<sup>[2,3]</sup>. Barrett's esophagus (BE), defined as metaplasia of the esophageal epithelium, in which squamous mucosa is replaced by a specialized columnar epithelium containing goblet cells, is a premalignant condition of the esophagus with increased risk of developing esophageal adenocarcinoma<sup>[4]</sup>. BE is based on the endoscopic findings of columnar epithelium lining the distal esophagus and diagnosis is confirmed by the presence of specialized intestinal metaplasia in esophageal biopsy specimens<sup>[5]</sup>. BE, sometimes also referred as intestinal metaplasia (IM), is caused by chronic inflammation and injury due to gastroesophageal reflux disease (GERD). BE may be present in as many as 10%-15% of patients with GERD and is associated with a significantly elevated risk of esophageal adenocarcinoma<sup>[4,6-9]</sup>.

Barrett's dysplasia (BD) is a histological diagnosis that one or more clones of epithelial cells have acquired genetic alterations rendering them neoplastic and prone to malignancy. Most recent study suggests the annual rate of cancer development for patients with BE is 0.12%<sup>[10]</sup>. While this rate increases to 6% for patients with BE and HGD<sup>[11]</sup>. Even among experienced pathologists, the extent of inter-observer agreement for the diagnosis of BD is a major problem<sup>[12-14]</sup>. Despite several imperfections such as sampling errors and inter-observer variability, pathologic classification of dysplasia on endoscopic biopsy specimens is the single most predictive variable for progression to cancer in patients with BD<sup>[15]</sup>. The degree of dysplasia has been shown to correlate with the risk of development cancer. The optimal care of patients with BD is unclear. In uncontrolled studies, satisfactory results have been noted in BE with HGD who were managed with esophagectomy<sup>[16]</sup>, intensive endoscopic surveillance<sup>[17]</sup> and various endoscopic ablation techniques<sup>[18,19]</sup>. Although current guidelines<sup>[11]</sup> endorse various strate-

gies, the relative efficacy and safety of the promising endoscopic ablation treatment modalities remain unclear. There is no previous head-to-head comparison of photodynamic therapy (PDT) *vs* radiofrequency ablation (RFA) exists. Therefore, we compare efficacy, safety, and cost-effectiveness of PDT *vs* RFA in patients with BD, in IRB-approved, prospectively collected BE outcome database at single center.

## MATERIALS AND METHODS

### Study population

The institutional review board at The Methodist Hospital, Houston, Texas approved this protocol. All patients signed an informed consent form prior to being enrolled in the study. We retrospectively evaluated prospective collected database of all patients with a diagnosis of BE containing dysplasia [low-grade dysplasia (LGD) and high-grade dysplasia (HGD)] between May 2000 and June 2009 to ascertain eligibility for surveillance and therapeutic intervention. Inclusion criteria were: age > 18 years of age and non-nodular BD at enrollment. Exclusion criteria were: active esophagitis, esophageal stricture preventing passage of a therapeutic endoscope, any history of esophageal cancer, esophageal varices, and uncontrolled coagulopathy. All patients with BD were counseled about antireflux measures and received twice daily oral proton pump inhibitors throughout the study. All HGD patients underwent surgical consultation and based on age, performance status and co-morbidities were either ineligible for surgery or were offered surgery and refused after multidisciplinary meeting with gastroenterologist and surgeon regarding the risk and benefits related to endoscopic therapy and surgery.

### Interventions

All endoscopic procedures (biopsy and ablation) were performed on an outpatient basis using intravenous conscious sedation (narcotic and benzodiazepine) or monitored anesthesia care (propofol). Endoscopic biopsies for baseline dysplastic grade confirmation, as well as for surveillance after ablative therapy, were performed using jumbo forceps in at least four quadrants every 1 cm of the BE segment. Post-ablative biopsies always encompassed the entire original extent of BE. Additional directed biopsies were obtained and placed in a separate container if any other visible abnormalities were noted during surveillance. Specimens from each level were fixed in formalin and embedded in paraffin to allow mapping of lesions. The blocks were sectioned, applied to glass slides, and stained with hematoxylin and eosin. All slides were independently evaluated by two gastrointestinal pathologists with expertise in the field of BE. Each specimen was assessed for the presence of BE, and if present, the worst pathologic grade noted per specimen as follows: non-dysplastic BE, LGD, HGD, or cancer. The worst pathologic grade used as the grade for that patient for that biopsy session. In cases of discordance between independent pathology readings, an open consensus di-

agnosis was obtained. Patients with visible nodule(s) who had endoscopic mucosal resection (EMR) were excluded from this study group.

Eligible patients with HGD who were enrolled between May 2000 and late 2007 were offered PDT, consisting of an intravenous photosensitizing agent (2.0 mg/kg, porfimer sodium, Axcan Pharma, Birmingham, AL) 40–50 h prior to endoscopy. During endoscopy, laser light (630 nm) was applied to the BE segment using a laser catheter without centering balloon (dose 130 J/cm<sup>2</sup>). A maximum of 7 cm of BE was treated per session. In longer segments, a second PDT session was performed 3 mo later to treat the remaining segment. PDT patients had upper endoscopy and biopsies at 2 and 12 mo after the primary PDT and then annually thereafter, provided no HGD or adenocarcinoma was found on biopsy. If HGD was detected, PDT was repeated for a maximum of 3 total PDT sessions.

Eligible patients with LGD or HGD who were enrolled between September 2007 and June 2010 were offered RFA, consisting of step-wise treatment with the HALO ablation system (Covidien Inc., Mansfield, MA). In cases where the BE segment was > 3 cm, the primary ablation treatment was performed with the HALO<sup>360</sup> ablation catheter, a balloon-based electrode which creates a circumferential 3-cm long ablation zone (40 W/cm<sup>2</sup>, 12 J/cm<sup>2</sup>). In cases where the BE segment was < 3 cm, the primary ablation treatment was performed with the HALO<sup>90</sup> ablation catheter, an endoscope-mounted electrode which creates a focal 1.3 cm by 2.0 cm ablation zone (40 W/cm<sup>2</sup>, 12 J/cm<sup>2</sup>). After primary ablation, patients had endoscopy every 2 mo with assessment of endoscopic response to therapy. If residual BE was detected visually, RFA touch-up with HALO-90 was performed (maximum 2 additional sessions). Upon achieving a complete visual response or exceeding the maximum allowed sessions, biopsies were performed to confirm a histological complete response. Thereafter, patients were biopsied at two and 12 mo, and then annually provided no BD or BE detected.

### Post-ablation care

After ablation, all patients were provided with double dose proton pump inhibitor medication to enable healing of the ablation zone and ensure long-term control of GERD. Patients were also provided with pain medication and anti-emetics to use as needed for 1–2 wk after therapy. PDT patients were educated to avoid exposure of eyes and skin to direct sunlight and high intensity visible light for at least 30 d or longer in patients with lighter skin color.

### Outcome measures

The primary outcomes for this study were complete histological response of BD (CR-BD), defined as no evidence of dysplasia at last available biopsy session. Detection of cancer during any follow-up was considered a censoring event for the primary outcome. Secondary outcomes include the occurrence of adverse events, occurrence of

**Table 1** Characteristics factors of the patients *n* (%)

Variable	PDT ( <i>n</i> = 33)	RFA ( <i>n</i> = 53)
Age (yr)		
Mean ± SD	70.7 ± 12.2	65.4 ± 12.7
Range	49–80	54–80
Sex		
Female	3 (9)	14 (26)
Male	30 (91)	39 (74)
Body-mass index		
Mean ± SE	27.8 ± 0.7	31.7 ± 1.3
Range	21–38	23–47
Length of Barrett's esophagus (cm)		
Mean ± SD	5.4 ± 3.2	5.7 ± 3.2
Range	1.0–8.0	1.0–9.0

PDT: Photodynamic therapy; RFA: Radiofrequency ablation.

subsquamous intestinal metaplasia at the last biopsy visit, and cost calculated per procedure and per patient.

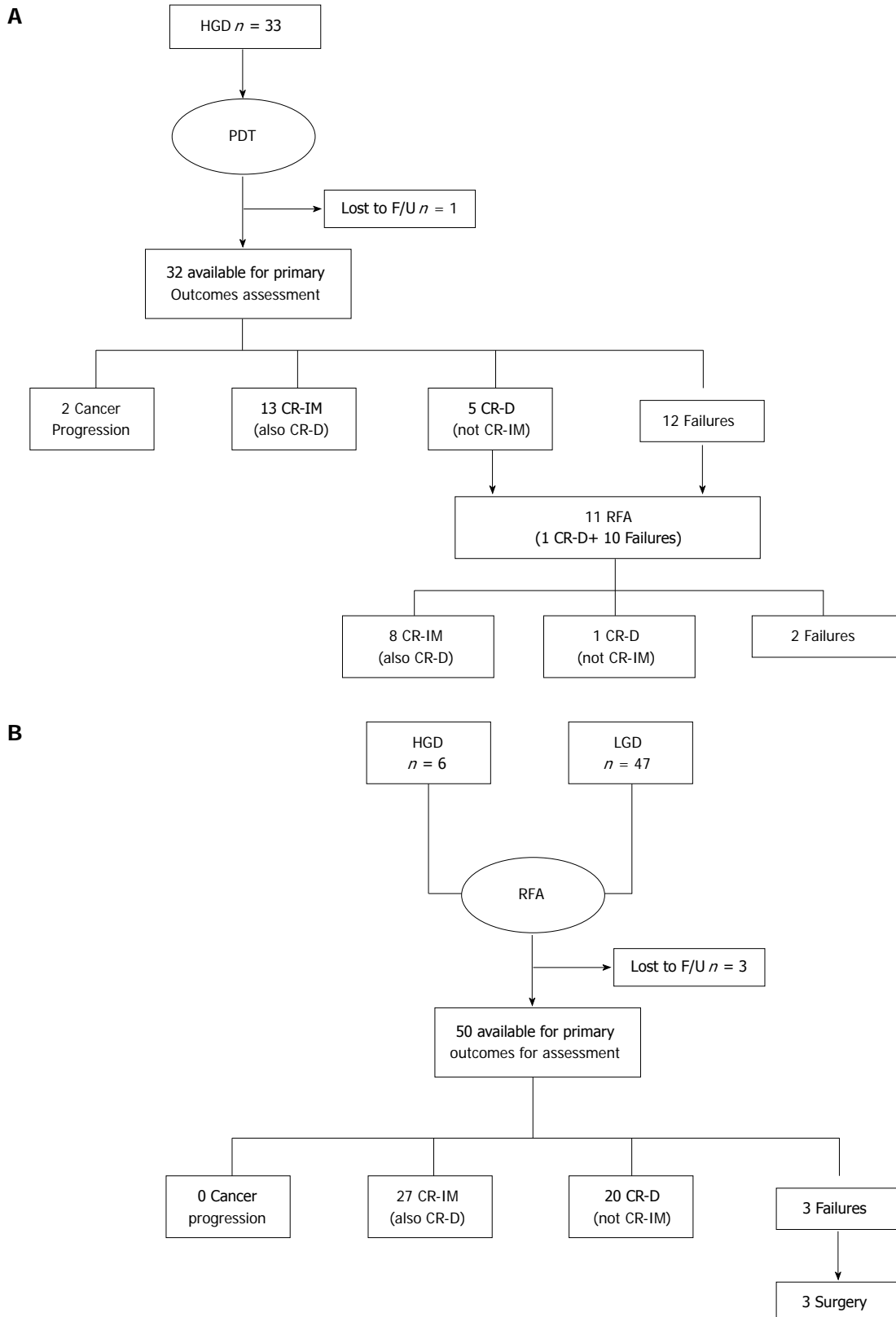
### Statistical analysis

The primary analysis was intention-to-treat (ITT), with all lost-to-follow-up patients being considered failures for the primary outcomes. The secondary analysis was per-protocol (PP), evaluating those patients with data available. Fisher's exact test and Student's *t* test were used to compare baseline variables. Fisher's exact test was used to assess differences between the two study groups for primary outcomes. For all outcomes, a two-sided *P* value of less than 0.05 was considered to indicate statistical significance. The statistical analysis was performed using the SPSS 12.0.1 software for Windows. The costs of the PDT and RFA were calculated with a non-bias formula by the Methodist Hospital Cost Center.

## RESULTS

There were 86 patients who fulfilled the study criteria and signed informed consent. Thirty-three patients underwent primary PDT (all with HGD). Fifty-three patients underwent primary RFA (6 with HGD and 47 with LGD). See Table 1 for baseline patient characteristics and Figure 1 for patient flow. Mean baseline BE length values were similar between groups (PDT: 5.4 ± 3.2, RFA: 5.7 ± 3.2, *P* = 0.53), while PDT patients tended to be older (PDT: 70.7 ± 12.2, RFA: 65.4 ± 12.8, *P* = 0.10) and more likely to have HGD (PDT: 33/33, RFA: 6/53) as entry diagnosis (*P* < 0.0001).

The average length of follow-up from primary ablative therapy to primary outcome biopsy session was 44 mo (range 24–60 mo) for PDT and 33 mo (range 24–48 mo) for RFA. PDT patients had an average of 1.4 PDT sessions, while RFA had an average of 2.6 sessions. CR-D was achieved in 18/33 (54.5%) PDT and 47/53 (88.7%) RFA patients (*P* = 0.0001), Table 2. Eight PDT patients demonstrated an initial complete response, but then recurred (1 HGD, 7 LGD) at later biopsy sessions and were considered failures for the primary outcomes with PDT. After recurrence, these patients could not un-



**Figure 1 Patient flow.** HGD: High-grade dysplasia; PDT: Photodynamic therapy; CR-D: Complete histological resolution of Barrett's dysplasia; IM: Intestinal metaplasia.

dergo further PDT due to exceeding the maximum PDT sessions achieved or intolerance to therapy, therefore all underwent salvage therapy with RFA with 100% CR-BD (post-hoc analysis). Two PDT patients developed cancer during follow-up at 18 and 24 mo. Both were considered

failures for the primary endpoint of this study and both underwent esophagectomy. Surgical pathology showed T1NOMO lesions. No RFA patient developed cancer progression.

In the PDT cohort, two patients reported photo-



**Table 2 Outcomes measures *n* (%)**

	PDT	RFA
<b>Primary outcomes</b>		
Complete Response IM		
ITT	13/33 (39.4)	27/53 (50.9)
PP	13/32 (40.6)	35/61 (57.4)
Complete Response Dysplasia		
ITT	18/33 (54.5)	47/53 (88.7)
PP	18/32 (56.3)	56/61 (91.8)
<b>Secondary outcomes</b>		
Complications		
Perforation	1/32 (3.1)	0/50 (0.00)
Stricture	9/32 (28.1)	2/50 (4.0)
Cost per session	\$9449	\$1888 (HALO-360) \$1486 (HALO-90)

PDT: Photodynamic therapy; RFA: Radiofrequency ablation; ITT: Intention-to-treat; PP: Per-protocol; IM: Intestinal metaplasia.

sensitivity reactions (none required therapy), 9 (28.13%) developed a stricture necessitating serial endoscopic dilations (mean 3 per patient) with two requiring temporary stent placement. One patient developed an esophageal perforation that was managed by nonsurgical measures in the PDT cohort. In the RFA cohort, 2 patients (4.0%) developed a stricture managed with endoscopic dilation (one dilation per patient). No perforation was seen in the RFA patients. Subsquamous intestinal metaplasia (SSIM, "buried glands") was seen in 4/32 (12.5%) of patients treated with PDT and 3/50 (6%) of those treated with RFA ( $P = 0.28$ ).

The facility cost for a HALO-360 RFA session *vs* a PDT session was \$1888 and \$9449, respectively. The facility cost of a HALO-90 RFA was slightly lower at \$1486. Cost per patient was also calculated, including primary ablation, follow-up ablation, and salvage ablation procedures, but excluding surgical salvage. According to a calculated cost analysis in our institution, PDT was approximately five times more costly than RFA per procedure.

## DISCUSSION

Professional guidelines endorse various strategies for management of BE with dysplasia<sup>[11]</sup>. Due to lack of head to head comparison, relative efficacy of interventions like PDT, RFA, and cryotherapy remains unclear. Based on the finding that over 40% of patients with HGD undergoing esophagectomy have invasive cancer in their pathology specimen<sup>[16,20]</sup>, esophagectomy is still been recommended for patients with HGD<sup>[11]</sup>. The prevalence of a missed cancer in patients may be less with recently used aggressive endoscopic biopsy protocols and better endoscopic visualization, however it is not been proven yet. Moreover, even when an esophagectomy is performed by experienced surgeons in high volume centers, it is associated with significant morbidity and mortality<sup>[16,20]</sup>. There are no RCTs comparing esophagectomy and endoscopic therapy for treatment of dysplastic BE<sup>[21]</sup>. Although debatable, intensive endoscopic surveillance

with rigorous biopsy protocol can detect the progression from BD to cancer at the curative stage<sup>[17]</sup>, but is associated with considerable anxiety for these patients with a negative impact to their quality of life. The accumulated evidence suggests that endoscopic resection (ER) therapy like endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) or endoscopic ablation modalities such as PDT and RFA have become attractive alternatives for the management of patients with BD<sup>[22-25]</sup>. ER of focal lesions can achieve complete eradication of HGD and IMC in 82.5%-95% of patients and also allows detection of occult invasive adenocarcinoma<sup>[21,26-28]</sup>. However, metachronous lesions or disease recurrence is been seen in up to 14% of patients within 12 mo, and 21.5% of patients over 5 years after ER<sup>[21,24,29]</sup>. International consortium of expert clinicians recommends ER for focal lesions followed by ablation of whole Barrett's segment to decrease recurrence rate<sup>[30]</sup>. Endoscopic ablation modalities are based on the hypothesis that injury to the metaplastic and dysplastic epithelium will reverse the pathologic progression of BD to invasive cancer and restore a normal squamous epithelium. Despite the attractiveness of these endoscopic ablation technologies, no previous head-to-head comparison of PDT *vs* RFA exists in patients with BD.

In our study, we compared outcomes in patients treated with PDT and RFA in a single center by a single gastroenterologist in a prospectively collected database. These two modalities were compared with regard to complete eradication of BE and BD, adverse events, and costs. Our data shows that PDT and RFA eradicated dysplasia in 54% and 89%, respectively. Complete reversal of intestinal metaplasia was noted in 39% cases for PDT and 51% cases for RFA.

Overholt *et al*<sup>[31]</sup> compared PDT with Photofrin (PHO) plus omeprazole (PHOPDT) to omeprazole (OM) only to eradicate BE-HGD in a multicenter, randomized (2:1 randomization), pathology-blinded trial. At 5 years PHOPDT was significantly more effective than OM in eliminating HGD [77% (106/138) *vs* 39% (27/70),  $P < 0.0001$ ]. A secondary outcome measure preventing progression to cancer showed a significant difference ( $P = 0.027$ ) with about half the likelihood of cancer occurring in PHOPDT [21/138 (15%)] *vs* OM [20/70 (29%)], with a significantly ( $P = 0.004$ ) longer time to progression to cancer favoring PHOPDT. In our experience, PDT eliminated HGD in 54.5% of patients compared to 77% on Overholt *et al*<sup>[31]</sup> study. In Overholt *et al*<sup>[31]</sup> study 36% of the patients developed stricture, and 69% had a photosensitivity reaction. Similarly, in our study 28% of patients developed strictures requiring serial endoscopic dilation. Shaheen *et al*<sup>[32]</sup> performed a multicenter, sham-controlled trial, and randomly assigned 127 patient with dysplastic BE in a 2:1 ratio to receive either RFA or a sham procedure (control group). In the intention-to-treat analyses, among patients with LGD, complete eradication of dysplasia occurred in 90.5% of those in the ablation group, as compared with 22.7% of those in the

control group ( $P < 0.001$ )<sup>[32]</sup>. Among patients with HGD, complete eradication occurred in 81.0% of those in the ablation group, as compared with 19.0% of those in the control group ( $P < 0.001$ ). Overall, 77.4% of patients in the ablation group had complete eradication of intestinal metaplasia, as compared with 2.3% of those in the control group ( $P < 0.001$ ). Patients in the ablation group had less disease progression (3.6% vs 16.3%,  $P = 0.03$ ) and fewer cancers (1.2% vs 9.3%,  $P = 0.045$ ). We observed similar rates of complete response as illustrated in Table 2.

While making decisions about the management of precancerous conditions, potential benefits, risks and costs associated with different competing strategies are the most important considerations. For less severe disease like BE with LGD compared to BE with HGD, safety and cost of the suggested intervention becomes even more important<sup>[32]</sup>. According to the calculated direct cost comparison analysis, PDT was five times more costly than RFA in our institution. Detail cost effective analysis requires calculation for direct and indirect cost related to each intervention, as well as cost associated with complication and treatment failure. PDT patients are required to avoid sun and high intensity visible light during four weeks after the procedure and this may add significant indirect cost, in addition to high direct cost with PDT therapy. In our experience, side effects and complications of RFA were also less than PDT therapy.

In principle, a head-to-head comparison of PDT and RFA requires a randomized, blinded study design but this is currently impossible given the limited availability of PDT ablation technology for the esophagus. The strengths of our study are the very low drop-out rate, tight control of technique by a single experienced gastroenterologist (AE), and a rigorous biopsy protocol with review of all pathology by two well known gastroenterology pathologists and long follow up duration. Our study also has several limitations including the lack of randomization, the significant disproportion of HGD between the PDT and RFA groups respectively and difference in follow up time for both PDT and RFA group and the results of our study should be interpreted accordingly. PDT therapy was approved for HGD only and till RFA technology became available, only patients with BE-HGD were offered treatment with PDT. The implications of LGD vs HGD in Barrett's patients are markedly different. LGD implies a risk of progression to cancer of less than 1% per patient-year<sup>[33]</sup>, whereas the risk associated with HGD may be as high as 10% per patient-year<sup>[34,35]</sup>.

In conclusion, in our experience both PDT and RFA were successful in eradicating dysplasia in BE. However, overall success rate of RFA was higher than PDT and RFA was very well tolerated without any major complications and fewer side effects. At our center, each session of RFA therapy was five times less costly than PDT therapy. However, for head-to-head comparison of PDT and RFA, prospective, randomized, blinded, multi-center studies are needed.

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## COMMENTS

### Background

The incidence of esophageal adenocarcinoma has increased by more than 500% since 1970 and it is considered one of the most rapidly increasing cancers in western society. Barrett's esophagus (BE) is a premalignant condition and predisposes to adenocarcinoma of the esophagus and gastro-esophageal junction.

### Research frontiers

Current guidelines endorse various strategies like esophagectomy, intensive endoscopic surveillance and various endoscopic resection and endoscopic ablation techniques for management of dysplastic BE. Significant controversies and disagreement exists in management of BE including the role of Barrett's surveillance, the optimal means of intervention, and the stage at which intervention is indicated.

### Innovations and breakthroughs

Various endoscopic ablation techniques like photodynamic therapy (PDT), radiofrequency ablation (RFA) and cryotherapy are currently available for treatment of dysplastic Barrett's esophagus. Both PDT and RFA have been proven to be superior to eradicate dysplastic Barrett's esophagus compared to routine antireflux measures and pharmacological anti-reflux measures in randomized trials. This study compared relative safety, efficacy and cost related to PDT and RFA therapy for dysplastic BE in a single center consecutive case series and found that RFA was more effective, safe and less costly than PDT therapy.

### Applications

With better understanding of relative efficacy, safety and cost between PDT and RFA, this study will help physicians to determine optimal endoscopic ablation technique for treatment of dysplastic BE.

### Peer review

It is a well written and accurate study. It shows a striking superiority of RFA vs PDT for Barrett esophagus and, even that the study has not been randomized and the number of patients is not very long, the results deserve its publication and must be known by practical endoscopists.

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## Children with celiac disease and high tTGA are genetically and phenotypically different

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### Abstract

**AIM:** To investigate whether celiac disease (CD) patients with tissue-transglutaminase antibody (tTGA)  $\geq 100$  U/mL are different from patients with lower tTGA levels.

**METHODS:** Biopsy-proven (Marsh III) pediatric CD patients ( $n = 116$ ) were prospectively included between March 2009 and October 2012. The biopsies were evaluated by a single pathologist who was blinded to all of the patients' clinical data. The patients were distributed into 2 groups according to their tTGA level, which was measured using enzyme-linked immunoassay: tTGA  $\geq 100$  U/mL and tTGA  $< 100$  U/mL. The patients' characteristics, symptoms, human leukocyte antigen (HLA) genotype and degree of histological involvement were compared between the 2 groups.

**RESULTS:** A total of 34 (29.3%) children had tTGA values  $< 100$  U/mL and 82 (70.7%) tTGA levels of  $\geq 100$  U/mL. Patients with high tTGA levels had lower average body weight-for-height standard deviation scores (SDS) than did patients with tTGA  $< 100$  U/mL ( $-0.20 \pm 1.19$  SDS vs  $0.23 \pm 1.03$  SDS,  $P = 0.025$ ). In the low tTGA group, gastrointestinal symptoms were more common (97.1% vs 75.6%,  $P = 0.006$ ). More specifically, abdominal pain (76.5% vs 51.2%;  $P = 0.012$ ) and nausea (17.6% vs 3.7%,  $P = 0.018$ ) were more frequent among patients with low tTGA. In contrast, patients with solely extraintestinal manifestations were only present in the high tTGA group (18.3%,  $P = 0.005$ ). These patients more commonly presented with aphthous stomatitis (15.9% vs 0.0%,  $P = 0.010$ ) and anemia (32.9% vs 11.8%,  $P = 0.019$ ). In addition, when evaluating the number of CD-associated HLA-DQ heterodimers (HLA-DQ2.5, HLA-DQ2.2 and HLA-DQ8), patients with low tTGA levels more commonly had only 1 disease-associated heterodimer (61.8% vs 31.7%,  $P = 0.005$ ), while patients with high tTGA more commonly had multiple heterodimers. Finally, patients with tTGA  $\geq 100$  U/mL more often had a Marsh IIIc lesion (73.2% vs 20.6%,  $P \leq 0.001$ ) while in patients with low tTGA patchy lesions were more common (42.4% vs 6.8%,  $P \leq 0.001$ ).

**CONCLUSION:** Patients with tTGA  $\geq 100$  U/mL show several signs of more advanced disease. They also carry a larger number of CD associated HLA-DQ heterodimers.

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**Key words:** Celiac disease; Serology; Anti-tissue transglutaminase antibodies; Human leukocyte antigen; Phenotype

**Core tip:** We prospectively investigated the differences between celiac disease (CD) (Marsh III) patients with tissue-transglutaminase antibody (tTGA) levels  $\geq 100$

U/mL and patients with lower tTGA levels. We found that patients with high tTGA more often carried multiple CD-associated heterodimers compared with patients with tTGA < 100 U/mL. In addition, high-tTGA patients have more advanced mucosal lesions that are also less patchy. Phenotypically, high-tTGA patients have a lower body weight and more often present with extraintestinal symptoms compared with patients with lower levels of tTGA, who more often have intestinal symptoms. These results provide further evidence that patients with tTGA  $\geq$  100 U/mL are truly a distinct group with more advanced disease.

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## INTRODUCTION

Celiac disease (CD) is a highly prevalent disorder with a strong genetic component. The disease has a complex and variable clinical presentation: some patients display symptoms ranging from severe malabsorption to vague intestinal or extraintestinal manifestations, while others have no symptoms at all<sup>[1-3]</sup>. The disease is caused by inappropriate immune responses to gluten, a storage protein in wheat and the related grain species barley and rye<sup>[4]</sup>. The immune reaction mainly affects the small intestine, where it typically causes lymphocyte invasion in the epithelium, hyperplasia of the crypts and various grades of villous atrophy<sup>[5,6]</sup>. These histological lesions can be patchily distributed throughout the small intestine and can even occasionally be localized exclusively in the duodenal bulb<sup>[7,8]</sup>. Serologically, signs of inflammation are also evidenced by the presence of disease-associated antibodies, including endomysium antibodies (EMA) and tissue transglutaminase antibodies (tTGA)<sup>[4,9,10]</sup>.

Until recently, these serological and histological manifestations were used in combination to detect CD, with histological evaluation being essential for establishing the diagnosis in all cases<sup>[9,11]</sup>. However, given the excellent sensitivity and specificity of serology, the new ESPGHAN guidelines now indicate that a biopsy can be omitted in symptomatic children with tTGA levels  $\geq$  100 U/mL (> 10 times the upper limit) and positive EMA, provided the patient also carries a disease-associated human leukocyte antigen (HLA) type and responds well to the diet<sup>[12]</sup>. In contrast, in patients with a tTGA < 100 U/mL, a biopsy is always necessary because a significant proportion of patients with these levels do not have CD.

It is unclear why patients with a tTGA  $\geq$  100 U/mL virtually always have CD. These high levels could be a sign of advanced disease. Patients with high serum tTGA may also have a different genetic risk profile. Because

HLA genes make the greatest genetic contribution, the aim of this study was to assess whether patients with a tTGA  $\geq$  100 U/mL have a different HLA distribution compared with patients with lower tTGA levels<sup>[13]</sup>. We also investigated whether more advanced small intestinal histological lesions were present in patients with a tTGA  $\geq$  100 U/mL. In addition, as it remains to be resolved whether patients with tTGA levels  $\geq$  100 U/mL are phenotypically distinct from those with a tTGA < 100 U/mL, we set out to detect differences in clinical presentation between both groups.

## MATERIALS AND METHODS

### Study population

Pediatric patients who had a histologically confirmed diagnosis of CD between March 2009 and October 2012 in the Wilhelmina Children's Hospital in Utrecht, The Netherlands, were prospectively included in the study. Patients were referred to us because of CD-associated symptoms or because they belonged to a group at risk for CD. Biopsies were collected from patients with abnormal serology. Biopsies were also collected from patients with negative serology but a strong clinical suspicion of the disease. Patients with immune globulin A (IgA) deficiency ( $n = 3$ ) were excluded from the study. The clinical symptoms at presentation were collected from the medical records. The study was performed according to the guidelines of the local medical ethics board.

### Histological evaluation

Biopsies were obtained using upper endoscopy. On average, 3.09 biopsies (range 1-5, SD = 0.75) were obtained from the distal duodenum, and 2.41 (range 0-5, SD = 1.03) were obtained from the duodenal bulb. The biopsies were evaluated by a single experienced pathologist who was blinded to all of the patients' clinical data and who used the Marsh classification, as modified by Oberhuber<sup>[5,6]</sup>. The duodenal bulb and the distal duodenum were scored separately, but the final Marsh score for each patient was graded according to the most affected site (highest Marsh score). Only Marsh III lesions (*i.e.*, those characterized by an increased number of intraepithelial lymphocytes, crypt hyperplasia and villous atrophy) were considered diagnostic for CD. Patients with other histological findings were not included. Marsh III lesions were further classified according to the degree of villous atrophy: Marsh IIIa (partial villous atrophy), Marsh IIIb (subtotal villous atrophy) and Marsh IIIc (total villous atrophy).

### Serological assessment

Serum IgA tTGA levels were measured using the ELiA Celikey IgA kit (Phadia AB, Uppsala, Sweden). Serum samples containing an antibody titer of more than 10 U/mL were considered positive, as recommended by the manufacturer. IgA EMA levels were detected *via* indirect immunofluorescence using sections of distal monkey



esophagus mounted on glass slides (IMMCO Diagnostics Inc., Buffalo NY). Total IgA was measured in all patients, and a serum IgA concentration below 0.07 g/L was regarded as IgA deficiency.

### HLA-typing

Genomic DNA was isolated from ethylenediaminetetraacetic acid-anticoagulated blood with a standardized DNAzol-based technique. The HLA-DQA1 and HLA-DQB1 alleles were typed using the sequence-specific oligonucleotide primed polymerase chain reaction technique with the Luminex-based OneLambda LABType SSO Class II DQA1/DQB1 typing kit, following the recommendations of the manufacturer (One Lambda Inc., Canoga Park, CA, United States). Samples were analyzed on a LABScan™ 100 System (Luminex, Austin TX, United States), and data were interpreted using the HLA-Fusion 2.0 Software package (OneLambda).

HLA-DQ2.5 (DQA1\*05:01, -DQB1\*02:01 or DQA1\*05:05, -DQB1\*02:02), HLA-DQ2.2 (DQA1\*02:01, -DQB1\*02:02) and HLA-DQ8 (DQA1\*03:01, -DQB1\*03:02 or DQA1\*03:02, -DQB1\*03:02) were considered CD-associated HLA-types. The patients were scored for the number of CD-associated heterodimers that they could form with their HLA-genotypes. For example, a patient who is homozygous for HLA-DQ2.5 (or HLA-DQ2.2 or HLA-DQ8) can form 4 different heterodimers that are associated with CD. The same is true of patients who are compound heterozygous for HLA-DQ2.5 and HLA-DQ2.2, because these patients can also make 4 different CD-associated heterodimers: HLA-DQA1\*05:01, -DQB1\*02:01; HLA-DQA1\*02:01, -DQB1\*02:02; HLA-DQA1\*05:01, -DQB1\*02:02 and HLA-DQA1\*02:01, -DQB1\*02:01 (the latter 2 of which are molecularly indistinguishable from the first 2). Patients who are heterozygous for HLA-DQ2.5 and HLA-DQ8 or HLA-DQ2.2 and HLA-DQ8 can only form 2 CD-associated heterodimers. Finally, patients with only 1 CD-associated HLA genotype can only generate 1 CD-associated heterodimer.

### Statistical analysis

The patients were divided in 2 groups: those with tTGA  $\geq 100$  U/mL and those with tTGA  $< 100$  U/mL. Subsequently, the differences between the 2 groups in terms of gender, average age at diagnosis, average height and weight, the presence of a CD-associated disease, the presence of a first-degree relative with CD, symptoms, HLA type, Marsh classification and histological differences between the duodenal bulb and the more distal duodenum were calculated using SPSS Version 20.0.

To test for statistical significance, the  $\chi^2$  or Fisher exact test was used for nominal variables. For continuous variables, the independent *t* test or the Mann-Whitney *U* test were used. A *P* value  $< 0.05$  was considered statistically significant.

## RESULTS

### Patient characteristics

A total of 116 patients met the study's inclusion criteria. Of those, 34 (29.3%) patients had tTGA values  $< 100$  U/mL and 82 (70.7%) had a serum tTGA of at least 100 U/mL. Within the low tTGA group, 2 patients, a 10-month-old girl and a 2-year-old boy, had a tTGA level  $< 10$  U/mL and negative EMA, which is not an uncommon finding in very young children<sup>[11,14-18]</sup>. All of the remaining patients had positive EMA levels.

Of the total study population, 32 (27.6%) were male and 84 (72.4%) female, with no difference in gender distribution between the high and low tTGA groups (Table 1). The mean age of the included patients at diagnosis was 6.5 years, ranging from 0.9 to 17.7 years. The average age at diagnosis was slightly higher (7.4 years) in the low tTGA group compared with the high tTGA group (6.1 years), but this was statistically not significant. The patients in the high tTGA group were slightly shorter (-0.83 standard deviation score, SDS) compared with the low tTGA group (-0.60 SDS), but this difference was not significant. In contrast, the average body weight-for-height was significantly lower (-0.20 SDS) in the high tTGA group compared with patients in the low tTGA group, who had an average weight of 0.23 SDS (*P* = 0.025).

Regarding comorbidity, 5 (4.3%) patients had Down syndrome, and 1 (0.86%) of those also had hypothyroidism. Another 4 (3.4%) patients had diabetes mellitus Type I, 1 (0.86%) patient had juvenile rheumatoid arthritis and 1 (0.86%) patient had Graves disease. Remarkably, all but 1 of the patients with comorbidity had tTGA  $\geq 100$  U/mL; however, this finding was not statistically significant. Finally, 9 (26.5%) patients in the low tTGA group had a first-degree relative with CD, compared with 14 (17.1%) patients in the high tTGA group; again, this difference was not statistically significant.

### Symptoms

Only 5 (4.3%) patients were asymptomatic, 4 of which had a tTGA  $\geq 100$  U/mL and 1 of which had a tTGA  $< 100$  U/mL (Table 2). The other 111 (95.7%) patients had various gastrointestinal and extraintestinal symptoms. Interestingly, gastrointestinal symptoms were significantly (*P* = 0.006) more common in the low tTGA group (*n* = 33; 97.1%) compared with the high tTGA group, in which 75.6% (*n* = 62) of the patients suffered from a gastrointestinal symptom. However, although patients with symptoms restricted to the gastrointestinal tract (without any extraintestinal manifestations) were also more common in the low tTGA group (23.5% *vs* 9.8%, respectively), this difference was not statistically significant (*P* = 0.074). In terms of specific gastrointestinal complaints, abdominal pain and nausea were significantly more common in the low tTGA group. Indeed, 76.5% (*n* = 26) of the patients in the low tTGA group had abdominal pain, compared

**Table 1 Characteristics of patients *n* (%)**

Patient characteristic	tTGA < 100 U/mL ( <i>n</i> = 34)	tTGA ≥ 100 U/mL ( <i>n</i> = 82)	<i>P</i> value
Gender (M)	8 (23.5)	24 (29.3)	0.529
Average age (yr)	7.40 ± 4.06	6.10 ± 3.82	0.114
Average height in SDS	-0.60 ± 1.15	-0.83 ± 1.22	0.331
Average weight for height in SDS	0.23 ± 1.03	-0.20 ± 1.19	0.025
CD associated comorbidity	1 (2.9)	10 (12.2)	0.171
First degree relative with CD	9 (26.5)	14 (17.1)	0.248

tTGA: Anti-tissue transglutaminase antibodies; SDS: Standard deviation scores; CD: Celiac disease; M: Male.

with 51.2% (*n* = 42) in the high tTGA group (*P* = 0.012). Similarly, in the low tTGA group, 17.6% (*n* = 6) of the patients suffered from nausea, compared with 3.7% (*n* = 3) in the high tTGA group (*P* = 0.018). Moreover, there was a statistically non-significant trend (*P* = 0.096) towards more constipation in the low tTGA group (*n* = 14; 41.2%) compared with the high tTGA group (*n* = 21; 25.6%). In contrast, diarrhea was more common in the high tTGA group (*n* = 27; 32.9%) compared with the low tTGA group (*n* = 8; 23.5%), but the difference was not significant (*P* = 0.316). Similarly, a comparable trend (*P* = 0.277) was seen for vomiting, which occurred more often in the high tTGA group (11.0% *vs* 2.9%). Finally, the presence of bloating was comparable in both groups with more than 1/3 of the patients suffering from this symptom.

Extraintestinal symptoms occurred in 25 (73.5%) of the patients with low tTGA compared with 70 (85.4%) patients in the high tTGA group, but this difference was not statistically significant (*P* = 0.132). However, patients with solely extraintestinal symptoms (*i.e.*, without gastrointestinal symptoms) were only present in the high tTGA group (*n* = 15; 18.3%), a finding that was statistically significant (*P* = 0.005). Similarly, aphthous stomatitis only occurred in patients with high tTGA (*n* = 13; 15.9%). This was statistically significant, with a *P* value of 0.010. Likewise, anemia was significantly (*P* = 0.019) more common in the high tTGA group: 27 (32.9%) of the patients with high tTGA had anemia, compared with 4 (11.8%) patients with low tTGA. There was also a trend towards more increased appetite (7.3% *vs* 2.9%), joint pain (11.0% *vs* 5.9%) and low weight (8.5% *vs* 5.9%) in the high tTGA group, but these differences were not statistically significant (*P* > 0.05). Tooth enamel defects were more common in the low tTGA group (5.9% *vs* 3.7%), but this was also not statistically significant (*P* = 0.629). Finally, the presence of fatigue, irritability, anorexia and short stature was comparable in both groups.

### HLA-types

All of the patients carried at least one of the CD-associated HLA types. In the high tTGA group, the patients more often carried multiple CD-associated heterodimers (*P* = 0.005; Table 3). Illustratively, in the low tTGA

**Table 2 Symptoms in celiac disease patients *n* (%)**

	tTGA < 100 U/mL ( <i>n</i> = 34)	tTGA ≥ 100 U/mL ( <i>n</i> = 82)	<i>P</i> value
Symptoms			
Asymptomatic	1 (2.9)	4 (4.9)	1.000
Gastrointestinal symptoms			
Any gastrointestinal symptom	33 (97.1)	62 (75.6)	0.006
Only gastrointestinal symptoms	8 (23.5)	8 (9.8)	0.074
Abdominal pain	26 (76.5)	42 (51.2)	0.012
Diarrhea	8 (23.5)	27 (32.9)	0.316
Constipation	14 (41.2)	21 (25.6)	0.096
Bloating	12 (35.3)	31 (37.8)	0.799
Nausea	6 (17.6)	3 (3.7)	0.018
Vomiting	1 (2.9)	9 (11.0)	0.277
Extraintestinal symptoms			
Any extraintestinal symptom	25 (73.5)	70 (85.4)	0.132
Only extraintestinal symptoms	0 (0.0)	15 (18.3)	0.005
Fatigue	16 (47.1)	35 (42.7)	0.666
Irritability	9 (26.5)	25 (30.5)	0.665
Anorexia	13 (38.2)	32 (39.0)	0.937
Increased appetite	1 (2.9)	6 (7.3)	0.672
Joint pain	2 (5.9)	9 (11.0)	0.504
Tooth enamel defects	2 (5.9)	3 (3.7)	0.629
Aphthous stomatitis	0 (0.0)	13 (15.9)	0.010
Anaemia	4 (11.8)	27 (32.9)	0.019
Short stature (height < -2 SDS)	4 (11.8)	11 (13.4)	1.000
Low weight (< -2 SDS)	2 (5.9)	7 (8.5)	1.000

tTGA: Anti-tissue transglutaminase antibodies; SDS: Standard deviation scores.

group, more than half of the patients (*n* = 21; 61.8%) had only one CD-associated heterodimer, compared with 26 (31.7%) in the high tTGA group. Two patients (5.9%) with low tTGA had 2 CD-associated heterodimers, compared with 20 (24.4%) patients in the high tTGA group. Finally, 36 (43.9%) patients in the high tTGA group had 4 CD-associated heterodimers, compared with 11 (32.4%) patients with low tTGA.

### Histology

In the low tTGA group, 5 (14.7%) patients had a Marsh IIIa lesion, 22 (64.7%) had a Marsh IIIb lesion, and only 7 (20.6%) had a Marsh IIIc lesion (Table 3). This was significantly different from the high tTGA group (*P* < 0.001). Illustratively, only 4 (4.9%) patients in the high tTGA group had a Marsh IIIa lesion; 18 (22.0%) had a Marsh IIIb lesion, and the largest proportion of the patients in the high tTGA group (*n* = 60; 73.2%) had flat mucosa (Marsh IIIc).

In 106 patients, both duodenal bulb and distal duodenum biopsies were taken. To assess the presence of patchy lesions, the Marsh classification in both locations was compared. A patchy lesion was defined as the absence of villous atrophy in either the duodenal bulb or the distal duodenum. In 7 (6.6%) patients, a Marsh III lesion was only found in the duodenal bulb, while the distal duodenum was spared. In 12 (11.3%) patients, the distal

**Table 3** Human leukocyte antigen distribution, Marsh classification in celiac disease patients *n* (%)

	tTGA < 100 U/mL ( <i>n</i> = 34)	tTGA ≥ 100 U/mL ( <i>n</i> = 82)	<i>P</i> value
HLA-score			
1 heterodimer	21 (61.8)	26 (31.7)	0.005
2 heterodimers	2 (5.9)	20 (24.4)	
4 heterodimers	11 (32.4)	36 (43.9)	
Marsh classification			< 0.001
Marsh IIIa	5 (14.7)	4 (4.9)	< 0.001
Marsh IIIb	22 (64.7)	18 (22.0)	
Marsh IIIc	7 (20.6)	60 (73.2)	
	<i>n</i> = 33 <sup>1</sup>	<i>n</i> = 73 <sup>1</sup>	
Patchy lesions <sup>2</sup>	14 (42.4)	5 (6.8)	

<sup>1</sup>Only 106 patients out of the total study population also underwent duodenal bulb biopsies; <sup>2</sup>Discrepancy in the diagnosis based on histology in the duodenal bulb *vs* in the distal duodenum. HLA: Human leukocyte antigen; tTGA: Anti-tissue transglutaminase antibodies.

duodenum was the only affected site. Interestingly, a discrepancy between the diagnosis in the distal duodenum *vs* the duodenal bulb was more common in patients with low tTGA than in patients with high tTGA (42.4% *vs* 6.8%, *P* < 0.001). In addition, patchy lesions were more common in patients with Marsh IIIa (in 5 of 9 patients, 55.6%) than in patients with Marsh IIIb (in 13 of 35 patients, 37.1%) or IIIc lesions (in 1 of 62 patients; 1.6%, *P* < 0.001).

## DISCUSSION

CD is defined as a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals<sup>[19]</sup>.

Patients with tTGA levels ≥ 100 U/mL (> 10 times the upper limit) virtually always have CD, whereas the disease can be histologically absent in a significant number of patients with a lower serum tTGA level. In the present study, we show in a pediatric population that patients with a tTGA level ≥ 100 U/mL also have a different HLA-pattern and a more severe histological lesion and seem to be phenotypically different, with more extraintestinal symptoms and a lower body weight.

Patients with high tTGA levels are more likely to have 2 and 4 CD-associated heterodimers compared with patients with lower tTGA levels, who more often only have 1 CD-associated heterodimer (Table 3). This seems pathophysiologically logical. In CD, HLA-molecules on antigen-presenting cells in the lamina propria present gluten peptides to CD4+ T-cells, which in turn further activate the immune system, including B-cells<sup>[20-22]</sup>. Thus, increased cell-surface expression of CD-associated heterodimers will lead to more antigen presentation and therefore more T- and B-cell stimulation, which will eventually generate a stronger antibody response. However, because not all patients with multiple heterodimers had a tTGA ≥ 100 U/mL, and some patients with a single HLA-heterodimer also had tTGA levels ≥ 100

U/mL, other factors, such as non-HLA genes or environmental factors, are likely to contribute to the tTGA-level response. This finding is in line with a previous study showing a correlation between antibody level and HLA-dose; patients homozygous for HLA-DQB1\*02 had significantly higher tTGA levels compared with patients with a single dose of HLA-DQB1\*02 and to patients not carrying any HLA-DQB1\*02<sup>[23]</sup>. In the current study, a comparable HLA-DQB1\*02 correlation was found, but the difference was not significant (*P* = 0.101; data not shown).

The current study also provided evidence that patients with high tTGA levels have more advanced mucosal lesions compared with CD patients with lower tTGA levels. First, patients with tTGA levels ≥ 100 U/mL had a more severe grade of villous atrophy, in line with previous studies showing an increasing tTGA titer with increasing villous atrophy<sup>[24,25]</sup>. However, we also showed that patchy lesions, defined as the absence of villous atrophy in either the duodenal bulb or the distal duodenum, were more common in patients with low tTGA than in patients with high tTGA, suggesting that in patients with high tTGA, the total area of mucosa involved is larger. In addition, patients with a lesser degree of villous atrophy, which is more common in the low tTGA group, also had a higher chance of patchy lesions, providing more evidence that the disease in these patients is truly less advanced.

Interestingly, we also found significant differences in clinical presentation between patients with high tTGA and those with levels < 100 U/mL. The group with high tTGA levels had lower body weight and more extraintestinal complaints than did patients with low tTGA (Table 2). This suggests that patients with high tTGA levels have more advanced or generalized disease. Other studies investigating the relationship between antibody levels and symptoms are rare. Dahlbom and colleagues found that children with an onset of CD in early childhood and/or severe malabsorption had higher tTGA levels than did patients with a late childhood onset of disease and/or moderate symptoms, and also when compared with patients presenting in adulthood<sup>[24]</sup>. Taavela *et al*<sup>[26]</sup> also showed that the serum levels of antibodies associated with CD correlated with gastrointestinal symptoms. None of these two studies specifically investigated the differences in intestinal and extraintestinal symptoms, so their results cannot be directly compared with our study. However, in both studies, a relationship between antibody levels and symptom severity was observed, once again suggesting that patients with a high tTGA have more advanced disease.

Finally, we showed that patients in the low tTGA group more often have a positive family history for CD (26.5% *vs* 17.1%), although this difference was not statistically significant. This difference could have resulted because patients with a positive family history are detected earlier than those without a positive history, before a very high tTGA level is reached. Conversely, patients with



comorbidity were found more frequently (although statistically not significant) in the high tTGA group (12.2% *vs* 2.9%), which might be due to a more advanced disease progression in this group.

Our combined data confirm, in a pediatric population, the hypothesis that patients with tTGA  $\geq 100$  U/mL have more advanced disease, given the more severe histological involvement and the increased incidence of extraintestinal manifestations and lower body weight. Pathophysiologically, these patients also express more CD-associated HLA-heterodimers on their cells. These findings should also be investigated in adults.

## COMMENTS

### Background

Genetically predisposed symptomatic children with positive endomysium antibodies (EMA) and tissue-transglutaminase antibody (tTGA) levels  $\geq 100$  U/mL virtually always have the classical histological triad of an increased number of intraepithelial lymphocytes, crypt hyperplasia and villous atrophy. These features are diagnostic for celiac disease (CD); therefore, in children with these high tTGA values, recent ESPGHAN guidelines have suggested that a biopsy is unnecessary to confirm the disease. In contrast, in patients with lower tTGA levels, a biopsy is still mandatory for histological confirmation because a significant number of these patients appear not to have CD.

### Research frontiers

It is unknown whether CD patients with high tTGA are phenotypically and genotypically different from CD patients with low tTGA.

### Innovations and breakthroughs

Authors prospectively investigated the differences between CD (Marsh III) patients with tTGA levels  $\geq 100$  U/mL and patients with lower levels. They found that patients with tTGA  $\geq 100$  U/mL more often carry multiple CD-associated heterodimers compared with patients with lower levels. In addition, these patients have more advanced mucosal lesions that are also less patchy. Phenotypically, they have a lower body weight and more often present with extraintestinal symptoms compared with patients with lower tTGA levels, who more often have intestinal symptoms.

### Applications

The findings of the current study provide further evidence that patients with high tTGA values are truly a distinct group with more advanced disease. These results therefore support the new European Society for Paediatric Gastroenterology, Hepatology and Nutrition criteria.

### Terminology

tTGA: these antibodies are directed against the enzyme tissue-transglutaminase, which is the auto-antigen in CD. This enzyme plays a key role in eliciting the immune response against gluten. EMA: the endomysium is the intercellular matrix that lies between the smooth muscle cells of the muscularis mucosae throughout the gastrointestinal tract. It is rich in the enzyme tissue-transglutaminase. Antibodies directed against the endomysium are actually directed against tissue-transglutaminase. Human leukocyte antigen (HLA)-DQ2/8: gluten-derived peptides, especially after enzymatic modification by the enzyme tissue-transglutaminase, show a very high affinity for HLA-DQ2/8. In contrast, gluten peptides barely show affinity to other HLA-DQ types. Therefore, having more of these CD-associated heterodimers will result in a stronger T- and B-cell response, while an absence of HLA-DQ2/8 excludes the presence of CD.

### Peer review

The paper by Mubarak *et al* investigated celiac children with high tTGA titres vs low titres. Main findings are a genetic diversity, extra-intestinal pathologies, lower height/weight ratio in high titre group. The paper is interesting and well written.

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## Reference genes for quantitative RT-PCR data in gastric tissues and cell lines

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### Abstract

**AIM:** To evaluate the suitability of reference genes in gastric tissue samples and cell lines.

**METHODS:** The suitability of genes *ACTB*, *B2M*, *GAPDH*, *RPL29*, and *18S rRNA* was assessed in 21 matched pairs of neoplastic and adjacent non-neoplastic gastric tissues from patients with gastric adenocarcinoma, 27 normal gastric tissues from patients without cancer, and 4 cell lines using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). The ranking of the best single and combination of reference genes was determined by NormFinder, geNorm™, BestKeeper, and DataAssist™. In addition, GenEx software was used to determine the optimal number of reference genes. To validate the results, the mRNA expression of a target gene, *DNMT1*, was quantified using the different reference gene combinations suggested by the various software packages for normalization.

**RESULTS:** *ACTB* was the best reference gene for all gastric tissues, cell lines and all gastric tissues plus cell lines. *GAPDH* + *B2M* or *ACTB* + *B2M* was the best combination of reference genes for all the gastric tissues. On the other hand, *ACTB* + *B2M* was the best combination for all the cell lines tested and was also the best combination for analyses involving all the gastric tissues plus cell lines. According to the GenEx software, 2 or 3 genes were the optimal number of references genes for all the gastric tissues. The relative quantification of *DNMT1* showed similar patterns when normalized by each combination of reference genes. The level of expression of *DNMT1* in neoplastic,



adjacent non-neoplastic and normal gastric tissues did not differ when these samples were normalized using *GAPDH* + *B2M* ( $P = 0.32$ ), *ACTB* + *B2M* ( $P = 0.61$ ), or *GAPDH* + *B2M* + *ACTB* ( $P = 0.44$ ).

**CONCLUSION:** *GAPDH* + *B2M* or *ACTB* + *B2M* is the best combination of reference gene for all the gastric tissues, and *ACTB* + *B2M* is the best combination for the cell lines tested.

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**Key words:** Gastric cancer; Reference gene; Normalization; Gene expression; Quantitative real-time polymerase chain reaction

**Core tip:** Gene expression studies have revealed much about the molecular basis of gastric cancer. However, the normalization of expression data using reference genes without validation may undermine the results. In the present study, we evaluated the suitability of possible reference genes in gastric tissues and cell lines. To our knowledge, our study is the first to determine and validate reference genes for gastric samples in a Western population. In addition, the inclusion of normal gastric tissues from patients without cancer in determining the best reference genes is original in the literature.

Wisniewski F, Calcagno DQ, Leal MF, Santos LC, Gigeck CO, Chen ES, Pontes TB, Assumpção PP, Assumpção MB, Demachki S, Burbano RR, Smith MAC. Reference genes for quantitative RT-PCR data in gastric tissues and cell lines. *World J Gastroenterol* 2013; 19(41): 7121-7128 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7121.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7121>

## INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death<sup>[1]</sup>. Although approximately 90% of gastric tumors are adenocarcinomas, the etiology and disease evolution may vary among populations, primary tumor location, histological subtypes of adenocarcinoma, and other variables. Among these factors, ethnicity can determine different levels of susceptibility and aggressiveness of gastric tumors<sup>[2,3]</sup>. An understanding of GC biology is important to identify cancer biomarkers, which may help in early diagnosis and in the development of new targets therapies and, therefore, contribute to reduce mortality or morbidity rates.

Although gene expression studies have revealed much about the molecular basis of GC, the detailed mechanisms remain unclear. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is currently considered the gold standard for accurate, sensitive, and rapid measurements of gene expression<sup>[4-6]</sup>. However, to

obtain reliable data, the gene expression levels must be normalized using two or more reference genes<sup>[7-9]</sup>. Ideally, reference genes should be stable, unregulated, and invariable under the conditions of the experiment<sup>[10,11]</sup>; therefore, a validation experiment for the evaluation of reference gene expression stability for each target tissue and disease is recommended<sup>[12,13]</sup>. To our knowledge, only one previous study has aimed to assess the best single and combination of reference genes for gastric adenocarcinoma and non-neoplastic samples in an East Asian population<sup>[14]</sup>. In contrast, there is no information about the stability of candidate reference genes in gastric samples from other populations.

In this study, we assessed the suitability of 5 possible reference genes in 21 matched pairs of neoplastic and non-neoplastic gastric tissues from patients with gastric adenocarcinoma and 27 normal gastric tissues from patients without cancer. We also included 4 cell lines in the analysis. The stability analysis was performed using 4 freely available software packages.

## MATERIALS AND METHODS

### Cell lines

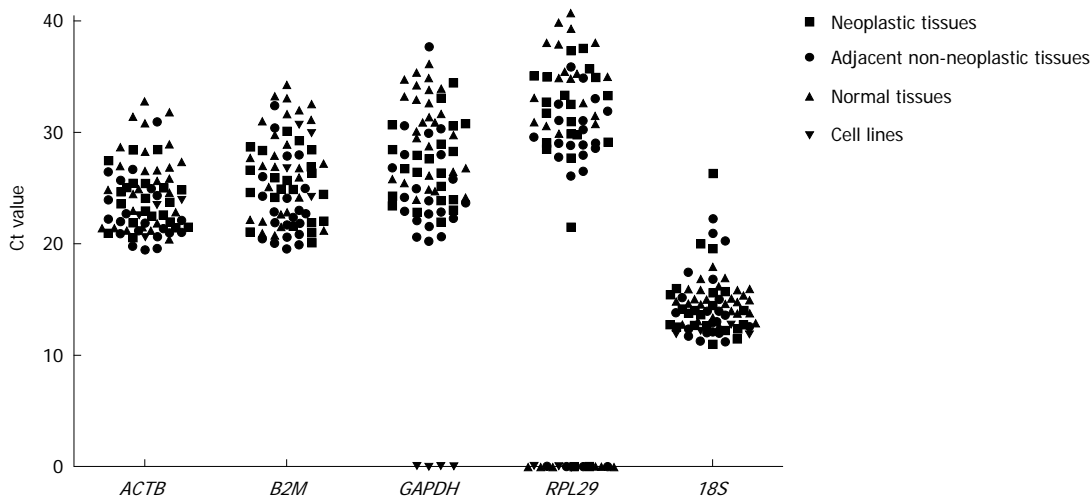
The ACP02 and ACP03 cell lines were established by our research group from primary gastric adenocarcinomas classified as diffuse and intestinal types, respectively<sup>[15]</sup>. The PG100 and MRC-5 cell lines were obtained from Rio de Janeiro Cell Bank, Brazil, and were established from a primary gastric adenocarcinoma and from normal human fibroblasts, respectively. All the cell lines were cultured at 37 °C in RPMI media 1640 (GIBCO®, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO®, Grand Island, NY), and 0.02 mg/mL kanamycin (GIBCO®, Grand Island, NY).

### Patients

Twenty-one matched pairs of neoplastic and adjacent non-neoplastic gastric tissues were obtained from patients with gastric adenocarcinoma who were subjected to gastric resection. Twenty-seven normal gastric tissues were obtained from patients subjected to routine endoscopic examination. Table 1 shows the clinicopathological features of the studied patients. All the gastric tissue samples were obtained from João de Barros Barreto University Hospital (HUIBB) in Pará State, Brazil, and were snap-frozen in liquid nitrogen and stored frozen until use. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery, and there was no other co-occurrence of diagnosed cancers. Written informed consent with approval of the ethics committee of HUIBB was obtained from all patients prior to sample collection.

### RNA extraction and cDNA synthesis

Total RNA was extracted from the cell lines and tissue samples using the AllPrep DNA/RNA/Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer's



**Figure 1** Expression level of five candidate reference genes detected by quantitative real-time polymerase chain reaction. A lower cycle threshold (Ct) value indicates higher gene expression.

**Table 1** Clinicopathological features of the studied patients  
*n* (%)

Clinicopathological feature	Patients with gastric adenocarcinoma	Patients without gastric adenocarcinoma
Age (yr, mean $\pm$ SD)	57 $\pm$ 15.6	49 $\pm$ 14.5
Gender		
Male	15 (71)	12 (44)
Female	6 (29)	15 (56)
Location		
Cardia	2 (9)	0 (0)
Non-cardia	19 (91)	27 (100)
Histopathological type <sup>1</sup>		
Intestinal	16 (76)	NA
Diffuse	5 (24)	NA
Stage <sup>2</sup>		
Early	4 (19)	NA
Advanced	17 (81)	NA
Tumor Invasion		
T1/T2	9 (43)	NA
T3/T4	12 (57)	NA
Lymph node metastasis		
Absent	3 (14)	NA
Present	18 (86)	NA
Distant metastasis		
Unknown/absent	18 (86)	NA
Present	3 (14)	NA

<sup>1</sup>According to the Lauren classification<sup>[29]</sup>; <sup>2</sup>according to AJCC<sup>[30]</sup>. NA: Not applicable.

instructions. The concentration and quality of the extracted RNA were measured using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE), and the integrity was determined by gel electrophoresis. The complementary DNA was synthesized using High-Capacity<sup>®</sup> cDNA Reverse Transcription (Life Technologies, Foster City, CA) following the manufacturer's protocol.

### RT-qPCR

The reaction to detect the expression range of the 5 candidate reference genes was performed in triplicate using TaqMan<sup>®</sup> inventoried Assays-on-Demand probes (Life

Technologies, Foster City, CA) and the Applied Biosystems 7500 fast real-time PCR system. We also quantified the mRNA expression of a target gene, *DNMT1*, using the possible candidate genes for normalization. For this analysis, we evaluated 18 matched pairs of adjacent non-neoplastic and neoplastic gastric tissues from patients with gastric adenocarcinoma and 19 normal gastric tissues from patients without cancer. The analyzed genes, their respective TaqMan<sup>®</sup> assay identification and efficiencies are provided in Table 2. The relative quantification (RQ) of *DNMT1* expression was calculated according to the Livak method<sup>[16]</sup>. A sample from a patient without cancer was designated as a calibrator.

### Analysis of reference gene stability

We categorized the gastric tissues and cell lines into the following groups: (1) neoplastic tissues; (2) adjacent non-neoplastic tissues; (3) matched pairs of adjacent non-neoplastic and neoplastic gastric tissues; (4) normal tissues; (5) all gastric tissues; (6) cell lines; and (7) all gastric tissues plus cell lines. For the stability comparisons of the candidate reference genes, we used the software NormFinder version 20 (<http://www.mdl.dk/publicationsnormfinder.htm>)<sup>[17]</sup>, geNorm<sup>™</sup> (<http://medgen.ugent.be/~jvdesomp/genorm/>)<sup>[7]</sup>, BestKeeper1 (<http://www.gene-quantification.de/bestkeeper.html>)<sup>[18]</sup>, and DataAssist<sup>™</sup> (<http://www.lifetechnologies.com/us/en/home/technical-resources/software-downloads/dataassist-software.html>) according to the recommendations of the authors. The software GenEx (<http://genex.gene-quantification.info/>) was used to determine the optimal number of reference genes by calculating the Accumulated Standard Deviation (Acc.S.D.).

In the analysis using geNorm, the reference genes were ranked according to the expression stability value M (average pair-wise variation of a gene with all other tested candidate reference genes). Using NormFinder, the set of candidate reference genes was ranked accord-

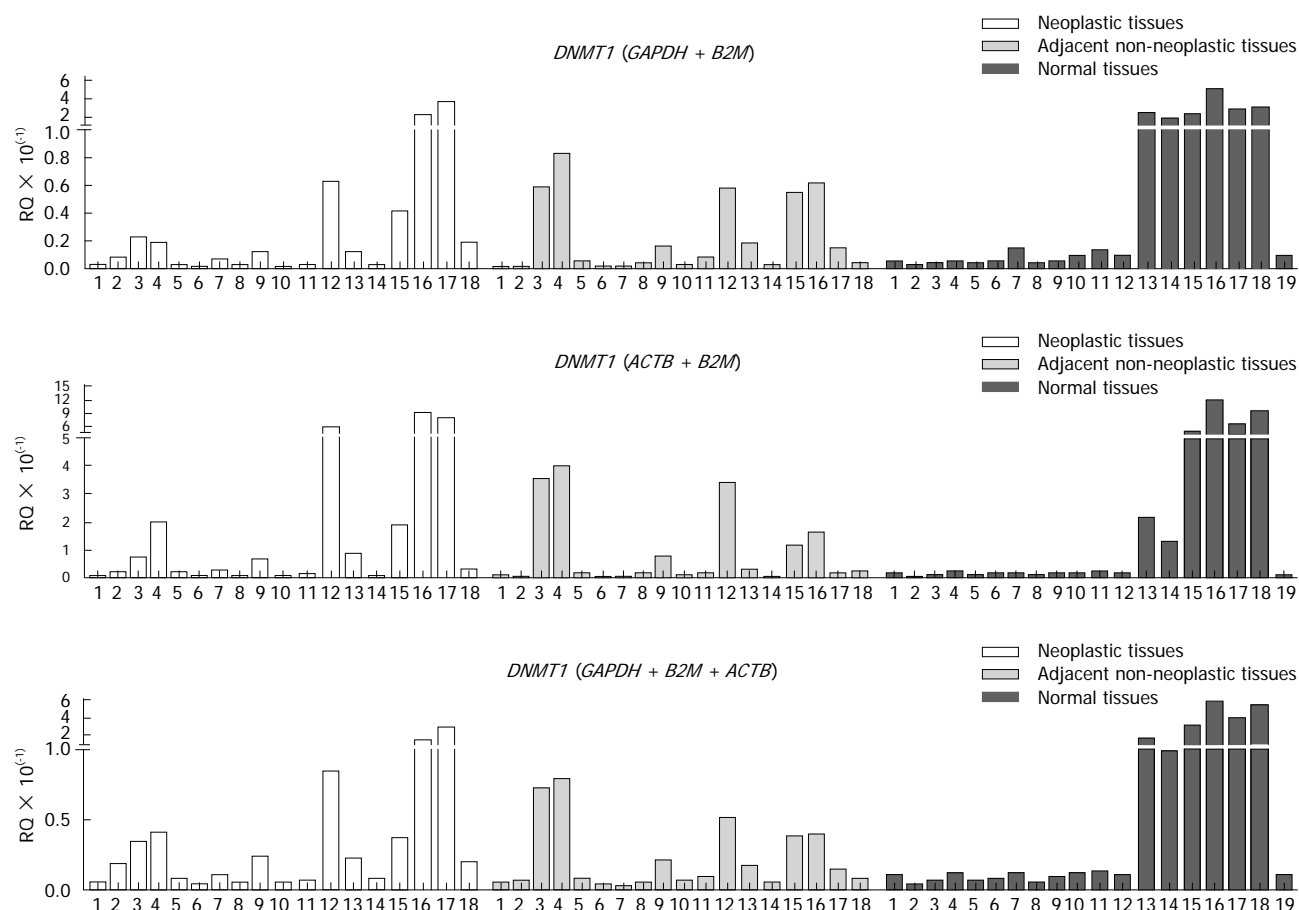


Figure 2 Relative quantification of *DNMT1*, as normalized by *GAPDH + B2M*, *ACTB + B2M*, and *GAPDH + B2M + ACTB* in gastric tissues.

ing to their expression stability (combination of the intra- and intergroup variation). The ranking of the 5 reference genes by Bestkeeper was based on the standard deviation (SD) and coefficient of variance (CV) expressed as a percentage of the cycle threshold (Ct) level. Lastly, DataAssist provides a metric to measure reference gene stability based on the geNorm algorithm. Unlike all the other programs, DataAssist uses RQ to calculate the stability value of individual candidate reference genes. The two genes that showed the highest stability were considered the best combination of reference genes.

## RESULTS

### Expression level of candidate reference genes

The expression levels of 5 candidate reference genes as the Ct value are shown in Figure 1. These genes displayed a wide range of expression levels. *18S rRNA* showed the highest expression level in the gastric tissues and cell lines. In contrast, *RPL29* showed the lowest expression level and did not amplify in 3 samples of neoplastic tissue, 2 samples of adjacent non-neoplastic tissue, and 9 samples of normal tissue. Similarly, *RPL29* and *GAPDH* did not amplify in any of the 4 cell lines studied. Therefore, *RPL29* was excluded from the ensuing analysis, and *GAPDH* was excluded from the set of

candidate reference genes in the cell line analysis.

### Expression stability of candidate reference genes

Table 3 demonstrates the stability value ranking of the single candidate reference genes calculated using the 4 different software packages. Although the various software packages suggested different reference genes, *ACTB* was the gene most cited as the best reference gene in the different gastric tissue categories, followed by *GAPDH* and *B2M*. *ACTB* was also the best reference gene in the cell line and all gastric tissues plus cell line categories.

Table 4 shows the best combination of reference genes suggested by the 4 software packages. Overall, for the different gastric tissue categories, *GAPDH + B2M* were the genes more cited as the best combination of reference gene, followed by *ACTB + B2M* and *GAPDH + ACTB*. *ACTB + B2M* was also the best combination of reference genes suggested for the cell lines and all gastric tissues plus cell line categories.

Although the software indicated up to 2 genes as the best combination of reference genes, we also used GenEx software to determine the optimal number of reference genes. This software revealed that an Acc. S.D. of 0.03 was the lowest when 2 or 3 reference genes were used for both the matched pairs of adjacent non-



**Table 2** Summary of five reference genes and a target gene

Symbol	Gene name (Assay ID <sup>1</sup> )	Location	Description	Efficiency
<i>ACTB</i>	β-Actin (Hs03023943_g1)	7p22	Cytoskeletal structural protein	109%
<i>B2M</i>	β-2-Microglobulin (Hs00984230_m1)	15q21	Beta-chain of major histocompatibility complex class I molecules	104%
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1)	12p13	Oxidoreductase in glycolysis and gluconeogenesis	102%
<i>RPL29</i>	Ribosomal protein L29 (Hs00426490_g1)	3p21	Structural constituent of the ribosome	100%
<i>18S rRNA</i>	18S ribosomal RNA (Hs99999901_s1)	22p12	Ribosome subunit	110%
<i>DNMT1</i>	DNA (cytosine-5-)-methyltransferase 1 (Hs00945875_m1)	19p13	Regulation of tissue-specific patterns of methylated cytosine residues	98%

<sup>1</sup>TaqMan® probes were purchased as Assays-on-Demand Products for Gene Expression (Life Technologies, Foster City, CA).

**Table 3** Ranking of the candidate single reference genes by each software package used

	NormFinder		geNorm™		BestKeeper		DataAssist™	
	Stability value <sup>1</sup>	Ranking	M value <sup>1</sup>	Ranking	Coefficient of variance <sup>1</sup>	Ranking	Score <sup>1</sup>	Ranking
Neoplastic tissues	0.24	<i>ACTB</i>	0.07	<i>GAPDH</i>	9.78	<i>ACTB</i>	1.58	<i>ACTB</i>
	0.73	<i>B2M</i>	0.07	<i>B2M</i>	11.77	<i>B2M</i>	1.70	<i>B2M</i>
	0.99	<i>GAPDH</i>	0.08	<i>ACTB</i>	13.39	<i>GAPDH</i>	1.88	<i>GAPDH</i>
	1.41	<i>18S rRNA</i>	0.12	<i>18S rRNA</i>	17.14	<i>18S rRNA</i>	2.25	<i>18S rRNA</i>
Adjacent non-neoplastic tissues	0.61	<i>B2M</i>	0.05	<i>GAPDH</i>	7.80	<i>ACTB</i>	1.65	<i>B2M</i>
	0.65	<i>GAPDH</i>	0.05	<i>B2M</i>	9.61	<i>B2M</i>	1.77	<i>ACTB</i>
	0.65	<i>ACTB</i>	0.07	<i>ACTB</i>	10.52	<i>GAPDH</i>	1.81	<i>GAPDH</i>
	1.53	<i>18S rRNA</i>	0.13	<i>18S rRNA</i>	16.57	<i>18S rRNA</i>	2.46	<i>18S rRNA</i>
Matched pairs of adjacent non-neoplastic and neoplastic gastric tissues	0.14	<i>ACTB</i>	0.06	<i>GAPDH</i>	9.24	<i>ACTB</i>	1.83	<i>GAPDH</i>
	0.29	<i>B2M</i>	0.06	<i>B2M</i>	11.31	<i>B2M</i>	1.83	<i>ACTB</i>
	0.41	<i>GAPDH</i>	0.07	<i>ACTB</i>	12.52	<i>GAPDH</i>	2.00	<i>B2M</i>
	0.54	<i>18S rRNA</i>	0.13	<i>18S rRNA</i>	16.86	<i>18S rRNA</i>	2.60	<i>18S rRNA</i>
Normal gastric tissues	0.38	<i>GAPDH</i>	0.06	<i>GAPDH</i>	7.38	<i>18S rRNA</i>	1.82	<i>GAPDH</i>
	0.41	<i>ACTB</i>	0.06	<i>ACTB</i>	11.08	<i>GAPDH</i>	2.24	<i>ACTB</i>
	1.07	<i>B2M</i>	0.07	<i>B2M</i>	11.46	<i>ACTB</i>	2.40	<i>B2M</i>
	2.24	<i>18S rRNA</i>	0.11	<i>18S rRNA</i>	13.34	<i>B2M</i>	3.91	<i>18S rRNA</i>
All gastric tissues	0.14	<i>ACTB</i>	0.07	<i>GAPDH</i>	10.89	<i>ACTB</i>	2.04	<i>ACTB</i>
	0.36	<i>B2M</i>	0.07	<i>B2M</i>	13.12	<i>B2M</i>	2.09	<i>B2M</i>
	0.69	<i>GAPDH</i>	0.08	<i>ACTB</i>	13.24	<i>18S rRNA</i>	2.12	<i>GAPDH</i>
	0.96	<i>18S rRNA</i>	0.13	<i>18S rRNA</i>	13.43	<i>GAPDH</i>	3.34	<i>18S rRNA</i>
Cell lines	0.53	<i>ACTB</i>	0.06	<i>ACTB</i>	2.36	<i>18S rRNA</i>	1.71	<i>ACTB</i>
	1.55	<i>B2M</i>	0.06	<i>B2M</i>	4.81	<i>ACTB</i>	2.45	<i>B2M</i>
	1.73	<i>18S rRNA</i>	0.13	<i>18S rRNA</i>	8.60	<i>B2M</i>	2.63	<i>18S rRNA</i>
All gastric tissues + cell lines	0.45	<i>ACTB</i>	0.09	<i>ACTB</i>	10.67	<i>ACTB</i>	2.73	<i>B2M</i>
	1.11	<i>B2M</i>	0.09	<i>B2M</i>	13.02	<i>B2M</i>	3.32	<i>ACTB</i>
	1.19	<i>18S rRNA</i>	0.16	<i>18S rRNA</i>	13.41	<i>18S rRNA</i>	3.38	<i>18S rRNA</i>

<sup>1</sup>A lower value indicates increased stability in gene expression.

neoplastic and neoplastic gastric tissues and all gastric tissues categories.

#### Target gene normalization using different combined reference genes

Because *GAPDH* + *B2M* and *ACTB* + *B2M* or *GAPDH* + *B2M* + *ACTB* were identified as the best combinations of reference genes for gastric tissues, we evalu-

ated the expression of *DNMT1*, as normalized by these combinations of reference genes. The RQ of *DNMT1* normalized by each combination of reference genes showed similar patterns (Figure 2). The level of expression of *DNMT1* in neoplastic, adjacent non-neoplastic and normal gastric tissues did not differ when these samples were normalized using *GAPDH* + *B2M* ( $P = 0.32$ , Kruskal-Wallis test), *ACTB* + *B2M* ( $P = 0.61$ ,

**Table 4** Best reference gene combinations according to each software

Neoplastic tissues	Adjacent non-neoplastic tissues	Normal tissues	Matched pairs of adjacent and neoplastic gastric tissues	nonneoplastic All gastric tissues	Cell lines	All gastric tissues <sup>+</sup> cell lines
<i>ACTB</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>ACTB</i>	<i>ACTB</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>	<i>ACTB</i> + <i>18S rRNA</i>
<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>ACTB</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>
<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>
<i>ACTB</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>GAPDH</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>	<i>ACTB</i> + <i>B2M</i>

Kruskal-Wallis test), or *GAPDH* + *B2M* + *ACTB* ( $P = 0.44$ , Kruskal-Wallis test).

## DISCUSSION

Reference genes have been described for RT-qPCR studies in several diseases and tissues<sup>[19-23]</sup>. However, with regard to gastric adenocarcinoma samples, there is only one previous study that evaluated the best single and combination of reference genes in an East Asian Population<sup>[14]</sup>. Because ethnicity can determine different levels of gene expression, it is important to determine the suitability of reference genes considering the population in addition to the disease type and target tissue. To our knowledge, our study is the first to determine and validate reference genes for gastric samples in a Western Population. The population in Pará State, Brazil, is composed of interethnic crosses between three main groups: European (mainly represented by Portuguese), Africans, and Amerindians<sup>[24]</sup>. In addition, to our knowledge, the study of normal gastric tissue from patients without gastric cancer and its inclusion in determining the best single and combination of reference genes is original in the literature.

The software packages NormFinder, geNorm<sup>TM</sup>, BestKeeper, and DataAssist<sup>TM</sup> are statistical tools that aid in the selection of appropriate reference genes. Although these software packages differed in the suggestion of the best single and combination of reference genes, at least two programs agreed with the results for each group evaluated (Tables 3 and 4), emphasizing the importance of using more than one software to assess the best reference genes among a set of candidate genes. When considering all gastric tissues, our results showed that *ACTB* and *GAPDH* + *B2M* or *ACTB* + *B2M* were the best single and combination of reference genes, respectively. Despite the software packages indicating up to 2 genes as the best combination, the GenEx software revealed that 2 or 3 reference genes were necessary for gene normalization in all gastric tissue. When the expression of the target gene *DNMT1* was evaluated using the 3 different combinations of reference genes for normalization (*GAPDH* + *B2M*, *ACTB* + *B2M*, and *GAPDH* + *B2M* + *ACTB*), no differences in *DNMT1* expression were detected among the neoplastic, adjacent non-neoplastic, and normal tissues. These results validated the combination of reference genes suggested by the software, proving that combinations of 2 genes can be used and that it is not necessary to use 3 or more reference genes for all gastric tissues. *ACTB* and *ACTB* + *B2M*

were the best single and combination of reference genes, respectively, for all the cell lines. Our results showed that *ACTB* + *B2M* was the best option under circumstances that require the use of the same combination of reference genes for all gastric tissues and cell lines. Although the measure of stability for *18S rRNA* was within the range of acceptance when using BestKeeper, it has repeatedly been documented that this is not a good reference gene because the regulation of its synthesis is not representative of mRNA levels<sup>[25-28]</sup>.

Rho *et al.*<sup>[14]</sup> proposed different reference genes for the study of gene expression in gastric tissues and cell lines, suggesting *RPL29* and *RPL29* + *B2M* and *B2M* and *GAPDH* + *B2M* as the best single and combination of reference genes, respectively. Interestingly, the genes suggested by Rho *et al.*<sup>[14]</sup>, *RPL29* and *GAPDH*, did not amplify in our cell lines and in some tissue samples. The different methodologies applied can explain the different results. In the present study, we evaluated gene expression using commercially available TaqMan<sup>®</sup> assays, whereas Rho *et al.*<sup>[14]</sup> evaluated gene expression using SYBR green and primers previously reported in the literature that can detect non-specific reaction products with variable sensitivity. In addition, it should be considered that samples obtained from different ethnicities could contribute to the different results of our group and Rho *et al.*<sup>[14]</sup>.

In conclusion, our suitability analysis suggested *ACTB* and *GAPDH* + *B2M* or *ACTB* + *B2M* as the best single and combination of reference genes for all gastric tissues, with *ACTB* and *ACTB* + *B2M* as the best single and combination of reference genes for all cell lines tested. When circumstances require the use of the same combination of reference genes for all gastric tissues and cell lines, our results showed that *ACTB* + *B2M* was the best option. The use of these genes for RT-qPCR data normalization may enhance the robustness of transcription level determination in gastric samples.

## COMMENTS

### Background

Gastric cancer is the fourth most common cancer worldwide, with high rates of mortality and morbidity. Reverse transcription quantitative polymerase chain reaction is currently considered the gold standard for the accurate, sensitive, and rapid measurement of gene expression. To obtain reliable data, a validation experiment to evaluate the best reference genes for the normalization of gene expression data is recommended for each target tissue and disease.

### Research frontiers

The etiology and disease evolution of gastric adenocarcinomas vary among patients due to several factors. Among them, ethnicity can determine different levels of gastric tumor susceptibility and aggressiveness. The understanding

of gastric cancer biology is important to identify cancer biomarkers, which may help in the early diagnosis and development of new targets therapies and, therefore, contribute to reduce mortality and morbidity rates.

### Innovations and breakthroughs

Only one previous study aimed to evaluate the best reference genes for gastric adenocarcinoma in an East Asian population. To their knowledge, the present study is the first to determine and validate reference genes for gastric samples in a Western population. In addition, the analysis of normal gastric tissue from patients without gastric cancer and its inclusion in determining the best reference genes is original in the literature.

### Applications

The use of the combination of reference genes determined and validated in our study for reverse transcriptional quantitative polymerase chain reaction data normalization may enhance the robustness of transcription level determination in gastric samples.

### Terminology

Reference genes are internal controls used in reverse transcription quantitative polymerase chain reaction analysis to avoid the sample biases related to variability in the total RNA content, RNA stability, and enzymatic efficiency. Ideal reference genes should be stable, unregulated, and invariable under the conditions of the experiment.

### Peer review

The authors evaluated the suitability of five possible reference genes in matched pairs of non-neoplastic and neoplastic gastric tissues from patients with gastric adenocarcinoma and normal gastric tissues from patients without cancer. Four cell lines were also included in this analysis. The stability analysis was performed using four freely available software packages. This study validated *GAPDH* + *B2M* or *ACTB* + *B2M* as the best combination of reference genes for all gastric tissues. In addition, *ACTB* + *B2M* were suggested as the best combination of reference genes for cell lines. When circumstances require the use of the same combination of reference genes for all gastric tissues and cell lines, the *ACTB* + *B2M* combination was found to be the best option.

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## Impact of cirrhosis on surgical outcome after pancreaticoduodenectomy

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### Abstract

**AIM:** To elucidate surgical outcomes of pancreaticoduodenectomy (PD) in patients with liver cirrhosis.

**METHODS:** We studied retrospectively all patients who underwent PD in our centre between January 2002 and December 2011. Group A comprised patients with cirrhotic livers, and Group B comprised patients with non-cirrhotic livers. The cirrhotic patients had Child-Pugh classes A and B (patient's score less than 8). Pre-operative demographic data, intra-operative data and postoperative details were collected. The primary outcome measure was hospital mortality rate. Secondary outcomes analysed included duration of the operation, postoperative hospital stay, postoperative morbidity and survival rate.

**RESULTS:** Only 67/442 patients (15.2%) had cirrhotic livers. Intraoperative blood loss and blood transfusion

were significantly higher in group A ( $P = 0.0001$ ). The mean surgical time in group A was significantly longer than that in group B ( $P = 0.0001$ ). Wound complications ( $P = 0.02$ ), internal haemorrhage ( $P = 0.05$ ), pancreatic fistula ( $P = 0.02$ ) and hospital mortality ( $P = 0.0001$ ) were significantly higher in the cirrhotic patients. Postoperative stay was significantly longer in group A ( $P = 0.03$ ). The median survival was 19 mo in group A and 24 mo in group B. Portal hypertension (PHT) was present in 16/67 cases of cirrhosis (23.9%). The intraoperative blood loss and blood transfusion were significantly higher in patients with PHT ( $P = 0.001$ ). Postoperative morbidity (0.07) and hospital mortality ( $P = 0.007$ ) were higher in cirrhotic patients with PHT.

**CONCLUSION:** Patients with periampullary tumours and well-compensated chronic liver disease should be routinely considered for PD at high volume centres with available expertise to manage liver cirrhosis. PD is associated with an increased risk of postoperative morbidity in patients with liver cirrhosis; therefore, it is only recommended in patients with Child A cirrhosis without portal hypertension.

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**Key words:** Periampullary tumour; Liver cirrhosis; Portal hypertension; Pancreaticoduodenectomy

**Core tip:** Traditionally, cirrhosis has been considered a contraindication to major gastrointestinal surgery. Hospital mortality rates have been reported to be 17.5 % to 38% for cirrhotic patients undergoing gastrointestinal surgery. Pancreaticoduodenectomy is associated with an increased risk of postoperative morbidity in patients with liver cirrhosis; therefore, it is recommended only in patients with Child A cirrhosis. Cirrhotic patients with portal hypertension were associated with poorer

outcome than cirrhotic patients without portal hypertension. Patients with periampullary tumours and well-compensated chronic liver disease should be routinely considered for radical surgery at high volume centres with available expertise to manage liver cirrhosis.

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## INTRODUCTION

Pancreaticoduodenectomy (PD) is a complex procedure; however, it is the only curative procedure for patients with malignant diseases of the pancreas and periampullary region. The procedure is also recommended in some benign pancreatic tumours<sup>[1-3]</sup>. Although PD is performed in many hospitals, its associated morbidity and mortality rates are still high. Recently, the operative mortality rate after PD has dramatically decreased to less than 5%, while the incidence of postoperative complications remains high: from 30% to 60%<sup>[3-7]</sup>. Recent studies have suggested that many factors influence postoperative morbidity and prognosis after PD, including age, sex, preoperative jaundice, operative time, intraoperative blood loss, type of pancreatic reconstruction, anastomotic technique, consistency of pancreatic stump, pancreatic duct diameter, use of somatostatin, mass size, safety margin, lymph node ratio and surgeon experience<sup>[5-9]</sup>.

Cirrhosis represents a common pathological pathway for a wide variety of chronic liver diseases. The progression of liver injury to cirrhosis can occur over weeks to years. Hepatitis C virus (HCV) is the most important cause of liver cirrhosis in Egypt<sup>[10,11]</sup>. Traditionally, cirrhosis has been considered a contraindication to major gastrointestinal surgery. The hospital mortality rates have been reported to be 17.5% to 38% for cirrhotic patients undergoing any gastrointestinal surgery<sup>[12-14]</sup>. Studies from the 1980s reported a mortality rate of approximately 25% and a morbidity rate of 35% for patients with cirrhosis undergoing open cholecystectomy<sup>[15,16]</sup>. Postoperative mortality for cirrhotic patients undergoing cholecystectomy has decreased significantly over the last two decades, partly because of the use of laparoscopy<sup>[17-19]</sup>.

Many studies have observed that patients with cirrhosis tend to have a significant risk of developing postoperative complications after major abdominal operations, frequently leading to their eventual death<sup>[15,16,20]</sup>. Patients with cirrhosis have an increased risk of complications during surgery (bleeding because of portal hypertension and coagulopathy, liver dysfunction, and ascites, which often lead to sepsis), which is related to the severity of

liver disease. It is a challenge to determine which patients are the best candidates for surgery<sup>[20,21]</sup>. Now, major surgical procedures can be safely performed in patients with cirrhosis with intensive preoperative care and with minimised intraoperative blood loss because surgical techniques and medical management have been improved significantly<sup>[16,19,20]</sup>.

The impact of cirrhosis on postoperative morbidity and mortality for gastrointestinal cancer resection has not been well described<sup>[12,22-25]</sup>. The aim of this study was to elucidate surgical outcomes of PD in patients with liver cirrhosis.

## MATERIALS AND METHODS

### Study design

We studied retrospectively all cirrhotic patients who underwent PD for malignant and benign diseases in pancreatic head and periampullary region in our Gastroenterology Surgical Center, Mansoura University, Egypt, from January 2002 to December 2011. The clinical condition of the patients was graded according to the Child-Pugh classification system<sup>[26]</sup>. In our centre, the inclusion criteria for PD included patients with resectable Periampullary tumours with CTP classes A and B (patient's score less than 8), and no other hepatic pathology. Exclusion criteria for surgery included patients with CTP classes B (patient's score > 8), and C, patients with poor liver function, patients with bleeding risky oesophageal varices or gastric varices, and patients with thrombosed portal veins, malnutrition or coagulopathy.

The medical records of patients, including their well-designed pancreatic surgical sheet, were reviewed. Informed consent for the surgical procedures was obtained from each patient. The local ethical committee approved this study.

The patients who underwent PD formed two groups: Group A (PD in patients with cirrhotic livers) and Group B (PD in patients with normal livers).

### Preoperative assessment

Preoperative diagnostic workup for all patients included clinical assessment, laboratory investigations (complete blood count, liver functions, HCV and HBV markers, creatinine, serum amylase and tumour markers, such as CEA and CA19-9) and radiological investigations [abdominal ultrasound, magnetic resonance cholangiopancreatography (MRCP) and abdominal computerised tomography CT].

Preoperative endoscopic retrograde cholangiopancreatography (ERCP) was carried out in selected patients (patients with serum levels of total bilirubin greater than 10 mg/dL or patients with hepatic dysfunction (transaminase: more than threefold the normal *i.e.*, more than 120 IU/mL).

The diagnosis of cirrhosis was proven on ultrasound findings, CT findings and intraoperatively. Liver biopsies were performed in some cases. The presence of preop-



erative portal hypertension was evaluated retrospectively: direct measurement of portal pressure was not performed routinely in this study, and portal hypertension was indirectly defined as oesophageal varices detected by endoscopy, or splenomegaly [major diameter > 12 cm with platelet count less than 100000/mm<sup>3</sup>, according to the Barcelona Clinic Liver Cancer (BCLC) group criteria]<sup>[22-25]</sup>.

### Surgical procedures

PD was carried out under general anaesthesia; hepatotoxic drugs were avoided. Standard Whipple type operation or pylorus preserving PD (PPPD) was performed. All patients underwent standard regional lymphadenectomy, which included resection of nodes within the outlines of the hepatoduodenal ligament, the right side of the superior mesenteric artery and the inferior vena cava. The presence of cirrhosis did not modify the standard procedure; however, we preferred to use suture ligature rather than cauterisation when possible. Pancreatic reconstruction was performed by either pancreaticogastrostomy (PG) or pancreaticojejunostomy (PJ), based on surgeon preference. Biliary drainage was achieved by end-to-side hepaticojejunostomy (retrocolic). However, gastric drainage was achieved by gastrojejunostomy (GJ) (antecolic or retrocolic) (manual or using a stapler) in the standard Whipple operation or duodenojejunostomy in PPPD (end-to-side or end-to-end).

### Postoperative management

All patients were managed in the intensive care unit (ICU) for at least one day before transfer to the ward. All patients received antibiotics intraoperatively and for 4 d postoperatively. Prophylactic sandostatin was given subcutaneously and continued postoperatively for 4 d in risky patients, which included patients with liver cirrhosis. The drain was removed in all enrolled patients if no bile leak, pancreatic leak or pus occurred. Outputs from Ryle tube were recorded daily, and it was removed if the patients passed flatus, had no distension or the daily output was less than 500 mL. The patients resumed oral feeding, started by a fluid diet, followed by a regular diet once the bowel movement restarted and they could tolerate oral feeding.

Liver functions were measured on post-operative day (POD) 1, and POD 6. Abdominal ultrasound was performed routinely for all patients, and repeated if we suspected intraabdominal collection. Ultrasound (US) guided tubal drainage was done if there was abdominal collection.

### Definitions

Postoperative pancreatic fistula was defined as proposed by the International Study Group of Pancreatic Fistula (ISGPF) as any measurable volume of fluid on or after POD 3 with amylase content greater than three times the serum amylase activity, and classified into grades A, B, and C<sup>[7,27,28]</sup>.

Biliary leak was defined as the presence of bile in the

drainage fluid that persisted to POD 4. Delayed gastric emptying was defined as output from a nasogastric tube of greater than 500 mL per day that persisted beyond POD 10, the failure to maintain oral intake by POD 14 or reinsertion of a nasogastric tube<sup>[7,23]</sup>. Postoperative ascites was defined as effusion of more than 400 mL/d through the drain after POD 4.

### Follow up

Patients were followed up after 2 wk, 1 mo, 6 mo, 1 year and then annually. Patients' follow-up was based on medical records, their last hospital visit and personal communication conducted by telephone calls.

### Data collection

Preoperative demographic and clinical data, surgical procedure, intra-operative data, pathologic diagnosis, post-operative course, early and late complications details and survival were collected.

### Statistical analysis

The primary outcome measure was hospital mortality rate. Secondary outcomes analysed included duration of the operation, postoperative hospital stay, postoperative morbidity and survival rate. Statistical analysis of the data in this study was performed using SPSS software, version 17. For continuous variables, descriptive statistics were calculated and were reported as the mean  $\pm$  SD. Categorical variables were described using frequency distributions. Independent sample *t*-test was used to detect differences in the means of continuous variables and  $\chi^2$  test was used in cases with low expected frequencies. Survival was calculated and plots constructed according to the Kaplan-Meier method and life table method. A log-rank test was used for comparison of survival in different subgroups. *P* values < 0.05 were considered to be significant.

## RESULTS

### Characteristics of patients

PD was performed in 442 patients in our Gastroenterology Surgical Center, Mansoura University, Egypt, between January 2002 and December 2011. During this study, only 67 patients (15.2%) had cirrhotic livers [48 (71.6%) men and 19 (28.4%) women, with a mean age  $54.07 \pm 9.43$  years] (group A), and the other 375 patients (84.8%) had non-cirrhotic livers (group B).

In group A, cirrhosis was diagnosed as secondary to hepatitis C in 44 patients (65.7%), hepatitis B in eight patients (11.9%), both hepatitis C and B in five patients (7.5%) and pure bilharzial periportal fibrosis in 10 patients (14.9%, Table 1).

Portal hypertension was present in 16/67 cases (23.9%) and absent in the remaining 51 (76.1%). Of these 16 cases, four patients had Child-Pugh class B (all patients had splenomegaly with platelet count less than 100000/mm<sup>3</sup> and oesophageal varices grade 1) and 12 patients had Child-Pugh class A (all patients had splenomegaly with

**Table 1** Demographic data for cirrhotic patients *n* (%)

Variables	Statistics
Number	67 (15.2)
Age	
Mean (yr)	54.07 ± 9.43
< 60 yr	48 (71.6)
> 60 yr	19 (28.4)
Sex	
Male	52 (77.6)
Female	15 (22.4)
Cause	
HCV	44 (65.7)
HBV	8 (11.9)
Combined	5 (7.5)
Pure periportal fibrosis	10 (14.9)
Child classification	
Child A	63 (94)
Child B (patient's score less than 8)	4 (6)
With portal hypertension	16 (23.9)
Without portal hypertension	51 (76.1)

HCV: Hepatitis C virus; HBV: Hepatitis B virus.

platelet count less than 100000/mm<sup>3</sup>, and two patients had oesophageal varices).

There was no statistical difference between patient groups with regard to age, BMI, Preoperative SGPT, bilirubin, haemoglobin, preoperative drainage or preoperative CA19-9. There was statistical difference between patient groups with regard to gender: more male patients were in cirrhotic group ( $P = 0.006$ ). Preoperative albumin values were significantly lower in group A than in group B ( $P = 0.01$ ). The mean preoperative CEA was significantly higher in group A than in group B ( $67.31 \pm 69.78$  *vs*  $30.97 \pm 62.57$  respectively,  $P = 0.0001$ , Table 2).

PD was decided for cirrhotic patients (Child A or B with patient's score less than 8) for whom surgery could be performed (the tumour was not locally advanced, and they had no distant metastases).

### Operative data

There was no statistical difference between the groups in terms of tumour size, pancreatic texture, pancreatic duct diameter and site of the tumour. The median intraoperative blood loss and blood transfusion were significantly different between the groups. Blood loss was 500 mL (100-2500 mL) in the cirrhotic group *vs* 200 mL (50-2000) in the non-cirrhotic group,  $P = 0.0001$  and blood transfusion was 1 (0-4 units) in the cirrhotic group *vs* 0 (0-4 units) in the non-cirrhotic group,  $P = 0.0001$ . Estimated intraoperative bleeding of more than 500 mL occurred in 36 patients (53.7%) in group A and 142 (37.9 %) patients in group B; the difference between the two groups was statistically significant ( $P = 0.015$ ). Thirty eight patients (58.7%) in group A required a blood transfusion, and 145 patients (38.7%) in group B required a blood transfusion intraoperatively. The mean operative time in group A was  $5.81 \pm 0.87$  h, which was significantly longer than that in group B, which was  $5.1 \pm 0.99$  h ( $P = 0.0001$ ) (Table 3).

**Table 2** Preoperative data *n* (%)

Variables	Cirrhotic group Group A	Non cirrhotic group Group B	<i>P</i> value
Age			
Mean (yr)	54.07 ± 9.43	52.47 ± 10.73	NS
< 60 yr	48 (71.6)	286 (76.3)	NS
> 60 yr	19 (28.4)	89 (23.7)	
Sex			
Male	52 (77.6)	225 (60)	0.006
Female	15 (22.4)	150 (40)	
BMI			
< 25	53 (79.1)	285 (76)	NS
> 25	14 (20.9)	90 (24)	
Preoperative albumin (gm%)	3.69 ± 0.5	3.96 ± 0.48	0.01
Preoperative SGPT (IU/L)	71.68 ± 45.74	77.4 ± 71.29	NS
Preoperative bilirubin (mg%)	6.72 ± 8.01	8.79 ± 8.9	NS
Preoperative HG (gm/dL)	13.26 ± 11.57	13.43 ± 13.05	NS
Preoperative CEA			
Mean (ng/mL)	67.31 ± 69.78	30.97 ± 62.57	0.0001
< 5 ng/mL	14 (20.9)	182 (48.5)	0.0001
> 5 ng/mL	53 (79.1)	193 (51.5)	
Preoperative CA19-9			
Mean (U/mL)	103.89 ± 202.18	100.23 ± 208.85	NS
< 37 U/mL	35 (52.2)	207 (55.2)	NS
> 37 U/mL	32 (47.8)	168 (44.8)	
Preoperative ERCP	42 (62.7)	231 (50.4)	NS

NS: Not significant; BMI: Body mass index; ERCP: Endoscopic retrograde cholangio-pancreatography; SGPT: Alanine transaminase.

### Postoperative data

The mean postoperative stay was significantly longer in group A ( $12.97 \pm 11.2$  d *vs*  $10.71 \pm 7.41$  d,  $P = 0.03$ ). In group A, 70 postoperative complications developed in 31 patients (46.26%). While in group B, 188 postoperative complications occurred in 85 patients (22.66%). Patient morbidity was more frequent in group A than in group B. A statistically significant difference was observed in wound complications ( $P = 0.02$ ), occurrence of internal haemorrhage ( $P = 0.05$ ) and development of a postoperative pancreatic fistula (POPF,  $P = 0.02$ ). POPF occurred in 13 patients (19.4%) in group A and in 37 patients (9.9%) in group B. The POPF was grade C in 11 patients, five of them in cirrhotic group who died from sepsis and six in the non cirrhotic group, three of whom died from sepsis (Table 4).

In group A, two patients developed encephalopathy, seven patients developed postoperative ascites that was treated by diuretics and three patients (4.5%) developed liver cell failure. Twelve patients (17.5%) developed intra-abdominal collection, for whom ultrasound guided tubal drainage was performed. Eight patients (11.5%) required re-exploration, four because of internal haemorrhage, one for bleeding gastrojejunostomy, one for bleeding PG and two for debridement and drainage.

In group B, 40 patients (10.7%) developed intra-abdominal collection and were managed by ultrasound guided tubal drainage. Twenty seven patients (7.2%) required re-exploration, seven because of internal haemorrhage, 12 for bleeding gastrojejunostomy, six for bleeding

**Table 3** Operative data *n* (%)

Variables	Cirrhotic group Group A	Non cirrhotic group Group B	<i>P</i> values
Median blood loss (mL)	500 (100-2500)	200 (50-2000)	0.0001
< 500 mL	31 (46.3)	233 (62.1)	0.01
> 500 mL	36 (53.7)	142 (37.9)	
Blood transfusion			
Median (unite)	1 (0-4)	0 (0-4)	0.0001
Number of patients	38 (56.7)	145 (38.7)	0.006
Tumour size			
Mean (cm)	2.61 ± 1.2	2.82 ± 1.13	NS
< 2 cm	33 (49.3)	144 (38.4)	
> 2 cm	34 (50.7)	231 (61.6)	
Pancreatic texture			
Soft	48 (71.6)	246 (65.6)	NS
Firm	19 (28.4)	129 (34.4)	
Pancreatic duct diameter			
Mean (mm)	4.04 ± 2.96	4.24 ± 2.68	NS
< 3 mm	39 (58.2)	208 (55.5)	NS
> 3 mm	28 (41.8)	195 (44.5)	
Site of tumour			
Ampullary	25 (37.3)	116 (30.9)	
Pancreatic head mass	34 (50.7)	222 (59.2)	NS
Cholangiocarcinoma	2 (3)	13 (3.5)	
Duodenal tumour	6 (9)	24 (6.4)	
Operative time (h)	5.81 ± 0.87	5.1 ± 0.99	0.0001

NS: Not significant.

PG, one for debridement and drainage (one patient) and completion spleno-pancreatectomy was required in one patient who had PF complicated by internal haemorrhage because of erosion of the gastroduodenal artery.

Postoperative complications, including delayed gastric emptying, intra-abdominal collection, biliary leakage, pulmonary complication, bleeding gastrosplenostomy and bleeding PG, were not significantly different between both groups (Table 4).

Cirrhotic patients with portal hypertension were associated with poorer outcome than cirrhotic patients without portal hypertension. The median intraoperative blood loss and blood transfusion were significantly more in cirrhotic patients with portal hypertension than in cirrhotic patients without portal hypertension [blood loss was 1000 mL (200-2500 mL) *vs* 300 mL (100-2500 mL), *P* = 0.001 respectively and blood transfusion was two units (0-4 units) *vs* one unite (0-4 units), *P* = 0.02 respectively]. Twelve (75%) of the cirrhotic patients with portal hypertension required a blood transfusion, while 26 (51%) cirrhotic patients without portal hypertension required a blood transfusion intraoperatively. Patients who had portal hypertension developed 30 postoperative complications in the form of pancreatic leakage in six patients (37.5%), ascites in three patients (18.8%), wound infection in four patients (25%), intra-abdominal collection in four patients (25%), delayed gastric emptying in four patients (25%) and deterioration of liver function in two cases (12.5%, Table 5).

Hospital mortality was significantly higher in group A than in group B [8 (11.9%) *vs* 6 (1.6%), *P* = 0.0001].

**Table 4** Postoperative data *n* (%)

Variables	Cirrhotic group Group A	Non cirrhotic group Group B	<i>P</i> values
Time to resume oral intake (d)	5.98 ± 5.11	6.08 ± 5.71	NS
Drain removal (d)	11.2 ± 9.89	9.41 ± 6.76	NS
Drain amount (mL)	1106.76 ± 1388.91	676.42 ± 1871.36	NS
Complications			
Pancreatic fistula	13 (19.4)	37 (9.9)	0.02
Grade A	5 (7.5)	16 (4.3)	
Grade B	3 (4.5)	15 (4)	NS
Grade C	5 (7.5)	6 (1.6)	
Delayed gastric emptying	10 (14.9)	41 (10.9)	NS
Biliary leakage	4 (6)	19 (5.1)	NS
Wound infection	10 (14.9)	24 (6.4)	0.02
Burst wound	4 (6)	2 (0.5)	0.0001
Internal haemorrhage	4 (6)	7 (1.9)	0.05
Bleeding	1 (1.5)	12 (3.2)	NS
gastrojejunostomy			
Bleeding	1 (1.5)	6 (1.6)	NS
pancreaticogastrostomy			
Abdominal collection	12 (17.9)	40 (10.7)	NS
Encephalopathy	2 (3)	0	0.001
Ascites	7 (10.44)	0	0.0001
Re-exploration	8 (11.9)	27 (7.2)	NS
Postoperative albumin (gm%)	2.81 ± 0.56	2.97 ± 0.43	0.01
Postoperative bilirubin (mg%)	4.54 ± 5.1	3.54 ± 3.89	NS
Postoperative SGPT (IU/L)	113.08 ± 50.1	58.35 ± 82.1	0.0001
Hospital mortality	8 (11.9)	6 (1.6)	0.0001
Liver cell failure	4 (6)	0	
Sepsis	4 (6)	4 (1)	
Pulmonary embolism	0	2 (0.5%)	
Postoperative stay (d)	12.97 ± 11.2	10.71 ± 7.41	0.03
Median survival (mo)	19	24	0.009
1 yr	42	59	
2 yr	13	29	
3 yr	8	19	

NS: Not significant.

Mortality in the cirrhotic group, 6/63 patients died had Child A and 2/4 patients had Child B (Table 4). Hospital mortality in cirrhotic patients with portal hypertension was higher than in cirrhotic patients without hypertension [4/16 (25%) *vs* 4/51 (7.8%), *P* = 0.07, Table 5].

### Long term survival

The median follow up time for this study was 22 mo (range; 1-123 mo).The median survival was 19 mo in group A and 24 mo in group B. The 1, 2, and 3 year survival rates were 42%, 13%, and 3% respectively in group A and 59%, 29%, and 19%, respectively, in group B. There was a statistically significant difference between the two groups (*P* = 0.009, Table 4).

## DISCUSSION

Recently, the operative mortality rate after PD has dramatically decreased to less than 5%, while the incidence



**Table 5 Outcomes of cirrhotic patients *n* (%)**

Variables	Cirrhotic Group	Patients without PHT (51 patients)	Patients with PHT (16 patients)	P values
Age				
Mean (yr)	54.07 ± 9.43	54.66 ± 10.05	52.18 ± 7.04	NS
< 60 yr	48 (71.6)	35 (68.6)	13 (81.3)	NS
> 60 yr	19 (28.4)	16 (31.4)	3 (18.8)	
Sex				
Male	52 (77.6)	40 (78.4)	12 (75)	NS
Female	15 (22.4)	11 (21.6)	4 (25)	
Median blood loss (mL)	500 (100-2500)	300 (100-2500)	1000 (200-2500)	0.001
< 500 mL	31 (46.3)	26 (51)	5 (31.3)	NS
> 500 mL	36 (53.7)	25 (49)	11 (68.8)	
Blood transfusion				
Median (unite)	1 (0-4)	1 (0-4)	2 (0-4)	0.020
Number of patients	38 (56.7)	26 (51)	12 (75)	
Operative time (h)	5.81 ± 0.87	5.8 ± 0.91	5.68 ± 0.72	NS
Hospital stay (d)	12.97 ± 11.2	14.5 ± 10.2	12.49 ± 11.55	NS
Pancreatic fistula	13 (19.4)	7 (13.7)	6 (37.5)	NS
Grade A	5 (7.5)	3 (5.9)	2 (12.5)	NS
Grade B	3 (4.5)	1 (2)	2 (12.5)	
Grade C	5 (7.5)	3 (5.9)	2 (12.5)	
Delayed gastric emptying	10 (14.9)	6 (11.8)	4 (25)	NS
Biliary leakage	4 (6)	3 (5.9)	1 (6.3)	NS
Wound infection	10 (14.9)	6 (11.8)	4 (25)	NS
Burst wound	4 (6)	2 (3.9)	2 (12.5)	NS
Internal haemorrhage	4 (6)	3 (5.9)	1 (6.3)	NS
Bleeding gastrojejunostomy	1 (1.5)	0	1 (6.3)	NS
Bleeding pancreaticogastrostomy	1 (1.5)	0	1 (6.3)	NS
Abdominal collection	12 (17.9)	8 (15.7)	4 (25)	NS
Encephalopathy	2 (3)	1 (2)	1 (6.3)	NS
Ascites	7 (10.44)	4 (7.8)	3 (18.8)	NS
Re-exploration	8 (11.9)	4 (7.8)	4 (25)	NS
Hospital mortality	8 (11.9)	4 (7.8)	4 (25)	NS
Liver cell failure	4 (6)	2 (3.9)	2 (12.5)	
Sepsis	4 (6)	2 (3.9)	2 (12.5)	
Pulmonary embolism	0			
Median survival (mo)	19	21	18	NS
1 yr	42	41	46	
2 yr	13	15	4	
3 yr	8	9	0	

NS: Not significant; PHT: Portal hypertension.

of postoperative complications remains high, from 30% to 60%<sup>[1-4]</sup>. However, PD remains the only curative treatment for periampullary tumours. In the majority of cases, morbidity and mortality after PD are related to surgical management of the pancreatic stump and anatomical feature of the stump<sup>[4-6]</sup>. PD may have a high risk of developing considerable complications, including POPF, intraabdominal bleeding, delayed gastric emptying, or intraabdominal collection<sup>[1-6]</sup>.

The incidence and prevalence of cirrhosis has been increasing in many countries for the past four decades, due to the increase incidence of viral hepatitis, alcoholic intake and non-alcohol related fatty liver disease<sup>[10,11,29,30]</sup>. These individuals are at an increased risk of bleeding, infection, hepatic decompensation, including hepatic coma after a major abdominal operation. Therefore, PD in these patients must be performed in a high volume center<sup>[29-34]</sup>. Indication for cancer treatment in cirrhotic patients has expanded, because surgical techniques and medical management have been improved remarkably. It has been suggested that PD in cirrhotic patient carries a

high risk of morbidity and mortality. Appropriate perioperative evaluation of cirrhotic patients will lead to their safer management<sup>[13,20,31-36]</sup>.

Few studies with small numbers of patients have been published to show the impact of cirrhosis on the surgical outcome after pancreatic resection<sup>[20,31-34,37]</sup>. No evidence-based guidelines were obtained regarding the management of resectable periampullary tumour in cirrhotic patients<sup>[1,35-40]</sup>. Artinyan *et al*<sup>[12]</sup> reported that cirrhosis is a risk factor for postoperative morbidity and mortality after general surgical procedures. However, the impact of cirrhosis on surgical outcome in gastrointestinal malignancies has not been described.

In our study, we decided to perform PD in child A and B patients with periampullary tumours. One way to avoid a high operative mortality in Child B and C is not to operate. The severity of liver disease is the most important factor predicting postoperative surgical outcome. Patients with liver cirrhosis have an inappropriate response to surgical stress secondary to the loss of liver reserve and because of other systemic derangements that

are the result of hepatic dysfunction (such as hemodynamic impairments)<sup>[32]</sup>. A case control study by Warnick *et al*<sup>[31]</sup> compared outcomes in 32 cirrhotic patients (30 Child A and Child B) *vs* matched controls (non cirrhotic) undergoing pancreatic resection surgery, they concluded that the cirrhotic group had a significantly higher rate of complications than the non-cirrhotic group (47% *vs* 22%;  $P = 0.035$ ), and required reoperation (34% *vs* 12%,  $P = 0.039$ ). These patients also had a prolonged hospital stay (27.9 d *vs* 24.3 d), a significantly longer ICU stay (8.6 d *vs* 3.7 d;  $P = 0.033$ ) and required twice as many transfusions. Overall, the hospital mortality was 3 patients, 1 with Child A (3% of all Child A patients) and 2 with Child B cirrhosis. The demanding medical efforts required by these patients demand that they are treated exclusively in high-volume centres<sup>[29]</sup>.

In this study, we found that intraoperative blood loss was significantly higher in cirrhotic patients. Estimated intraoperative bleeding of more than 500 ml occurred in 36 patients (53.7%) in group A and 142 (37.9%) patients in group B, the difference between the two groups was statistically significant ( $P = 0.015$ ). In cirrhotic patients, there are a bleeding tendency and portal hypertension explained the increased intraoperative blood loss. We have overcome this bleeding tendency using vitamin K injection and fresh frozen plasma. We used suture ligation rather than cauterisation when possible. Currently, the ultrasonically activated (Harmonic) scalpel and LigaSure™ have proved to be effective and safe instruments for dissection and haemostasis in both open and laparoscopic surgical procedures<sup>[17,19,38,40]</sup>.

Pancreatic leakage remains the most important cause of morbidity, and also contributes significantly to prolonged hospitalization, increased health care costs and mortality. It remains a challenge at high volume centres for pancreatic surgery<sup>[4,5]</sup>. The incidence of pancreatic anastomotic leakage after PD among different series ranged from 5% to 30%<sup>[2,6]</sup>. Many factors influence PF after PD, including age, sex, preoperative jaundice, operative time, intraoperative blood loss, type of pancreatic reconstruction, anastomotic technique, consistency of pancreatic stump, pancreatic duct diameter, use of somatostatin and surgeon experience<sup>[6-11]</sup>. In our study, POPF occurred in 13 patients (19.4%) in group A and in 37 patients (9.9%) in group B ( $P = 0.02$ ). The POPF was grade C in 11 patients, five in the cirrhotic group who died from sepsis, and six in the non-cirrhotic group, three of whom died from sepsis. This is because the healing power of cirrhotic patients is reduced compared with non-cirrhotic patients.

In this study, two patients developed encephalopathy and seven patients developed postoperative ascites in the cirrhotic group. Encephalopathy may be induced by infection, diuretics, metabolic alkalosis, constipation, hypoxia, sepsis, bleeding and electrolyte imbalance in the perioperative period. Correction of electrolyte imbalance, treatment of infection, branched chain amino acid and restriction of sedatives help to prevent encephalopa-

thy<sup>[32-34]</sup>.

The degree of portal hypertension can be correlated with the severity of cirrhosis, which is estimated by the Child-Pugh score. As a result, an improvement in liver function is associated with decrease in portal hypertension. Cucchetti *et al*<sup>[24]</sup> reported that cirrhotic patients with portal hypertension were associated with poorer outcome. Patients with portal hypertension were often Child-Pugh B and C patients, and when considering only Child-Pugh A class, the results were similar with or without portal hypertension. Some authors concluded that portal hypertension should not be considered as a contraindication for hepatic resection<sup>[24,25]</sup>. In our study, cirrhotic patients with portal hypertension were associated with poorer outcome than cirrhotic patients without portal hypertension. Intraoperative blood loss and blood transfusion were significantly higher in cirrhotic patients with portal hypertension than in cirrhotic patients without portal hypertension. Patients who had portal hypertension developed 30 postoperative complications in the form of pancreatic leakage, ascites, wound infection, intra-abdominal collection, delayed gastric emptying and deterioration of liver function.

The hospital mortality rates after various surgical operations among cirrhotic patients range from 8.3% to 25% (even in well selected cases) compared to 1.1% in non-cirrhotic patients<sup>[34-37]</sup>. Mortality is the consequence of a high rate of postoperative liver cell failure (especially in cases of intra-abdominal surgery) and an increased risk of bacterial infection<sup>[36]</sup>. Warnick *et al*<sup>[31]</sup> reported that, overall, 3 patients died following surgery. In Child A cirrhotic patient, the mortality is, however, comparable to non-cirrhotic patients. Artinyan *et al*<sup>[12]</sup> reported that a query of the National Inpatient Sample Database (2005-2008) identified 106729 patients who underwent resection for GI malignancy; 1479 (1.4%) had cirrhosis. Cirrhotic patients had higher risk of hospital mortality (8.9% *vs* 2.8%,  $P < 0.001$ ) and longer postoperative stay ( $11.5 \pm 0.26$  d *vs*  $10.0 \pm 0.03$  d,  $P < 0.001$ ). Mortality was highest in patients with moderate to severe liver cirrhosis (21.5% *vs* 6.5%,  $P < 0.001$ ). On multivariate analysis, cirrhosis was an independent predictor of hospital mortality. That study also suggested that resection of gastrointestinal malignancy can be performed safely in well-selected cirrhotic patients with mild liver dysfunction. In our study, the hospital mortality was significantly higher in cirrhotic patients than in non-cirrhotic patients [8/67 (11.9%) *vs* 6/375 (1.6%),  $P = 0.0001$ ] and the hospital mortality was higher in Child B patients than Child A. The cause of death in cirrhotic group was liver cell failure in four patients (6%) and sepsis in four patients (6%). Hospital mortality in cirrhotic patients with portal hypertension was higher than in cirrhotic patients without hypertension [4/16 (25%) *vs* 4/51 (7.8%),  $P = 0.07$ ]. In our series, postoperative mortality was low probably because we have experience in pancreatic surgery: in our centre we perform around forty cases per year<sup>[41,42]</sup>.

In this study, the median survival was 19 mo for cir-

rhctic patients, which is comparable to the 15.8 mo reported by Fuks *et al*<sup>[39]</sup> for PD in patients with cirrhosis.

The limitations of this study were the retrospective design and the limited number of cases. Further studies are needed to confirm the impact of cirrhosis and portal hypertension in surgical outcome after PD and to show risk factors.

In a conclusion, PD is associated with an increased risk of postoperative morbidity in patients with liver cirrhosis; therefore, it is recommended only in patients with Child A cirrhosis. Cirrhotic patients with portal hypertension were associated with poorer outcome than cirrhotic patients without portal hypertension. Patients with periampullary tumour and well-compensated chronic liver disease should be routinely considered for radical surgery at high volume centres with available expertise to manage liver cirrhosis.

## COMMENTS

### Background

Indication for cancer treatment in cirrhotic patients has expanded. Patients with periampullary tumours and well-compensated chronic liver disease should be routinely considered for pancreaticoduodenectomy (PD) at high volume centres with available expertise to manage liver cirrhosis. PD is associated with an increased risk of postoperative morbidity in patients with liver cirrhosis; therefore, it is only recommended in patients with Child A cirrhosis without portal hypertension.

### Research frontiers

Intraoperative blood loss and blood transfusion were significantly more in cirrhotic. The mean surgical time in group A was significantly longer than that in group B. Wound complications, internal haemorrhage, pancreatic fistula and hospital mortality were significantly higher in cirrhotic patients. Postoperative stay was significantly longer in cirrhotic. The median survival was in cirrhotic patients after PD. Portal hypertension (PHT) was present in 16/67 cirrhotic patients (23.9%). The intraoperative blood loss and blood transfusion were significantly more in portal hypertension (PHT). Postoperative morbidity and hospital mortality were higher in cirrhotic with PHT.

### Innovations and breakthroughs

Patients with periampullary tumour and well-compensated chronic liver disease should routinely be considered for radical surgery at high volume centres with expertise available to manage liver cirrhosis.

### Applications

PD is associated with an increased risk of postoperative morbidity in patients with liver cirrhosis, and therefore it is recommended only in patients with Child A cirrhosis. Cirrhotic patients with portal hypertension were associated with poorer outcome than cirrhotic patients without portal hypertension. Patients with periampullary tumour and well-compensated chronic liver disease should routinely be considered for radical surgery at high volume centres with expertise available to manage liver cirrhosis.

### Peer review

It is interesting and important to investigate surgical outcomes of PD in patients with liver cirrhosis. PD is recommended only in patients with Child A cirrhosis.

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## Complications and survival in patients undergoing colonic stenting for malignant obstruction

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### Abstract

**AIM:** To investigate whether predicting patients that might be at a higher risk for complications might serve to improve the selection of patients undergoing colonic stenting.

**METHODS:** A retrospective review of consecutive patients who underwent an attempted self-expandable metal stent (SEMS) insertion for malignant colonic obstruction between November 2006 and March 2013. All patients were either referred for preoperative colonic decompression with the intent of a single surgical procedure, or for palliation of the malignant colorectal obstruction for unresectable cancer. Fisher's test or  $\chi^2$  test was performed on categorical variables, and the  $t$  test

for continuous variables. Univariable and multivariable logistic regression were used to examine the association between independent variables and the presence of complications from SEMS insertion.

**RESULTS:** SEMS insertion was attempted in 73 patients. Males comprised 55.71% and the mean age was  $67.41 \pm 12.41$  years. Of these, 65.15% underwent subsequent surgery, while 34.85% received SEMS as palliation for advanced disease. Extracolonic tumors were only 4.76%. The majority of patients had stage IV disease (63.83%), while the remainder had stage III (36.17%). SEMS were successfully inserted in 93.85% (95%CI: 87.85%-99.85%). Perforations occurred in 4.10%, SEMS migration in 8.21%, and stent re-occlusion from ingrowth occurred in 2.74% of patients. The mean duration of follow up for the patients was  $13.52 \pm 17.48$  mo (range 0-73 mo). None of the variables: age, sex, time between the onset of symptoms to SEMS insertion, time between SEMS insertion and surgery, length of the stenosis, location of the stenosis, albumin level, or receiving neoadjuvant chemotherapy, could predict the development of complications from either SEMS insertion nor prolonged survival.

**CONCLUSION:** None of the variables could predict the development of complications or survival. Further studies are required to identify patients who would benefit the most from SEMS.

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**Key words:** Colonic obstruction; Colorectal cancer; Palliative interventions; Self-expanding metal stent; Colonic stents; Enteric stenting; Emergency surgery; Complications; Endoscopy

**Core tip:** Despite the debate as to whether there is an added benefit from the use of self-expandable metal stents (SEMS), when compared to surgery, as an ini-

tial management strategy in patients with malignant colorectal obstruction, this study found that SEMS insertion for malignant colonic obstruction is a safe option with an acceptable risk profile. We could not identify factors that would predict the development of complications or factors that might impact long-term survival. Nonetheless, based on current guidelines, SEMS insertion for malignant colorectal obstruction is the best option for palliation or as a bridge to surgery when technical skills for such a procedure are available.

Almadi MA, Azzam N, Alharbi O, Mohammed AH, Sadaf N, Aljebreen AM. Complications and survival in patients undergoing colonic stenting for malignant obstruction. *World J Gastroenterol* 2013; 19(41): 7138-7145 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i41/7138.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7138>

## INTRODUCTION

The use of self-expandable metal stents (SEMS) has increased in recent years, mostly either as a palliative measure or as a bridge to surgery<sup>[1-4]</sup>. There are risks associated with the use of SEMS, such as perforation<sup>[2-6]</sup>, migration<sup>[2-6]</sup> and reobstruction<sup>[2-4]</sup>, as well as a debate as to whether there is an added benefit from the use of SEMS when compared to surgery as an initial management strategy<sup>[7,8]</sup>, and even possibly a negative effect on survival<sup>[8,9]</sup>. However, there are study design considerations that might account for such results<sup>[8,9]</sup>. As a consequence of the variability in individual study designs as well as the lack of standardization in reporting outcomes, results of numerous meta-analyses conducted on this topic have been variable<sup>[10-16]</sup>. SEMS remain an attractive option due to the avoidance of emergency surgeries, the advantage of undergoing a single operation with the avoidance of stomas<sup>[17,18]</sup>, a lower early morbidity, a shorter hospital stay<sup>[11,17,18]</sup>, and decreased cost<sup>[2]</sup>. Attempting to predict patients that might develop complications and identifying factors that might impact long-term survival from the insertion of SEMS for the management of malignant colorectal obstruction might aid in the better selection of patients who undergo this management strategy and who would benefit the most from SEMS.

## MATERIALS AND METHODS

A retrospective review was conducted using an endoscopic reporting database of individuals seen at a major tertiary care university hospital: King Khalid University Hospital in Riyadh, Saudi Arabia. The medical records of consecutive patients who underwent an attempted SEMS insertion between November 2006 and March 2013 were included. All patients were either referred for preoperative colonic decompression with the intent of a single surgical procedure, or for palliation of the malignant colorectal obstruction of unresectable cancer.

Based on a computerized tomography (CT) scan that was performed for the patients, the stage of the tumor was determined, and the SEMS insertion would be either as a bridge to surgery in patients that were deemed resectable or as a palliative procedure in those who had metastatic disease or were poor surgical candidates. All demographic features were collected through a chart review, which included: age, sex, symptoms, comorbidities, indication for SEMS insertion, date of the procedure, date of subsequent surgery (if performed), location, length of stenosis, stage of the tumor, whether the patient received neo-adjuvant chemotherapy, whether the SEMS insertion was successful (as well as the reason if it did not succeed), length of the SEMS used, number of SEMS used (if more than one SEMS was used for a patient), any complications that occurred after the SEMS insertion, duration between the initial symptoms and the SEMS insertion, and the duration between SEMS insertion and last date of follow up. Patients with any of the following were excluded: clinical evidence of bowel perforation or peritonitis, free intraperitoneal air on abdominal imaging, significant coagulopathy, hemodynamic or pulmonary instability, non-malignant strictures (*e.g.*, those with inflammatory strictures due to diverticulitis), those where the endoscopist found a patent lumen not requiring SEMS insertion, or rectal cancer within 5 cm from the anocutaneous line.

### Endoscopic technique

Before insertion of colonic SEMS, the patients underwent a CT scan of the chest, abdomen, and pelvis to evaluate the location and extent of the tumor, and to assess the area of the stenosis. SEMS were inserted by 1 of 3 therapeutic endoscopists. All endoscopies were performed under fluoroscopic guidance and were inserted through the working channel of the endoscope, which was either a therapeutic gastroscope, a colonoscope, or a duodenoscope depending on the location of the tumor and its angulation compared to the lumen of the colon. All the SEMS used were uncovered (WallFlex colonic stent), 22 mm in diameter, and 60 or 90 mm in length. The length of the stent used was dictated by the judgment of the endoscopist. The majority of the procedures were performed with the patient under conscious sedation, with intravenous midazolam and fentanyl administered by the endoscopist. Cleansing enemas were used until the washing water became clear. No oral bowel preparation was given. The endoscope was carefully inserted to the site of obstruction, then a straight tip guidewire (0.035 in diameter and 450 cm long) was inserted through a triple-lumen 5.5 French ERCP cannula through the stricture, and water soluble contrast was injected to delineate the length of the stricture, as well as the anatomy, and to confirm the intraluminal position of the guidewire. After the guidewire was passed through the stricture, a colonic SEMS assembly was advanced over the guidewire through the working channel and inserted through the obstruction site under combined fluoroscopic and endo-



scopic guidance. The stent was deployed at the stricture site while pulling back the outer sheath. If a SEMS did not expand, no dilation was attempted but a second stent inserted co-axially within the initial SEMS might have been used. If there was clinical suspicion of a complication, a plain abdominal radiograph was performed post-procedure. Stool softeners were routinely prescribed to prevent stool impaction in the stent. A plain abdominal radiograph the day after the procedure was performed, to confirm correct positioning and expansion of the SEMS. Successful SEMS insertion was defined as deployment and expansion of the SEMS across the stricture, radiologic and clinical relief of obstruction, and the ability to defecate. After the SEMS insertion, patients were observed for any procedure-related complications. Patients who had SEMS inserted with a palliative intent or as a bridge to surgery were followed until their last visits or death. The ethics committee of King Khalid University Hospital approved the study.

### Statistical analysis

Descriptive statistics were computed for continuous variables including mean  $\pm$  SD, and minimum and maximum values. Frequencies and inter-quintile ranges were used for categorical variables. Fisher's test or  $\chi^2$  test was performed on categorical variables, and the *t* test for continuous variables. Univariable and multivariable logistic regression were used to examine the association between independent variables and the presence of complications from SEMS insertion. OR and 95%CI were estimated. Cox proportional hazard ratio was used for survival analysis. We used the software STATA 11.2 (StataCorp, TX, United States) in our analysis. A *P* value of  $< 0.05$  was considered statistically significant.

## RESULTS

SEMS insertion was attempted in 73 patients. Males comprised 55.71% of the cohort and the mean age was  $67.41 \pm 12.41$  years (95%CI: 63.50-71.33). Clinical and laboratory values for these patients are summarized in Table 1.

Of these, 65.15% (95%CI: 53.35%-76.95%) underwent subsequent surgery while 34.85% (95%CI: 23.05%-46.65%) received SEMS for palliation for advanced disease or were not surgical candidates. The majority of the tumors were adenocarcinomas of the colon or rectum, while extracolonic tumors were only 4.76% (95%CI: 0.01%-14.70%). The obstruction in the sigmoid colon was found in 69.57%, the rectum and splenic flexure each comprising 8.70%, descending colon 7.25%, transverse colon 4.35%, and ascending colon in 1.45% (Table 1). The mean length of the strictures was  $5.16 \pm 0.32$  cm.

Looking at time trends, there was an increased use of SEMS for malignant colorectal obstruction over the duration of the study (Figure 1). The majority (63.83%) of the patients had stage IV disease (95%CI: 49.57%-78.09%), while the remainder (36.17%) had stage III (95%CI:

**Table 1** Description of the study population

Variable	Mean	95%CI
Age (yr)	67.41	63.50-71.33
Male	55.71%	43.78%-67.64%
Female	44.29%	32.36%-56.22%
Hemoglobin	103	92-114
Platelets	273	238-308
Creatinine	92	76-109
Urea	6.76	4.85-8.67
ALT	37	30-44
AST	37	24-51
ALP	192	106-277
Albumin	30	29-32
Total bilirubin	17	8-26
INR	1.4	1.2-1.6
CEA	90	33-148
Indication		
Palliation of colonic tumors	57.14%	34.06%-80.23%
Complete intestinal obstruction	38.10%	15.44%-60.75%
Extracolonic tumor causing obstruction	4.76%	0.01%-14.70%
Location of the obstruction		
Ascending colon	1.45%	0.01%-4.34%
Transverse colon	4.35%	0.01%-9.28%
Splenic flexure	8.70%	1.88%-15.51%
Descending colon	7.25%	0.97%-13.52%
Sigmoid colon	69.57%	58.44%-80.70%
Rectum	8.70%	1.88%-15.51%
Length of stricture (cm)	5.16	4.52-5.82
Stage of the tumor		
Stage III	36.17%	21.91%-50.43%
Stage IV	63.83%	49.57%-78.09%
Successful SEMS insertion	93.85%	87.85%-99.85%
Failed SEMS insertion	6.15%	0.15%-12.15%
Number of SEMS inserted		
A single SEMS	87.32%	79.39%-95.25%
Two SEMS	12.68%	4.75%-20.61%
Complications		
Perforation	4.10%	0.01%-8.77%
Migration	8.21%	0.02%-14.67%
Stent re-occlusion	2.74%	0.01%-6.57%
Went for surgery	65.15%	53.35%-76.95%
No surgery	34.85%	23.05%-46.65%
Received neoadjuvant chemotherapy	52.38%	29.09%-75.68%
From symptom onset to SEMS insertion	5	3-6
From SEMS insertion to surgery	34	19-49
From SEMS insertion to last follow-up or death (d)		
Full cohort	425	297-554
Patients who had surgery	608	420-796
Patients who had palliative therapy	137	83-191

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio; CEA: Carcino-embryonic antigen; SEMS: Self-expandable metal stents.

21.91%-50.43%). SEMS were successfully inserted in 93.85% (95%CI: 87.85%-99.85%) of patients, while insertion failed in 6.15% (95%CI: 0.15%-12.15%). SEMS technical failure occurred in 4 patients; in 3 the guidewire could not be passed through the stricture, while in the fourth patient the SEMS would not expand. The majority of patients required one SEMS insertion 87.32% (95%CI: 79.39%-95.25%), while two SEMSs inserted in a co-

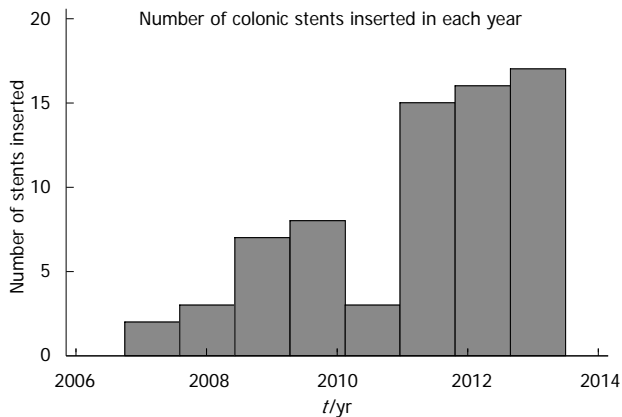


Figure 1 The number of colonic stents inserted for malignant colonic obstruction over the study period.

axial fashion for long strictures were required in 12.68% (95%CI: 4.75%-20.61%). The mean duration from the onset of symptoms to SEMS insertion was 4.7 d, and from SEMS insertion to surgery was 33.8 d. The mean duration of follow up for the patients was  $13.52 \pm 17.48$  mo (range 0-73 mo). Of the cohort of patients included in the study, 52.38% (95%CI: 29.09%-75.95%) received neo-adjuvant chemotherapy in our institution. Perforations occurred in 4.10%, SEMS migration in 8.21% and stent re-occlusion from ingrowth occurred in 2.74% of patients.

#### Predictors of complications from SEMS insertion:

On hypothesis testing, there was no association between any of the measured variables and the development of complications (Table 2). On univariable analysis, none of the following variables predicted the development of complications (perforation, migration, and stent re-occlusion) from SEMS insertion: patient age (OR = 1.02, 95%CI: 0.95-1.10), patient gender (OR = 2.37, 95%CI: 0.69-8.14), time between the onset of symptoms to SEMS insertion (OR = 1.01, 95%CI: 0.99-1.03), time between SEMS insertion and surgery (OR = 1.02, 95%CI: 0.85-1.22), length of the stenosis (OR = 1.12, 95%CI: 0.70-1.80), location of the stenosis (OR = 1.03, 95%CI: 0.97-1.08), albumin level (OR = 0.98, 95%CI: 0.90-1.06), or receiving neoadjuvant chemotherapy (OR = 1.38, 95%CI: 0.39-4.88). Also, on multivariable analysis, none of the variables were associated with the development of complications from SEMS insertion in malignant colorectal obstruction.

#### Predictors of survival

There was a difference in survival between the patients receiving SEMS as a palliative therapy ( $4.1 \pm 3.08$  mo) and those who had SEMS inserted as a bridge to surgery ( $19.4 \pm 0.83$  mo) (Figure 1). We think that this is a function of the stage of the disease; stage III with a mean duration of  $21.88 \pm 5.98$  mo *vs* stage IV with a mean duration of follow-up of  $7.36 \pm 1.93$  mo (Figure 2A). On univariable analysis, the albumin level was associated

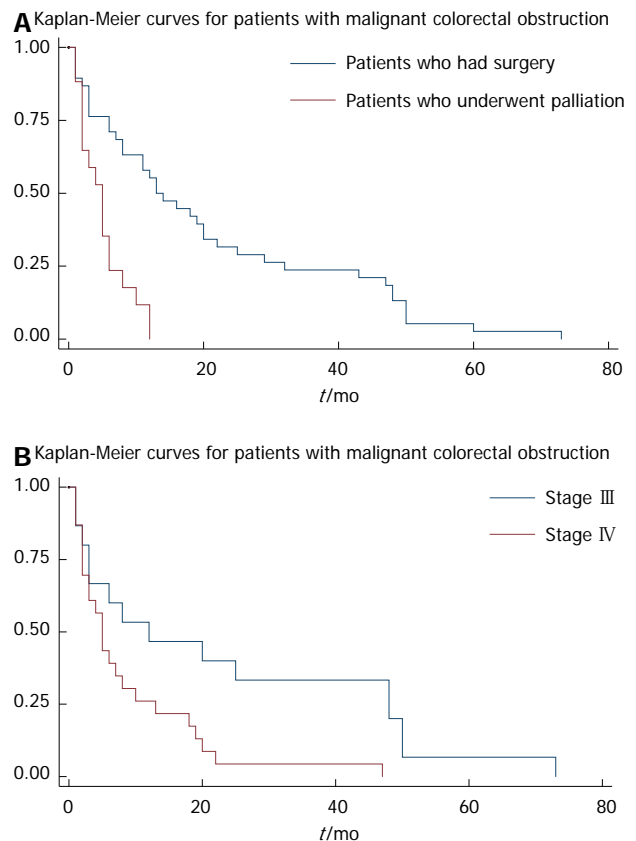


Figure 2 Survival curves. A: Survival curves for patients who received self-expandable metal stents for malignant colorectal obstruction stratified by those who had surgery and those who had palliation; B: Survival curves for patients who received self-expandable metal stents for malignant colorectal obstruction that stratified the stage of their disease.

with a survival advantage ( $P < 0.01$ ). None of the following predicted long term survival: time between the onset of symptoms to SEMS insertion ( $P = 0.91$ ), time between SEMS insertion and surgery ( $P = 0.44$ ), location of the stenosis ( $P = 0.43$ ), length of the stenosis ( $P = 0.95$ ), development of complications from SEMS insertion ( $P = 0.07$ ), carcinoembryonic antigen level ( $P = 0.10$ ), or neoadjuvant chemotherapy ( $P = 0.71$ ). On multivariate analysis, none of the variables were associated with long-term survival.

## DISCUSSION

SEMS are a reasonably safe option for patients with malignant colorectal obstruction<sup>[19]</sup>. The aim for the insertion of SEMS includes decreasing the need for emergency surgeries, reducing the rate of stomas, facilitation of laparoscopic resection when surgery is indicated, decreasing morbidity, shortening the time to chemotherapy, improving the quality of life for patients, and being more cost-effective<sup>[3,15-21]</sup>. Despite these potential and important endpoints and numerous studies addressing the use of SEMS for malignant colorectal obstruction, there still remains considerable controversy concerning their added benefit when compared to surgery as a dominant strategy

**Table 2** Factors associated with the development of complications (perforation, migration, and stent re-occlusion)

Variables	Complication				P value
	Yes		No		
	Mean	95%CI	Mean	95%CI	
Age	70	58.37-81.63	66.97	62.60- 71.34	0.56
Sex					
Male	12.82%	2.00%-23.64%	87.18%	76.36%-98.00%	0.17
Female	25.80%	9.87%-41.74%	74.19%	58.26%-90.13%	
Hemoglobin	106	73-140	102	92-114	0.84
Platelet count	287	176-398	271	234-308	0.38
Creatinine	90	76-104	93	73-113	0.76
Urea	5.44	2.78-8.09	7.04	4.77-9.32	0.83
Albumin	29.31	25.11-33.50	30.49	28.56-32.43	0.59
Alanine aminotransferase	53	17-88	34	30-37	0.28
Aspartate aminotransferase	86	7-165	28	21-34	0.13
Alkaline phosphatase	439	1-930	144	92-196	0.21
Total bilirubin	37	1-88	13	8-17	0.33
International normalized ratio	1.15	1.06-1.24	1.44	1.19-1.68	0.03
Carcinoembryonic antigen	188	0-439	69	19-148	0.32
Length of stenosis (cm)	5.43	4.75-6.11	5.09	4.24-5.93	0.49
Duration between symptoms and stenting (d)	5	1.62-8.38	4.59	2.94-6.24	0.80
Duration between stenting and surgery (d)	51.33	10.96-91.70	28.34	11.76-44.93	0.26
Neo-adjuvant chemotherapy	17.14%	4.22%-30.07%	22.22%	5.92%-38.53%	0.62
Number of stents	1.15	0.93-1.38	1.1	1.03-1.20	0.66

for managing these patients<sup>[5,7,20,22]</sup>. This is mostly due to methodological issues in these studies that are inherited in their design, as well as the possibility of selection bias, being underpowered in detecting differences between study arms, lack of standardized outcomes (as well as definitions), heterogeneity of the patients included, underlying origin of the tumor, stage of the disease, and the use of covered or uncovered SEMS<sup>[20]</sup>.

In a meta-analysis that included 601 patients, of which 38.6% underwent colonic SEMS insertion compared to emergency surgery, the SEMS group had a reduced risk of requiring intensive care with a risk ratio (RR = 0.42, 95%CI: 0.19-0.93), the need for a stoma (RR = 0.70, 95%CI: 0.50-0.99), reduced anastomotic leakage (RR = 0.31 (95%CI: 0.14-0.69), and reduced complications (RR = 0.42, 95%CI: 0.24-0.71)<sup>[15]</sup>. Furthermore, SEMS insertion prior to surgery did not affect the mortality or long-term survival<sup>[15]</sup>. Although encouraging, the meta-analysis by Zhang *et al*<sup>[15]</sup> had considerable heterogeneity, and included various study designs between observational and randomized studies<sup>[20]</sup>. In an editorial by Dayyeh *et al*<sup>[20]</sup>, a meta-analysis was attempted and included only randomized trials<sup>[23-26]</sup>. It demonstrated that there was a decrease in the rate of stomas with the use of SEMS for malignant colorectal obstruction (RR = 0.68, 95%CI: 0.53-0.89), and no difference in complications when compared to emergent surgery (RR = 0.88, 95%CI: 0.66-1.18)<sup>[20]</sup>. The authors correctly pointed out that when examining studies with a high technical success rate in inserting the SEMS, they had a more favorable complication profile when compared to emergent surgery<sup>[20]</sup>, and that possibly the focus should be on the probability of inserting a SEMS in patients with malignant colorectal obstruction, as a determinant for using it as a management strategy<sup>[4,20]</sup>.

The benefits of SEMS for right-sided malignant colorectal obstruction are less than that of distal lesions, as right hemicolectomy with primary anastomosis is possible even in an unprepared colon, although it avoids emergent surgery and possibly permits preoperative medical optimization of patients<sup>[4]</sup>.

Our cohort had a mean age similar to other studies<sup>[1,3,23,27]</sup>, with about half the study population being female, which is higher than in some series<sup>[1,27]</sup>. Also, in other series, 27%-41% of patients who underwent colonic SEMS insertion with a palliative intent had extracolonic tumor origins<sup>[1,27,28]</sup>, while only 4.8% of our series had extracolonic tumors, which is similar to the cohort by Jiménez-Pérez *et al*<sup>[3]</sup>. These salient differences in patient characteristics can explain some of the variation in study results, as patients who had SEMS inserted for malignant colonic obstruction from extraintestinal tumor origins were more likely to be unsuccessful<sup>[6,29]</sup>, but in those receiving SEMS with a palliative intent there was no difference in the SEMS patency and reobstruction rate (21.9% *vs* 30%, *P* = 0.29)<sup>[28]</sup>.

The location of the obstruction was mostly in the sigmoid colon and the majority was on the left side of the colon, this is also in keeping with the literature<sup>[1,3,27,28]</sup>.

The rate of patients with complete obstruction in this cohort (38.1%) was lower than that by Yoon *et al*<sup>[11]</sup> (73%), but a number of series have included patients with incomplete obstruction, defined as a state with narrow stool caliber or the ability to pass only small amounts of liquid stool or gas. We had a lower rate of patients with stage IV (64%) disease when compared to others (92%)<sup>[27]</sup>, although it was still similar to some series<sup>[30]</sup>. One of the concerns with some of the series, and which limits the generalization of their results, is a lack in reporting the stage of the disease; a known independent factor affect-



ing the overall survival of patients.

The success rate for SEMS insertion in our cohort was 93.85%. This is similar to that reported in the literature (83%-100%)<sup>[27,28,31]</sup>. Our study exclusively used uncovered SEMS, since covered SEMS had no added benefit when compared to uncovered SEMS with regards to technical or clinical success. Covered SEMS had a higher proportion of late migration (40% *vs* 0%) and loss of function during the long-term follow-up (60% *vs* 18.8%)<sup>[32]</sup>. A randomized trial also demonstrated that although there was a higher rate of ingrowth when using uncovered SEMS (14.5% *vs* 3.8%), the rate of migration was higher in the covered SEMS group (21.1% *vs* 1.8%), with no difference in the mean patency rate<sup>[33]</sup>. The migration rate in this study was 8.21%; this is in keeping with that reported by others (1%-6%)<sup>[3,27]</sup>, as well as a pooled analysis that found the migration rate to be 11.81%<sup>[34]</sup>. Kim *et al*<sup>[30]</sup> found that covered SEMS and those with a diameter of less than 24 mm had a higher risk of migration. The perforation rate was 4.1%. A pooled analysis of 2287 patients found the perforation rate to be 4.9%, with no statistical difference between the use of stents as a bridge to surgery or palliation<sup>[34]</sup>. The rate of silent perforations could not be assessed in our study, although it was been reported to be as high as 20% in a randomized trial<sup>[23]</sup>.

In our series, none of the patient or tumor characteristics were a predictor for complications from SEMS insertion. This may well be due to the low number of events in this cohort. A study by Kim *et al*<sup>[28]</sup> did not find any predictors for failed SEMS insertion, while in a series of 412 patients, Yoon *et al*<sup>[11]</sup> described predictors for technical and clinical failure in patients who underwent SEMS insertion. Factors associated with technical failure were: right-sided obstruction (OR = 2.25, 95%CI: 1.06-4.75), extrinsic origin of malignancy (OR = 2.57, 95%CI: 1.25-5.32), and the presence of carcinomatosis (OR = 2.83, 95%CI: 1.19-6.75). Factors associated with long term clinical failure, defined as the recurrence of obstructive symptoms requiring re-intervention after initial relief, were when balloon dilatation was used in combination with SEMS insertion (OR = 3.58, 95%CI: 1.25-5.32), while it was decreased when the patient received additional chemotherapy (OR = 0.52, 95%CI: 0.31-0.88)<sup>[11]</sup>.

Since we did not have the exact mortality data, we conducted the survival analysis until the patients' death, when known, or till the last date of follow up. The mean duration of follow up for the complete cohort was 425 d (95%CI: 297-554). As expected, the patients receiving subsequent surgery after SEMS insertion had a longer survival than those who had SEMS inserted with a palliative intent; 608 d *vs* 137 d respectively (Figure 2A). The authors think this is mainly due to the stage of the disease (Figure 2B), and also probably due to unmeasured factors like the functional status of the patients. On univariable analysis, the albumin level was found to be associated with a better survival, but this probably reflected the overall health of the patient. Thus, on mul-

tivariable analysis, this variable was not associated with a survival advantage. Jung *et al*<sup>[35]</sup> found that the location of the tumor affected the mean event-free survival, with distal obstructions being better than proximal lesions; 122.9 ± 18.6 d *vs* 35.8 ± 12.8 d respectively<sup>[35]</sup>. Additionally, SEMS < 10 cm long had a better mean event-free survival when compared to those > 10 cm; 151.0 ± 24.5 d *vs* 59.5 ± 14.4 d respectively<sup>[35]</sup>. The median duration of stent patency in patients with malignant colorectal obstruction was 193 ± 42 - 200 d<sup>[27,28]</sup>. This was not affected by patient demographics<sup>[27]</sup>, site of obstruction<sup>[27]</sup>, or the administration of palliative chemotherapy<sup>[27,28]</sup>.

We also demonstrated through this study that side-viewing duodenoscopes can be used successfully (as was the case in at least three patients in this series) for better visualizing of the tumor and targeting the insertion of a guidewire in areas where the tumor is situated in a tight angle in the distal colon<sup>[4,36]</sup>.

Guidelines on the management of left-sided colonic obstructions state that, in facilities where SEMS insertion is possible, they should be preferred to colostomy, since SEMS have a similar mortality/morbidity rate and a shorter hospital stay (grade of recommendation 2B)<sup>[37]</sup>. The guidelines also suggested considering alternative treatments to SEMS in patients eligible for further bevacizumab-based therapy, due to the potentially increased perforation rates<sup>[37]</sup>. Furthermore, the guidelines state that SEMS should be used as a bridge to elective surgery in referral centers with specific expertise and in selected patients, as their use seems to be associated with a lower mortality rate, a shorter hospital stay, and a lower colostomy rate (grade of recommendation 1B)<sup>[37]</sup>.

In conclusion, none of the variables in our study could predict the occurrence of complications (perforation, migration, and stent re-occlusion) from the insertion of SEMS or long-term survival in cases with malignant colonic obstruction. This may well be due to the size of the cohort in this study. Based on current guidelines<sup>[37]</sup>, as well as in a technical review<sup>[4]</sup>, SEMS insertion for malignant colorectal obstruction is the best option for palliation or as a bridge to surgery when technical skills for such a procedure are available<sup>[4]</sup>.

## COMMENTS

### Background

In patients presenting with malignant colorectal obstruction there is a debate in the literature about the best management strategy that would translate to decreased morbidity, mortality, and cost to the health care system. Despite some randomized controlled trials on the use of self-expandable metal stents (SEMS) or surgery, the answer is not clear due to the variability in the results, as well as the wide variability in the frequency of adverse events from the use of SEMS.

### Research frontiers

SEMS are an attractive management strategy for the management of malignant colorectal obstruction, as it allows for the avoidance of emergency surgeries as providing a single operation without the need for stomas, lower early morbidity, a shorter hospital stay, and decreased costs when compared to emergency surgery. In this study, the authors have demonstrated that the insertion of SEMS in cases with malignant colorectal obstruction is effective with an acceptable risk profile, but could not find any predictors that could determine the development

of complications or survival.

### Innovations and breakthroughs

Despite the conduction of randomized controlled trials with regards to an emergent surgery strategy compared to the insertion of SEMS as a bridge to surgery, there are considerable arguments with regards to the outcomes and the proportion of complications encountered during the insertion of SEMS. This study, although retrospective, replicates that the insertion of SEMS is relatively safe and the rate of complications is less than that reported in some randomized trials.

### Applications

By identifying factors that might predict complications or a survival advantage from one treatment modality compared to another, the authors could individually tailor the best management strategy for patients who present with malignant colorectal obstruction.

### Terminology

SEMS: tubes that are made of a metallic material and are inserted into the colon through the use of endoscopes. These are usually deployed in individuals who have developed a blockage of the colon, most commonly due to malignancy. Migration: when the stent has moved from its intended position to an area either before or after the area of obstruction. Ingrowth: when the tumor tissue extends through the mesh network of the stent and causes occlusion of the stent.

### Peer review

This is a well-written manuscript on an important topic. The authors aimed to predict complications after stent placement for colonic obstruction either in a palliative or a curative attempt. The purpose of the study is interesting and could help in selecting patients who present with malignant colorectal obstruction and would be good candidates for the insertion of SEMS.

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## Efficacy of a novel auto-fluorescence imaging system with computer-assisted color analysis for assessment of colorectal lesions

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### Abstract

**AIM:** To evaluate the efficacy of computer-assisted color analysis of colorectal lesions using a novel auto-fluorescence imaging (AFI) system to distinguish neoplastic lesions from non-neoplastic lesions and to predict the depth of invasion.

**METHODS:** From January 2013 to April 2013, consecutive patients with known polyps greater than 5 mm in size who were scheduled to undergo endoscopic treatment at The Jikei University Hospital were prospectively recruited for this study. All lesions were evaluated using a novel AFI system, and color-tone sampling was performed in a region of interest determined from narrow band imaging or from chromoendoscopy findings without magnification. The green/red (G/R) ratio for each lesion on the AFI images was calculated automatically using a computer-assisted color analysis system that permits real-time color analysis during endoscopic procedures.

**RESULTS:** A total of 88 patients with 163 lesions were enrolled in this study. There were significant differences in the G/R ratios of hyperplastic polyps (non-neoplastic lesions), adenoma/intramucosal cancer/submucosal (SM) superficial cancer, and SM deep cancer ( $P < 0.0001$ ). The mean  $\pm$  SD G/R ratios were  $0.984 \pm 0.118$  in hyperplastic polyps and  $0.827 \pm 0.081$  in neoplastic lesions. The G/R ratios of hyperplastic polyps were significantly higher than those of neoplastic lesions ( $P < 0.001$ ). When a G/R ratio cut-off value of  $> 0.89$  was applied to determine non-neoplastic lesions, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were 83.9%, 82.6%, 53.1%, 95.6% and 82.8%, respectively. For neoplastic lesions, the mean G/R ratio was  $0.834 \pm 0.080$  in adenoma/intramucosal cancer/SM superficial cancer and  $0.746 \pm 0.045$  in SM deep cancer. The G/R ratio of adenoma/intramucosal cancer/SM superficial cancer was significantly higher than that of SM deep cancer ( $P < 0.01$ ). When a G/R ratio cut-off value of  $< 0.77$  was applied to distinguish SM deep cancers, the sensitivity, specificity, PPV, NPV, and accuracy were 80.0%, 84.4%, 29.6%, 98.1% and 84.1%, respectively.

**CONCLUSION:** The novel AFI system with color analysis was effective in distinguishing non-neoplastic lesions from neoplastic lesions and might allow determination of the depth of invasion.

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**Key words:** Autofluorescence imaging; Computer-aided diagnosis; Colon; Neoplasia; Colonoscopy

**Core tip:** Recently, a novel auto-fluorescence imaging (AFI) system with has higher resolution and higher flame rate than those offered by previously used AFI systems has become commercially available in Japan. We evaluated the efficacy of computer-assisted color

analysis using the novel AFI system for distinguishing colorectal neoplasia and non-neoplasia and for predicting depth of invasion. The green/red ratios, which were obtained by dividing green color intensity by red color intensity, were significantly different between hyperplastic polyps, adenoma/intramucosal cancer/SM superficial cancer, and SM deep cancer. The novel AFI system was effective in distinguishing non-neoplastic lesions from neoplastic lesions and might have potential to predict the depth of invasion.

Inomata H, Tamai N, Aihara H, Sumiyama K, Saito S, Kato T, Tajiri H. Efficacy of a novel auto-fluorescence imaging system with computer-assisted color analysis for assessment of colorectal lesions. *World J Gastroenterol* 2013; 19(41): 7146-7153 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7146.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7146>

## INTRODUCTION

Colonoscopy is the most effective method for the detection of colonic neoplastic lesions in the earlier and more curable stages. According to the National Polyp Study, colorectal cancer incidence was decreased by the endoscopic removal of polyps detected during colonoscopy screenings<sup>[1]</sup>.

Accurate endoscopic determination of the histology of colorectal polyps could prevent unnecessary polypectomies and may allow the proposal of adequate surveillance recommendations. The strategy of “resect and discard” for diminutive colorectal polyps has been suggested by The American Society for Gastrointestinal Endoscopy<sup>[2]</sup>. However, a report evaluating real-time optical biopsy analysis of polyps with narrow band imaging (NBI) concluded that only 25% of community-based gastroenterologists assessed polyps with > 90% accuracy<sup>[3]</sup>. Therefore, easy and objective methods for distinguishing colorectal neoplastic lesions from non-neoplastic lesions are required.

We previously reported the effectiveness of auto-fluorescence imaging (AFI) systems in distinguishing colorectal non-neoplastic lesions from neoplastic lesions<sup>[4,5]</sup>; however, in that study, the number of lesions examined was limited.

In an AFI system, white light is separated into excitation light and green light and used to irradiate onto the colon mucosa. After blocking the reflected light, a false color image is displayed on the monitor based on the balance of autofluorescence intensity and green light intensity. Recently, a novel AFI system composed of light sources (EVIS LUCERA ELITE CLV-290SL; Olympus Medical Systems, Tokyo, Japan) and video processors (EVIS LUCERA ELITE CV-290; Olympus Medical Systems, Tokyo, Japan) was developed. The newer AFI systems feature two major improvements over previously available AFI systems (EVIS LUCERA CLV-260SL, EVIS LUCERA CV-260S; Olympus Medical Systems, Tokyo, Japan). The first improvement is a brighter lamp

that allows a higher frame rate, although the overall brightness of the endoscopic images is the same as that in first-generation AFI systems. The use of a brighter lamp produces less flickering and color splitting in the endoscopic images. The second major improvement is in the image-processing algorithm used, specifically the noise-reduction algorithm, which results in higher resolution images with less noise interference (Figure 1). In addition, software that enables real-time color analysis has been developed and is available.

The aim of this study was to prospectively evaluate the efficacy of computer-assisted color analysis of colorectal lesions by using the novel AFI system to attempt to distinguish non-neoplastic lesions from neoplastic lesions. In addition, if a colorectal neoplasm invades the submucosal layer, which contains where autofluorescent substances such as, collagen, nicotinamide adenine dinucleotide hydrate (NADH), flavin adenine dinucleotide, lysosome granules, and porphyrin<sup>[6-8]</sup>, autofluorescence would be expected to be attenuated. Therefore, we hypothesized that the AFI system would be useful in determining the depth of invasion of colorectal neoplasms. To verify our hypothesis, we evaluated the effectiveness of the novel system in permitting diagnosis of the depth of invasion of colorectal neoplasia.

## MATERIALS AND METHODS

This study was approved by the ethical committee of our institution [registration number 24-188(6954)] and was registered in the University Hospital Medical Information Network (UMIN) in Japan under registration number 24-188(6954): UMIN R000011404. Written informed consent for examination and treatment was obtained from all patients prior to the procedures. From January 2013 to April 2013, consecutive patients with known polyps over 5 mm in size who were scheduled to undergo endoscopic treatment at The Jikei University Hospital were prospectively recruited for this study.

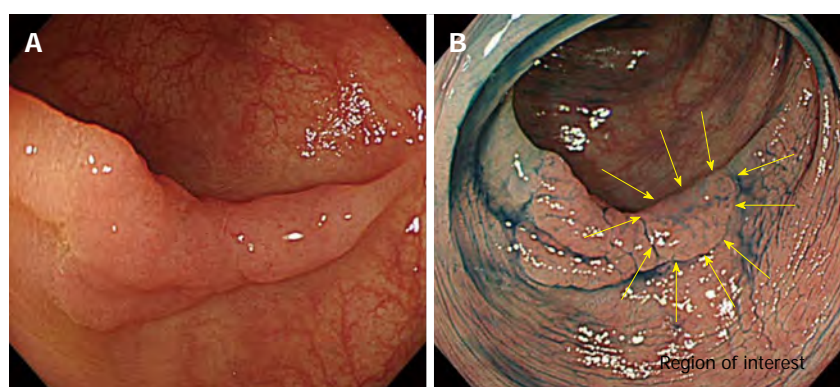
The exclusion criteria were refusal to participate, ages less than 19 or more than 91 years, and the presence of inflammatory bowel disease (IBD), familial adenomatous polyposis, or serrated sessile adenoma/polyp (SSA/P) lesions confirmed by pathological findings after endoscopic resection. Patients with SSA/P lesions were excluded because the histological diagnostic criteria for SSA/P are controversial in Japan at present<sup>[9]</sup>.

### Endoscopic procedure

All patients received total bowel cleansing with polyethylene glycol solutions (Niflec; Ajinomoto Pharmaceuticals Co. Ltd., Tokyo, Japan) after 12 h of nil per os (NPO) status. All colonoscopies were performed using the novel AFI system with a colonoscope designed for AFI observation (CF-FH260AZI; Olympus Medical Systems, Tokyo, Japan) by 5 endoscopists, each of whom possesses more than 5 years of experience in colonoscopy and treats more than 500 cases a year.



**Figure 1** Comparisons of images obtained with the previous autofluorescence imaging system and with the novel autofluorescence imaging system. The major improvement in the novel autofluorescence imaging (AFI) system is in the image-processing algorithm, specifically the noise-reduction algorithm, which results in higher resolution images with less noise interference. An AFI image from the previous AFI system (A) is clearer than an image from the novel AFI system (B).



**Figure 2** A determination of region of interest. A: A white light image of a flat-elevated lesion located in the sigmoid colon; B: A depressed area observed on chromoendoscopy (the area surrounded by yellow arrows). The region of interest for color tone analysis was chosen by the endoscopist based on the findings of non-magnifying narrow-band imaging or chromoendoscopy.

All endoscopic procedures were performed under conditions of CO<sub>2</sub> insufflation<sup>[10]</sup>. First, colonoscopy was performed using the white light imaging (WLI) function of the AFI system. When colorectal lesions were detected, they were observed by switching to the NBI mode using a button on the control head of the endoscope. Subsequently, the lesions were observed by chromoendoscopy (CE) using 0.4% indigo carmine and the WLI mode. The region of interest (ROI) was then determined from the NBI findings without magnification or CE. We determined the placement of the ROI in regions where characteristic endoscopic findings for SM deep invasion, such as expansion appearance, a large nodule equal to or greater than 10 mm in size, redness, or demarcated depressed areas<sup>[11,12]</sup> were found, we determined the placement of the ROI based on the individual characteristics of the lesion (Figure 2). In lesions without these findings, the ROI was placed at the center of the lesion. After washing the indigo carmine with water insufflation, real-time color analysis of the ROI on the AFI images was conducted using a personal computer with software for color analysis connected to the endoscopy system. On

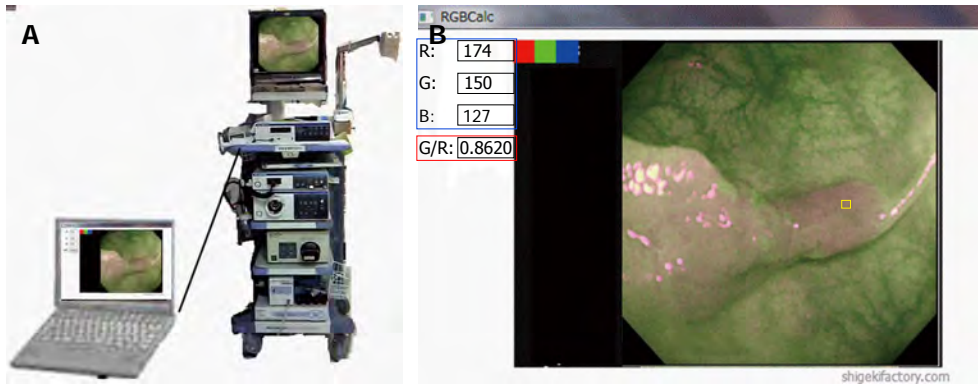
the computer monitor, the ROI was placed using a cursor (10 × 10 pixels). The green/red (G/R) ratio, which is obtained by dividing the green color tone intensity by the red color tone intensity, was calculated automatically during the endoscopic procedures (Figure 3). Finally, magnifying endoscopy with NBI and CE using crystal violet was performed to estimate the depth of invasion of the encountered lesion<sup>[13]</sup>.

If the estimated depth of invasion was limited to the mucosa or superficial SM (less than 1000 μm from the muscularis mucosae), the lesions were endoscopically removed. Lesions diagnosed as SM deep cancer (1000 μm or more from the muscularis mucosae) from magnified endoscopic findings were surgically resected.

### Histological assessment

All tissue specimens were fixed in 10% buffered formalin and cut into 2-mm slices. The specimens were then examined microscopically to determine depth of invasion and histological type. Histological diagnoses were based on the Japanese classification system for cancer of the colon and rectum<sup>[14]</sup> and on the Vienna classification system<sup>[15]</sup>.





**Figure 3** A method for real-time color intensity analysis calculated software. A: A personal computer with software that enables real-time color tone analysis was connected to the endoscopy system. B: The user interface of the personal computer for color tone analysis. The region of interest was chosen using the cursor (yellow square). The color tone intensity (blue square) and the green/red ratio (red square), which is obtained by dividing the green color tone intensity by the red color tone intensity, were calculated automatically.

The final pathological assessments were conducted by a pathologist who was blinded to this study and to the results of the color analyses.

### Endoscopic diagnosis

The endoscopist assessed whether the lesions were neoplastic or non-neoplastic and determined the depth of invasion (SM deep or not) on the basis of the findings of magnified observation using NBI and CE. After completion the color analysis, the diagnoses were related to the study coordinator by the endoscopist.

### Statistical analysis

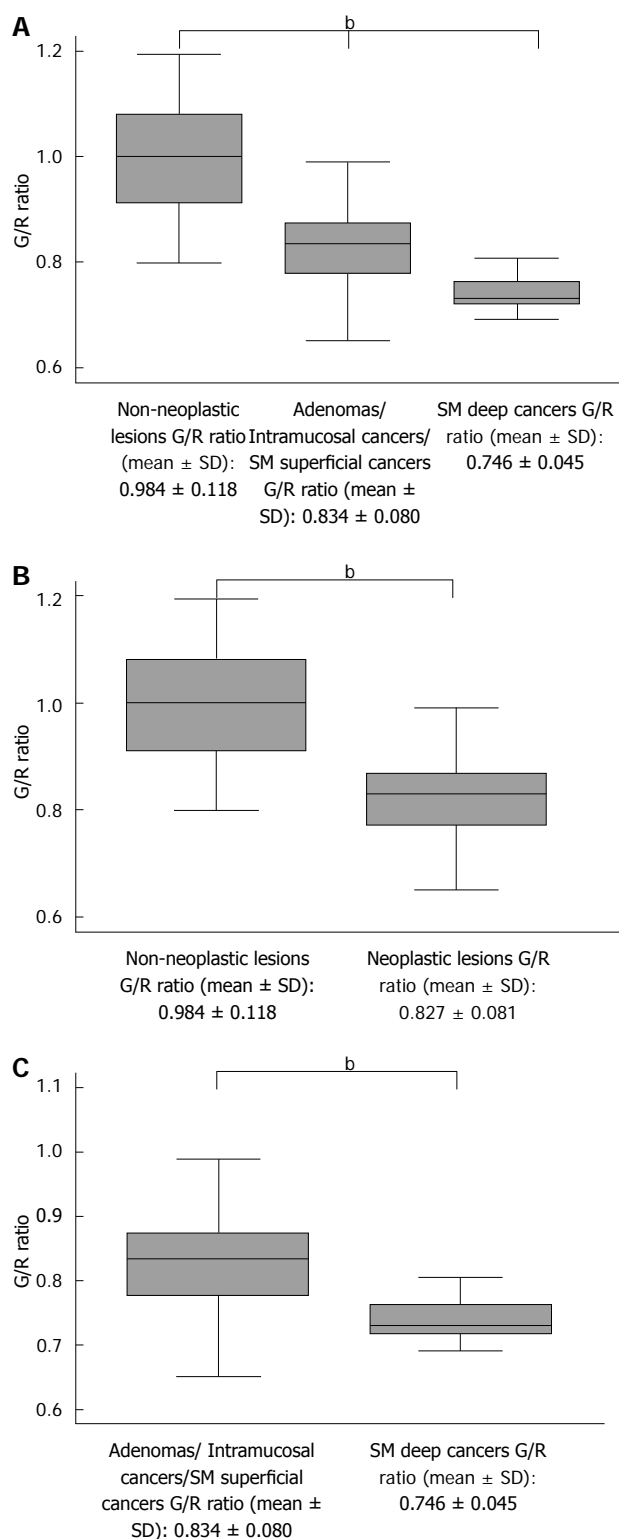
Statistical analysis was performed using SPSS for Windows (SPSS, release 6.0, 1993; SPSS Inc., Chicago, Illinois, United States). Data are expressed as the mean  $\pm$  SD. To determine differences in the mean G/R ratio, comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by multiple comparison testing using the Bonferroni-Dunn method. Continuous variables were analyzed using a *t* test. A *P* value  $< 0.05$  was considered statistically significant.

## RESULTS

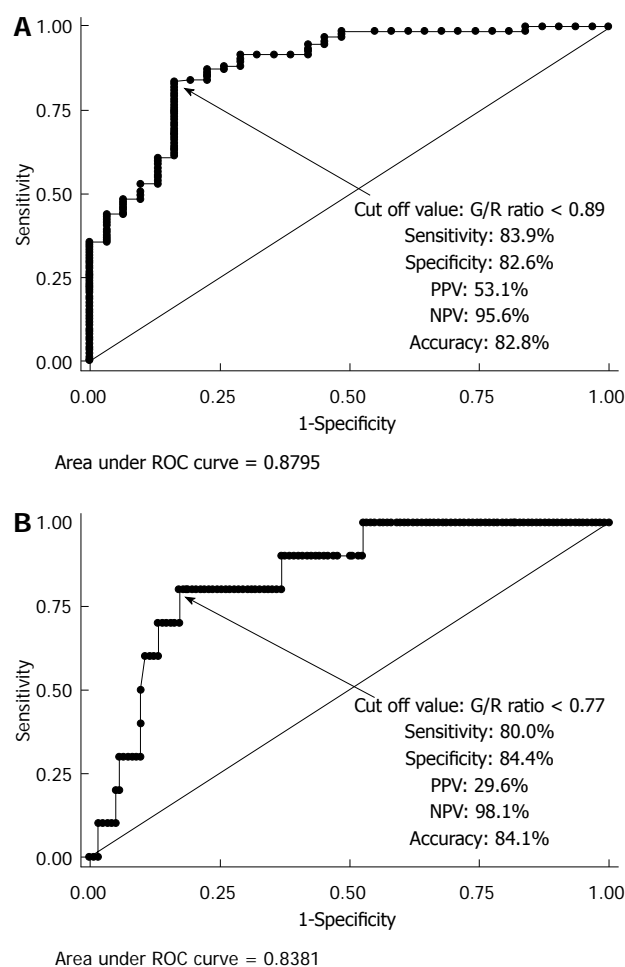
A total of 191 patients were recruited for this study. The following patients were excluded from this study based on the exclusion criteria: 89 patients who refused to participate, 9 patients who had SSA/P lesions, and 5 patients diagnosed with IBD. Finally, 88 patients with 163 lesions were enrolled in this study. The mean patient age was  $63.0 \pm 10.4$  years, the male-to-female ratio was 1:0.51, and the mean lesion size was  $15.0 \pm 12.6$  mm. Histologically, of the 163 lesions, there were 31 hyperplastic polyps (19.0%), 82 tubular/tubulovillous adenomas (50.3%), 40 mucosal or SM superficial cancers (24.5%), and 10 SM deep cancers (6.1%). Macroscopic types included 72 protruded (44.2%) and 91 flat elevated or depressed (55.8%) lesions. Tumor locations included 13 in the cecum (8.0%), 63 in the right colon (38.7%), 64 in the left colon (39.3%), and 23 in the rectum (14.1%). There

were significant differences between the G/R ratios of the hyperplastic polyps (non-neoplastic lesions), adenoma/intramucosal cancer/SM superficial cancer, and SM deep cancer ( $P < 0.0001$ ) (Figure 4A). The mean G/R ratios were  $0.984 \pm 0.118$  in hyperplastic polyps and  $0.827 \pm 0.081$  in neoplastic lesions. The G/R ratios of hyperplastic polyps were significantly higher than those of neoplastic lesions ( $P < 0.001$ ) (Figure 4B), and the area under the receiver operating characteristic (ROC) curve sensitivity and 1-specificity of the G/R ratio in discriminating between hyperplastic polyps and neoplastic lesions was 0.8795. When a G/R ratio cut-off value of  $> 0.89$  was applied to determine non-neoplastic lesions, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were 83.9%, 82.6%, 53.1%, 95.6%, and 82.8%, respectively (Figure 5A). Regarding the use of endoscopic diagnosis by experienced endoscopists to distinguish neoplastic lesions from non-neoplastic lesions using magnifying endoscopy with NBI and/or CE, the sensitivity, specificity, PPV, NPV, and accuracy were 93.5%, 97.0%, 87.9%, 98.5% and 96.3%, respectively. The accuracy of color analysis for distinguishing neoplastic lesions from non-neoplastic lesions was 90.1% for flat/depressed types and 70.8% for protruded types. The diagnostic accuracy for the flat/depressed type was significantly higher than that for the protruded type (Table 1).

For neoplastic lesions, the mean G/R ratio was  $0.834 \pm 0.080$  in adenoma/intramucosal cancer/SM superficial cancer and  $0.746 \pm 0.045$  in SM deep cancer (Figure 4C). The G/R ratio of adenoma/intramucosal cancer/SM superficial cancer was significantly higher than that of SM deep cancer ( $P < 0.01$ ), and the area under the ROC curve sensitivity and 1-specificity of the G/R ratio in discriminating between adenoma/intramucosal cancer/SM superficial cancer and SM deep cancer was 0.8381. When a G/R ratio cut-off value of  $< 0.77$  was applied to distinguish SM deep cancers, the sensitivity, specificity, PPV, NPV, and accuracy were 80.0%, 84.4%, 29.6%, 98.1% and 84.1%, respectively (Figure 5B). Regarding the endoscopic diagnosis of depth of invasion by experi-



**Figure 4** Distribution of green/red ratios. A: Between non-neoplastic lesions, adenoma/intramucosal cancer/submucosal (SM) superficial cancer, and SM deep cancer, there was a significant difference in the green/red (G/R) ratios of non-neoplastic lesions, adenomas/intramucosal cancer/SM superficial cancer, and SM deep cancer ( $^bP < 0.01$ ); B: Between non-neoplastic lesions and neoplastic lesions, There was a significant difference between non-neoplastic and neoplastic lesions ( $^bP < 0.01$ ); C: Between adenoma/intramucosal cancer/SM superficial cancer, and SM deep cancer, there was a significant difference between the two groups ( $^bP < 0.01$ ). SM superficial cancer: Submucosal invasion was defined as less than 1000  $\mu$ m from the muscularis mucosae; SM deep cancer: Submucosal invasion was defined as 1000  $\mu$ m or more from the muscularis mucosae.



**Figure 5** Receiver operating characteristic curve. A: For discriminating between hyperplastic polyps and neoplastic lesions, the area under the receiver operating characteristic curve (AUC) was 0.8795; B: For discriminating between adenoma/intramucosal cancer/SM superficial cancer and SM deep cancer, the area under AUC was 0.8381. G/R: Green/red.

enced endoscopists using magnifying endoscopy for SM deep cancers, the sensitivity, specificity, PPV, NPV, and accuracy were 60.0%, 100%, 100%, 96.8%, and 84.1%, respectively (Table 2).

## DISCUSSION

The NBI system, which enables precise observation of microvasculature and surface strictures of colorectal lesions<sup>[16-18]</sup>, is considered an effective method for distinguishing non-neoplastic lesions from neoplastic lesions, and an international expert group has proposed the NBI International Colorectal Endoscopic (NICE) classification to distinguish adenomas from hyperplastic polyps<sup>[19]</sup>. However, because diagnosis of colorectal neoplasia using the NBI system is based on the subjective judgment of the endoscopist, the accuracy of diagnosis differs between expert endoscopists and community-based gastroenterologists<sup>[3]</sup>. Therefore, a method that permits objective and real-time diagnosis with respect to for distinguishing colorectal non-neoplastic lesions from neoplastic lesions is needed.

**Table 1 Diagnostic value of colorectal lesions (for distinguishing neoplastic lesions from non-neoplastic lesions)**

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
Diagnosis based on the color analysis	83.9%	82.6%	53.1%	95.6%	82.8%
Flat or depressed type	93.1%	79.0%	94.4%	75.0%	90.1%
Protruded type	66.7%	91.7%	97.6%	35.5%	70.8%
Diagnosis based on the endoscopic findings <sup>1</sup>	93.5%	97.0%	87.9%	98.5%	96.3%

<sup>1</sup>Using magnifying endoscopy with narrow band imaging and chromoendoscopy.

**Table 2 Diagnostic value of colorectal lesions (For distinguishing intramucosal lesions/SM superficial cancer from SM deep cancer)**

	Sensitivity	Specificity	Negative predictive value	Positive predictive value	Accuracy
Diagnosis based on the color analysis	80.00%	84.40%	29.60%	98.10%	84.10%
Diagnosis based on the endoscopic findings <sup>1</sup>	60.00%	100%	100%	96.80%	84.10%

<sup>1</sup>Using magnifying endoscopy with narrow band imaging and/or chromoendoscopy. Submucosal (SM) superficial cancer: Submucosal invasion was defined as less than 1000  $\mu$ m from the muscularis mucosae; SM deep cancer: Submucosal invasion was defined as 1000  $\mu$ m or more from the muscularis mucosae.

The results of the present study indicate that color analysis using the novel AFI system is an effective method for the objective diagnosis of colorectal lesions, although the sensitivity/specificity/PPV/NPV for determining neoplastic lesions obtained in this study were inferior to those previously reported<sup>[5]</sup> using an earlier AFI system. The mean G/R ratios of colorectal lesions (non-neoplastic lesions, 0.984; neoplastic lesions, 0.827) in the present study were lower than the ratios reported in a previous study (non-neoplastic, 1.12; neoplastic, 0.86)<sup>[5]</sup>. The improvements in the novel AFI system, including the noise-reduction algorithm, higher frame rate, and brighter light source, may affect color tone intensity. In addition, in a previous report, color tone sampling was performed in one area of the lesion that was judged to represent a particular AFI color tone<sup>[5]</sup>; however, in the present study, color tone sampling was performed on an ROI, that, was determined using chromoendoscopy under WLI. Moreover, the present study was limited to patients with polyps over 5 mm in size; however, there were no size limitations on polyps in the previous report<sup>[5]</sup>. The use of different methods of color tone sampling and different inclusion criteria are possible reasons for the differences in the results. However, the lower G/R ratios obtained in the present study might indicate that the novel AFI system permits clearer visualization of colorectal lesions than the former AFI system.

The results of the present study also indicate the potential of color tone analysis to predict the depth of invasion of colorectal neoplasia. Standard methods in use for predicting the depth of invasion of colorectal neoplasia for planning treatment strategies are pit pattern classification using magnifying endoscopy and/or endoscopic ultrasonography<sup>[20-22]</sup>, both of which require long procedure times. A color tone analysis that involves a shorter procedure time and that permits objective diagnosis of the depth of invasion of colorectal neoplasia may provide significant benefits, especially to less endoscopists. However, because the number of SM deep lesions exam-

ined in this study was limited, larger studies are needed to further validate this method. The current study has several limitations. First, because it was difficult to estimate the effectiveness of color tone analysis in distinguishing neoplastic lesions from non-neoplastic lesions using the novel AFI system, the sample size was not calculated for this study. Second, the colonoscopies and ROI determinations performed in this study were conducted by experienced endoscopists; therefore, the results of this study might not be reproducible when patients are assessed by non-experienced clinicians. Finally, the lesions included in this study were limited to those over 5 mm in size, and the number of SM deep lesions was limited.

In conclusion, the results of the present study indicate that computer-assisted real-time color analysis using the novel AFI system was effective in distinguishing non-neoplastic lesions from neoplastic lesions. Color tone analysis, which is an objective and time-saving method, may have potential as a methods for predicting the depth of invasion of colorectal neoplasia, although the number of SM deep lesions examined in the present study was limited. Further studies are needed to determine the effectiveness of color tone analysis for predicting the depth of invasion of colorectal neoplasia.

## COMMENTS

### Background

Colonoscopy is the most effective method for the detection of colonic neoplastic lesions in their earlier and more curable stages. Accurate endoscopic determination of the histology of colorectal polyps could prevent unnecessary polypectomies and may allow the proposal of adequate surveillance recommendations. The authors have reported the effectiveness of autofluorescence imaging (AFI) systems in distinguishing colorectal non-neoplastic lesions from neoplastic lesions. Recently, a novel AFI system that employs second-generation light sources (EVIS LUCERA ELITE CLV-290SL; Olympus Medical Systems, Tokyo, Japan) and video processors (EVIS LUCERA ELITE CV-290; Olympus Medical Systems, Tokyo, Japan) was developed. In addition, software that enables real-time color analysis was developed.

### Research frontiers

AFI systems can be used to visualize fluorophores (autofluorescent substanc-



es) in the gastrointestinal (GI) tract. Several studies have demonstrated that the autofluorescence emitted by neoplastic lesions in the colon has a relatively longer wavelength than the autofluorescence emitted by surrounding normal mucosa. The aim of this study was to prospectively evaluate the efficacy of computer-assisted color analysis of colorectal lesions using a novel AFI system to distinguish non-neoplastic lesions from neoplastic lesions. The authors also evaluated the effectiveness of the system in determining the depth of invasion of colorectal neoplasms.

### Innovations and breakthroughs

The results of the present study indicate that color tone analysis using the novel AFI system is an effective method for the objective diagnosis of colorectal lesions. The improvements in the novel AFI system, including the noise reduction algorithm, higher frame rate, and brighter light source may affect color tone intensity. In addition, in the present study, color tone sampling was performed on the region of interest, which was determined using chromoendoscopy under white light imaging. The results of the present study also indicate the potential of color tone analysis for use in predicting the depth of invasion of colorectal neoplasia. A color tone analysis that involves a shorter procedure time and permits objective diagnosis of the depth of invasion of colorectal neoplasia may provide significant benefits to endoscopists, especially to those who are less experienced.

### Applications

The study results suggest that computer-assisted real-time color analysis using the novel AFI system was effective in distinguishing non-neoplastic lesions from neoplastic lesions. In addition, color tone analysis, which allows objective and time-saving prediction of the depth of invasion of colorectal neoplasia, has potential for use in determining appropriate treatment strategies for colorectal neoplasia.

### Terminology

AFI systems can be used to visualize fluorophores (autofluorescent substances) in the GI tract. In an AFI system, a rotation filter divides the light from the xenon light source and permits only blue and green light to pass. The returning reflection from the tissue, including fluorescent light, is captured by the CCD via a special barrier filter. Image processing then generates the AFI image. The novel AFI system has a brighter lamp that permits a higher frame rate, and produces, less flickering and color splitting, resultings in higher-resolution images with less noise interference.

### Peer review

This study prospectively evaluated the efficacy of computer-assisted color analysis of colorectal lesions using a novel AFI system to distinguish non-neoplastic lesions from neoplastic lesions. In addition, the authors also evaluated the effectiveness of the system in diagnosing the depth of invasion of colorectal neoplasia. They found that the novel AFI system with color analysis was effective in distinguishing non-neoplastic lesions from neoplastic lesions and that it may allow determination of the depth of invasion. The study is interesting, and the manuscript is well-written.

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## Characteristics and prognosis of synchronous multiple early gastric cancer

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### Abstract

**AIM:** To assess the clinicopathologic characteristics, risk factors, and prognosis for synchronous multiple early gastric cancer (SMGC).

**METHODS:** A total of 146 patients with SMGC and 1194 patients with single gastric cancer who had undergone gastrectomy between 1989 and 2008 were retrospectively analyzed to determine their clinicopathologic characteristics and postoperative survival. Tumors were classified into groups on the basis of location and histology. Smoking habits were evaluated using the Brinkman index. Clinical and pathological factors were compared using either Fisher's exact test or Pearson's  $\chi^2$  test. Logistic regression analysis was performed to identify independent risk factors. Survival rate was calculated using the Kaplan-Meier method.

**RESULTS:** SMGCs accounted for 10.9% of gastric cancer cases and occurred predominantly in elderly male patients with a family history of gastric cancer who were both smokers and drinkers. These tumors were typically

macroscopically elevated and histologically differentiated. There were no significant differences between SMGC and single gastric cancer patients with respect to tumor location, tumor size, lymph node metastasis, the number of metastatic lymph nodes, venous invasion, or tumor stage ( $P = 0.052$ ,  $P = 0.347$ ,  $P = 0.595$ ,  $P = 0.805$ ,  $P = 0.559$ , and  $P = 0.408$ , respectively). Further, there was no significant difference in postoperative survival between the patient groups ( $P = 0.200$ ). Of the 146 SMGC patients, a single patient had remnant cancer.

**CONCLUSION:** A careful preoperative endoscopy is necessary for patients who are at high risk of SMGC, and minimally invasive treatment may be indicated in some cases.

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**Key words:** Gastric cancer; Synchronous; Multiple; Endoscopy; Prognosis

**Core tip:** This study compares the clinicopathologic characteristics of synchronous multiple gastric cancer (SMGC) and single gastric cancer. Further, we identified risk factors for SMGC and assessed whether they can be treated with a minimally invasive approach. We found that SMGC occurred predominantly in elderly male patients who had a family history of gastric cancer, and who were both smokers and drinkers. The tumors were macroscopically elevated and histologically differentiated. Lymph node metastasis and vascular invasion were equally prevalent, and there was no significant difference in postoperative survival between these patient groups. We suggest minimally invasive approach may be applicable.

Isobe T, Hashimoto K, Kizaki J, Murakami N, Aoyagi K, Koufuji K, Akagi Y, Shirouzu K. Characteristics and prognosis of



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## INTRODUCTION

Due to the recent technical advances in endoscopic examinations, the number of patients with synchronous multiple gastric cancer (SMGC) has increased, and SMGC has been reported to account for 6%-14% of all gastric cancer cases<sup>[1-3]</sup>. A previous study reported that multiple gastric cancers have several clinicopathologic features, including incidence in old age, well differentiated tumors, and early stage tumors, and that the prognosis is similar to that of single gastric cancers<sup>[4]</sup>. Minimally invasive resection procedures, such as endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), and laparoscopic surgery, are known to improve the quality of life of early gastric cancer patients<sup>[5,6]</sup>. However, the presence of SMGC may increase the risk of missing a remnant gastric lesion and may make it more difficult to determine the range of gastrectomy required. Furthermore, it is unclear whether the same indication criteria for endoscopic resection can be applied to SMGC because comparative studies on the incidence of recurrence and prognosis between multiple and single gastric cancers are limited. The aim of this study was to compare the clinicopathologic characteristics between SMGC and single gastric cancer, to identify risk factors for SMGC, and to assess whether SMGC can be safely treated with a minimally invasive approach.

## MATERIALS AND METHODS

SMGC was defined in accordance with Moertel's criteria<sup>[7]</sup> as follows: (1) each lesion must have a pathologically proven malignancy; (2) all lesions must be clearly separated by intervals of microscopically normal gastric wall; and (3) the possibility that one of the lesions represents a metastatic tumor must be ruled out beyond any reasonable doubt. Between 1989 and 2008, we identified a total of 1406 patients who underwent gastric resection surgery for early gastric cancer at the Department of Surgery at Kurume University School of Medicine. The patients were excluded from the study if they had undergone gastrectomy after neoadjuvant chemotherapy, if they had surgery after EMR or ESD, or if they had gastric cancer in the remnant stomach after a previous gastrectomy. Therefore, a total cohort of 1340 patients with gastric cancer was analyzed. Microscopic examination of surgically resected specimens revealed SMGC in 146 patients (group A) and a single gastric cancer in the remaining 1194 patients (group B). We retrospectively analyzed the patients' clinicopathologic characteristics, including age, sex, family history, the presence of cancer in other organs (synchronous or metachronous), smoking and drinking

habits, tumor location, tumor size, macroscopic type, histological type, depth of invasion, number of metastatic lymph nodes, lymphovascular invasion, and tumor stage as defined by the Japanese Classification of Gastric Carcinoma (3<sup>rd</sup> English edition)<sup>[8]</sup>. The design of this study and the procedures for obtaining informed consent were based on the principles of the Declaration of Helsinki. Our study was approved by The Ethical Committee of Kurume University (No. 13091).

Tumors were classified into groups based on whether they were located in the upper (U), middle (M), or lower (L) third of the stomach, and the macroscopic type of each cancer was classified as I (protruding), II a (superficial, elevated), II b (flat), II c (superficial, depressed), III (excavated), or a combination of these (I + II a, II a + II c, II c + II a, or II c + III). All cases were regrouped into the elevated, flat, depressed, or mixed type. The histological type was classified as either differentiated (papillary adenocarcinoma, well-differentiated and moderately differentiated adenocarcinomas) or undifferentiated (poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma). If the depth of invasion of 2 or more lesions was equal, the largest was regarded as the primary lesion, with all other lesions regarded as accessory lesions.

Smoking habits were evaluated using the Brinkman index (BI)<sup>[9,10]</sup>, which is defined as (number of cigarettes per day) × (number of years for which the patient smoked). Using this index, we divided the patients into 3 groups: nonsmokers, light smokers (BI < 400), and heavy smokers (BI ≥ 400). To assess drinking habits, all patients were asked about their frequency of drinking, the amount (1 go = 22.8 g ethanol) typically consumed on any one occasion, and the type of beverage usually consumed (sake, shochu, beer, whisky, wine, or others). From these data, we calculated the amount of ethanol (in grams) consumed per day, and we classified patients into 3 groups: non-drinkers, occasional drinkers, and daily drinkers (< 22.8, 22.8-45.5, and > 45.5 g of ethanol per day, respectively). For each factor assessed, we used the same questionnaire for all patients when they were admitted.

Clinical and pathological factors were compared using either Fisher's exact test or Pearson's  $\chi^2$  test, as appropriate. Logistic regression analysis was performed to identify independent risk factors with odds ratios and 95%CI. The survival rate was calculated using the Kaplan-Meier method.  $P < 0.05$  was considered statistically significant. Data analysis was performed using the statistical program JMP 8 (SAS Institute, Cary, NC).

## RESULTS

A total of 1340 patients [146 patients with SMGC (group A) and 1194 patients with single gastric cancers (group B)] were included in this study. In group A, 120 patients had 2 lesions each, 22 had 3 lesions, 3 had 4 lesions, and 1 patient had 8 lesions, making a total of 326 lesions (Table 1).

The clinical differences between the groups are sum-

**Table 1** Incidence of multiple lesions in synchronous multiple early gastric cancer patients

No. of tumors	No. of patients	No. of lesions
2	120	240
3	22	66
4	3	12
8	1	8
Total	146	326

**Table 2** Clinical features of synchronous multiple early gastric cancer (group A) and single gastric cancer patients (group B) *n* (%)

Factors	Group A ( <i>n</i> = 146)	Group B ( <i>n</i> = 1194)	<i>P</i> value
Tumor location			
Upper	30 (20.6)	183 (15.3)	0.052
Middle	40 (27.4)	450 (37.7)	
Lower	76 (52.1)	554 (46.4)	
Entire	0 (0.0)	7 (0.5)	
Tumor size (mm) (mean ± SD)	30.8 ± 15.9	32.5 ± 21.5	0.347
Macroscopic type			
Elevated	39 (26.7)	218 (18.3)	0.019
Flat	2 (1.4)	16 (1.3)	
Depressed	81 (55.5)	815 (68.3)	
Mixed	24 (16.4)	145 (12.1)	
Histological type			
Differentiated	118 (80.8)	771 (64.6)	< 0.001
Undifferentiated	28 (19.2)	423 (35.4)	
Depth of invasion			
Mucosa	66 (45.2)	672 (56.1)	0.011
Submucosa	80 (54.8)	522 (43.9)	
LN metastasis			
0	134 (91.8)	1110 (93.0)	0.595
1	8 (5.5)	63 (5.3)	
2	4 (2.7)	15 (1.3)	
3	0 (0.0)	6 (0.5)	
No. of metastatic LNs	0.18 ± 0.72	0.21 ± 1.30	0.805
Lymphatic invasion	61 (41.8)	394 (33.0)	0.034
Venous invasion	13 (8.9)	90 (7.5)	0.559
Stage			
I	142 (97.3)	1173 (98.2)	0.408
II	4 (2.7)	21 (1.8)	

marized in Table 2. The patients in group A were older (68.0 years *vs* 64.3 years,  $P < 0.001$ ) and more likely to be male (81.5% *vs* 67.1%,  $P < 0.001$ ) than those in group B. Furthermore, patients in group A had a significantly greater number of family members with gastric cancer compared with those in group B ( $P = 0.046$ ), and SMGC patients also smoked ( $P = 0.020$ ) and drank more ( $P = 0.005$ ). However, there were no significant differences with respect to the presence of synchronous and metachronous cancers in other organs.

The pathological features of each group are listed in Table 3. Macroscopically, more than 50% of the tumors in both groups were of the depressed type, and this type was more common in group B than in group A. Elevated-type tumors were present more often in group A than in group B (68.3% *vs* 55.5% and 26.7% *vs* 18.3%,

**Table 3** Pathological features of lesions observed in groups A and B *n* (%)

Factors	Group A ( <i>n</i> = 146)	Group B ( <i>n</i> = 1194)	<i>P</i> value
Age (mean ± SD) (yr)	68.0 ± 9.9	64.3 ± 11.1	< 0.001
Gender			
Male	119 (81.5)	801 (67.1)	< 0.001
Female	27 (18.5)	393 (32.9)	
No. of family members with gastric cancer			
None	103 (70.6)	917 (76.8)	0.046
1	30 (20.6)	224 (18.7)	
≥ 2	13 (8.9)	53 (4.4)	
Presence of cancer in other organs			
Synchronous	4 (2.7)	48 (4.0)	0.450
Metachronous	20 (13.7)	107 (9.0)	0.065
Smoking habits			
Never	49 (33.6)	536 (44.9)	0.020
BI < 400	19 (13.0)	157 (13.2)	
BI ≥ 400	78 (53.4)	501 (42.0)	
Drinking habits			
Non-drinker	51 (34.9)	587 (49.2)	0.005
Occasional-drinker	55 (37.7)	340 (28.5)	
Daily-drinker	40 (27.4)	267 (22.4)	

respectively). The differentiated type was present more often in group A than in group B (80.8% *vs* 64.6%,  $P < 0.001$ ). Furthermore, the depth of invasion and the rate of lymphatic invasion in group A were both significantly higher than those in group B ( $P = 0.011$ ,  $P = 0.034$ , respectively). There were no significant differences between the groups with respect to tumor location, tumor size, lymph node metastasis, the number of metastatic lymph nodes, venous invasion, or tumor stage ( $P = 0.052$ ,  $P = 0.347$ ,  $P = 0.595$ ,  $P = 0.805$ ,  $P = 0.559$ , and  $P = 0.408$ , respectively). Of the 9 factors that were statistically significant in the univariate analyses, multivariate logistic regression analysis showed that the important risk factors for SMGC were age (OR = 1.93, 95%CI: 1.27-2.99,  $P = 0.002$ ) and sex (OR = 1.86, 95%CI: 1.07-3.32,  $P = 0.028$ , Table 4).

The overall median follow-up period was 78.1 months. Of the 146 patients in group A, a single patient had remnant cancer. This patient was diagnosed 1 year after distal gastrectomy, and the lesion was located in the cardia, necessitating a total gastrectomy. One patient died of the disease (hepatic metastasis), and there were 18 other deaths: 3 from an unknown cause, 14 from other diseases (including 5 deaths from cancer of another organ), and 1 from a traffic accident. The 3-year overall survival rates in group A and group B were 95.8% and 96.6%, respectively, and the 5-year overall survival rates in group A and group B were 90.3 and 93.2%, respectively (Figure 1). There was no significant difference in postoperative survival between the groups ( $P = 0.200$ ).

## DISCUSSION

Although the histogenesis of normal gastric cancer has been addressed in numerous studies, relatively little is known about the development of multiple gastric can-

**Table 4** Multivariate analysis of risk factors for synchronous multiple early gastric cancer

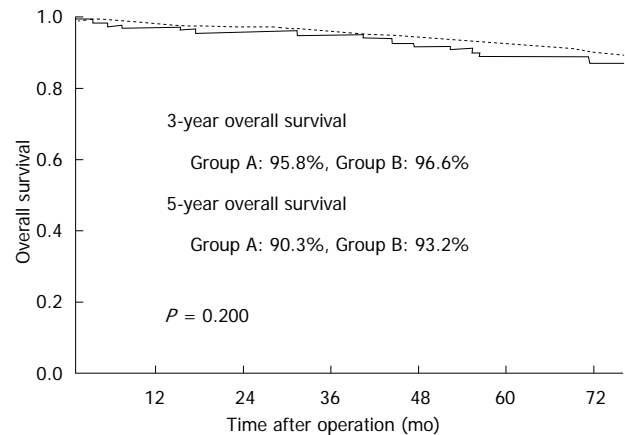
Factor	Odds ratio	95%CI <sup>1</sup>	P value
Age (yr)			
≥ 65 vs < 65	1.93	1.27-2.99	0.002
Sex			
Male vs female	1.86	1.07-3.32	0.028
Family history			
(+) vs (-)	1.35	0.87-2.06	0.182
Smoking habit			
(+) vs (-)	1.15	0.73-1.89	0.531
Drinking habit			
(+) vs (-)	1.51	0.79-3.14	0.218
Macroscopic type			
Elevated vs depressed	1.44	0.92-2.23	0.108
Histological type			
Differentiated vs undifferentiated	1.53	0.94-2.61	0.088
Depth of invasion			
Submucosa vs mucosa	1.61	0.90-2.81	0.109
Lymphatic invasion			
(+) vs (-)	0.879	0.50-1.60	0.664

<sup>1</sup>Determined using logistic regression analysis.

cers. Recent advances in endoscopy have made it possible to detect early gastric cancer, for which EMR and ESD have become common treatment modalities<sup>[2,11]</sup>. Such therapies conserve function; however, after resection, a large quantity of the gastric mucosa remains, which can give rise to further cancers. Concurrent multiple cancers and the development of post-therapeutic asynchronous multiple cancers represent serious problems. Nasu *et al.*<sup>[12]</sup> followed 143 patients with early gastric cancer who had undergone EMR and found that 16 (11%) patients developed SMGC within 1 year of the initial EMR. It is important to identify patients who are at a high risk of developing multiple gastric lesions due to the complexity involved in fully diagnosing and treating these patients.

Based on previous reports, SMGC accounts for 6%-14% of all early gastric cancers<sup>[1-4]</sup>. The incidence of SMGC among patients in this study was 10.9%, which is within this range. In the present study, we found that patients with SMGC were significantly older than those with single gastric cancer. The predominance of SMGC in elderly patients may be explained by the pathogenetic importance of intestinal metaplasia<sup>[13]</sup>, as gastric glands generally show atrophic change with a concomitant increase in intestinal metaplasia in the stomachs of elderly people.

Patients with a family history of carcinoma were reported to have a high incidence of gastric cancer<sup>[14,15]</sup>. One report reviewed 15 case-control studies of family history and gastric cancer, all of which indicated a positive relationship between these parameters, with risk ratios that ranged from 1.5- to 3.5-fold<sup>[16]</sup>. The relationship between gastric cancer and genetics has been demonstrated by the non-random involvement of certain chromosomes and related oncogenes, especially Ras and p53<sup>[17,18]</sup>. Another study showed that gastric cancer was associated with intestinal metaplasia in 52% of the included



**Figure 1** Survival curve for patients with synchronous multiple early gastric cancer (group A: solid line) and those with single gastric cancer (group B: dotted line). The 3-year survival rates of group A and group B were 95.8% and 96.6%, respectively, and the 5-year survival rates of group A and group B were 90.3% and 93.2%, respectively ( $P = 0.200$ ).

patients<sup>[19]</sup>. In this study, compared with single gastric cancer patients, a significantly greater number of SMGC patients had a family history of gastric cancer based on univariate analysis. Furthermore, the number of family members affected also appears to be significant. This may be attributed to genetic factors, in addition to the environmental conditions presumably shared by members of same family, which result in familial clustering of gastric cancer. However, we cannot determine whether this result is due to environmental or genetic factors.

Smoking and alcohol consumption have been reported to be risk factors for gastric cancer<sup>[20-22]</sup>, although a study by Morita *et al.* found that there was no significant association between the occurrence of multiple gastric cancer and either of these factors<sup>[3]</sup>. In this study, we also examined these factors in patients with SMGC and those with single gastric cancer. We found that there were significant differences in smoking and drinking habits between patients with SMGC and those with single gastric cancer based on univariate analysis. However, none of these habits were found to be independent risk factors in multivariate analysis. Therefore, the effects of smoking and alcohol on the occurrence of multiple gastric cancers remain controversial.

Tumor location is an important determinant of treatment, and a major concern for patients undergoing subtotal gastrectomy or endoscopic resection is the difficulty of detecting a lesion in the remnant portion of the stomach. In this study, 39.0% of patients with SMGC were found to have tumors located in different thirds of the stomach. In a report concerning the localization of SMGCs, Kitamura *et al.* stated that early multiple-tumor cases more frequently involved the upper region of the stomach than early single-tumor cases<sup>[23]</sup>. We also found that SMGC patients were more likely to have a tumor located in the upper third of the stomach compared with single gastric cancer patients. It is difficult to find lesions located in the upper third of the stomach because of



the technical limitations of forward-viewing endoscopy. Therefore, this area should be observed very carefully, and caution should be exercised before performing a proximal gastrectomy for early gastric cancer in the cardia or high body.

Most SMGCs were shown to have a differentiated histological type in previous studies<sup>[2,3,12]</sup>. Correspondingly, we found that, among 326 lesions in the 146 patients with SMGC, 271 (83.1%) lesions were of the differentiated type. Lymphovascular involvement has also been shown to be significantly associated with lymph node metastasis in gastric cancer<sup>[24]</sup>. Although lymphatic invasion occurred more frequently in the SMGC cases in this study, the frequency of lymph node metastasis and venous invasion in these cases did not differ significantly from that in the single gastric cancer group.

The prognosis of early gastric cancer is generally favorable, and a 5-year relative postoperative survival of  $\geq 90\%$  has been reported<sup>[2]</sup>. In this study, there were no significant differences in the 3- and 5-year survival rates between patients with SMGC and those with single gastric cancer, and the 5-year survival rate was  $\geq 90\%$  in both groups.

A total gastrectomy has been recommended for the treatment of multiple gastric cancer because it is believed that the remnant stomach of patients who have undergone a partial gastrectomy is at increased risk for ongoing carcinogenesis<sup>[7]</sup>. However, prophylactic total gastrectomy reportedly did not improve outcome in patients with multiple gastric cancer<sup>[2]</sup>. The cumulative prevalence of gastric remnant cancer after ESD or surgical partial gastrectomy for early gastric cancer is reportedly 2.4%-14% at 5 years<sup>[12,25,26]</sup>. In the present study, 1 patient had remnant cancer, and 2 patients died of gastric cancer after gastrectomy. Early gastric cancer patients treated using EMR or ESD have been identified as a high-risk group for remnant gastric cancer. However, most metachronous lesions were intramucosal tumors with no lymphovascular involvement and no lymph node metastasis, indicating the potential for additional ESD or other local therapies rather than gastrectomy<sup>[5]</sup>. Endoscopists should recognize the characteristics of SMGCs, and special attention should be given to cases of EMR or ESD to avoid missing synchronous lesions, as SMGC patients are at a high risk of developing metachronous cancer in the remnant stomach after treatment<sup>[12,25-27]</sup>. Based on our findings, EMR or ESD may be applicable for SMGC cases if they meet the criteria for endoscopic resection.

The limitations of this study include the small number of patients enrolled, its retrospective design, and the fact that not every patient received total gastrectomy; therefore, whole stomach pathology could not be evaluated.

In conclusion, on the basis of the results from our study, we suggest that elderly male patients with a family history of gastric cancer who also have a history of smoking and alcohol consumption should be carefully examined preoperatively, especially if their tumor is macroscopically elevated and histologically differentiated.

Additionally, a minimally invasive approach may be applicable for cases of SMGC if they meet the criteria.

## COMMENTS

### Background

Due to improved diagnostic procedures, more cases of gastric cancer have been diagnosed. Minimally invasive procedures, such as endoscopic resection and laparoscopic surgery, have also gained worldwide acceptance as they improve the quality of life of early gastric cancer patients. However, the benefits of such procedures have not been demonstrated for synchronous multiple early gastric cancer (SMGC).

### Research frontiers

It is questionable whether the same indication criteria for minimally invasive procedures can be applied to SMGC because comparative studies on the incidence of LN metastasis and prognosis between multiple and single gastric cancers are limited. Here, we compare the clinicopathologic characteristics of SMGC and single gastric cancer to identify risk factors for SMGC and to assess whether SMGC patients can be safely treated with a minimally invasive approach.

### Innovations and breakthroughs

There have been very few studies regarding the risk factors for SMGC. The clinicopathologic characteristics of SMGC and single gastric cancer were addressed in this retrospective study, using a long term follow-up of a large number of patients. Furthermore, this is the first demonstration that there are significant differences in the smoking habits, alcohol consumption, and family history of gastric cancer between patients with SMGC and those with single gastric cancer.

### Applications

The findings of this study may lead to an improvement in the post-surgical prognosis of gastric cancer, especially in older men with a family history of smoking and alcohol consumption. Furthermore, if the tumor is macroscopically elevated and histologically differentiated, a minimally invasive approach may be applicable for SMGC cases, provided the criteria are met.

### Terminology

SMGC was defined as follows: (1) each lesion must have a pathologically proven malignancy; (2) all lesions must be clearly separated by intervals of microscopically normal gastric wall; and (3) the possibility that one of the lesions represents a metastatic tumor must be ruled out beyond any reasonable doubt. Endoscopic mucosal resection is an endoscopic technique of resection of a lesion that requires the separation of the submucosa using normal saline solution. Endoscopic submucosal dissection is a new method of resection, allowing the dissection of the lesion within the thickness of the submucosa or the interface between the submucosa and the muscularis propria.

### Peer review

This is a good retrospective study in which authors analyze the clinicopathologic characteristics and the risks factors of SMGC. The results are interesting and suggest that careful preoperative endoscopy and minimally invasive treatment must be used for patients with high risk of SMGC.

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## Forward-viewing endoscopic ultrasound-guided NOTES interventions: A study on peritoneoscopic potential

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### Abstract

**AIM:** To evaluate the feasibility of diagnostic and therapeutic transgastric (TG) peritoneoscopic interventions with a forward-viewing endoscopic ultrasound (FV-EUS).

**METHODS:** This prospective endoscopic experimental study used an animal model. Combined TG peritoneoscopic interventions and EUS examination of the intra-abdominal organs were performed using an FV-EUS on 10 animal models (1 porcine and 9 canine). The procedures carried out include EUS evaluation and endoscopic biopsy of intraperitoneal organs, EUS-guided fine needle aspiration (EUS-FNA), EUS-guided radiofre-

quency ablation (EUS-RFA), and argon plasma coagulation (APC) for hemostatic control. The animals were kept alive for 7 d, and then necropsy was performed to evaluate results and complications.

**RESULTS:** In all 10 animals, TG peritoneoscopy, followed by endoscopic biopsy for the liver, spleen, abdominal wall, and omentum, was performed successfully. APC helped control minor bleeding. Visualization of intra-abdominal solid organs with real-time EUS was accomplished with ease. Intraperitoneal EUS-FNA was successfully performed on the liver, spleen, and kidney. Similarly, a successful outcome was achieved with EUS-RFA of the hepatic parenchyma. No adverse events were recorded during the study.

**CONCLUSION:** Peritoneoscopic natural orifice trans-luminal endoscopic surgery (NOTES) interventions through FV-EUS were feasible in providing evaluation and performing endoscopic procedures. It promises potential as a platform for future EUS-based NOTES.

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**Key words:** Forward-viewing endoscopic ultrasound; Oblique-viewing endoscopic ultrasound; Endoscopic ultrasound guided intervention; Peritoneoscopy; Natural orifice transluminal endoscopic surgery

**Core tip:** Recently, the forward-viewing endoscopic ultrasound (FV-EUS) was developed, however, peritoneoscopic natural orifice transluminal endoscopic surgery (NOTES) interventions with an FV-EUS has never been discussed. In this study, transgastric peritoneoscopy with FV-EUS, real-time EUS, EUS-guided fine needle aspiration, EUS-guided radiofrequency ablation, and bleeding control were successfully undertaken. FV-EUS will broaden the prospects of NOTES interventions to endoscopists, and the NOTES interventions with an FV-



EUS might be performed in the various conditions.

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## INTRODUCTION

Natural orifice transluminal endoscopic surgery (NOTES) reaches the target organ by inserting the endoscope through a natural orifice (*e.g.*, mouth, anus, vagina, or urethra) and entering the peritoneal cavity by making an incision on the luminal wall. In the years after the first described NOTES by Kalloo *et al.*<sup>[1]</sup> in 2004, a wide range of NOTES procedures with a transgastric (TG) endoscopic approach to access the peritoneal cavity have been reported. Several studies, mainly performed using animal models, have been feasible for a variety of procedures, including fallopian tube ligation<sup>[2]</sup>, cholecystectomy<sup>[3]</sup>, biliary anastomosis<sup>[4]</sup>, gastrojejunostomy<sup>[5]</sup>, splenectomy<sup>[6]</sup>, and partial hysterectomy<sup>[7]</sup>. NOTES with flexible peritoneoscopy enables the examination of the peritoneal cavity with minimal invasiveness. By avoiding abdominal incisions, these successful NOTES procedures have the potential to offer less postoperative pain and reduced postoperative recovery time while avoiding hernia formation, adhesions, surface incision infection and scarring<sup>[8]</sup>. Primarily confined to the proponents of NOTES in the surgical discipline, these procedures offered a viable alternative to laparoscopic surgery, especially in patients deemed at high risk for complications.

In assessing the peritoneal cavity, the anterior wall of the stomach is usually the ideal incision site while the posterior wall may be selected to explore the retroperitoneum<sup>[9-11]</sup>. However, the TG approach has inherent risks, such as access site bleeding, adjacent organ injury during gastrotomy creation, or gastric content leakage, giving rise to infection in the peritoneal cavity. Apart from infection, bleeding is one of the most common complications. Given these considerations, the endoscopic ultrasound (EUS) has been used to avoid and mitigate the risk of injury to extraluminal structures, as well as to detect neighboring vessels by using color Doppler imaging<sup>[12,13]</sup>. In these studies, an oblique-viewing, curved, linear-array endoscopic ultrasound (OV-EUS) was used to acquire real-time images of the vessels and structures outside the gastrointestinal tract during access into the peritoneal cavity. After making an incision on the gastric wall, the OV-EUS must be exchanged for endoscopy to perform the subsequent NOTES procedures, because it provides oblique-viewing images different from the direction of the echoendoscopic movement.

To overcome several limitations of OV-EUS in EUS-

interventions, a forward-viewing endoscopic ultrasound (FV-EUS) was developed. The FV-EUS simultaneously offers a straight endoscopic view and an ultrasound image. In several studies, FV-EUS was successfully tested in EUS-interventions, such as the drainage of pancreatic pseudocysts<sup>[14,15]</sup>, EUS-guided fine needle aspiration (EUS-FNA)<sup>[16]</sup>, and celiac plexus neurolysis<sup>[17]</sup>. In addition, it is now possible to go beyond the gut wall with the FV-EUS for intraluminal to intraperitoneal EUS evaluation. Although it showed advantages in other EUS-interventions, studies have yet to suggest the possibility of FV-EUS in NOTES procedures. Hence, this study was conducted to evaluate the technical feasibility and safety profile of the FV-EUS in a variety of procedures related to diagnostic and therapeutic TG peritoneoscopic interventions.

## MATERIALS AND METHODS

### Animals

A mini pig (40 kg) and 9 dogs (mean weight, 18 kg; weight range, 15-20 kg) were used. Approval of the Institutional Animal Care and Use Committee was obtained before initiation of the study. All animals were fasted for 24 h but permitted water *ad libitum*. Anesthetic induction was achieved with a drug combination of tiletamine and Zolazepam (7.5 mg/kg) (Zoletil 50, Virbac, South Korea) and Xylazine Hydrochloride (2 mg/kg) (Rompun; Bayer, South Korea) and maintained on 1.5% isoflurane (Forane, JW pharmaceutical, South Korea) following endotracheal intubation. Cardiopulmonary parameters were monitored throughout the procedure.

### FV-EUS-guided transgastric access

Under general anesthesia, an FV-EUS (UCT 160J-AL5, Olympus, Tokyo, Japan) was advanced into the esophagus and stomach. The access site on the anterior gastric wall was first evaluated under real-time image and Doppler guidance to exclude adjacent organs and interfering vessels. A single-lumen microknife needle (Boston Scientific, Natick, MA) was used to create and puncture a small hole in the anterior gastric wall. Through the puncture site, a standard 0.035-guidewire (Jagwire; Boston Scientific, Natick, MA) was advanced through the microknife into the abdominal cavity. The microknife was then withdrawn with the guidewire left *in situ*.

This portal of access was then dilated with a 20-mm controlled radial expansion (CRE) balloon (Boston Scientific, Natick, MA). The balloon was held in place for 1 min. The radially expanded puncture formed a circular gastrotomy that granted passage of the FV-EUS into the peritoneum. The resultant entry allowed air insufflation through the echoendoscope to expand the peritoneal cavity for improved visualization.

### Peritoneoscopic interventions

After access into the peritoneal cavity with the FV-EUS, the following procedures were performed: (1) Peritoneoscopy and endoscopic biopsy of the liver, spleen,

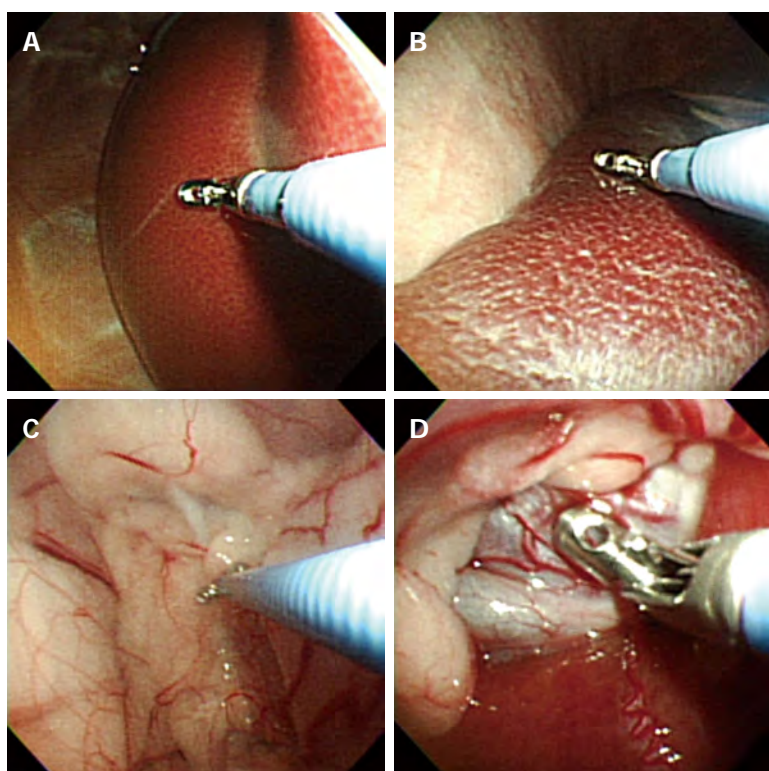


Figure 1 Peritoneoscopy and endoscopic biopsy of the intraperitoneal organs. A: Liver; B: Spleen; C: Abdominal wall; D: Omentum.

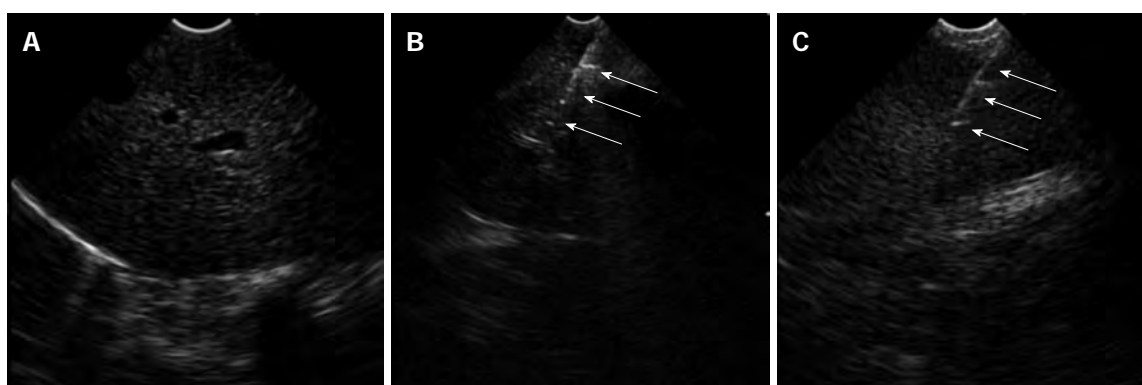


Figure 2 Forward-viewing endoscopic ultrasound image of the intraperitoneal organs and endoscopic ultrasound-guided fine needle aspiration using a 19G aspiration needle (white arrow). A: Liver; B: Kidney; C: Spleen.

abdominal wall, and omentum using a rat-tooth biopsy forceps (Olympus, Tokyo, Japan) (Figure 1); (2) Real-time FV-EUS examination of the intraperitoneal solid organs (Figure 2); (3) EUS-FNA with a 19G (Cook Medical Inc., Winston-Salem, NC) aspiration needle on the liver, spleen, and kidney (Figure 2); (4) FV-EUS-guided radio-frequency ablation (EUS-RFA) with the newly developed 18G RFA needle (Starmed, Seoul, South Korea) on the hepatic parenchyma (Figure 3); and (5) Argon plasma coagulation (APC) for hemostatic control of artificially induced bleeding at the liver and spleen (Figure 4).

#### Gastrotomy closure and post-procedure assessment

The gastrotomy site was closed with endoscopic hemoclips. After the procedure, antibiotics and analgesics were

administered and the regular diet was introduced 24 h later. The animals were kept alive for 7 d and then sacrificed. Necropsy was performed to evaluate macroscopically the EUS-FNA and EUS-RFA lesions, as well as any gross anatomical injuries to the intraperitoneal organs and infective complications.

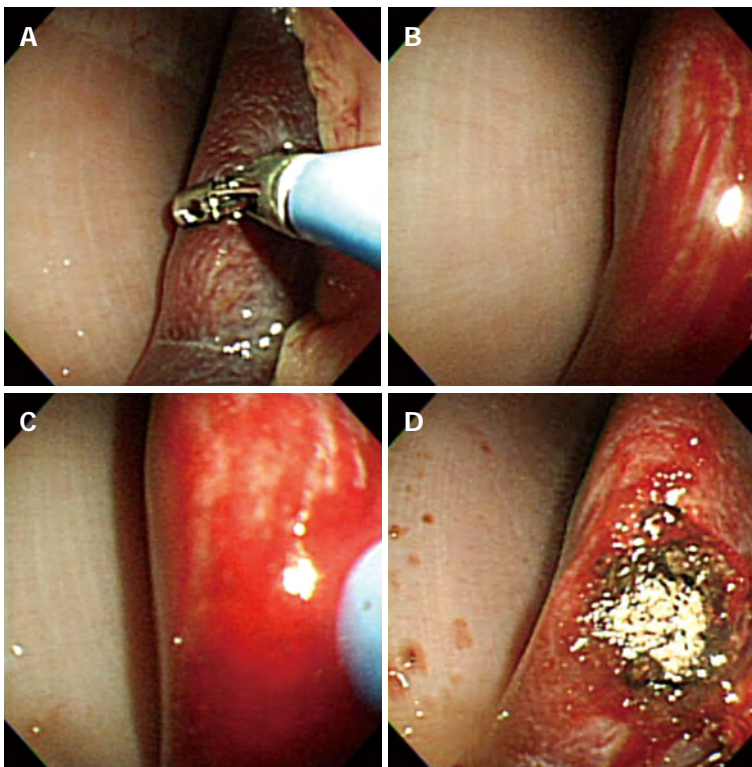
## RESULTS

### Feasibility

This study was performed on 1 pig and 9 dogs. After gastrotomy, the FV-EUS, using forward optic view to enter the peritoneal cavity, and diagnostic TG peritoneoscopy for various intraperitoneal organs was undertaken safely and easily in all animals. Endoscopic biopsies of the liver,



**Figure 3** Endoscopic ultrasound-guided radio frequency ablation using an 18G radiofrequency ablation needle on the hepatic parenchyma. A: Radiofrequency ablation (RFA) needle (white arrow) in the hepatic parenchyma with echogenic ablation zone (red arrow); B: RFA needle tip with ablation zone (red arrow) echogenic marker (white arrow); C: Gross pathology of ablated tissue in the liver parenchyma.



**Figure 4** Argon plasma coagulation for hemostatic control of artificially induced bleeding at the spleen. A: Bleeding induced by a biopsy forcep; B: Surface bleeding seen at the spleen; C: Argon plasma coagulation catheter introduced to achieve thermal coagulation; D: Hemostasis successfully achieved.

spleen, abdominal wall, and omentum were also completed successfully without complications in all 10 animals.

APC was successfully used to control minor artificial bleeding caused by deliberate multiple-forceps biopsy and



**Table 1** Diagnostic and therapeutic peritoneoscopic procedures performed on the animals

Procedure	1 (pig)	2 (dog)	3 (dog)	4 (dog)	5 (dog)	6 (dog)	7 (dog)	8 (dog)	9 (dog)	10 (dog)
Peritoneoscopy	+	+	+	+	+	+	+	+	+	+
Multiple biopsies	+	+	+	+	+	+	+	+	+	+
EUS-FNA		Liver	Liver, kidney	Liver, spleen	Liver, spleen	Liver, spleen	Liver, spleen, kidney,	Liver, spleen, kidney	Liver, spleen, kidney	Liver, spleen, kidney
EUS-RFA	Liver			Liver	Liver	Liver	Liver	Liver		
Argon plasma coagulation		+	+	+	+					

EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; EUS-RFA: Endoscopic ultrasound-guided radiofrequency ablation.

poking on the liver and spleen in 4 animals. Real-time EUS images were acquired with ample clarity and ease while observing the deeper portions of the intra-abdominal organs. When the scope contacted the target organ, the endoscopic view was switched to the sonographic view, and EUS-FNA from the peritoneal cavity was successfully performed on the liver, spleen, and kidney in the 9 dogs.

The EUS-RFA was undertaken when the equipment was made available. In the EUS-RFA, the power was set to 50 watts, and the duration was 1 min. The EUS-RFA of the hepatic parenchyma was equally successful in 6 animals by using the RFA needle (Table 1).

### Evaluation of post-procedure outcomes

All the animals survived for 7 d without any obvious pattern of behavioral distress. Necropsy revealed no apparent or gross anatomical damage to the intraperitoneal organs related to these diagnostic and therapeutic procedures. The closure of the gastrotomy orifice was accomplished using 6 to 7 endoscopic hemoclips. No significant peritoneal adhesions or peritonitis were seen in the necropsies. In addition, neither intraperitoneal infectious complications nor abscesses were detected in the animals.

## DISCUSSION

EUS-guided therapeutic interventions are performed with the OV-EUS. The major disadvantage of the OV-EUS is that the echoendoscope occasionally accesses the targeted area at an acute angle. Because of the acute angle, the force of accessory advancement may cause the scope to push away from the target organ. Another limitation of the OV-EUS is the lack of forward-viewing endoscopy. It requires reorientation in switching from a sonographic to endoscopic view. As a result of technological advances and a surge in new therapeutic modalities for EUS-guided procedures, FV-EUS was developed to overcome the disadvantages of OV-EUS.

The FV-EUS facilitated needle or device insertion and deployment<sup>[18]</sup>. Unlike the OV-EUS an important advantage offered with the FV-EUS is that the axis and optics of the echoendoscope is in line with the accessory channel. This straight alignment not only provides the operator easier deployment and manipulation of needle and devices through the working channel but also renders better transmission of force to the tip of the accessory

device or needle<sup>[17]</sup>. Furthermore, the FV-EUS could be manipulated to secure a perpendicular puncture trajectory instead of the angulated puncture direction in OV-EUS, thereby, preventing the “pushback” phenomenon or moving away from the gut wall<sup>[19]</sup>. This ensures that the echoendoscope could be kept more easily in its intended position during therapeutic interventions.

Of course, the FV-EUS has disadvantages: narrow ultrasound scanning range, absence of an elevator, and incapability of using a balloon at the tip of the echoendoscope. However, these disadvantages of FV-EUS did not affect its maneuverability or outcomes<sup>[15,19,20]</sup>. Overall, the FV-EUS facilitates EUS-guided therapeutic procedures.

With the introduction of the FV-EUS, its use in TG NOTES peritoneoscopy could mark the evolution from mainly a diagnostic modality to the prospect of carrying out a wide range of peritoneoscopic interventional procedures. This is possible because of certain advantages afforded by the FV-EUS, namely, improved maneuverability guided by forward optics, wider distal-end range of angulation, a shorter and smaller distal tip in front of the view, the facility to deploy needles and other accessory devices along the axis of the scope, and the ability to switch readily between sonographic and endoscopic views without the need for frequent echoendoscope re-orientation<sup>[14]</sup>. By using FV-EUS to identify and avoid extraluminal organs and vessels, the gastrotomy site created on the anterior abdominal wall was accomplished without intra- or post-procedural complications. Neither bleeding from the gastric wall during its incision nor injury to the contiguous organs on entry into the peritoneal cavity was observed. FV-EUS enhanced safety by providing real-time images of the anticipated path of the microknife puncture. Hence, with the advantages mentioned earlier, the transmural microknife puncture through the gastric layers, advancement of the guidewire *via* the fistula into the peritoneal cavity, and fistula dilatation with the CRE balloon were successful in all 10 animal cases. In particular, the FV-EUS circumvents the need of a second endoscopic procedure for peritoneal cavity entry and, thereby, reducing overall procedure time<sup>[12]</sup>.

In the current animal study, biopsies were taken from the liver, spleen, anterior abdominal wall, and omentum successfully with the FV-EUS. This was exemplified in the minimal resistance and enhanced facility the operator encountered during the procedure. In the event of

bleeding, APC was used to attain control. Hemostasis was achieved effectively for minor bleeding, which was deliberately induced by the forceps biopsy at the liver and spleen in 4 dogs. Electrocautery could also be used to prevent further bleeding from the biopsy sites<sup>[21]</sup>. These findings suggest that liver and splenic biopsies *via* the TG peritoneoscopic approach could be accomplished uneventfully and without major bleeding complications.

The EUS-FNA and EUS-RFA needles were clearly visualized extruding from the working channel of the FV-EUS and then they were inserted directly into the various organs under real-time EUS imaging. The FV-EUS, with its ability to switch readily between endoscopic and sonographic views, diminished manipulative reorientation of the echoendoscope during EUS-FNA or EUS-RFA, greatly improving technical performance. This translated into successful attempts at EUS-FNA with a 19G needle on the liver, spleen, and kidney in all 9 dogs. Likewise, EUS-RFA with an 18G RFA needle at 50W for 1 min to the hepatic parenchyma was successfully duplicated in 5 dogs and 1 pig.

Necropsy findings revealed a well-demarcated RFA ablation zone in the hepatic parenchyma while FNA needle puncture marks were seen on the intra-abdominal organs. Therefore, the design of the FV-EUS enabled the operator to target lesions within and external to the organs with relative ease, which greatly improved the ability to perform diagnostic and therapeutic procedures. This suggests that the new EUS-RFA method is able to treat the mass of intraperitoneal solid organs by using the newly developed RFA-needle.

The improvement in intraperitoneal maneuverability of the FV-EUS results in adequate visualization of all four abdominal quadrants and intestinal loops<sup>[22]</sup>. As modern imaging techniques tend to understage around 10%-40% of GI malignancies<sup>[23,24]</sup>, peritoneoscopy with intraperitoneal FV-EUS could provide adequate minimally invasive staging of GI malignancies, especially for pancreatic and stomach cancers, prior to surgical resection. Therefore, by providing better diagnostic accuracy, the FV-EUS, with its extra ability to see through solid organs, might be a preferred substitute to staging laparoscopy in detecting peritoneal carcinomatosis and small metastatic tumors. In addition, endoscopic visualization of the anterior abdominal wall could be easily achieved by looking up to the abdominal wall rather than looking back with angled laparoscopes.

Necropsy findings in this study did not reveal any organ injury or infective complications related to the TG peritoneoscopy. Nevertheless, bacterial contamination and infection in the abdomen is a genuine concern for a gastrotomy site. Donatsky *et al.*<sup>[25]</sup> reported that in TG NOTES with over-the-scope-clip closure, intra-abdominal chronic abscesses were discovered in 3 of 10 pigs at necropsy, although all the animals survived during the study period. The study concluded that peritoneal contamination did occur, which warranted implementation other than the use of single-dose prophylactic anti-

biotics to prevent infective complications. In contrast, a study by Narula *et al.*<sup>[26]</sup> revealed that despite the presence of contamination measured by an increase in the bacterial colony-forming units, no clinically significant spillage into the peritoneum that resulted in abscess formation was seen. These conflicting results would require further evaluation to prevent post-gastrotomy septic complications, including peritonitis.

The effectiveness of current suture techniques and the perforation risk following closure of the gastrotomy site remain unsettled issues. Unsatisfactory closure of the transluminal access site has resulted in several animal cases of microabscesses, peritonitis, and death<sup>[27]</sup>. Although the endoclips used in this study did not give rise to any adverse complications, mucosal closure with endoclips has been shown unreliable and it could result in substantial air and gastric fluid leakage<sup>[28]</sup>. The presence of tissue edema and widely opposing incisional edges considerably impede satisfactory tissue approximation. Without achieving full thickness closure, the potential for gastric fluid leakage and spontaneous perforation risk definitely exist. Until now, the unavailability of a simple and safe closure technique continued to impede the progress of NOTES procedure.

This study was limited in its small number of animal cases, as well the substantial difference in porcine and canine abdominal anatomy that may limit the relevance of the study findings in relation to clinical human applicability. Despite the limitation, this animal study is the first report that suggested the possibility of FV-EUS in NOTES procedures and show that the FV-EUS was very efficient as a modality of NOTE interventions. It is possible to go beyond the gut wall with the FV-EUS from intraluminal to intraperitoneal EUS evaluation, enabling freedom to assess many areas within the abdominal cavity, including the pelvic region. Armed with a sonographic window, extraluminal peritoneoscopic evaluation with the FV-EUS would enable assessment beyond visual inspection by providing views and accessibility to lesions within solid intraperitoneal organs and structures, thereby, broadening the appeal of NOTES peritoneoscopic interventional procedures to the endoscopist.

In conclusion, TG NOTES combined with EUS-guided peritoneoscopic interventions and intraperitoneal, as well as intraluminal, EUS could be achieved with the FV-EUS. This study ably demonstrated the utility and success of FV-EUS in both diagnostic and therapeutic peritoneoscopic interventions in animal models, which adds to the growing armamentarium available for NOTES procedures. Even though concerns remain, embracing this strategy is essential for further development of EUS-guided NOTES interventions.

## ACKNOWLEDGMENTS

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## COMMENTS

## Background

Natural orifice transluminal endoscopic surgery (NOTES) is a new surgical technique and it has the potential to offer less operation related complications. However, the therapeutic transgastric (TG) approach to access the peritoneal cavity has inherent risks, such as bleeding and adjacent organ injury. The endoscopic ultrasound (EUS) has been used to avoid the risk of injury to extraluminal structures and oblique-viewing, curved, linear-array echoendoscope (OV-EUS) have been used in NOTES interventions. Recently, the forward-viewing endoscopic ultrasound (FV-EUS) was developed and successfully tested in EUS-guided interventions. The FV-EUS is regarded as an ergonomic and viable endoscopic modality to perform TG peritoneoscopic interventions via NOTES. However, peritoneoscopic NOTES interventions through FV-EUS have never been discussed.

## Research frontiers

The FV-EUS was developed to overcome the limitations of OV-EUS which provides oblique-viewing images different from the direction of the echoendoscopic movement. Several experimental studies through FV-EUS have been feasible for a variety of EUS-interventions, such as the drainage of pancreatic pseudocysts, EUS-FNA, and celiac plexus neurolysis.

## Innovations and breakthroughs

FV-EUS has not been used previously for NOTES access. In their animal experiments, the authors aim to investigate the use of the FV-EUS for the performance of standard NOTES interventions. This study ably demonstrates the utility and success of FV-EUS in both diagnostic and therapeutic peritoneoscopic interventions, and it suggests that FV-EUS can improve safety of the NOTES access to the peritoneal cavity. In conclusion, this animal study is the first report that suggested the possibility of FV-EUS in NOTES procedures and show that the FV-EUS was very efficient as a modality of NOTES interventions.

## Applications

Armed with a sonographic window, extraluminal peritoneoscopic evaluation with the FV-EUS would enable assessment beyond visual inspection by providing views and accessibility to lesions within various intraperitoneal structures. Therefore, FV-EUS will broaden the prospects of NOTES interventions to endoscopists and gastroenterologists, and the NOTES interventions through a FV-EUS might be performed in the various conditions.

## Terminology

NOTES: NOTES is an experimental surgical technique. NOTES reaches the target organ by inserting the endoscope through a natural orifice (e.g., mouth, anus, vagina, or urethra) and entering the peritoneal cavity by making an incision on the luminal wall, thus avoiding any external incisions. FV-EUS: FV-EUS has both an forward endoscopic view and a sonographic view, plus a working channel in alignment with the endoscope shaft. It is able to deploy needles and other accessory devices along the axis of the scope, and has a wider angulation range of the tip.

## Peer review

The authors performed the first animal study of NOTES procedures using forward view EUS guidance. The study confirm the feasibility of FV-EUS guided NOTES. It is an interesting study that provide important information on future studies of NOTES under FV-EUS guidance.

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## Endoscopic papillary large balloon dilation in patients with periampullary diverticula

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### Abstract

**AIM:** To evaluate the safety and effectiveness of endoscopic papillary large balloon dilation (EPLBD) for bile duct stone extraction in patients with periampullary diverticula.

**METHODS:** The records of 223 patients with large common bile duct stones ( $\geq 10$  mm) who underwent EPLBD (12-20 mm balloon diameter) with or without limited endoscopic sphincterotomy (ES) from July 2006 to April 2011 were retrospectively reviewed. Of these patients, 93 (41.7%) had periampullary diverticula (PAD), which was categorized into three types. The clinical variables of EPLBD with limited ES (EPLBD + ES) and EPLBD alone were analyzed according to the presence of PAD.

**RESULTS:** Patients with PAD were significantly older than those without ( $75.2 \pm 8.8$  years *vs*  $69.7 \pm 10.9$  years,  $P = 0.000$ ). The rates of overall stone removal and complete stone removal in the first session were not significantly different between the PAD and non-PAD groups, however, there was significantly less need for mechanical lithotripsy in the PAD group (3.2% *vs*

11.5%,  $P = 0.026$ ). Overall stone removal rates, complete stone removal rates in the first session and the use of mechanical lithotripsy were not significantly different between EPLBD + ES and EPLBD alone in patients with PAD (96.6% *vs* 97.1%; 72.9% *vs* 88.2%; and 5.1% *vs* 0%, respectively). No significant differences with respect to the rates of pancreatitis, perforation, and bleeding were observed between EPLBD + ES and EPLBD alone in the PAD group (3.4% *vs* 14.7%,  $P = 0.095$ ; 0% *vs* 0%; and 3.4% *vs* 8.8%,  $P = 0.351$ , respectively).

**CONCLUSION:** EPLBD with limited ES and EPLBD alone are safe and effective modalities for common bile duct stone removal in patients with PAD, regardless of PAD subtypes.

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**Key words:** Endoscopic papillary large balloon dilation; Endoscopic sphincterotomy; Periampullary diverticula

**Core tip:** Endoscopic papillary large balloon dilation (EPLBD) is a highly effective technique for treating difficult bile duct stones. However, the safety of EPLBD is of concern, especially in patients with periampullary diverticula (PAD). In the present study, the clinical outcomes and complications of EPLBD with limited endoscopic sphincterotomy (ES) (EPLBD + ES) and EPLBD alone according to the presence of PAD were not significantly different. We suggest that EPLBD + ES and EPLBD alone are safe and feasible modalities for large bile duct stone removal in patients with PAD. Furthermore, the presence of PAD was not found to affect therapeutic outcomes.

Kim KH, Kim TN. Endoscopic papillary large balloon dilation in patients with periampullary diverticula. *World J Gastroenterol* 2013; 19(41): 7168-7176 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i41/7168.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7168>

## INTRODUCTION

Although endoscopic sphincterotomy (ES) is the standard therapy for choledocholithiasis, it has been reported to produce several serious complications, such as bleeding and perforation<sup>[1-3]</sup>. Endoscopic papillary balloon dilation (EPBD), which was introduced by Staritz *et al*<sup>[4]</sup>, has been used as an alternative procedure for the removal of common bile duct (CBD) stones. However, several cases of post-procedural pancreatitis and two deaths associated with EPBD have been documented<sup>[5]</sup>. Furthermore, it has been reported that mechanical lithotripsy is more frequently required during EPBD than during ES<sup>[6,7]</sup>.

Some bile duct stones are difficult to remove due to a large size, a rectangular shape, or anatomical difficulties, and these remain challenges for endoscopists despite advancements in expertise and the developments of various accessories. Several recent series have demonstrated that endoscopic papillary large balloon dilation (EPLBD) combined with limited endoscopic sphincterotomy (EPLBD + ES) has a similar therapeutic effect, but a lower complication rate than standard ES for the removal of difficult bile duct stones<sup>[8-12]</sup>. In particular, anatomical variations, such as periampullary diverticula (PAD), which can influence endoscopic outcomes because of the high risk of procedure-associated complications. Duodenal diverticula are outpouchings of mucosa, submucosa, and partially speckled muscle along the intestinal wall. Reported locations and incidences of PAD in the general population vary<sup>[13-18]</sup>. PAD is known to be associated with an increased frequency of pancreatobiliary diseases, and it is widely accepted that the presence of diverticula can be a technical obstacle to cannulation and requires skillful endoscopy manipulation<sup>[13,14]</sup>. However, recent two studies have shown that rates of successful cannulation are not dependent on the presence of diverticula<sup>[17,19]</sup>.

Nevertheless, when EPLBD is performed in patients with PAD, the potential risks of perforation and bleeding are of concern, because the ampullary area in PAD is composed of thin mucosa without sphincter muscle. Recent studies have indicated that EPLBD without ES is as effective and safe as ES for the removal of large bile duct stones, regardless of the presence of PAD<sup>[20-22]</sup>. Accordingly, the aim of this study was to evaluate the technical feasibility and safety of EPLBD with or without ES for the removal of CBD stones in patients with PAD.

## MATERIALS AND METHODS

The records of 223 patients with CBD stones  $\geq 10$  mm in diameter who underwent EPLBD combined with antecedent ES (EPLBD + ES) or EPLBD alone (EPLBD - ES) for the removal of bile duct stones from July 2006 to April 2011 were retrospectively reviewed. Patients with a history of endoscopic sphincterotomy or Roux-

en-Y gastrojejunostomy were excluded. All procedures were performed using side-viewing endoscopes (TJF-240; Olympus Optical Corporation, Tokyo, Japan). Endoscopic retrograde cholangiopancreatography (ERCP) was performed by experienced endoscopists at a single center. Cannulation was attempted using an ERCP catheter or a pull-type sphincterotome. When conventional cannulation failed, a pre-cut technique using a needle knife was applied. EPLBD was performed using a dilating balloon catheter (CRE balloon, Boston Scientific Cork, Ireland) positioned at the center of the balloon across the ampullary orifice (Figure 1). Dilating balloon catheters with a diameter of 12-20 mm were used. Ballooning size was determined based on stone sizes and CBD diameter, but should not exceed 2 mm of the diameter of the distal CBD. Balloons were inflated with caution until balloon notches disappeared. Mechanical lithotripsy was attempted when stones were too difficult to remove intact. When incomplete stone removal was suspected, a nasobiliary tube or a plastic stent was placed to prevent cholangitis. Complete stone removal was confirmed either by cholangiogram at the end of each procedure or by follow-up cholangiogram through a nasobiliary tube. The presence and types of diverticula were documented. Duodenal diverticula were categorized into three subtypes based on the locations of the major papilla with respect to diverticula: (1) type 1, when papilla was located inside the diverticulum; (2) type 2, when papilla was located in the margin of the diverticulum; and (3) type 3, when papilla was located outside the diverticulum (Figure 2). Patients were thoroughly observed for possible complications including bleeding, pancreatitis, and perforation during and after ERCP. Post-ERCP pancreatitis was defined as a serum amylase level exceeding three times the upper normal limit and the development of abdominal pain after ERCP. Hyperamylasemia was defined as a serum amylase level exceeding three times the normal upper limit without any abdominal pain. Post-ERCP bleeding was classified as major or minor based on amounts of hemorrhage. Major bleeding was defined as moderate to severe hemorrhage necessitating transfusion or intervention, and minor bleeding was defined as mild hemorrhage not requiring transfusion. Clinical and endoscopic factors were retrospectively evaluated. The study was approved by the institutional review board of our hospital.

### Statistical analysis

Statistical analysis was carried out using the Student's *t* test, the  $\chi^2$  test, and one-way ANOVA in SPSS version 17.0 (SPSS, Inc., Chicago, IL, United States). A *P* value < 0.05 was considered statistically significant.

## RESULTS

Demographic characteristics of the 223 patients (122 men, 101 women; mean age  $72.0 \pm 10.4$  years) are presented in Table 1. The incidence of PAD was 41.7% (93/223). The mean age was significantly higher in the



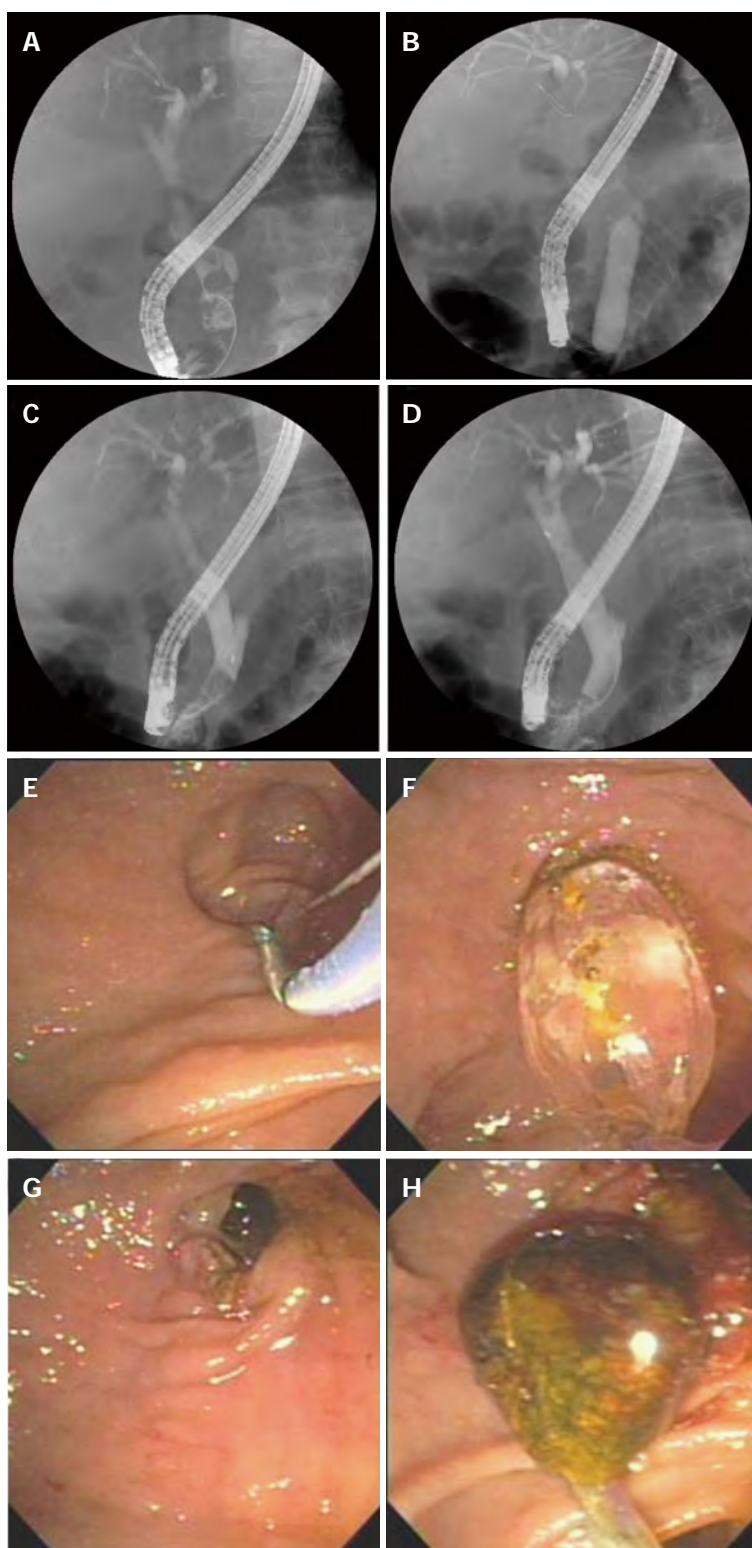
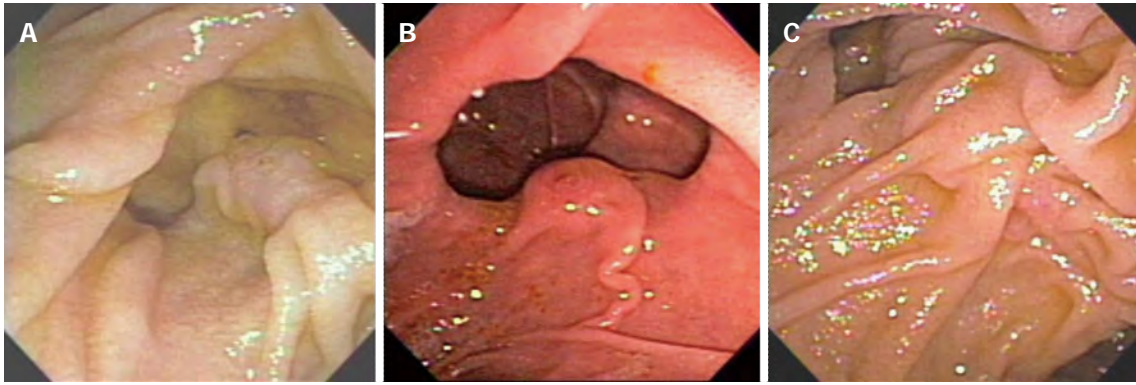


Figure 1 Endoscopic papillary large balloon dilation in a patient with periampullary diverticulum. A: Cholangiogram showing multiple movable filling defects in the common bile duct; B: Fluoroscopic view showing the disappearance of balloon waist after gradual inflation with contrast media; C: Cholangiogram showing a large stone captured in a basket; D: Cholangiogram showing no residual filling defects in the common bile duct; E: Endoscopic sphincterotomy was carefully performed due to the presence of a huge diverticulum; F: Endoscopic view showing a dilating balloon inflated to 15 mm; G: Endoscopic view showing an enlarged ampullary opening; H: Endoscopic view showing a large brown pigment stone being extracted with a basket.

PAD group ( $75.2 \pm 8.8$  years *vs*  $69.7 \pm 10.9$  years,  $P = 0.000$ ), and no difference was found between genders. Of the 93 cases of diverticula, 18 cases (19.3%) were

of type 1, 41 cases (44.0%) were of type 2, and 34 cases (36.7%) were of type 3. Billroth II gastrectomy state was documented in 7 patients (7.5%) in the PAD group



**Figure 2** Endoscopic classification of periampullary diverticula. A: Type 1 with major papilla located inside the diverticulum; B: Type 2 with major papilla located in the margin of the diverticulum; C: Type 3 with major papilla located outside the diverticulum.

**Table 1** Baseline characteristics of patients *n* (%)

Variable	Total ( <i>n</i> = 223)	PAD group ( <i>n</i> = 93)	Non-PAD group ( <i>n</i> = 130)	<i>P</i> value
Mean age (yr)	72.0 ± 10.4 (39-92)	75.2 ± 8.8 (51-92)	69.7 ± 10.9 (39-90)	0.000
Sex (male/female)	122:101 (54.7:45.3)	46:47 (49.5:50.5)	76:54 (58.5:41.5)	0.220
BMI (kg/m <sup>2</sup> )	22.6 ± 3.1	22.5 ± 2.8	22.6 ± 3.3	0.947
Billroth II gastrectomy	26 (11.7)	7 (7.5)	19 (14.6)	0.138
Large balloon dilation				
With ES	132 (59.2)	59 (63.4)	73 (56.2)	0.334
Without ES	91 (40.8)	34 (36.6)	57 (43.8)	
Precutting with needle knife	25 (11.2)	9 (9.7)	16 (12.3)	0.668
CBD stones				
Mean diameter of stone (mm)	15.9 ± 5.8 (10-40)	16.4 ± 6.0 (10-37)	15.5 ± 5.7 (10-40)	0.252
Number of stones				
1/2/≥ 3	119/35/68	49/15/29	70/20/39	0.835
Types of stones				
Brown/black/cholesterol	182/33/2 (83.9/15.2/0.9)	82/9/0 (90.1/9.9/0)	100/24/2 (79.4/19.0/1.6)	0.144
Mean diameter of CBD (mm)	19.9 ± 5.3 (10-37)	20.0 ± 5.0 (10-35)	19.8 ± 5.4 (10-37)	0.743
Distal CBD angulation (degree)	140.6 ± 21.9	142.1 ± 21.8	139.4 ± 22.0	0.444
Balloon dilation				
Dilating balloon size (mm)	15.6 ± 2.4 (12-20)	15.5 ± 2.4 (12-20)	15.8 ± 2.4 (12-20)	0.374
Duration of ballooning (s)	38.3 ± 16.1(10-60)	36.1 ± 15.0 (10-60)	39.9 ± 16.8 (10-60)	0.081

Values are presented as mean ± SD (range). PAD: Periampullary diverticula; BMI: Body mass index; ES: Endoscopic sphincterotomy; CBD: Common bile duct.

and in 19 patients (14.6%) in the non-PAD group ( $P = 0.138$ ). The frequencies of EPLBD + ES and EPLBD - ES were not significantly different between the PAD and non-PAD groups (EPLBD + ES, 63.4% *vs* 56.2%; and EPLBD - ES, 36.6% *vs* 43.8%,  $P = 0.334$ , respectively). The mean stone size in the 223 patients was  $15.9 \pm 5.8$  mm (range, 10-40 mm), the mean bile duct diameter was  $19.9 \pm 5.3$  mm (range, 10-37 mm), and the mean dilated balloon size was  $15.6 \pm 2.4$  mm (range, 12-20 mm). The mean stone(s) and CBD diameters were not significantly different between the PAD and non-PAD groups ( $16.4 \pm 6.0$  mm *vs*  $15.5 \pm 5.7$  mm,  $P = 0.252$ ; and  $20.0 \pm 5.0$  mm *vs*  $19.8 \pm 5.4$  mm,  $P = 0.743$ , respectively). Mean balloon diameter for EPLBD was  $15.5 \pm 2.4$  mm in the PAD group and  $15.8 \pm 2.4$  mm in the non-PAD group ( $P = 0.374$ ).

Of the 223 patients, stone removal was completed in 96.9% (216/223). The rates of overall stone removal and stone removal in the first session were not significantly

different between the PAD and non-PAD groups [90/93 (96.8%) *vs* 126/130 (96.9%),  $P = 1.000$ ; and 73/93 (78.5%) *vs* 93/130 (71.5%),  $P = 0.277$ , respectively], but the frequency of mechanical lithotripsy was significantly lower in the PAD group [3/93 (3.2%) *vs* 15/130 (11.5%),  $P = 0.026$ ]. When the PAD and non-PAD groups were further divided by EPLBD + ES or EPLBD - ES, no significant differences with respect to overall stone removal rates, stone removal rates in the first session or needs for mechanical lithotripsy were observed [57/59 (96.6%) *vs* 33/34 (97.1%),  $P = 1.000$ ; 43/59 (72.9%) *vs* 30/34 (88.2%),  $P = 0.016$ ; and 3/59 (5.1%) *vs* 0 (0%),  $P = 0.297$ , respectively, Table 2]. Sub-analysis by PAD type revealed no significant difference with regard to age, stone size, CBD diameter, distal CBD angle, dilated balloon size, or duration of ballooning (Table 3). When comparing types of PAD, overall stone removal rates, complete stone removal rates in the first session, and the use of mechanical lithotripsy were not significantly different (Table 3).

**Table 2 Comparison of outcomes between the two groups *n* (%)**

	PAD group ( <i>n</i> = 93)			Non-PAD group ( <i>n</i> = 130)			<i>P</i> value <sup>1</sup>
	EPLBD + ES ( <i>n</i> = 59)	EPLBD - ES ( <i>n</i> = 34)	<i>P</i> value	EPLBD + ES ( <i>n</i> = 73)	EPLBD - ES ( <i>n</i> = 57)	<i>P</i> value	
Overall stone removal	57 (96.6)	33 (97.1)	1.000	72 (98.6)	54 (94.7)	0.319	1.000
Complete stone removal in 1 <sup>st</sup> session	43 (72.9)	30 (88.2)	0.116	51 (69.9)	42 (73.7)	0.632	0.277
Mechanical lithotripsy	3 (5.1)	0 (0.0)	0.297	9 (12.3)	6 (10.5)	0.750	0.026

<sup>1</sup>Comparison between the periampullary diverticula (PAD) and non-PAD groups. EPLBD: Endoscopic papillary large balloon dilation; ES: Endoscopic sphincterotomy; EPLBD+ES: Endoscopic papillary large balloon dilation with limited endoscopic sphincterotomy; EPLBD-ES: Endoscopic papillary large balloon dilation without endoscopic sphincterotomy.

**Table 3 Baseline characteristics and outcomes of patients by periampullary diverticula type *n* (%)**

	Type 1 ( <i>n</i> = 18)	Type 2 ( <i>n</i> = 41)	Type 3 ( <i>n</i> = 34)	<i>P</i> value
Mean age (yr)	75.9 ± 6.7	76.5 ± 8.7	73.2 ± 9.9	0.268
Large balloon dilation				
With ES	9 (50.0)	27 (65.9)	23 (67.6)	0.257
Without ES	9 (50.0)	14 (34.1)	11 (32.4)	
Mean diameter of stone (mm)	17.8 ± 6.7	15.9 ± 5.5	16.1 ± 6.2	0.514
Mean diameter of CBD (mm)	20.8 ± 4.9	20.5 ± 5.6	19.0 ± 4.3	0.324
Distal CBD angulation (degree)	141.8 ± 9.6	143.6 ± 29.0	140.5 ± 16.6	0.869
Balloon dilation				
Dilating balloon size (mm)	14.7 ± 2.5	15.8 ± 2.4	15.5 ± 2.4	0.261
Duration of ballooning (s)	30.6 ± 12.6	36.3 ± 12.8	38.8 ± 17.9	0.165
Overall stone removal	17 (94.4)	41 (100)	32 (94.1)	0.698
Complete stone removal in 1 <sup>st</sup> session	14 (77.8)	33 (80.5)	26 (76.5)	0.912
Mechanical lithotripsy	1 (5.6)	1 (2.4)	1 (2.9)	0.679

Values are presented as mean ± SD. Type 1: When major papilla is located inside the diverticulum, Type 2: In the margin of the diverticulum; and Type 3: Outside the diverticulum. PAD: Periampullary diverticula; ES: Endoscopic sphincterotomy; CBD: Common bile duct.

**Table 4 Comparison of complications between the two groups *n* (%)**

	PAD ( <i>n</i> = 93)			Non-PAD ( <i>n</i> = 130)			<i>P</i> value <sup>1</sup>
	EPLBD + ES ( <i>n</i> = 59)	EPLBD - ES ( <i>n</i> = 34)	<i>P</i> value	EPLBD + ES ( <i>n</i> = 73)	EPLBD - ES ( <i>n</i> = 57)	<i>P</i> value	
Pancreatitis	2 (3.4)	5 (14.7)	0.095	7 (9.6)	5 (8.8)	0.873	0.809
Perforation	0 (0.0)	0 (0.0)	NA	0 (0.0)	1 (1.8)	0.438	1.000
Bleeding	2 (3.4)	3 (8.8)	0.351	7 (9.6)	4 (7.0)	0.601	0.548
Major	0 (0.0)	0 (0.0)		1 (1.4)	0 (0.0)		
Minor	2 (13.4)	3 (8.8)		6 (8.2)	4 (7.0)		
Hyperamylasemia	4 (6.8)	5 (14.7)	0.279	15 (20.5)	6 (10.5)	0.123	0.232
Mortality	0 (0.0)	0 (0.0)	NA	1 (1.4)	1 (1.8)	1.000	0.230

<sup>1</sup>Comparison of the periampullary diverticula (PAD) and non-PAD groups. EPLBD: Endoscopic papillary large balloon dilation; ES: Endoscopic sphincterotomy; EPLBD + ES: Endoscopic papillary large balloon dilation with limited ES; EPLBD - ES: Endoscopic papillary large balloon dilation without ES; NA: Not applicable.

Procedure-related complications are listed in Table 4. The rates of post-ERCP pancreatitis, perforation, and bleeding were not significantly different between the PAD and non-PAD groups [7/93 (7.5%) *vs* 12/130 (9.2%), *P* = 0.809; 0% *vs* 1/130 (0.8%), *P* = 1.000; and 5/93 (5.4%) *vs* 11/130 (8.5%), *P* = 0.548, respectively]. When complications of EPLBD with or without ES were compared in the PAD group, the rates of pancreatitis, perforation, and bleeding did not differ significantly [2/59 (3.4%) *vs* 5/34 (14.7%), *P* = 0.095; 0% *vs* 0%; and 2/59 (3.4%) *vs* 3/34 (8.8%), *P* = 0.351, respectively]. Moreover, complication rates were not significantly different for PAD subtypes (Table 5). All cases of pancreatitis were mild and they

were treated conservatively. There were 16 (7.1%) bleeding cases, including one case (0.4%) of major bleeding and 15 cases (6.7%) of minor bleeding. The one major hemorrhage, which required more than 5 pints of blood, occurred in the non-PAD group. This patient was referred for emergency surgery for bleeding control and was managed effectively, with no further consequence. In addition, there were two in-hospital deaths in the non-PAD group. One in the EPLBD - ES group was caused by retroperitoneal perforation directly attributed to ERCP, and the other occurred in cirrhotic patients with fulminant hepatic failure and multi-organ failure due to sepsis in the EPLBD + ES group.



**Table 5** Complications of endoscopic papillary large balloon dilation by periampullary diverticula subtype *n* (%)

	PAD subtype ( <i>n</i> = 93)			<i>P</i> value
	Type 1 ( <i>n</i> = 18)	Type 2 ( <i>n</i> = 41)	Type 3 ( <i>n</i> = 34)	
Pancreatitis	1 (5.6)	4 (9.8)	2 (5.9)	0.913
Perforation	0 (0.0)	0 (0.0)	0 (0.0)	NA
Bleeding				
Major	0	0	0 (0.0)	NA
Minor	1 (5.6)	2 (4.9)	2 (5.9)	0.930
Hyperamylasemia	0 (0.0)	5 (12.2)	4 (11.8)	0.240
Mortality	0 (0.0)	0 (0.0)	0 (0.0)	NA

Type 1: When major papilla is located inside the diverticulum; Type 2: In the margin of the diverticulum; and Type 3: Outside the diverticulum. EPLBD: Endoscopic papillary large balloon dilation; PAD: Periampullary diverticula; NA: Not applicable.

## DISCUSSION

Currently, EPLBD combined with limited ES (EPLBD + ES) is regarded as an effective modality for treating difficult common bile duct stones. Complications, such as, hemorrhage and perforation, have been reported to be less frequent in EPLBD + ES than in standard ES<sup>[9-12]</sup>. Furthermore, mechanical lithotripsy is less required during EPLBD + ES, because it provides spacious ampullary opening, and thus, facilitates complete bile duct stone removal. More recently, it has been suggested that EPLBD without ES is as safe and effective as ES for the removal of large bile duct stones<sup>[20-22]</sup>. Nevertheless, the safety of EPLBD with or without antecedent ES is still a matter of concern. In particular, risks of complications might be greater in cases of PAD.

Most periampullary diverticula are asymptomatic and are found incidentally during ERCP<sup>[15]</sup>. Although duodenal diverticula are known to be acquired lesions, the etiology of PAD has not been established<sup>[18]</sup>. Several studies have mentioned that PAD is rarely found in patients younger than 40 years old, and that the incidence of PAD shows an increasing tendency with age<sup>[15,17,18,23]</sup>. In the present study, the mean age of patients in the PAD group was significantly higher than in the non-PAD group ( $75.2 \pm 8.8$  years *vs*  $69.7 \pm 10.9$  years,  $P = 0.000$ ), which concurs with previous reports and supports a substantive relationship between age and the formation of PAD<sup>[15,17,18,23]</sup>. However, previous studies have reported a prevalence of PAD at ERCP ranging from 5% to 32%, whereas the prevalence of PAD in the present study was higher at 41.7%, which might be explained by the relatively high proportion of older individuals in the present study<sup>[13-18]</sup>.

It is known that PAD represents a technical barrier during ERCP, and that the rate of cannulation failure is higher in PAD patients than in non-PAD patients. Several studies have addressed the influence of PAD on the technical difficulties of ERCP and on complications, and it appears that endoscopist skill, diverticula size, the

location of ampulla with respect to the diverticulum, CBD angulation, bowel motility, and patient cooperation contribute to the technical success of cannulation<sup>[16,17]</sup>. In order to facilitate cannulation of the bile duct in patients with PAD, a number of clever techniques, such as precutting with a needle knife and delicate handling of the scope, have been tried. Several series have reported successful cannulation rates ranging from 94.2% to 97.0%, complete stone removal in the first session ranging from 69.9% to 76.2%, and overall stone removal in up to 95.2% of patients with PAD<sup>[15,17,24,25]</sup>. In the present study, the rates of overall CBD stone removal and complete stone removal in the first session did not differ significantly between the PAD and non-PAD groups, which concurs with previous studies<sup>[17,25]</sup>. Moreover, overall stone removal and complete stone removal rates in the first session were not significantly different between PAD subtypes in the present study, which suggests that PAD types do not influence the clinical outcomes of EPLBD.

Mechanical lithotripsy is a rather labor-intensive but necessary technique for removing difficult stones, although EPLBD + ES reportedly reduces the need for mechanical lithotripsy<sup>[25,26]</sup>. Lowering the frequency of mechanical lithotripsy is important to prevent recurrent duct stones because remnant stone fragments following lithotripsy can act as nidi for stone recurrence. Theoretically, if EPLBD with a large-diameter balloon (12-20 mm) is applied, mechanical lithotripsy might be less required than after EPBD with a small-sized balloon ( $\leq 10$  mm). In the present study, mechanical lithotripsy was used significantly less in the PAD group than in the non-PAD group (3.2% *vs* 11.5%,  $P = 0.026$ ). Furthermore, when EPLBD alone was performed, bile duct stones were successfully removed without mechanical lithotripsy in the PAD group. However, when EPLBD + ES was applied, mechanical lithotripsy was required for three patients (5.1%) in the PAD group, although this did not represent a significant difference. One possible explanation of these results is that ampullary enlargement can easily be achieved by EPLBD alone because of the lack of sphincter muscle in patients with PAD, which may explain the reduced need for mechanical lithotripsy after EPLBD without ES. Furthermore, ease of ampullary widening by EPLBD could explain the tendency toward a shorter ballooning time in the PAD group. These findings suggest that EPLBD - ES could be an appropriate technique for CBD stone retrieval in the presence of PAD, as long as safety is guaranteed.

Post ERCP pancreatitis, perforation and bleeding are the most important complications related to EPLBD. According to the present study and previous reports, ERCP-related complication rates are similar in patients with or without PAD and for different PAD types<sup>[16,24,25]</sup>. Pancreatitis is the most concerning complication, and EPBD is not widely recommended for stone removal due to the possibility of severe pancreatitis<sup>[5,7]</sup>. This increased risk of pancreatitis is associated with an im-

pairment of pancreatic duct flow or direct pancreatic damage caused by physical compression during balloon dilation<sup>[3,26]</sup>. Although the pathogenesis of pancreatitis following EPLBD is not clear, it is suggested that ES prior to EPLBD could prevent potential injury of the main pancreatic duct, because ES can steer the direction of balloon dilation toward the CBD and minimize the pressure overload on the pancreatic orifice<sup>[8,20-22]</sup>. However, the recent studies have proposed that EPLBD alone can be an alternative for the removal of large stones<sup>[20-22]</sup>. Repeated cannulation attempts and excessive contrast injection due to anatomical difficulties could be major culprits of post-ERCP pancreatitis in patients with PAD. However, in the present study, no significant difference was observed in rates of post-ERCP pancreatitis between the PAD and non-PAD groups (7.5% *vs* 9.2%,  $P = 0.809$ ) or among types of PAD (5.6% *vs* 9.8% *vs* 5.9% for types 1, 2, and 3, respectively,  $P = 0.913$ ). Furthermore, the rate of post-ERCP pancreatitis in the PAD group (7.5%) was lower in the present study than in previous studies (9.6%-14.5%), which could have been due to cautious and gentle cannulation<sup>[24,25]</sup>. The relatively high percentage of older patients in this study might be associated with the observed lower incidence of pancreatitis<sup>[21]</sup>. In particular, it has been suggested that longstanding CBD stones can cause gradual bile duct dilation, and subsequently, a patulous ampullary orifice<sup>[22]</sup>. In a recent study, a very low incidence of pancreatitis (1.4%) was observed following EPLBD in patients with recurrent CBD stones after ES, which is similar to the pathophysiology of the process of patulous ampulla<sup>[27]</sup>.

Perforation is one of the most serious complications associated with EPLBD. Hypothetically, the risk of perforation should be higher in patients with PAD due to lack of sphincter muscle components around the ampulla. Balloon diameter and duration of ballooning are the important parameters for perforation after EPLBD. In most previous studies, balloon dilators sized from 12 to 20 mm were applied for 10 to 60 seconds, and in the present study, balloon dilators not exceeding CBD diameters were used and balloons were dilated gradually to minimize the risk of perforation<sup>[25-27]</sup>. As a result, the overall incidence of perforation was 0.4% and the risk of perforation was found to be not higher in the presence of PAD. In fact, only one case of perforation occurred in the non-PAD group. The size of sphincterotomy is another issue to consider before EPLBD. In the present study, after deep cannulation, the length of cutting was adjusted according to the locations of the ampulla and the bile duct axis. Ampullary distension without mucosal rupture around the ampulla can be obtained by gradual inflation and ballooning to an appropriate size. Because the weakest point is vulnerable to rupture by the radial forces produced by ballooning, overdilation of the ampulla exceeding CBD diameter should be avoided to prevent perforation, especially in cases of biliary stricture<sup>[27]</sup>.

Bleeding is another complication and is possibly related to excessive ampullary dilation. Theoretically, bleeding

risk can be increased if ES is performed. Previous studies have reported incidences of bleeding after EPLBD + ES ranging from 0% to 9%<sup>[5,8-12,25]</sup>. However, recent studies involving EPLBD without ES have reported lower rates of post-ERCP bleeding (0%-2.4%), which suggests that EPLBD without ES is suitable in patients with coagulopathies<sup>[20,21]</sup>. In the present study, we experienced one episode of major bleeding after sphincterotomy in the non-PAD group. It is presumed that most cases of post-ERCP bleeding are associated with small caliber vessels surrounding ampulla that are liable to be injured by radial pressure caused by balloon dilation, whereas large vessels are so elastic that they tend to be repelled by gradual balloon dilation<sup>[27]</sup>. The rates of adverse events in patients with PAD were acceptable in the present study.

In conclusion, EPLBD combined with limited ES and EPLBD alone appear to be safe and effective modalities for CBD stone removal in patients with PAD. PAD is commonly found in patients undergoing ERCP, and this study shows that the presence of PAD does not affect therapeutic outcome. Furthermore, an incidental finding of PAD presents no additional technical challenge to the achievement of successful EPLBD. This result complements those of previous studies on the management of CBD stones in patients with PAD. Nevertheless, because the present study is limited by its retrospective nature and a relatively small cohort, a large prospective study is needed to analyze the clinical feasibility of EPLBD with or without limited ES in patients with PAD.

## COMMENTS

### Background

Periampullary diverticula (PAD) may influence endoscopic outcomes because of the high risk of complications associated with anatomical variations. Endoscopic papillary large balloon dilation (EPLBD) is a useful method to remove difficult common bile duct (CBD) stones. However, the effectiveness and safety of this procedure in patients with PAD is not fully established. Accordingly, authors conducted this study to investigate the feasibility and safety of EPLBD with or without ES for the removal of CBD stones in patients with PAD.

### Research frontiers

It has been reported that 10%-15% of bile duct stones are difficult to retrieve by conventional techniques. Challenging cases of stone removal are as follows: a rectangular shape, a large size (> 15 mm), Billroth-II gastrectomy, Roux-en-Y gastrojejunostomy, and PAD. PAD can infrequently present a technical barrier during endoscopic retrograde cholangiopancreatography (ERCP). In particular, diverticula size and the location of ampulla may pose technical difficulties associated with ERCP and complications. Much conflicting data regarding the technical success and complications of ERCP have been published, and recently, EPLBD has been introduced for CBD stone removal in patients with PAD.

### Innovations and breakthroughs

Endoscopic papillary large balloon dilation following limited ES (EPLBD+ES) by Ersoz *et al* has been advocated as an alternative to ES for removal of large CBD stones. According to a few recent reports, EPLBD + ES showed a similar therapeutic effect but a lower complication rate than ES. EPLBD + ES requires mechanical lithotripsy less often due to enlargement of the ampullary opening. However, when EPLBD is performed in patients with PAD, the potential risks of perforation and hemorrhage are of particular concern. Nevertheless, several recent studies have suggested that EPLBD without ES is easy to perform for the removal of large bile duct stones, with similar therapeutic outcomes.

### Applications

EPLBD combined with limited ES and without ES were found to be safe and

effective for treating difficult CBD stones in patients with PAD. An incidental finding of PAD presents no additional technical challenge to the achievement of successful EPLBD.

### Terminology

EPLBD + ES is defined as endoscopic papillary balloon dilation (usually  $\geq 12$  mm in diameter) after limited sphincterotomy, using a dilating balloon catheter (CRE Balloon, Boston Scientific Cork, Ireland). The balloon is positioned across the orifice of ampulla, gradually inflated up to an appropriate size. Duodenal diverticula (PAD) are categorized into three subtypes based on the locations of the major papilla: (1) type 1, when papilla was located inside the diverticulum; (2) type 2, in the margin of the diverticulum; and (3) type 3, located outside the diverticulum

### Peer review

This retrospective study was conducted to investigate the technical feasibility and safety of EPLBD for the removal of difficult CBD stones in patients with PAD. The authors conclude that EPLBD + ES and EPLBD alone appear to be safe and effective procedures for CBD stone removal in patients with PAD. This result is encouraging and provides valuable information for other researchers.

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## Quantification of pancreatic exocrine function of chronic pancreatitis with secretin-enhanced MRCP

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### Abstract

**AIM:** To obtain reference values for pancreatic flow output rate (PFR) and peak time (PT) in healthy volunteers and chronic pancreatitis (CP); to correlate quantification of secretin enhanced magnetic resonance cholangiopancreatography (SMRCP) of pancreatic fluid output following secretin with fecal elastase-1 (FE-1) tests.

**METHODS:** The present study includes 53 subjects comprised of 17 healthy individuals and 36 patients with CP from April 2011 to January 2013. The 36 patients with CP were divided into three groups of mild CP ( $n = 14$ ), moderate CP ( $n = 19$ ) and advanced CP ( $n = 3$ ) by M-ANNHEIM classification for CP. Fifty-three cases underwent FE-1 test and magnetic resonance imaging using 3.0 T-device (Signa EXCITE, GE Healthcare). Coronal T<sub>2</sub>-weighted single-shot turbo spin-echo, spiratory triggered, covering the papillae, duodenum and small bowel. MRCP was performed with a heavily T<sub>2</sub>-weighted fat-suppressed long TE HASTE sequence

(thick slab 2D MRCP sequence), repeated every 2 min up to 11 min after 0.1 mL/kg secretin injection (Secrelux, Sanochemia®, Germany). FE-1 test used sandwich enzyme-linked immunosorbent assay (ELISA) test (ScheBo. Tech®, Germany).

**RESULTS:** A good linear correlation showed between the calculated volume and the actual volume by Phantom experiments. Fifty-three paired Quantification of secretin enhanced magnetic resonance cholangiopancreatography (MRCPQ) and FE-1 data sets were analyzed. The mean FE-1 of 53 cases was  $525.41 \pm 94.44$   $\mu\text{g/g}$  for 17 healthy volunteers,  $464.95 \pm 136.13$   $\mu\text{g/g}$  for mild CP,  $301.55 \pm 181.55$   $\mu\text{g/g}$  for moderate CP,  $229.30 \pm 146.60$   $\mu\text{g/g}$  for advanced CP. Also, there was statistically significant difference in FE-1 ( $P = 0.0001$ ) between health and CP. The mean values of PFR and PT were  $8.18 \pm 1.11$  mL/min,  $5.76 \pm 1.71$  min for normal;  $7.27 \pm 2.04$  mL/min,  $7.71 \pm 2.55$  min for mild CP;  $4.98 \pm 2.57$  mL/min,  $9.10 \pm 3.00$  min for moderate CP;  $4.13 \pm 1.83$  mL/min,  $12.33 \pm 1.55$  min for advanced CP. Further, statistically significant difference in PFR ( $P = 0.0001$ ) and PT ( $P = 0.0001$ ) was observed between health and CP. Besides, there was correlation ( $r = 0.79$ ) and consistency ( $K = 0.6$ ) between MRCPQ and ELISA Test. It was related between M-ANNHEIM classification and PFR ( $r = 0.55$ ), FE-1 ( $r = 0.57$ ).

**CONCLUSION:** SMRCP can provide a safe, non-invasive and efficient method to evaluate the exocrine function of the pancreas.

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**Key words:** Secretin; Magnetic resonance cholangiopancreatography; Pancreatic exocrine function; Chronic pancreatitis; Magnetic resonance imaging

**Core tip:** After all subjects were injected secretin, the results of secretin enhanced magnetic resonance chol-

angiopancreatography (SMRCP) were that the mean values of PFR and PT were  $8.18 \pm 1.11$  mL/min,  $5.76 \pm 1.71$  min for normal;  $7.27 \pm 2.04$  mL/min,  $7.71 \pm 2.55$  min for mild chronic pancreatitis (CP);  $4.98 \pm 2.57$  mL/min,  $9.10 \pm 3.00$  min for moderate CP;  $4.13 \pm 1.83$  mL/min,  $12.33 \pm 1.55$  min for advanced CP. Statistically significant difference in PFR and PT was observed between health and CP. Also, there was correlation and consistency between Quantification of secretin enhanced magnetic resonance cholangiopancreatography and enzyme-linked immunosorbent assay test. SMRCP can provide a safe, non-invasive and efficient method to evaluate the exocrine function of the pancreas.

Bian Y, Wang L, Chen C, Lu JP, Fan JB, Chen SY, Zhao BH. Quantification of pancreatic exocrine function of chronic pancreatitis with secretin-enhanced MRCP. *World J Gastroenterol* 2013; 19(41): 7177-7182 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i41/7177.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7177>

## INTRODUCTION

Pancreatic exocrine function test plays an important role of in the diagnosis of chronic pancreatitis. It can guide the clinical diagnosis and therapy. At present, the "tubed" secretin test is the standard reference method for functional investigations. Unfortunately, this test is hampered by lack of test standardization and is uncomfortable for the patient due to prolonged duodenal intubation<sup>[1,2]</sup>. Indirect pancreatic function test is non-invasive, safe and cost-effective, but it is positive only in a serious shortage of pancreatic exocrine function<sup>[3,4]</sup>. FE-1 (fecal elastase-1) test is one of the indirect pancreatic exocrine tests, and widely used in the clinical. Its role in diagnosing chronic pancreatitis is controversial<sup>[5-7]</sup>. However, previous studies have proven a positive correlation between the secretin test and FE-1, suggesting that it has a possible role in evaluating exocrine pancreatic function<sup>[8-10]</sup>.

Magnetic resonance cholangiopancreatography (MRCP) has been a reliable noninvasive method for the diagnosis of both pancreatic and biliary disease<sup>[11,12]</sup>. Magnetic resonance imaging is a noninvasive diagnostic imaging method without ionizing radiation and provides details of pancreatic parenchymal and ductal morphology with potential relevance for pancreatic exocrine function<sup>[13]</sup>.

Secretin enhanced magnetic resonance cholangiopancreatography (SMRCP) is routinely performed as part of the work up of patients with known or suspected pancreatic disease in many centers. Recently, SMRCP has gained increasing interest as a noninvasive imaging method enabling visualization and quantitative assessment of various aspects of pancreatic exocrine function<sup>[14-19]</sup>.

In this study we propose a similar quantification method using S-MRCP to evaluate pancreatic exocrine function during secretin stimulation by measuring pancreatic flow rate (PFR). Our study has both primary and

secondary purposes: (1) to obtain reference values for PFR in healthy volunteers and CP; and (2) to correlate quantification of secretin enhanced magnetic resonance cholangiopancreatography (MRCPQ) of pancreatic fluid output following secretin with FE-1 tests.

## MATERIALS AND METHODS

### Ethics

This work has been supported by Natural Science Foundation of China. This study was approved ethically by the Second Military Medical University. All patients provided informed written consent.

### Patients

From April 2011 to January 2013, the study included 53 subjects (18 females and 35 males) composed of 17 healthy volunteers and 36 patients with CP. Twelve health were male and five health were female, with a mean age of  $43.71 \pm 14.48$  years (range, 64-24 years) and a mean body mass index (BMI) of  $24.60 \pm 3.45$  kg/m<sup>2</sup> (range, 31.12-24.60 kg/m<sup>2</sup>). Twenty-three patients were male and thirteen patients were female, with a mean age of  $41.73 \pm 14.13$  years (range, 15-78 years) and a mean BMI of  $23.60 \pm 4.42$  kg/m<sup>2</sup> (range, 15.39-34.65 kg/m<sup>2</sup>). Thirty six patients with CP were divided into three groups of mild CP ( $n = 14$ ), moderate CP ( $n = 19$ ) and advanced CP ( $n = 3$ ) by M-ANNHEIM classification for CP<sup>[20-24]</sup>.

### Phantom experiments

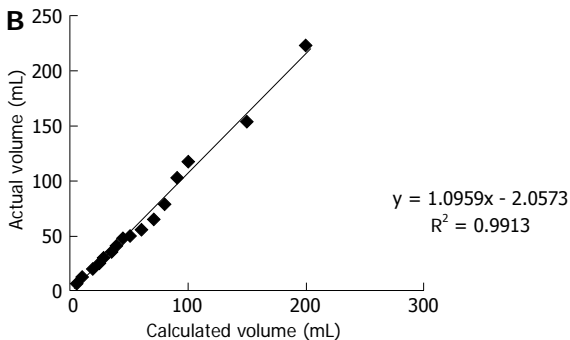
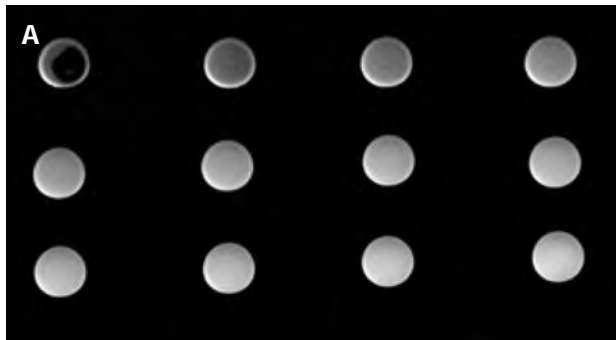
Measurement phantoms (MP) had a cylindrical shape and were filled with water. MPs were moved to isocenter. All images were acquired with the same the positions ensuring all imaging volume included matching phantoms. Volumes from 0 mL up to 200 mL water were applied. Put 200 mL of water into MP with different increments (0-50 mL in 5 mL increments, 50-100 mL in 10 mL increments, 100-200 mL in 50 mL increments). Then, the correlation between actual volume and calculated volume was examined.

Voxels containing 100% water were identified from the middle slice of the last volume step, when MP underwent maximal filling<sup>[17]</sup>. Commercially available software provided to measure the mean signal intensity/voxel.

### FE-1 Test

For the FE-1 test, a single random stool sample was required with no need to stop enzyme supplements. Pancreatic exocrine reserve was in all patients using the FE-1 test determined. Results were determined using the sandwich enzyme-linked immunosorbent assay (ELISA) test, with two monoclonal antibodies that only bind to human elastase-1. A commercial kit was used (ScheBo Tech®, Germany) containing a human elastase-specific antibody-sensitized ELISA plate. Elastase concentration was then determined by a photometric method. The FE-1 values were separately scored as 1 for  $FE-1 \geq 200$  µg/g, and 0 for  $FE-1 < 200$  µg/g.





**Figure 1** Images of Phantom experiments. A: Measurement phantoms (MP) images with 5-70 mL of water in the MP; B: Correlation between the calculated and actual volumes of water *in vitro* study.

### MR technique

All examinations were performed using a 3.0-T MR (Signa EXCITE, GE Healthcare) with a phased array body coil. All examinations were fasted for at least 4 h before examination. Immediately, before imaging patients drank 100 mL of water ensuring the presence of a voxel containing 100% water within the imaging volume.

The pancreatic MR examination protocol consisted of: (1) coronal T<sub>2</sub>-weighted single-shot turbo spin-echo, respiratory-triggered, covering the papillae, duodenum and small bowel. The parameters for coronal T<sub>2</sub>-weighted sequences were the following: TR/TE 1375/119 ms, slice thickness 64 mm, no gap, echo time 119 ms, FA 90°, field of view (FOV) 400 × 400, matrix 224 × 288; and (2) MRCP was performed with a heavily T<sub>2</sub>-weighted fat-suppressed long-TE HASTE sequence (thick slab 2D MRCP sequence). Imaging parameters were TR/TE 7000 ms/1270 ms, flip angle 90°, 64 mm slice thickness, FOV 320 mm × 320 mm - 420 mm × 420 mm, matrix 288 × 288, echo time 1274 ms. After the first dynamic acquisition, a bolus of secretin was injected intravenously at a dose of 1 CU/kg body weight. The secretin (Secrelux Sanochemia®, Germany) dose 0.1 mL/kg was administered and the exact same sequence repeated at 1, 3, 5, 7, 9 and 11 min. All positions before and after injection remained unchanged.

### Imaging analysis

Two single radiologists assessed pancreatic imaging without knowledge of clinical and FE-1 data.

A semi-quantitative evaluation of duodenal filling (DF), a function of pancreatic exocrine secretion, was

**Table 1** Results in normal and chronic pancreatitis

	<i>n</i>	FE-1 (μg/g)	PFR (mL/min)	PT (min)
Normal	17	525.41 ± 94.44	8.18 ± 1.11	5.76 ± 1.71
Mild	14	464.95 ± 136.13	7.27 ± 2.04	7.71 ± 2.55
Moderate	19	301.55 ± 181.55	4.98 ± 2.57	9.10 ± 3.00
Advanced	3	229.30 ± 146.60	4.13 ± 1.83	12.33 ± 1.15
<i>F</i> value		9.45	9.59	9.06
<i>P</i> value		0.000	0.000	0.000

FE-1: Fecal elastase-1; PFR: Pancreatic flow output rate; PT: Peak time.

performed on MRCP images obtained 10 min after secretin injection and compared with MRCP images acquired before secretin administration. MRCP images were assessed as follows described by Matos *et al.*<sup>[25]</sup>, grade 0 for absence of DF; grade 1 for fluid limited into the duodenal bulb; grade 2 for DF up to the genu inferius; grade 3 for filling beyond to the genu inferius. In agreement within the literature, pancreatic exocrine function reduced when DF was less than grade 3.

Subsequently, a quantitative analysis of DF was performed by drawing an appropriate region of interest (ROI). Changes in signal intensity in the imaging volume were plotted against time, and the flow rate derived from the gradient. PFR were calculated using the method previously described in<sup>[17,19,25]</sup>. Pancreatic exocrine secretions were quantified by PFR and PT. Then the PFR values were separately scored as 1 for PFR ≥ 5 mL/min, and 0 for PFR < 5 mL/min. The volume of water was calculated with the following formula: Volume = (Mean signal intensity/Voxel × Size of the region of interest)/Signal intensity (100% water).

### Statistical analysis

Statistical analyses were performed in SPSS 20.0 for Windows software. Data are reported as the median or the mean ± one SD. Mean flow rates and FE-1 were compared using the one-way ANOVA LSD test and Student's *t* test. It was statistically significant different at the 0.05 level. Linear regression analysis was used to compare the values between PFR and FE-1. Correlations between DF grades, M-ANNHEIM classifications and PFR, FE-1 were tested using the Spearman's rank correlation coefficients respectively. Correlation was significant at the 0.01 level. The consistency of the two methods was compared with the test where MRCPQ < 5 mL/min, FE-1 < 200 μg/g as abnormal.

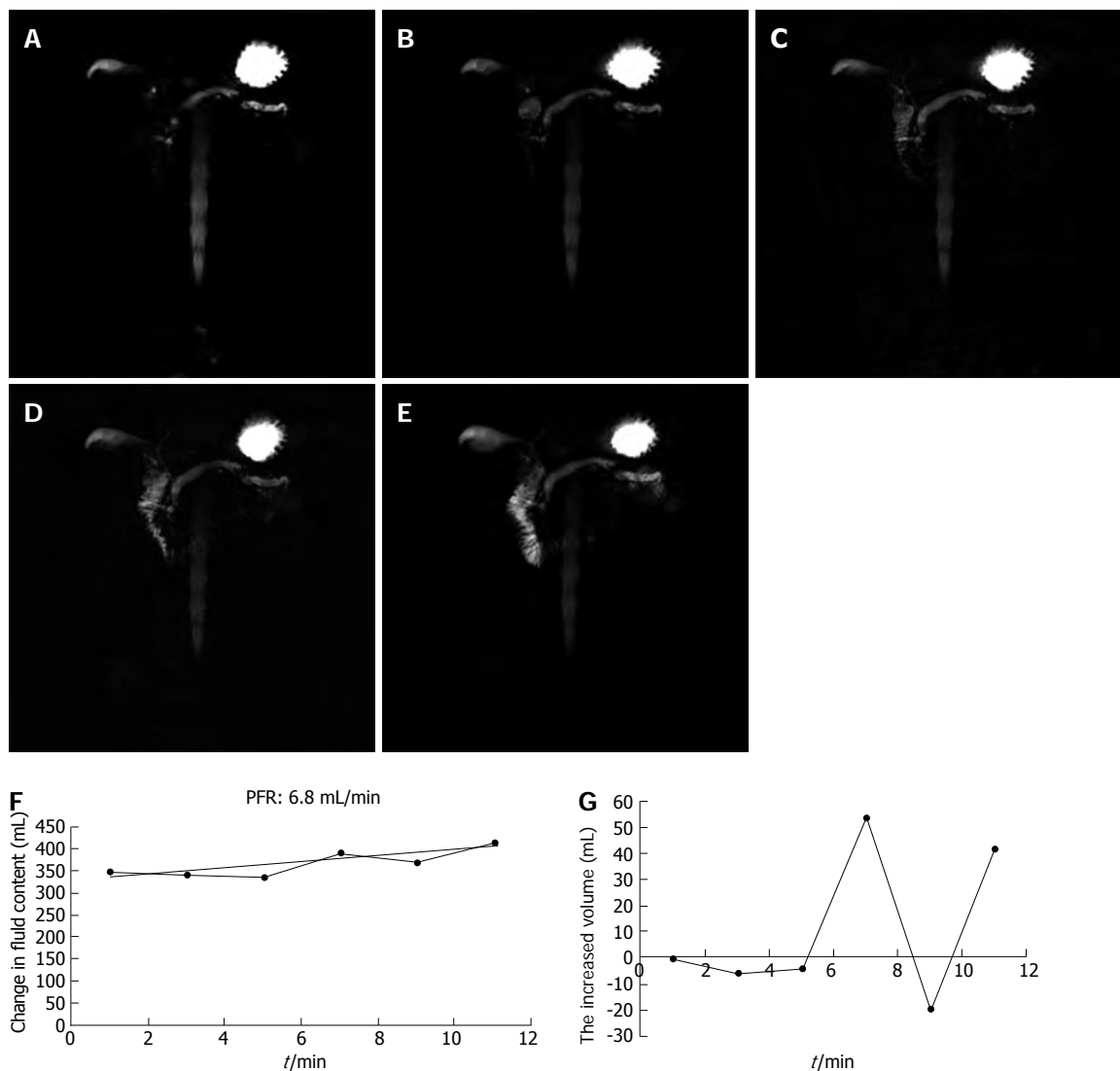
## RESULTS

### The result of phantom experiments

Phantom experiments showed a good correlation between known and calculated volumes of water (Figure 1).

### Semi-quantitative image analysis

Ten minutes after secretin administration, 17 volunteers and 23/33 patients showed a DF beyond the genu inferius (grade 3); whereas 8/36 showed a DF up to the



**Figure 2** An illustration of the secretin effect with the calculated pancreatic flow rate in a 54-year-old man with chronic pancreatitis. A-E: There was a visible increase in DF at 1, 3, 5, 7, 9, 11 min after secretin, grade 3 DF was present on SMRCP at 10 min; F, G: A plot of change in volume over time from patient data, normal response with a flow rate of 6.8 mL/s and PT of 7 min.

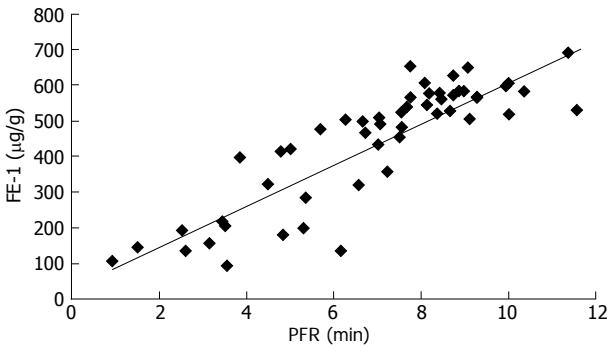
Table 2 Results in normal and reduced duodenal filling				
	n	FE-1 (μg/g)	PFR (mL/min)	PT (min)
Normal DF (grade 3)	40	453.64 ± 162.74	7.16 ± 2.34	7.28 ± 2.56
Reduced DF (grade 1, 2)	13	285.59 ± 158.03	4.71 ± 1.92	9.61 ± 3.57
P value		0.002	0.001	0.012

FE-1: Fecal elastase-1; PFR: Pancreatic flow output rate; PT: Peak time; DF: Duodenal filling.

genu inferius (grade 2); 5/36 showed a DF limited to the duodenal bulb (grade 1); None of the patients showed absent filling (grade 0).

**Quantitative image analysis**

Table 1 demonstrated results of the mean FE-1, PFR and PT of volunteers and patients based on M-ANNHEIM classification for CP. Table 2 demonstrated results of the mean FE-1, PFR and PT of volunteers and patients



**Figure 3** Scatter plot of pancreatic flow output rate and fecal elastase-1.

based on DF volume for CP. There was correlation ( $r = 0.79$ ) and consistency ( $K = 0.6$ ) between MRC PQ and FE-1. Also, it was related between M-ANNHEIM classification and PFR ( $r = 0.55$ ), FE-1 ( $r = 0.57$ ). There were correlations between DF grades and PFR ( $r = 0.36$ ), but

**Table 3** Quantification of secretin enhanced magnetic resonance cholangiopancreatography vs Fecal elastase-1 *n* (%)

	Normal FE-1	Abnormal FE-1
Normal MRCPQ	18 (51.4)	3 (8.6)
Abnormal MRCPQ	4 (11.4)	10 (28.6)

MRCPQ (quantification of secretin enhanced magnetic resonance cholangiopancreatography) < 5 mL/min and FE-1 < 200 µg/g are abnormal *K* = 0.6. FE-1: Fecal elastase-1.

**Table 4** Results of correlation

	M-ANNHEIM		DF	
	<i>r</i> value	<i>P</i> value	<i>r</i> value	<i>P</i> value
PFR	0.55	0.000	0.36	0.009
FE-1	0.57	0.000	0.29	0.038

Correlation is significant at the 0.01 level (2-tailed). DF: duodenal filling.

no FE-1 (*r* = 0.29, Figures 2 and 3, Tables 3 and 4).

## DISCUSSION

In this study, we quantified pancreatic exocrine function of CP. Some technical details are worth emphasis. The coil we used was the body coil not torso phased-array coil, because the body receiver coil provided a uniform sensitivity<sup>[26,27]</sup>. Second, Voxels containing 100% water was chosen in order to reduce the impaction of surrounding. If not, it would cause a high PFR<sup>[19,20]</sup>. Third, all cases fasted for at least four hours before examination. Immediately, patients were given 100 mL of water to drink before imaging, to ensure the presence of a voxel containing 100% water within the imaging volume. Keeping positions constant is also critical before and after the injection of secretin. Fourth, the scanning images should be along the long axis of the pancreas<sup>[28-31]</sup>. Lastly, we chose 64 mm slice thickness and large ROI including the papillae, duodenum and small bowel to prevent fluid loss.

There are some limitations in this study. The study sample is relatively small, in particular, the subgroup. Besides, it is distinguishing between water filled in measurement phantoms and pancreatic juice. Additionally, phantom experiments are only valid in vitro. The other data<sup>[15,16,18,29]</sup> show that a thin multilayer coronal T2WI images were obtained after the injection of secretin; based on change of signal, the volume of pancreatic secretion by the equation is calculated. This method needed patients to drink water many times. Finally, what we calculated is PFR, not pancreatic flow volume.

In summary, we have shown a good linear correlation between the calculated volume and the actual volume by Phantom experiments, which also has been reported in the literature<sup>[32-34]</sup>. The data gathered by the phantom studies strongly support the feasibility of this technique. Using the same technique, we have been able to measure pancreatic flow rates in health and CP. Moreover, there are correlation and consistency between MRCPQ and

FE-1. Our study confirmed the capability of MRCP images to quantitatively evaluate the pancreatic exocrine reserve. Nevertheless, considering the limitations of this study, we need to confirm our results with further trials.

## COMMENTS

### Background

Pancreatic exocrine function test plays an important role of in the diagnosis of chronic pancreatitis. It can guide the clinical diagnosis and therapy. At present, the "tubed" secretin test as the standard reference method is hampered by lack of test standardization and is uncomfortable for the patient due to prolonged duodenal intubation. Indirect pancreatic function test is non-invasive, safe and cost-effective, but it is positive only in a serious shortage of pancreatic exocrine function. Recently, secretin enhanced magnetic resonance cholangiopancreatography (SMRCP) has gained increasing interest as a non-invasive imaging method enabling visualization and quantitative assessment of various aspects of pancreatic exocrine function.

### Research frontiers

The authors examined the published literatures on quantification of pancreatic exocrine function and there were no other related or similar studies in China.

### Innovations and breakthroughs

In this study, the authors chose 64 mm slice thickness and large region of interest to quantify pancreatic exocrine function of chronic pancreatitis. This way is non-invasive, safe and effective and above all, directly measure pancreatic fluid output.

### Applications

The results of the present study indicate that SMRCP has been a reliable non-invasive method for the diagnosis of pancreatic exocrine function, whereas it can displace the "tubed" secretin test and other indirect tests.

### Terminology

Quantification of secretin enhanced-MRCPQ was acquired as follows: The secretin dose 0.1 mL/kg was administered and the exact same sequence repeated at 1, 3, 5, 7, 9 and 11 min. All positions before and after injection remained unchanged.

### Peer review

Pancreatic exocrine function test plays an important role of in the diagnosis of chronic pancreatitis. It can guide the clinical diagnosis and therapy. Recent developments in SMRCP techniques have shown the feasibility of a quantitative assessment of the pancreatic exocrine function.

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## Clinical features and prognosis in colorectal cancer patients with different ethnicities in Northwest China

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### Abstract

**AIM:** To compare the clinical factors and tumor characteristics that predict survival in colorectal cancer (CRC) patients with different ethnicities in Xin Jiang area.

**METHODS:** A total of 1421 histopathologically confirmed sporadic CRC patients who were either Han/Chinese or Uyghur were identified and enrolled from a database of both diagnoses and operative procedures from Xin Jiang Tumor Hospital, which is affiliated to Xin Jiang Medical University between 2000 and 2007. Patients with family histories of CRC, hereditary nonpolyposis CRC, familial adenomatous polyposis, inflammatory bowel disease, carcinoid, squamous carcinoma or melanoma were excluded. The two ethnic groups were compared with regard to clinical features, tumor characteristics, disease stage, overall survival rate, disease-free survival rate and cancer-specific survival rate. The factors predicting long-term survival were assessed *via*

both univariate and multivariate analysis.

**RESULTS:** Among the 1421 patients with CRC enrolled in this study, 1210 patients were Han/Chinese (mean age,  $62.3 \pm 4.5$  years; range, 19-92 years), while 211 patients were Uyghur (mean age,  $52.4 \pm 15.6$  years; range, 17-87 years). There were significant differences in proportions of gender, age, blood type, occupation and histopathological type between the Han/Chinese and Uyghur patients ( $P < 0.05$ ). The median overall, disease-free and cancer-specific survival time were 45, 62 and 65 mo for the Han/Chinese patients and 42, 49 and 61 mo for the Uyghur patients ( $P = 0.000$ ,  $P = 0.005$ ,  $P = 0.007$ ). The cumulative 5-year survival of the Uyghur patients was significantly worse than that of the Han patients ( $P = 0.000$ ). A multivariate analysis showed that age, ethnicity, histopathological type, differentiation, T (Infiltration depth), N (Lymph node metastasis), staging, postoperative metastasis and metastatic site ( $P < 0.05$ ) were found to be the prognostic factors.

**CONCLUSION:** The Uyghur CRC patients are associated with significantly younger age, more aggressive histopathologic characteristics and have significantly worse prognosis than the Han/Chinese patients.

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**Key words:** Colorectal cancer; Ethnicity; Clinicopathological factor; Survival

**Core tip:** Racial/ethnic differences in colorectal cancer (CRC) survival have been documented in the literatures. However, the reasons for these disparities are difficult to decipher. These disparities may be attributed to many factors, including differences in socioeconomic status, tumor biology, stage at diagnosis, treatment, post-treatment surveillance, physician characteristics and hospital factors. This is the first comparative study on the clinicopathological factors and survival of CRC

patients with different ethnicities in Xin Jiang. We found some marked differences and performed preliminary analysis for possible reasons.

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## INTRODUCTION

The incidence and mortality rates of colorectal cancer (CRC) worldwide are continuously rising. There were approximately 1.2 million new cases and 630000 deaths world-wide in 2007, which is increased compared with that in 2000 (by 27% and 28%, separately). The average increases for these figures are 3.9% and 4.0% annually<sup>[1,2]</sup>. With the development of the economy and the improvement of living conditions and eating habits, the incidence of CRC in China has been increasing continuously and has become the second most common malignancy<sup>[3,4]</sup>. According to WHO reports, the death rate of CRC has increased by 70.7% in 2005 compared with that in 1991, annually increasing by 4.9%<sup>[5]</sup>.

Disproportionately high mortality rates are observed among African Americans for cancers of the lung, breast, prostate, colon and rectum, oral cavity and pharynx, cervix<sup>[6]</sup>, and esophagus<sup>[7]</sup>. In addition, the 5-year survival rates for African Americans are lower than those for whites for all of the major cancer sites. Similarly, mortality rates for cancers of the cervix, liver, and stomach are higher among Asians/Pacific Islanders and Native Americans than among non-Hispanic whites. Overall breast cancer mortality rates for Native Hawaiians are the highest of all racial/ethnic groups<sup>[8]</sup>. Colon cancer incidence and mortality vary markedly by race/ethnicity; specifically, African Americans have a higher incidence and mortality for colon cancer compared with other population groups<sup>[9]</sup>.

The Xinjiang Uyghur Autonomous Region is a part of the People's Republic of China located in the northwest of the country. This region is the home to a number of different ethnic groups, including Uyghur, Han/Chinese, Kazakh, Hui, Kyrgyz, and Mongols, accounting for the a majority of the population. The Uyghurs are a population with Eastern and Western Eurasian anthropometric and genetic traits and one of the many populations of Central Eurasia that can be considered to be genetically related to European and East Asian populations. Uyghur and Han/Chinese people are the two major ethnic groups in this area and have different origins, cultures, religions as well as living and eating habits. The purpose of this study was to determine whether racial disparities in clinicopathological features and survival exist among CRC patients in two ethnic groups in Xin Jiang.

## MATERIALS AND METHODS

The Xin Jiang Tumor hospital is a unique hospital capable of comprehensive treatment for cancers in Xin Jiang that serves the patients from all parts of the Xin jiang Uyghur Autonomous Region and functions as a primary cancer registration unit. Thus, data from this hospital possess relative representativeness and reliability. Between 2000 and 2007, there were 1759 newly diagnosed cases of histopathologically confirmed colorectal malignancies at Xin Jiang Tumor Hospital affiliated to Xin Jiang Medical University. A total of 1421 patients who were either Uyghur or Han/Chinese were identified and enrolled using a computer-generated search from a database with information on both diagnoses and operative procedures. Patients with family histories of CRC, hereditary non-polyposis CRC, familial adenomatous polyposis, inflammatory bowel disease, carcinoid, squamous carcinoma or melanoma were excluded. The clinicopathological characteristics included age, gender, ethnicity, occupation, blood type, onset tumor location, tumor size, histopathological types, gross type, pathological staging, recurrence, metastasis, metastatic site and so on were retrospectively analyzed. The right colon included the cecum, ascending colon, hepatic flexure, and transverse colon, while the left colon included the splenic flexure, descending colon, and sigmoid colon. A surgical metachronous tumor was considered the presence of another tumor found away from the primary tumor, when detected at least 6 mo after the primary operation. Resection was considered radical when there was macroscopic resection of all malignant tissue and no microscopic evidence of surgical margin spread. The tumor, node and metastasis (TNM) staging system<sup>[10]</sup> was used for tumor staging. Postoperatively, all patients were followed up at 3-mo intervals for the first year, 6-mo intervals from years 2 to 5, and annually thereafter. The end of follow-up was December 31, 2010. The two ethnic groups were compared in regard to clinical features, tumor characteristics, disease stage, overall survival rate, disease-free survival rate, and cancer-specific survival rate.

### Statistical analysis

Differences in the distribution of baseline characteristics between ethnic groups were assessed using Students T test or  $\chi^2$  or Fisher's exact tests. The overall survival was calculated as the time from diagnosis to death due to any cause. Colon cancer-specific survival was calculated as the time from diagnosis to death due to colon cancer. Disease-free survival was calculated as the time from surgical resection of the tumor to recurrence due to colon cancer. The cumulative survival rates were calculated using the Kaplan-Meier method and compared with the log rank test. The Cox technique was used for univariate and multivariate survival analyses. Significance was established at  $P < 0.05$ . Statistical calculations were performed using Stata Statistical Software, version 15 (SPSS, Inc., Chicago, IL).



**Table 1** Characteristics of colorectal cancer patients of different ethnicities *n* (%)

Characteristic	Han ( <i>n</i> = 1210)	Uyghur ( <i>n</i> = 211)	<i>P</i> value
Age (yr)			0.000
< 30	9 (0.7)	21 (10.0)	
30-50	230 (19.0)	71 (33.6)	
50-70	580 (47.9)	94 (44.5)	
≥ 70	391 (32.3)	25 (11.8)	
Gender			0.040
Male	694 (57.4)	105 (49.8)	
Female	516 (42.6)	106 (50.2)	
Occupation			0.000
Cadre (Office worker)	511 (42.2)	61 (28.9)	
Physical worker	337 (27.9)	30 (14.2)	
Farmer and herder	205 (16.9)	90 (42.7)	
Unemployed	157 (13.0)	30 (14.2)	
Blood type			0.013
A	378 (31.2)	44 (20.9)	
B	386 (31.9)	81 (38.4)	
AB	139 (11.5)	32 (15.2)	
O	307 (25.4)	54 (25.6)	
Tumor location			0.054
Rectum	704 (58.2)	141 (66.8)	
Left hemi colon	300 (24.8)	39 (18.5)	
Right hemi colon	206 (17.0)	31 (14.7)	
Tumor size			0.183
< 4	196 (16.2)	24 (11.4)	
4-8	838 (69.3)	157 (74.4)	
> 8	176 (14.5)	30 (14.2)	
Tumor differentiation			0.360
Poorly	203 (21.1)	42 (24.6)	
Moderate	606 (63.0)	108 (63.1)	
Well	153 (15.9)	21 (13.3)	
Histopathology			0.012
Adenocarcinoma	1031 (85.2)	175 (82.9)	
Mucinous cell	146 (12.1)	22 (10.4)	
Signet-ring cell	33 (2.7)	14 (6.6)	
Gross type			0.395
Invasive	153 (12.6)	20 (9.5)	
Ulcerous	576 (47.6)	101 (47.9)	
Fungus	481 (39.8)	90 (42.7)	
T			0.673
1	34 (2.8)	4 (1.9)	
2	249 (20.6)	40 (19.0)	
3	520 (43.0)	99 (46.9)	
4	407 (33.6)	68 (32.2)	
N			0.071
No	494 (40.8)	77 (36.5)	
≤ 4	538 (44.5)	111 (52.6)	
> 4	178 (14.7)	23 (10.9)	
M			0.692
No	1039 (85.9)	179 (84.8)	
Yes	171 (14.1)	32 (15.2)	
Staging			0.669
I	129 (10.7)	20 (9.5)	
II	362 (29.9)	56 (26.5)	
III	548 (45.3)	103 (48.8)	
IV	171 (14.1)	32 (15.2)	
CEA			0.992
≤ 3.4	301 (27.8)	52 (27.8)	
> 3.4	780 (72.2)	135 (72.2)	

T: Tumor infiltration depth; N: Number of lymph node; M: Metastasis at diagnosis; CEA: Carcinoembryonic antigen.

## RESULTS

### Patients and clinical data

A total of 1421 patients with sporadic colorectal adeno-

carcinoma were enrolled, of which 1210 patients were Han/Chinese (mean age,  $62.3 \pm 4.5$  years; range, 19-92 years), while 211 patients were Uyghur (mean age,  $52.4 \pm 15.6$  years; range, 17-87 years, Table 1). There were more patients who were over 50 years of age in the Han/Chinese group (80.2% *vs* 56.3%,  $P = 0.000$ ) and more patients who were under 50 years of age in the Uyghur group (43.6% *vs* 26%,  $P = 0.000$ ). The proportion of male patients in the Han/Chinese group was higher than that of the Uyghur group ( $P < 0.05$ ). There were differences between occupational proportions in the two ethnic groups. Cadre (office workers) and physical workers were the most prevalent in the Han/Chinese group (70.1% *vs* 43.1%) and farmers and herders were more prevalent in the Uyghur group (42.7% *vs* 16.9%). There were more A blood type patients in the Han/Chinese group (31.2% *vs* 20.9%,  $P < 0.05$ ), while there were more B blood type patients in the Uyghur (38.4% *vs* 31.9%,  $P < 0.05$ ) group. Although there was no significant difference in tumor sites between the two ethnic groups, the most common sites for cancer occurrence were the sigmoid colon and the rectum in both groups (73.0% in the Han/Chinese group *vs* 79.1% in the Uyghur group,  $P = 0.196$ ). There was no statistically significant difference in treatment including surgery, post-operative chemotherapy or radiotherapy between the two ethnic groups ( $P > 0.05$ , Table 2).

### Tumor characteristics

The histopathologic differences between the two ethnic groups are summarized in Table 1. Compared with the Han/Chinese group, the Uyghur patients had a greater proportion of suffering from signet-ring cell type (6.6% *vs* 2.7%,  $P < 0.05$ ). A comparison of the Han/Chinese and Uyghur patients revealed that they exhibited almost the same incidence of any stage, presence of metastasis at the initial diagnosis and recurrence rate after resection ( $P > 0.05$ ). There were no significant differences in gross type, tumor size or carcinoembryonic antigen between the two groups, respectively.

### Recurrence and survival

The median follow-up in all patients was 42 mo (range, 7-122 mo; mean, 48.73 mo in the Han/Chinese group and 42.37 mo in the Uyghur group). A total of 175 patients (12.3%) were lost to follow-up; in total, 543 patients (44.9%) in the Han/Chinese group and 104 in the Uyghur group (49.3%) developed either local or distant recurrence ( $P = 0.235$ , Table 2). The Han/Chinese group had respective 1-year, 3-year, and 5-year overall survival rates of 87.2%, 46.8%, and 29.2%, whereas the respective rates were 80.4%, 42.9%, and 10.1% in the Uyghur group. There was significant difference in median survival time between the two groups ( $P = 0.000$ ). As shown in Figure 1, overall survival, disease-free survival and cancer-specific survival in the Uyghur group were significantly lower than the Han/Chinese group ( $P = 0.000$ ,  $P = 0.005$ ,  $P = 0.007$ , respectively). Using univariate Cox proportional-hazards regression analyses, the related factors of age, ethnicity, tumor site, histopathological type,

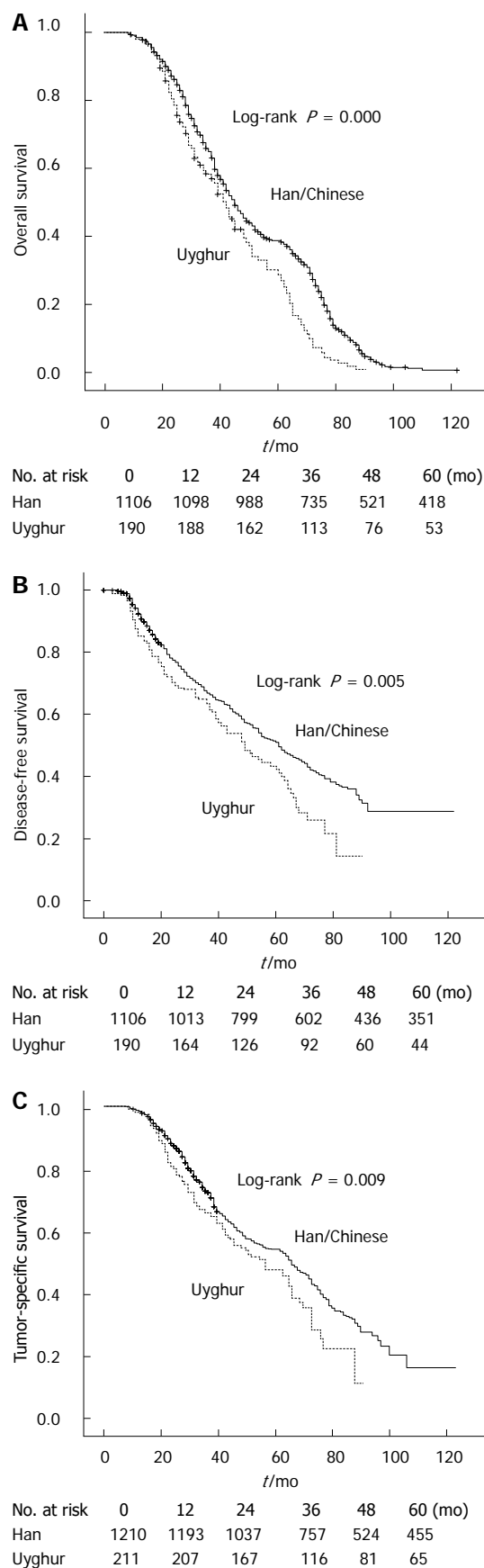
**Table 2** Treatment, recurrence and survival *n* (%)

	Han ( <i>n</i> = 1210)	Uyghur ( <i>n</i> = 211)	<i>P</i> value
Chemotherapy			0.493
Yes	692 (57.2)	126 (59.7)	
No	518 (42.8)	85 (40.3)	
Radiotherapy			0.216
Yes	247 (20.4)	51 (24.2)	
No	963 (79.6)	160 (75.8)	
Surgery			0.872
Radical	1091 (90.2)	191 (90.5)	
Palliative	119 (9.8)	20 (9.5)	
Local recurrence			0.282
Yes	151 (12.5)	32 (15.2)	
No	1059 (87.5)	179 (84.8)	
Postoperative metastasis			0.535
Yes	620 (51.2)	113 (53.6)	
No	753 (48.8)	98 (46.4)	
Metastatic site			0.224
No	591 (48.8)	97 (46.0)	
Liver	394 (32.6)	81 (38.4)	
Non-liver	225 (18.6)	33 (15.6)	
Median overall survival	45	42	0.000
Median dis-free survival	62	49	0.005
Median tumor-specific survival	65	61	0.007

differentiation, tumor infiltration depth (T), numbers of lymph node metastasis (N), metastasis (M), TNM staging, postoperative metastasis and the metastatic site were found to be the prognostic factors ( $P < 0.05$ ); multivariate analysis showed that age, ethnicity, histopathological type, differentiation, T, N, TNM staging, postoperative metastasis and the metastatic site were the prognostic factors ( $P < 0.05$ , Table 3).

## DISCUSSION

Our results provide the first single center-based comparison of clinicopathological factors and hazard ratios regarding the survival of patients with CRC between the two ethnic groups in Xin Jiang. The ethnic distribution significantly differed in Xin Jiang; the majority of Uyghurs inhabited in the south of Xin Jiang and most of the Han/Chinese population resided in northern Xin Jiang. The economic development of southern and northern Xin Jiang is not consistent and thus is related to level of education. Northern Xin Jiang is undergoing more rapid economic and educational developments, while socioeconomic status and health care knowledge are higher than those in southern Xin Jiang. Additionally, the quality of health care services in northern Xin Jiang is superior to those in southern Xin Jiang. This circumstance may be able to explain the difference between occupational proportions in the two ethnic groups. Our study showed that the proportion of male patients in the Han/Chinese group was higher than that in the Uyghur group ( $P < 0.05$ ). In a study by Brenner *et al.*<sup>[11]</sup>, gender is also shown to be a contributing factor to the development of CRC in young individuals. The risk of CRC in male patients is higher at an earlier age than in women; accordingly, their recommendation is that the optimal age for initiating



**Figure 1** Kaplan-Meier estimates. A: Overall survival for different ethnic patients with colorectal cancer (CRC); B: Disease-free survival for different ethnic patients with CRC; C: Tumor-specific survival for different ethnic patients with CRC.

**Table 3** Significant predictive factors for cancer-specific survival in a Cox proportional-hazard analysis

Prognostic factor	Univariate HR (95%CI)	P value	Multivariate HR (95%CI)	P value
Age	0.768 (0.706-0.836)	0.000	0.828 (0.751-0.913)	0.000
Ethnicity	1.494 (1.277-1.749)	0.000	1.316 (1.107-1.565)	0.002
Tumor site	0.918 (0.852-0.988)	0.023	0.972 (0.889-1.063)	0.531
Histopathological type	0.584 (0.537-0.634)	0.000	0.479 (0.307-0.748)	0.001
Tumor differentiation	1.201 (0.976-1.479)	0.028	1.421 (1.126-1.795)	0.003
T	1.475 (1.374-1.582)	0.000	1.378 (1.268-1.498)	0.000
N	1.684 (1.561-1.817)	0.000	1.169 (1.007-1.357)	0.040
M	3.829 (3.264-4.492)	0.000	1.179 (0.895-1.554)	0.243
Staging	2.009 (1.865-2.164)	0.000	1.579 (1.418-1.798)	0.000
Postoperative metastasis	2.538 (2.260-2.850)	0.000	2.329 (1.805-3.004)	0.000
Metastatic site	1.513 (1.416-1.617)	0.000	0.837 (0.716-0.978)	0.025

T: Tumor infiltration depth; N: Number of lymph node; M: Metastasis at diagnosis.

CRC screening in women is approximately 5 years older than that for men<sup>[10,11]</sup>. In terms of dietary habit, Uyghurs primarily consume meats (roasted, fried and boiled) and pastas every day, while Hans (Chinese) primarily consume rice and vegetables. The incidence of CRC is generally higher in populations with a high intake of meat and a low intake of staple plant foods<sup>[12]</sup>. A comprehensive review by the WCRF and the American Institute for Cancer Research concluded that “the evidence that red meat and processed meat are a cause of CRC is convincing”<sup>[13]</sup>. The disparities regarding age, blood type and histological features may also suggest some biological or genetic differences between the two ethnic groups, which is the area of study of our future investigations. Because of this prominent bias, it was particularly important to take regional, social and conventional gaps into consideration during the analysis.

Ethnic disparities have been demonstrated at each step of the cancer care continuum. Although these disparities are multifactorial in nature, they have a profound impact on cancer-related survival. Increased mortality among African American colon cancer patients has been attributed to decreased screening, more advanced stage at diagnosis, the disproportional receipt of standard surgical therapy and the unequal receipt of adjuvant therapy and postoperative surveillance<sup>[14,21]</sup>. Additionally, African American patients present at more advanced disease stages and are more likely to have an undetermined stage at presentation<sup>[22]</sup>. In contrast, East Asian American patients had significantly better 5-year overall and colon cancer-specific survival than non-Hispanic white, Hispanic white, and African American patients, which persisted when separately analyzed in patients with stage II and stage III diseases<sup>[23]</sup>. Several studies have suggested that there may be variations in CRC stage and survival between the Asian/Pacific Islander subgroups<sup>[24]</sup>.

In our study, the Uyghur patients had significantly worse prognosis than Han/Chinese patients given no significant differences in staging, recurrence or treatment. Numerous reports have suggested that socioeconomic status is a important factor in the poor prognosis of African American patients<sup>[25,26]</sup>. However, Wudel *et al.*<sup>[27]</sup> suggested that the marked reductions in survival of African

American patients did not appear to be related to variations in treatment but may have been due to biological factors or non-cancer-related health conditions. Uyghurs are more reluctant to screen with colonoscopy due to their traditional and religious beliefs and are more likely to live in a low-income community with limited access to gastroenterologists. Uyghurs also may have a lower incidence of insurance to cover a colonoscopy. In the present study, a poor prognosis in the Uyghur patients was consistently observed in overall survival, disease free survival and colon cancer-specific survival across nearly all stages. Therefore, it is likely that biological differences, socioeconomic status, disparities in health care service and ideology all contributed to the poor prognosis. A further epidemiological multicenter survey is needed to better quantify and determine whether dietary, regional, educational, socioeconomic status or genetic background could impact the incidence and mortality of CRC patients in these ethnic groups.

In a conclusion, Uyghur CRC patients are associated with a much younger age at disease onset, more aggressive histopathologic characteristics and a significantly worse prognosis than those in the Han/Chinese patients. Therefore, more attention should be paid to enhancing health care education and regular screening for the Uyghur population as well as promoting comprehensive treatment techniques in an effort to improve survival for both ethnic groups.

## COMMENTS

### Background

Cancer disparities between racial and ethnic groups are major public health concerns. It has been well established that the rates of cancer incidence and colorectal cancer (CRC)-related death are variable between patients from different racial and ethnic groups. Despite the great advances in medicine over recent decades, racial disparities in cancer-related mortality remain a challenging problem. The authors attempted to identify whether there are any differences that may contribute to survival between these ethnic groups.

### Research frontiers

The survival of patients with colon cancer reportedly differs according to race/ethnicity in the United States. Several studies have shown that African Americans are more likely to be diagnosed with advanced stage CRC and to have poorer survival rates after diagnosis compared with Caucasians. Several factors



including lack of health insurance, inability to access medical care, poverty, and low education level, are attributable to such findings. There has been no formal research thus far regarding the disparities between CRC patients with different ethnicities in the Xin Jiang area. This is the first report on clinicopathological factors and survival of CRC patients with differing ethnicity in Xin Jiang.

### Innovations and breakthroughs

This study has several strengths. First, the sample size was relatively large and the reliability and representativity of data were strong. Second, when the authors compared the survival rates of two different ethnic groups, the comparability between other confounding factors, such as staging, recurrence and treatment were guaranteed. Third, Uyghur CRC patients were found to be associated with significantly younger age, more aggressive histopathologic characteristics and significantly worse prognosis than Han/Chinese patients. These disparities may suggest that some biological or genetic differences exist between the two ethnic groups or that several factors, including a lack of health insurance, reluctance to access medical care, poverty, and low education level, in Uyghur patients contributed to such findings.

### Applications

This article demonstrated that there were disparities between the two ethnic groups in regard to clinicopathological factors and survival. The possible reasons for poor survival were preliminarily analyzed. These findings are valuable for CRC detection and prevention in the Xin Jiang region.

### Peer review

This is a retrospective study to assess clinical features and prognosis in CRC patients of different ethnicity in a medical center in Xinjiang province. The results provide valuable information for communities and public health programs.

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## Association between Ras association domain family 1A promoter methylation and hepatocellular carcinoma: A meta-analysis

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### Abstract

**AIM:** To assess diagnostic accuracy of Ras association domain family 1A (RASSF1A) promoter methylation in body fluids (serum, plasma and whole blood) for hepatocellular carcinoma (HCC).

**METHODS:** Relative information about study characteristics and incidence of RASSF1A methylation was collected. Quality of all included studies was evaluated by Quality Assessment of Diagnostic Accuracy Studies-2. Sensitivity and specificity were pooled using a random-effect model, and a summary receiver operating characteristic curve was used to demonstrate the overall diagnostic performance. Positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) with 95%CI were also calculated. Meta-regression was applied to analyze observed heterogeneity, and

Deeks' test was performed to detect publication bias.

**RESULTS:** After a systematic literature review, seven studies with a total of 302 cases of HCC and 250 cases of chronic liver diseases were included in the analysis. The pooled sensitivity and specificity were 0.70 (95%CI: 0.49-0.85) and 0.72 (95%CI: 0.54-0.85), respectively. The PLR was 2.51 (95%CI: 1.64-3.86), NLR was 0.41 (95%CI: 0.25-0.68), and DOR was 6.13 (95%CI: 3.17-11.84). The  $\chi^2$  values of sensitivity, specificity, PLR, NLR and DOR were 59.41 ( $P < 0.001$ ), 50.50 ( $P < 0.001$ ), 17.40 ( $P = 0.010$ ), 31.24 ( $P < 0.001$ ) and 80.51 ( $P < 0.001$ ), respectively. The area under the curve was 0.77 (95%CI: 0.73-0.81). Three factors were analyzed by univariate meta-regression and none was significant to interpret the observed heterogeneity ( $P > 0.05$ ). No significant publication bias was detected by Deeks' test ( $P = 0.346$ ).

**CONCLUSION:** We showed the potential diagnostic value of RASSF1A methylation in body fluids in HCC patients and it may improve diagnostic accuracy combined with the  $\alpha$ -fetoprotein test.

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**Key words:** Methylation; Ras association domain family 1A; Hepatocellular carcinoma; Biomarker; Diagnostic sensitivity; Diagnostic specificity

**Core tip:** The published results on the diagnostic potential of Ras association domain family 1A (RASSF1A) promoter methylation for detection of hepatocellular carcinoma (HCC) are not consistent. We performed a comprehensive literature search to assess the diagnostic accuracy of RASSF1A promoter methylation. We rigorously selected patients with chronic liver diseases as controls to mimic clinical practice, and we only included studies that used body fluids as samples for detection

because such an approach is non-invasive and promising for clinical application. Our meta-analysis demonstrated good sensitivity and specificity of RASSF1A methylation and may complement the  $\alpha$ -fetoprotein test to improve HCC detection.

Zhao ZH, Fan YC, Yang Y, Wang K. Association between Ras association domain family 1A promoter methylation and hepatocellular carcinoma: A meta-analysis. *World J Gastroenterol* 2013; 19(41): 7189-7196 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i41/7189.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7189>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third most frequent cause of cancer-related death worldwide<sup>[1]</sup>. With a dramatic increase in incidence, HCC has become a major health challenge and has aroused growing concern. Due to the fact that most HCC patients at the advanced stage are prone to present a poor prognosis, early HCC detection is urgently needed. Currently, the serum  $\alpha$ -fetoprotein (AFP) test is widely used, however, with a relatively low sensitivity, the application is barely satisfactory and its function is limited<sup>[2]</sup>.

Recent studies have revealed that inactivation of multiple tumor suppressor genes underlain by hypermethylation of promoter CpG islands can be a critical event in hepatocarcinogenesis<sup>[3,4]</sup>. Thus, DNA methylation may provide an ideal route for screening because methylated DNA can be detected with a high sensitivity and specificity<sup>[5]</sup>. Furthermore, the circulating DNA in cell-free serum/plasma is becoming a new focus. Increasing studies have attempted to identify abnormal methylation pattern in cfDNA of patients with human cancers<sup>[6-10]</sup>. Several tumor-associated alterations including plasma/serum DNA methylation have been well demonstrated in liver cancer<sup>[11]</sup> and such cfDNA is thought to be derived from apoptosis and necrosis of cancer cells in tumor microenvironment<sup>[12]</sup>. Thus, approaches have been developed to analyze promoter methylation using DNA isolated from fluid samples, which makes it possible to perform noninvasively and allows patients to avoid physical discomfort and other complications.

Among the biomarkers studied previously, the Ras association domain family 1A (RASSF1A) gene has been extensively investigated. RASSF1A is located at 3p21.3 and is implicated in the Ras signaling pathway, which plays a pivotal role in cell cycle control, microtubule stabilization, cellular adhesion, cell motility, and apoptosis<sup>[13]</sup>. The tumor suppressor function of RASSF1A has been identified by both *in vivo* and *in vitro* observations in which re-expression of the gene in RASSF1A-negative cancer cells results in reduced colony formation in soft agar and reduced tumorigenicity in nude mice<sup>[14]</sup>. Loss of RASSF1A expression is one of the most common events in human cancer, with aberrant promoter methylation

reported in a variety of tumor types, including HCC<sup>[15]</sup>.

During the past decade, there have been an increasing number of investigations focusing on the diagnostic role of RASSF1A promoter methylation in HCC and many of them utilized body fluids as samples, which can be used for clinical application. Although the results of the studies are encouraging, there was some disagreement in relation to diagnostic accuracy. Therefore, we conducted a meta-analysis on the diagnostic sensitivity and specificity of RASSF1A methylation in body fluids for diagnosis of HCC. The results of this study indicate the potential diagnostic value of RASSF1A methylation and provide evidence for a reliable biomarker to discriminate HCC.

## MATERIALS AND METHODS

### Study selection

We performed a comprehensive literature search of articles through the following databases without date limitation: PubMed, Embase, Web of Science, and the Cochrane Library. The search terms used were: "hepatocellular carcinoma/ HCC/liver cancer", "Ras association domain family protein 1A/RASSF1A", "sensitivity", "specificity" and "diagnosis". The search was updated to May 24, 2013. The reference lists of the publications were also manually searched for additional related studies.

### Inclusion and exclusion criteria

We considered studies eligible for inclusion if they met the following criteria: (1) measurement of DNA methylation in one of the following samples: whole blood, plasma, serum, and buffy coat; (2) designed as a cohort study or a case-control study; (3) published in the English language; and (4) conducted in adults.

We excluded studies that tested RASSF1A methylation in liver tissues and cell lines. We also excluded studies in which body fluid samples were not collected before surgery or other treatment which may have reduced RASSF1A methylation dramatically. In clinical practice, patients who are recommended for advanced imaging examination (such as computed tomography and magnetic resonance imaging) and biopsies typically, have elevated AFP levels, aberrant ultrasonic images, and abnormal liver function or other related symptoms, and can differ from healthy controls<sup>[1]</sup>. Therefore, we rigorously classified the controls into two categories: (1) patients who had negative biopsies but suffered from chronic liver diseases such as liver cirrhosis, chronic hepatitis B, and chronic hepatitis C; and (2) healthy controls. In particular, we excluded healthy controls in order to diminish selection bias and avoid exaggerating diagnostic power. The selection process for studies included in this review is shown in Figure 1.

### Data extraction and quality assessment

For each study included, two reviewers (ZH Zhao and YC Fan) independently extracted the following information: authors' names, year of publication, sample type, detection technique, primer sequence, annealing tempera-



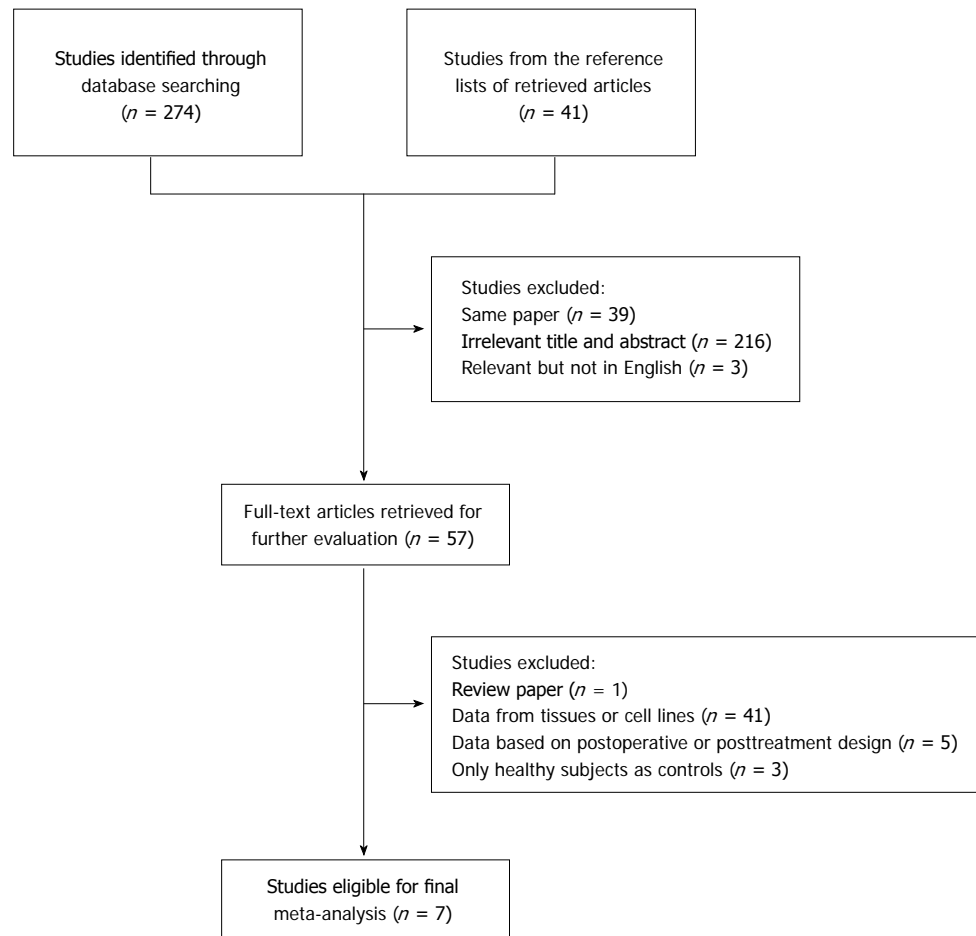


Figure 1 Flowchart of study selection.

ture, country, race, number of positive and negative results among cases and controls, and other characteristics of the study population. All disparities were resolved by discussion.

An updated quality evaluation tool Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) guideline was consulted to assess the methodological quality of each study. The newly revised tool is reported to perform better because it offers additional and improved features, including distinguishing between bias and applicability, identifying four key domains supported by signaling questions to aid judgment on risk of bias, and rating risk of bias and concerns about applicability as “high” and “low”<sup>[16]</sup>. The results were presented in a recommended way.

### Statistical analysis

We referred to a standard procedure recommended for meta-analysis of diagnostic test accuracy (DTA) studies<sup>[17]</sup>. Before the statistical analysis was conducted, we gathered the number of cases and controls with RASSF1A methylation. The true-positive (TP) ones were indicated to have RASSF1A methylation within cases, and false-negative (FN) ones were without RASSF1A methylation. A similar

definition was given to FP and TN controls. The sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) estimates with 95%CI from each study were analyzed using a random-effect model<sup>[17,18]</sup>. The pooled sensitivity and specificity were illustrated with a coupled forest plot. We also created a summary receiver operation characteristic (SROC) curve that displayed the results of individual studies in ROC space and reflected the discriminating ability<sup>[19]</sup>. The area under the curve (AUC) was calculated to present the general performance of the test and could be interpreted as the probability that the test would correctly rank a randomly chosen case/non-case pair with respect to their test values<sup>[20]</sup>. To assess the heterogeneity between studies, the  $\chi^2$ -based Cochrane  $Q$  test of heterogeneity was performed for each analysis. The  $I^2$  statistic, which measured the extent of inconsistency between studies, was also assessed. For detecting publication bias, Deeks’ test was conducted to examine funnel plot asymmetry, which was reported to be more appropriate for reviews of DTA studies<sup>[21]</sup>. The analysis was conducted using Stata version 12.0 (Stata Corporation, College Station, TX, United States). All  $P$  values were two-tailed and  $P < 0.05$  was considered significant.

**Table 1** Characteristics of included studies in meta-analysis

Author	Year	Country/area	Race	Sample	Method	Case type	Cases (n)	Control type	Controls (n)
Chan <i>et al</i> <sup>[22]</sup>	2008	Hong Kong	Asian	Serum	MSRE-qPCR	HCC	63	HBV infection	63
Chang <i>et al</i> <sup>[23]</sup>	2008	China	Asian	Plasma	MSP	HCC	26	Liver cirrhosis	16
Maurizio <i>et al</i> <sup>[24]</sup>	2011	Italy	Caucasian	Whole blood	MSP	HCC	31	Liver cirrhosis/ chronic hepatitis C	33/30
Mohamed <i>et al</i> <sup>[25]</sup>	2012	Egypt	Caucasian	Plasma	QMSP	HCC	40	HCV infection	40
Azab <i>et al</i> <sup>[26]</sup>	2011	Egypt	Caucasian	Whole blood	MSP	HCC	20	Liver cirrhosis	14
Zhang <i>et al</i> <sup>[27]</sup>	2007	Taiwan	Asian	Serum	MSP	HCC	50	HBV infection/ HCV infection/coinfection	9/6/2
Huang <i>et al</i> <sup>[28]</sup>	2011	China	Asian	Plasma	MSRE-qPCR	HCC	72	Liver cirrhosis/ chronic inactive hepatitis	25/12

MSP: Methylation-specific polymerase chain reaction (PCR); QMSP: Quantitative methylation-specific PCR; MSRE-qPCR: Methylation-sensitive endonuclease-qPCR; HCC: Hepatocellular carcinoma.

**Table 2** Primer sequences used in included studies for detection of Ras association domain family 1A methylation

Author	Year	Method	Forward	Reverse	Annealing T (°C)
Chan <i>et al</i> <sup>[22]</sup>	2008	MSRE-qPCR	5'-AGCCTGAGCTCATTGAGCTG-3'	5'-ACCAGCTGCCGTGTGG-3'	60
Chang <i>et al</i> <sup>[23]</sup>	2008	MSP	M 5'-GTGTTAACGCGTTGCGTATC-3' U 5'-TTTGGTTGGAGTGTGTTAATGTG-3'	M 5'-AACCCCGCGAACTAAAAACGA-3' U 5'-CAAACCCACAAACTAAAAACAA-3'	60
Maurizio <i>et al</i> <sup>[24]</sup>	2011	MSP	NA	NA	NA
Mohamed <i>et al</i> <sup>[25]</sup>	2012	QMSP	5'-AGCCTGAGCTCATTGAGCTG-3'	5'-ACCAGCTGCCGTGTGG-3'	60
Azab <i>et al</i> <sup>[26]</sup>	2011	MSP	M 5'-GTGTTAACGCGTTGCGTATC-3'; U 5'-TTTGGTTGGAGTGTGTTAATGTG-3'	M 5'-AACCCCGCGAACTAAAAACGA-3'; U 5'-CAAACCCACAAACTAAAAACAA-3'	54
Zhang <i>et al</i> <sup>[27]</sup>	2007	MSP	M 5'-GTGTTAACGCGTTGCGTATC-3' U 5'-TTTGGTTGGAGTGTGTTAATGTG-3'	M 5'-AACCCCGCGAACTAAAAACGA-3' U 5'-CAAACCCACAAACTAAAAACAA-3'	60
Huang <i>et al</i> <sup>[28]</sup>	2011	MSRE-qPCR	5'-AGCCTGAGCTCATTGAGCTG-3'	5'-ACCAGCTGCCGTGTGG-3'	58

M: Methylated sequence; NA: Not available; U: Unmethylated sequence; MSP: Methylation-specific polymerase chain reaction (PCR); QMSP: Quantitative methylation-specific PCR; MSRE-qPCR: Methylation-sensitive endonuclease-qPCR.

## RESULTS

### Study characteristics

According to our search strategy and inclusion and exclusion criteria, seven studies with a total of 302 cases and 250 controls were included in the final meta-analysis (Table 1)<sup>[22-28]</sup>. The eligible studies were published between 2007 and 2012. Among the studies, two used sera, three used plasma, and two used whole blood as samples. Three methods were applied to detect the methylation status of RASSF1A promoter: four studies used methylation-specific polymerase chain reaction (MSP); one study used quantitative MSP (QMSP); and two used methylation-sensitive endonuclease-quantitative polymerase chain reaction (qPCR). The primer sequences used in the studies are summarized in Table 2.

### Quality assessment

Quality assessment results based on the updated QUADAS-2 are shown in Table 3. According to the guidelines, if the case-control design was not avoided, the risk of bias should be considered to be high in the patient selection domain. All seven studies included in our analysis were case-control studies and could have introduced selection bias. For other domains, the studies were basically satisfactory.

### Diagnostic accuracy analysis

The coupled forest plot of sensitivity and specificity for RASSF1A methylation assays in the diagnosis of HCC of the seven studies is shown in Figure 2. The sensitivity ranged from 0.27 to 0.94 (pooled: 0.70; 95%CI: 0.49-0.85) and the specificity ranged from 0.38 to 0.95 (pooled: 0.72; 95%CI: 0.54-0.85). The PLR was 2.51 (95%CI: 1.64-3.86), NLR was 0.41 (95%CI: 0.25-0.68), and DOR was 6.13 (95%CI: 3.17-11.84). The  $\chi^2$  values of sensitivity, specificity, PLR, NLR, and DOR were 59.41 ( $P < 0.001$ ), 50.50 ( $P < 0.001$ ), 17.40 ( $P = 0.010$ ), 31.24 ( $P < 0.001$ ), and 80.51 ( $P < 0.001$ ), respectively, which indicated significant heterogeneity between studies. The graph of the SROC curve is shown in Figure 3. We noted that the curve was positioned near the desirable upper left corner. The AUC was 0.77 (95%CI: 0.73-0.81), which represented a relatively high level of overall accuracy.

### Meta-regression and publication bias

Due to the heterogeneity observed in both analyses, we conducted meta-regression to search the sources<sup>[29]</sup>. The accuracy estimate we used was DOR, because it could demonstrate the diagnostic performance combining both sensitivity and specificity. The assay methods used to detect RASSF1A methylation could have affected the diagnostic accuracy directly for discriminating HCC.

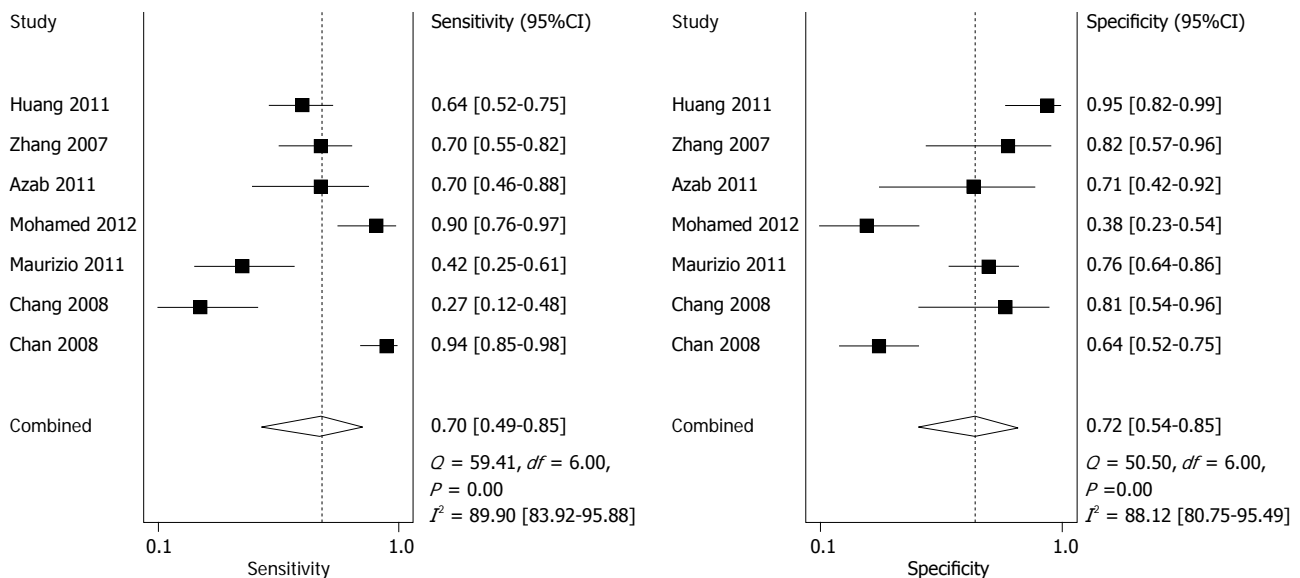
**Table 3** Quality Assessment of Diagnostic Accuracy Studies-2 results of all included studies

Study	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Chan <i>et al</i> <sup>[22]</sup>	↑	?	↓	↓	↓	?	↓
Chang <i>et al</i> <sup>[23]</sup>	↑	?	↓	↓	↓	?	↓
Maurizio <i>et al</i> <sup>[24]</sup>	↑	?	↓	?	↓	?	↓
Mohamed <i>et al</i> <sup>[25]</sup>	↑	↓	↓	↓	↓	↓	↓
Azab <i>et al</i> <sup>[26]</sup>	↑	↓	↓	↓	↓	↓	↓
Zhang <i>et al</i> <sup>[27]</sup>	↑	↓	↓	↓	↓	↓	↓
Huang <i>et al</i> <sup>[28]</sup>	↑	?	↓	↓	↓	?	↓

↓: Low risk; ↑: High risk; ?: Unclear risk.

**Table 4** Meta-regression results of meta-analysis

Sources	Coefficient (95%CI)	SE	T	P value	$\tau^2$	$I^2$ Res (%)	Adjusted $R^2$ (%)
Method	1.09 (-0.55-2.73)	0.64	1.71	0.148	0.24	37.16	50.47
Race	0.83 (-1.00-2.66)	0.71	1.17	0.294	0.36	46.68	26.54
Sample	0.88 (-1.09-2.85)	0.77	1.87	0.301	0.36	45.96	26.46



**Figure 2** Coupled forest plot showing the sensitivity and specificity of Ras association domain family 1A methylation in diagnosis of hepatocellular carcinoma. Forest plots document estimates of sensitivity and specificity for each study together with 95%CI. The point estimates of sensitivity and specificity from each study are shown as solid squares.

Also, race and sample type may have had an influence to a varying degree. Therefore, we considered the three factors above as covariates and performed univariate meta-regression analysis. However, none of the factors was statistically significant ( $P > 0.05$ , Table 4).

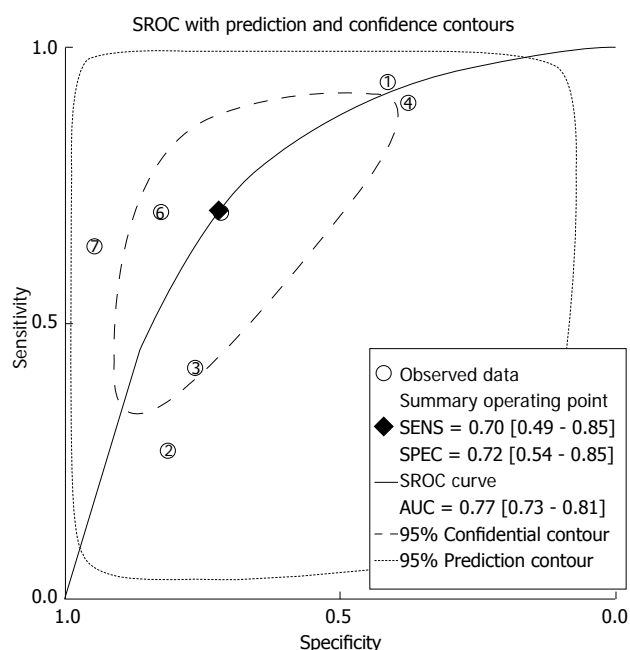
Publication bias was evaluated using Deeks' test (Figure 4). We found no significant publication bias among the studies that detected RASSF1A methylation in body fluids from patients with HCC ( $P = 0.346$ ).

## DISCUSSION

Although there have been many studies about sensitivity and specificity of RASSF1A promoter methylation

in diagnosis of HCC, to the best of our knowledge, no meta-analysis has been reported. For the traditional serum marker AFP, the specificity varied, but the sensitivity was generally low. A large multi-center survey including 1158 patients with HCC reported that the sensitivity for the most used cutoff value (20 ng/mL) was only 0.54<sup>[2]</sup>. Another recent study showed that the sensitivity and specificity of the AFP test was 0.42 and 0.95 when the cutoff value was 20 ng/mL<sup>[30]</sup>. In comparison, our analysis showed a relatively high sensitivity (0.70) of RASSF1A promoter methylation, which, if combined, may complement the AFP test and reduce FNs by performing detection of RASSF1A methylation status and the AFP test simultaneously. Positive results for either of

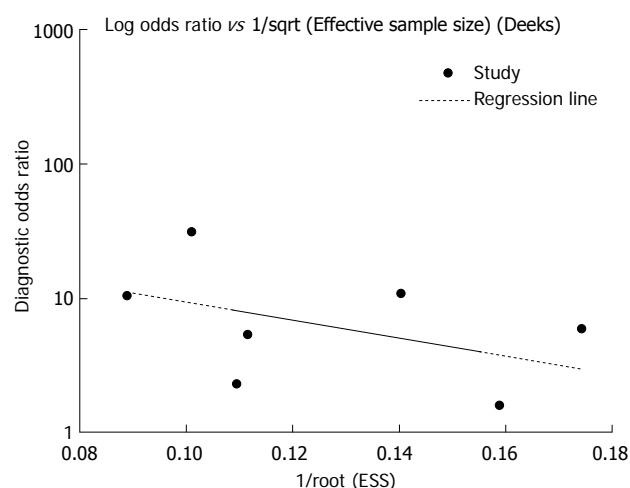




**Figure 3 Summary receiver operation characteristic curve for Ras association domain family 1A methylation assays.** Hollow numbered circles represent included studies. Summary receiver operation characteristic (SROC) curve summarizes the overall diagnostic accuracy.

the two tests demonstrate a high likelihood of HCC and patients should be recommended for advanced imaging or percutaneous liver biopsies. Nevertheless, more evidence on the correlation between RASSF1A methylation and AFP level should be collected. In our analysis, we observed FPs in some of included studies. *RASSF1A* gene promoter methylation might occur in controls including chronic liver disease and/or in preneoplastic (cirrhotic) liver to hepatocellular nodules to HCC<sup>[25]</sup>.

The *RASSF1A* gene has been long noted and intensively studied for its role in tumor suppression, and aberrant methylation in the promoter region is suspected as the main mechanism of downregulation and silencing that is widely observed in human malignancies<sup>[15]</sup>. However, in this meta-analysis, we aimed to verify the feasibility of body fluid RASSF1A methylation in identifying HCC from high risk population rather than healthy population. So we selected patients with chronic liver diseases which were in high risk of developing HCC as controls. Under this condition, HCC was of greater possibility than other types of cancer. Thus, methylation of RASSF1A is relatively specific to HCC and the detection was still meaningful in distinguishing HCC from benign liver diseases. The potential clinical use of RASSF1A methylation as a biomarker for HCC diagnosis has not been fully exploited, and studies measuring RASSF1A methylation in body fluids are rare. Nevertheless, the non-invasive test possesses considerable advantages. It avoids the risk of complications and sampling error caused by imaging or percutaneous liver biopsies and is more acceptable to patients<sup>[31]</sup>. Although tumor-specific cfDNA is thought to be mainly derive from apoptosis and necrosis of cancer



**Figure 4 Assessment of the potential publication bias in Ras association domain family 1A methylation detection.** We found no significant publication bias ( $P > 0.05$ ). Each spot represents one study and the regression line is shown.

cells in tumor microenvironment, discordance between frequencies of alterations found in DNA extracted from tumor tissue and cfDNA do exist<sup>[12]</sup>. Zhang *et al.*<sup>[32]</sup> showed that the frequency of RASSF1A methylation in 48 HCC tissues was 100%, which was greatly higher than our results.

Moreover, rapidly developed technologies for DNA methylation detection make it easier, faster and cheaper to measure RASSF1A methylation accurately<sup>[5,33-35]</sup>. The technologies applicable to the analysis of the small amounts of DNA present in the body fluids are mainly real-time PCR-based approaches such as MethyLight, HeavyMethyl, methylation-sensitive high-resolution melting analysis, and methylation-sensitive melting analysis after real-time methylation specific PCR<sup>[36]</sup>. However, some methodological issues should be resolved. The primer sequences and PCR procedures are varied in studies applying qualitative MSP. Also, MSP may have difficulties in distinguishing TP from FP due to the methodological limitations. Besides, the different cut-off values chosen could affect judgment of positive or negative results in detection using QMSP. We summarized the primer sequences for RASSF1A methylation detection in all the included studies to facilitate further confirmation. Before the assay can be put into clinical practice, more evidence based on methodological investigations should be collected and a standard protocol developed.

Exploring the sources of heterogeneity is one major purpose of meta-analysis<sup>[37]</sup>. Due to the significant heterogeneity observed in our analysis, we performed meta-regression. However, we failed to figure out the sources as all the covariates we took into consideration could not significantly interpret the heterogeneity. We surmise that the limited number of included studies was the main factor that hampered the analysis of heterogeneity, which also made subgroup analysis not possible.

In addition to the small number of studies, there were

some other limitations. First, the case-control design used in all our included studies was likely to have introduced bias in patient selection<sup>[16]</sup> and more prospective studies are needed. Second, although we classified the controls and excluded healthy controls, the remaining patients still presented a spectrum of diseases. Due to the insufficient detailed information provided by the publications, we failed to subdivide the chronic liver diseases and specific stages of one single disease, which may have introduced considerable heterogeneity. Third, it has been reported that time of sampling influences the level of methylation dramatically<sup>[38,39]</sup>. The studies included in our analysis varied greatly in time of sampling, which may have caused heterogeneity.

In conclusion, RASSF1A promoter methylation is a valuable diagnostic biomarker with a qualified sensitivity, which may complement the AFP test in screening for HCC. More prospective diagnostic trials with strictly defined controls are needed to elucidate further the accuracy of RASSF1A methylation for diagnosis of HCC.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most prevalent human malignancies. HCC patients are prone to present a poor prognosis due to failure of early detection. The currently used serum  $\alpha$ -fetoprotein (AFP) test has a relatively low sensitivity, which limits its application. Ras association domain family 1A (RASSF1A) methylation has been shown to be a diagnostic marker for HCC.

### Research frontiers

Recently, there has been an increasing number of investigations focusing on the diagnostic role of RASSF1A promoter methylation in HCC. However, there is disagreement about the actual diagnostic accuracy. This study was conducted to assess pooled estimates of the diagnostic accuracy of RASSF1A methylation in HCC.

### Innovations and breakthroughs

The authors assessed RASSF1A methylation for HCC diagnosis by meta-analysis. The controls were rigorously defined as patients with chronic liver diseases to mimic clinical practice and only studies that used body fluids as samples for detection were included because they were non-invasive. The results showed a better sensitivity compared to the AFP test. The findings may improve HCC diagnostic accuracy.

### Applications

RASSF1A methylation may have diagnostic potential for HCC and could be a new candidate marker. Furthermore, detection in body fluids makes it possible to be non-invasive and more acceptable to patients.

### Terminology

DNA methylation is a conversion of the cytosine to 5-methylcytosine which typically occurs at CpG islands in the promoter region with the help of DNA methyltransferases. This process may result in gene silencing without changing its coding sequence.

### Peer review

The authors showed that RASSF1A methylation in body fluids in HCC patients can improve HCC diagnostic accuracy using meta-analysis. The idea of this study is novel and important as they continue to evaluate novel potential biomarkers for the early diagnosis of HCC.

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## Magnetic endoscopic imaging vs standard colonoscopy: Meta-analysis of randomized controlled trials

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### Abstract

**AIM:** To assess the theoretical advantages of magnetic endoscope imaging (MEI) over standard colonoscopies (SCs) and to compare their efficacies.

**METHODS:** Electronic databases, including PubMed, EMBASE, the Cochrane library and the Science Citation Index, were searched to retrieve relevant trials. In addition, abstracts from papers presented at professional meetings and the reference lists of retrieved articles were reviewed to identify additional studies. The meta-analyses were performed using RevMan 5.1. A random effect model with the Mantel-Haenszel method was used for pooling dichotomous and continuous data. A sensitivity analysis was performed by excluding the trials with a small number of patients and by excluding the trials performed by inexperienced providers.

**RESULTS:** Eight randomized controlled trials (RCTs), including 2967 patients, were included in the meta-analysis to compare cecal intubation rates and times, sedation dose, abdominal pain scores and the use of ancillary maneuvers between MEI and SC. The overall OR was 1.92 (95%CI: 1.13-3.27, eight RCTs), as indicated by the cecal intubation rate of MEI compared with SC, but MEI did not have any distinct advantage over SC for cecal intubation time (MD = -0.07, 95%CI: -0.16-0.02; three RCTs). MEI did not generally result in lower pain scores. Outcomes were also analyzed for the two subgroups based on the endoscopists' experience level to evaluate cecal intubation rates. MEI presented better outcomes for non-experienced colonoscopists than experienced colonoscopists.

**CONCLUSION:** The real-time magnetic imaging system is of benefit in training and educating inexperienced endoscopists and improves the cecal intubation rate for experienced and inexperienced endoscopists.

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**Key words:** Colonoscope; Magnetic endoscope imaging; Magnetic; Standard colonoscope; Meta-analysis

**Core tip:** This study aimed to assess the theoretical advantages of magnetic endoscopic imaging (MEI) over standard colonoscopy (SC) and to compare the efficacies of MEI and SC. The meta-analyses compared the cecal intubation rate and time, sedation dose used, abdominal pain scores and the use of ancillary maneuvers between MEI and SC. The real-time magnetic imaging system is of benefit in training and educating inexperienced endoscopists, and it improved the cecal intubation rate for both experienced and inexperienced endoscopists.

Chen Y, Duan YT, Xie Q, Qin XP, Chen B, Xia L, Zhou Y, Li NN, Wu XT. Magnetic endoscopic imaging vs standard colonoscopy: Meta-analysis of randomized controlled trials. *World J*

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## INTRODUCTION

Colonoscopy is the gold standard and the most common and accurate tool for detecting important structural lesions of the lower gastrointestinal tract and for diagnosing colonic diseases, such as polyps, colorectal cancer and inflammatory bowel disease<sup>[1-3]</sup>. However, the existence of sharp angulation or looping of the colon increases the difficulty of the procedure and causes distinct discomfort for patients. The failure rate of initially reaching the cecum remains significant at 2%-10%<sup>[4-6]</sup>. In addition, there is still a small but definite risk of procedure-related complications, notably bleeding and perforation<sup>[7,8]</sup>. Thus, technological advances in colonoscopy have continued over the last decade<sup>[9,10]</sup>.

Magnetic endoscopic imaging (MEI) is a non-radiographic imaging technique that has been developed in recent years that is capable of displaying real-time three-dimensional images of the colonoscope shaft within the abdominal cavity<sup>[11-12]</sup>. The MEI system has previously been described in detail<sup>[13]</sup>. A pulsed low-magnetic field is sequentially produced by a series of electromagnetic generator coils spaced 10 cm apart along a catheter inserted through the accessory channel of the endoscope. The imager view is updated every 0.2 s to make the system essentially real time, and the images are subsequently recorded on a computer disk for subsequent replay or analysis<sup>[14]</sup>. The MEI system has been shown to be beneficial in increasing the cecal intubation rate<sup>[15,16]</sup>, reducing the number of attempts to straighten loops<sup>[16,17]</sup>, and in reducing the duration of looping, especially with trainees, when compared with no visualization. To date, a few studies have compared MEI with standard colonoscopy (SC); however, the results have not been uniform.

The aim of the present meta-analysis was to evaluate the effect of the two different methods.

## MATERIALS AND METHODS

### Data sources

First, electronic databases, including PubMed (1966 to June 2012), EMBASE (1980 to June 2012), the Cochrane Central Register of Controlled Trials (CENTRAL, The Cochrane Library, Issue 6 of 12, June 2012), and the Science Citation Index, were searched. The search was performed with the following search terms as free-text terms as well as MeSH terms: colonoscope, colonoscopy, magnetic and magnetic endoscopic imaging. Second, meeting abstracts and the reference lists of the retrieved articles were reviewed for additional relevant studies. No language restrictions were imposed.

### Equipment type

The instruments used in the trials included the Olym-

pus CF-1T200L scope (160 cm)<sup>[16,17]</sup>, the ScopeGuide endoscope insertion tube system<sup>[18-20]</sup>, the Olympus CF-Q160DI with the Olympus ScopeGuide system<sup>[15,21]</sup>, the Olympus CF-Q180AL, the Olympus CF-Q160AL and the CF-Q140DL/I with the Olympus ScopeGuide system.

### Study selection

Randomized controlled trials (RCTs) comparing MEI with SC were included in this analysis. Only the most recent study was included if more than one study was published using the same study population. Thirty-five papers were uncontrolled, observational studies and case reports and were thus excluded from the meta-analysis.

### Data extraction

All the data were tabulated with standard data abstractions sheets. For each study and each type of intervention, the following characteristics were extracted: study design and conduct, numbers of patients, endoscopist characteristics, instrument features and study outcomes. The study outcomes included the cecal intubation rate, cecal intubation time, sedation dose used, abdominal pain score, and ancillary maneuvers during the procedure (manual pressure used and position changes made).

Two investigators (Chen Y and Xie Q) independently extracted details of the study population, interventions and outcomes. A paper was reviewed if either of the two investigators thought its abstract was relevant. If there were any discrepancies in the information provided in a title and the corresponding abstract, the full article was reviewed for clarification. Differences in opinion were resolved by discussion with the third author of this paper (Chen B).

### Assessment of risk of bias in included studies

To avoid the risk of bias in the assessment, two investigators independently used an assessment form recommended by the Cochrane Handbook. Any disagreements were resolved by discussion with a third author until consensus was obtained. We considered the following criteria: (1) Sequence generation: was the allocation sequence adequately generated? (2) Allocation concealment: was the allocation adequately concealed? (3) Blinding: was knowledge of the allocated intervention adequately prevented during the study? (4) Incomplete outcome data: were incomplete outcome data adequately addressed? (5) Selective outcome reporting: were reports of the study free of the suggestion of selective outcome reporting? and (6) Other sources of bias: was the study apparently free of other problems that could place it at a high risk of bias?

Each domain was graded as yes (low risk of bias), no (high risk of bias), or unclear (uncertain risk of bias) according to the criteria.

For rating the strength and quality of the evidence for a given comparison, the Working Group grades of evidence and Summary of Findings tables recommended by the Cochrane Collaboration were used.

### Assessment of reporting biases

For the assessment of publication bias, a funnel plot was

constructed if sufficient data were available.

### Statistical analysis

Meta-analyses were conducted for trials comparing MEI with SC using the statistical tool Revman 5.1. Dichotomous data were expressed as an OR, and continuous outcomes were expressed as the mean difference (MD) with a 95%CI. A random effects model was used for the pooling of data.

We used a random effect model with the Mantel-Haenszel method for pooling dichotomous and continuous data. We assessed the heterogeneity of the trial results by calculating the  $I^2$  measure of inconsistency with a cutoff point of  $I^2 = 50\%$ .

A sensitivity analysis was performed by excluding the trials with small numbers of patients and by excluding the trials performed by inexperienced providers.

## RESULTS

### Search results

Overall, our searches identified 43 articles that compared MEI with SC. After reading the abstracts and full-texts, we excluded 35 of these articles because they were reviews or were not RCTs or case reports. Finally, eight studies met the criteria for inclusion in the review<sup>[9-15,22]</sup>.

### Trial characteristics

The characteristics of these studies are summarized in Table 1. All of these studies were RCTs, containing a total of 2967 participants (1566 male, 1401 female) of 7 to 90 years of age.

The instruments used in the trials included the Olympus CF-1T200L scope (160 cm)<sup>[16,17]</sup>, the ScopeGuide endoscope insertion tube system<sup>[18-20]</sup>, the Olympus CF-Q160DI with the Olympus ScopeGuide system<sup>[15,21]</sup>, the Olympus CF-Q180AL, the Olympus CF-Q160AL and the CF-Q140DL/I with the Olympus ScopeGuide system.

The experience levels of the endoscopists were evaluated either by years of experience (more than six years) or by the number of procedures performed (more than 200 procedures). In the retrieved articles, eight trials evaluated MEI procedures performed by experienced colonoscopists; while four studies evaluated MEI procedures performed by less experienced colonoscopists (four studies included both experienced and less experienced colonoscopists).

### Risk of bias in included studies

Among the eight RCTs that were included in this meta-analysis, an allocation sequence was generated using a computer-generated random number table<sup>[15-17,20,23]</sup>. Four of the eight trials reported adequate allocation concealment<sup>[15,20,21,23]</sup>, while in another four trials, the allocation concealment was unclear. In all eight trials, all patients were blinded, but the endoscopists were not blinded in any of these trials because of the nature of the interventions.

The quality of the evidence for the outcomes in the included studies is shown in the Summary of Findings

tables (Table 2).

### Outcomes

**Cecal intubation rate:** There were eight research papers that reported on this topic. MEI with a colonoscope showed a higher cecal intubation rate compared with SC (OR = 1.92, 95%CI: 1.13-3.27, Figure 1A).

**Cecal intubation time:** Only three studies included the cecal intubation time, and all of these studies were included in the analysis. The meta-analysis of these three trials showed no significant difference in the cecal intubation time between MEI and SC (MD = -0.07, 95%CI: -0.16-0.02; Figure 1B). There was no heterogeneity among these three studies ( $I^2 = 2\%$ ,  $P = 0.36$ ).

**Sedation dosage:** Five studies reported the sedation dose used during the colonoscopic procedure. One trial used a patient-controlled analgesia (PCA) pump consisting of a mixture of midazolam and meperidine<sup>[17]</sup>; another one employed a combination of midazolam and pethidine<sup>[16]</sup>. Franciosi JP *et al*<sup>[19]</sup> reported the use of a mixture of midazolam and fentanyl<sup>[19]</sup>, and Dechène A *et al*<sup>[20]</sup> used midazolam, pethidine and propofol together. The other studies used midazolam, pethidine and diazepam<sup>[21]</sup>.

**Abdominal pain:** Eight studies presented pain scores as the mean and standard deviation or median. However, the scales used for scoring pain were different. In two studies<sup>[18,19]</sup>, a 0 to 10 score scale was used, and the other six studies used a 0 to 100 score scale, a 1 to 7 visual analogue scale, a validated questionnaire or abdominal compression<sup>[15-17,20,23]</sup>. Due to the differences in the scales, we did not pool the data for these studies.

**Ancillary maneuvers:** Four trials reported ancillary maneuvers during colonoscopy. Only two trials listed the amount of abdominal pressure applied, and only two trials reported the position changes made during colonoscopy; therefore, these data were not pooled for analysis.

### Subgroup and sensitivity analysis

A subgroup analysis was performed to evaluate the cecal intubation rate during colonoscopy according to the experience level of the endoscopists. The cecal intubation rate of MEI with experienced endoscopists was similar to that of SC (OR = 1.84, 95%CI: 0.97-3.48, seven trials, Figure 1C), while the chance of achieving cecal intubation was clearly higher with MEI than SC for inexperienced endoscopists (RR = 3.63, 95%CI: 1.96-6.74, three trials, Figure 1D).

The sensitivity analysis that excluded the studies with a small number of patients (less than 100) resulted in insignificant changes to the ORs and Weighted Mean Difference (WMDs). Additionally, we used the fixed-effect model to reanalyze all the data previously analyzed using the random-effect model. There were no significant changes to the ORs or RRs and WMDs when the fixed-effect model was used.



**Table 1** Characteristics of the included studies comparing use of the magnetic endoscopic imaging colonoscope and standard colonoscope

Study	Number of patients (n)	Endoscopists' experience level	Colonoscope type	Cecal intubation rate	Cecal intubation time	Sedation dose	Pain score	Ancillary maneuvers
Shah <i>et al</i> <sup>[16]</sup>	296 (male 138, female 158)	Trainees, skilled endoscopists	MEI, SC	Total MEI: 100% (150/150) SC: 90.4% (132/146) Trainees: MEI: 100% (58/58) SC: 89% (49/55) <i>P</i> = 0.0115 Skilled endoscopists: MEI: 100% (92/92) SC: 91% (83/91) <i>P</i> = 0.0032	Trainees: Median, min MEI: 11.8 (4.3-31.5) SC: 15.3 (4-67) <i>P</i> = 0.0092 Skilled endoscopists: MEI: 8.0 (2.6-40.8) SC: 9.3 (2.5-52.6) <i>P</i> = 0.0484	Trainees: Mean (SD) Midazolam, mg MEI: 1.2 (0.4) SC: 1.2 (0.4) <i>P</i> = 0.4013 Pethidine, mg MEI: 26 (14.5) SC: 30 (15.5) <i>P</i> = 0.1674 Skilled endoscopists Mean (SD) Midazolam, mg MEI: 1.3 (1.1) SC: 1.6 (1.0) <i>P</i> = 0.0724 pethidine, mg MEI: 30 (23.9) SC: 34 (25.6) <i>P</i> = 0.2036	Trainees: Mean (SD) 0-100 VAS MEI: 28.5 (20.2) SC: 30.1 (24.4) <i>P</i> = 0.553 Skilled endoscopists: MEI: 28.6 (23.1) SC: 24.8 (24.2) <i>P</i> = 0.30	Abdominal hand pressure used: Trainees: MEI: 78 SC: 61 Skilled endoscopists: MEI: 93 SC: 147
Shah <i>et al</i> <sup>[17]</sup>	122 (male 62, female 60)	Experienced	MEI, SC	MEI: 97% (61/62) SC: 95% (57/60) <i>P</i> = 0.3606	Median, min MEI: 10.6 (7.6-17.03) SC: 13.1 (9.01-26.47) <i>P</i> = 0.0664	Midazolam (mg), median MEI: 0.44 (0-1.48) SC: 0.88 (0-1.47) <i>P</i> = 0.2875 Meperidine (mg), median MEI: 16.75 (0-59) SC: 32.5 (0-59) <i>P</i> = 0.2643	Patient pain score (100 mm VAS) MEI: 19 (9-29) SC: 29 (10-50) <i>P</i> = 0.0662	Not stated
Cheung <i>et al</i> <sup>[18]</sup>	120 (male, 64 female 56)	Experienced	MEI, SC	MEI: 95% (57/60) SC: 93% (56/60) <i>P</i> = 1.0	Median, min MEI: 5 (2-46) SC: 5 (3-15) <i>P</i> = 0.32	Not stated	Median (range), pain score from patients MEI: 5 (0-10) SC: 4 (0-10) <i>P</i> = 0.13	Abdominal hand pressure MEI: 0 SC: 0 Position change made MEI: 6.7% SC: 0% <i>P</i> = 0.12
Hoff <i>et al</i> <sup>[15]</sup>	419 (male 202, female 217)	Experienced, inexperienced	MEI, SC	MEI: 90% (190/212) SC: 74% (153/207) <i>P</i> < 0.001 experienced: MEI: 90% (137/152) SC: 78% (115/148) <i>P</i> = 0.003 Inexperienced: MEI: 88% (53/60) SC: 64% (38/59) <i>P</i> = 0.002	Mean (95%CI), min MEI: 19.1 (17.2-21.0) SC: 17.6 (15.8-19.5) <i>P</i> = 0.28	Not stated	Severe pain during Examination: experienced MEI: 7.3% (10/137) SC: 16% (21/132) <i>P</i> = 0.03 Inexperienced MEI: 14% (8/56) SC: 15% (7/47) <i>P</i> = 0.93	Not stated
Franciosi <i>et al</i> <sup>[19]</sup>	40 (male 16, female 24)	Experienced	MEI, SC	MEI: 95% (19/20) SC: 94.4% (17/18) <i>P</i> = ns	Mean (range), min MEI: 16.5 (6-52) SC: 12 (6-33) <i>P</i> = ns	Not stated	Median, 0-10 pointscale MEI: 7 (2-10) SC: 19 (3-10) <i>P</i> = ns	Not stated
Dechène <i>et al</i> <sup>[20]</sup>	1000 (male 550, female 450)	Experienced, inexperienced	MEI, SC	MEI: 98.2% (481/490) SC: 98.0% (500/510) <i>P</i> = ns	Mean time, (s) MEI: 507 ± 384 (8.45 ± 6.4) SC: 538 ± 428 (8.97 ± 7.13) <i>P</i> = ns Inexperienced: MEI: 613 ± 435 (225) SC: 660 ± 458 (245) <i>P</i> = ns Experienced: MEI: 415 ± 304 (256) SC: 421 ± 361 (255) <i>P</i> = ns	Not stated	Not stated	Position change made MEI: 1.5% (7/481) SC: 3.0% (15/500) <i>P</i> = ns Manual pressure used MEI: 4.2% (20/481) SC: 6.4% (32/500) <i>P</i> = ns

Holme <i>et al</i> <sup>[21]</sup>	810 (male 378, female 432)	Experienced, MEI, SC inexperienced	MEI: 91.9% (385/419) SC: 89.5% (350/391) <i>P</i> = 0.28 Inexperienced: MEI: 77.8% (42/54) SC: 56.0% (28/51) <i>P</i> = 0.022 Experienced: MEI: 94.0% (343/365) SC: 96.0% (321/340) <i>P</i> = 0.87	Mean ± SD MEI: 14.0 ± 12.2 SC: 15.3 ± 14.2 <i>P</i> = 0.67 Experienced: MEI: 11.4 ± 7.2 SC: 12.3 ± 9.4 <i>P</i> = 0.78 Inexperienced: MEI: 31.7 ± 21.3 SC: 35.7 ± 22.1 <i>P</i> = 0.42	Not stated	No pain during examination: MEI: 24% (82/341) SC: 20.8% (66/318) Severe pain during examination: MEI: 0 SC: 0	Need for assistance experienced: MEI: 1.1% (4/365) SC: 1.5% (5/340) <i>P</i> = 0.75 Inexperienced: MEI: 18.5% (10/54) SC: 40% (20/51) <i>P</i> = 0.018
Shergill <i>et al</i> <sup>[23]</sup>	160 (male 156, female 4)	Experienced MEI, SC	MEI: 100% (65/65) SC: 97% (73/75) <i>P</i> = 0.19	Mean ± SD MEI: 9.4 ± 5.7 SC: 8.5 ± 5.4 <i>P</i> = 0.31	Not stated	Mean (SD) MEI: 3.06 (1.13) SC: 3.12 (1.22) <i>P</i> = 0.60	Not stated

MEI: Magnetic endoscopic imaging; SC: Standard colonoscopy VAS: Visual Analogue Scale.

**Table 2 Summary of findings for the main comparison of magnetic endoscopic imaging colonoscopy and standard colonoscopy**

Outcomes	Illustrative comparative risks <sup>1</sup> (95%CI)		Relative effect (95%CI)	No of Participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk Standard colonoscopy	Corresponding risk Magnetic endoscope imaging colonoscopy				
Cecal intubation rate	Study population 912 per 1000	952 per 1000 (921 to 971)	OR = 1.92 (1.13-3.27)	2945 (8 studies)	+ + + - Moderate <sup>1</sup>	
	Moderate 939 per 1000	967 per 1000 (946 to 981)				
Cecal intubation time		The mean cecal intubation time in the intervention groups was 0.43 lower (0.13 lower to 0.28 higher)		1934 (3 studies)	+ + + + High <sup>1</sup>	

<sup>1</sup>The basis for the assumed risk (e.g., the median control group risk across studies) is provided in the footnotes. The corresponding risk (and its 95%CI) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95%CI). GRADE: Working Group grades of evidence. High quality: Further research is very unlikely to change our confidence in the estimate of the effect; Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of the effect and may change the estimate; Low quality: Further research is very likely to have an important impact on our confidence in the estimate of the effect and is likely to change the estimate; Very low quality: We are very uncertain about the estimate.

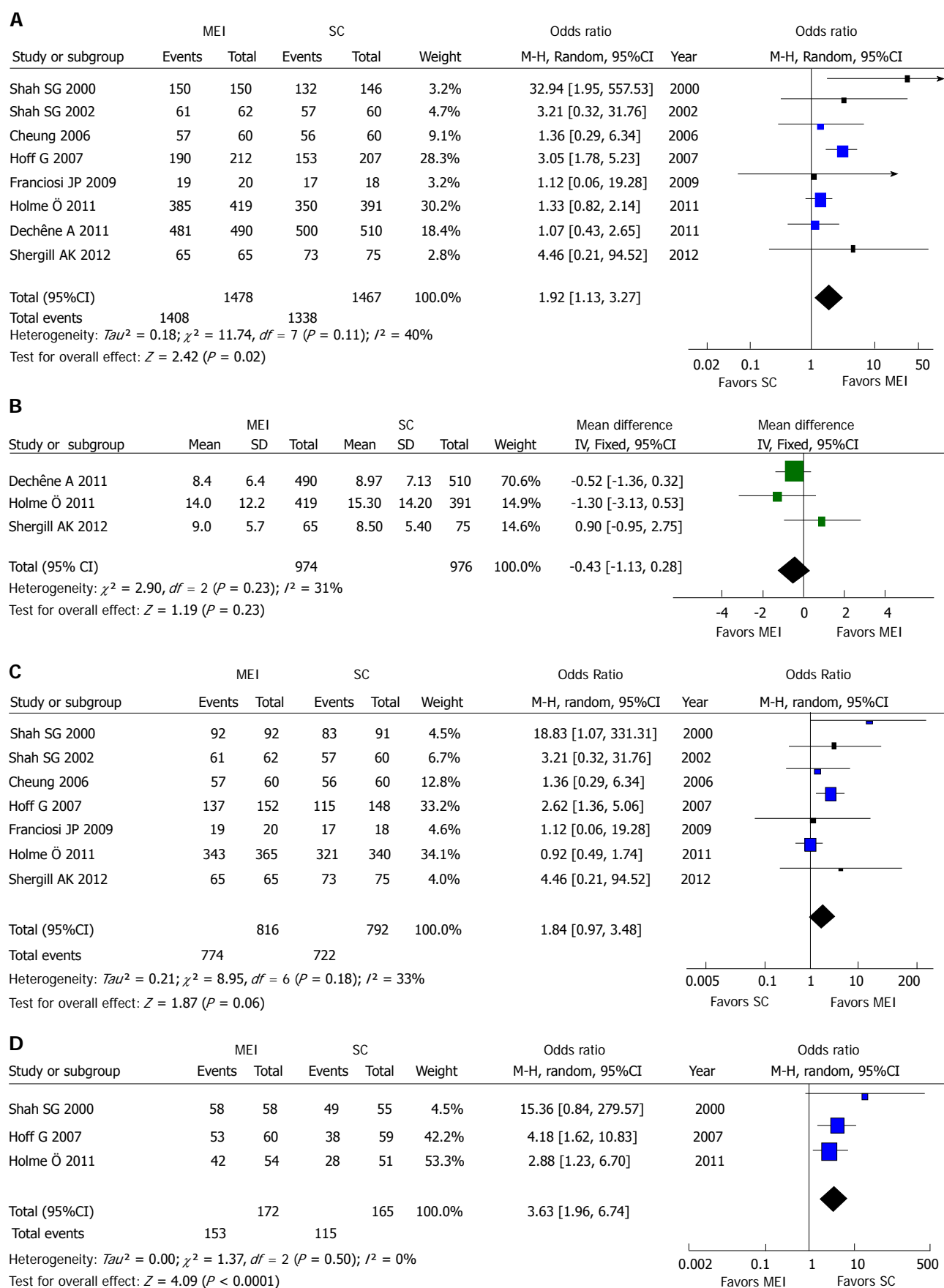
To detect publication bias, asymmetry was explored using a funnel plot. The distribution of the results of each study in the funnel plot excluded any potential publication bias.

## DISCUSSION

The meta-analysis included eight RCTs published up to June 2012, including a total of 2967 participants who received MEI or SC. MEI has replaced X-ray imaging during colonoscopic procedures in many situations and therefore has a proven benefit for patients and staff<sup>[24-26]</sup>. MEI exhibited higher cecal intubation rates compared with standard colonoscopy but did not have any distinct advantage over standard colonoscopy in terms of cecal intubation time. Considering the potential advantage of real-time imaging, we were surprised to find that most of the individual studies showed no difference between the MEI and standard groups in the time required to reach the cecum but that the pooled data favored MEI. The in-

creased sample size is the most likely explanation for the difference in the cecal intubation rate. A larger number of participants reduced the sampling error and directly affected the cecal intubation rate between MEI and SC. These results are meaningful in clinical practice. As is known, the failure rate of cecal intubation remains high in day-to-day SC. This means that part of the colon of some patients is not clearly visualized, which can prevent the early diagnosis and treatment of colonic diseases. MEI has increased the intubation rate and has made the early and accurate diagnosis of colonic issues, such as colorectal cancers, polyps and inflammatory bowel disease, possible.

The cecal intubation rate was also analyzed in two subgroups based on the experience level of the endoscopists (experienced and inexperienced). For inexperienced endoscopists, the MEI system appears to be advantageous. The cecal intubation rate for inexperienced endoscopists was higher in patients randomized to MEI than in the standard group. It is possible that inexperienced



**Figure 1 Meta-analysis.** A: Cecal intubation rate comparison for the magnetic endoscopic imaging (MEI) colonoscopy and standard colonoscopy (SC); OR with 95%CI; B: Cecal intubation time comparison for the MEI colonoscopy and SC; MDs with 95%CI; C: Cecal intubation rate: subgroup analysis of trials comparing the MEI colonoscopy and SC with experienced endoscopists; OR with 95%CI; D: Cecal intubation rate: subgroup analysis of trials comparing the MEI colonoscopy and SC with inexperienced endoscopists; OR with 95%CI.

endoscopists are capable of identifying and minimizing loops with the continuous real-time imaging system. However, experienced endoscopists are likely able to recognize and resolve loops quickly without the need for MEI visualization. Therefore, whether MEI actually makes both inexperienced and experienced physicians better endoscopists remains to be determined.

The individual studies included in this meta-analysis showed concordance in the cecal intubation times between the two groups, and the pooled results for all trials also showed no significance.

There were four complications reported in two studies included in this meta-analysis<sup>[15,21]</sup>, and they all occurred in the standard group. Three patients had a vasovagal reaction with rapid spontaneous recovery, and there was one case of bleeding following a polypectomy. To this point, no safety concerns have been raised with the use of MEI. During the procedure, precise judgment and caution are necessary, especially when advancing through a narrowed colon or pushing through loops.

A potential limitation of the meta-analysis is that these studies could not be performed in a way that would 'blind' the endoscopists to the scope used because of the nature of the interventions. Additionally, different models and manufacturers of MEI equipment were used in the studies included in the analysis. Finally, in several studies, specific patient subsets, such as colonic cancer patients and patients who had undergone prior colonic surgery, were excluded.

In conclusion, the present results indicated that the real-time magnetic imaging system is safe and beneficial in training and educating inexperienced endoscopists, as well as improving the cecal intubation rate for both experienced and inexperienced endoscopists. However, only a few studies have reported the advantages of MEI because it is a new technique, and further studies should be performed to confirm the role of the MEI colonoscope.

## COMMENTS

### Background

Colonoscopy is the gold standard and the most common and accurate tool for detecting important structural lesions of the lower gastrointestinal tract and diagnosing colonic diseases, such as polyps, colorectal cancer and inflammatory bowel disease. Magnetic endoscopic imaging (MEI) is a non-radiographic imaging technique that has been developed in recent years that is capable of displaying real-time three-dimensional images of the colonoscope shaft within the abdominal cavity. A pulsed low-magnetic field is sequentially produced by a series of electromagnetic generator coils spaced 10 cm apart along a catheter inserted through the accessory channel of the endoscope. The imager view is updated every 0.2 s to make the system essentially real-time, and the images are then recorded on a computer disk for subsequent replay or analysis.

### Research frontiers

The MEI system, when compared to standard colonoscopy (SC) with no visualization, has been shown to be beneficial in increasing the cecal intubation rate, reducing the number of attempts to straighten loops, and in reducing the duration of looping, especially with trainees. A few studies have compared MEI with SC; however, the results of these studies have not been uniform.

### Innovations and breakthroughs

A few studies have compared MEI with SC; however, the results of these studies have not been uniform. Thus, this was the first meta-analysis to assess

the theoretical advantages of MEI over SC and to compare the efficacies of MEI and SC. Through this study, we found that the real-time magnetic imaging system is of benefit in training and educating inexperienced endoscopists and improves the cecal intubation rate for experienced and inexperienced endoscopists.

### Applications

The results indicated that the real-time magnetic imaging system is safe and of benefit in training and educating inexperienced endoscopists, as well as improving the cecal intubation rate for both experienced and inexperienced endoscopists.

### Peer review

The authors report a meta-analysis of trials that have compared MEI colonoscopy with standard colonoscopy for cecal intubation rates and cecal intubation times. Although colonoscopy supported by MEI was first reported in 1993, this technique has not been widely adopted either because it is expensive or because gastroenterologists are uncertain of its benefits.

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## Spontaneous intramural duodenal hematoma in type 2B von Willebrand disease

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Patient was managed conservatively with bowel rest, continuous nasogastric decompression, total parenteral nutrition, recombinant factor VIII (humateP) and transfusion. Symptoms resolved over the course of the hospitalization. This case highlights an important complication of an inherited coagulopathy.

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**Key words:** Duodenal hematoma; von Willebrand disease

**Core tip:** Spontaneous non-traumatic duodenal hematoma has been linked to coagulopathy, but this case includes a rare form of coagulopathy in von Willebrand type 2B. Early identification with computed tomography imaging and treatment of the coagulopathy aided the usage of conservative therapy which allowed the patient to avoid surgical intervention consistent with prior successful cases of duodenal hematomas.

### Abstract

Intramural duodenal hematoma is a rare cause of a proximal gastrointestinal tract obstruction. Presentation of intramural duodenal hematoma most often occurs following blunt abdominal trauma in children, but spontaneous non-traumatic cases have been linked to anticoagulant therapy, pancreatitis, malignancy, vasculitis and endoscopy. We report an unusual case of spontaneous intramural duodenal hematoma presenting as an intestinal obstruction associated with acute pancreatitis in a patient with established von Willebrand disease, type 2B. The patient presented with abrupt onset of abdominal pain, nausea, and vomiting. Computed tomography imaging identified an intramural duodenal mass consistent with blood measuring 4.7 cm × 8.7 cm in the second portion of the duodenum abutting on the head of the pancreas. Serum lipase was 3828 units/L.

Eichele DD, Ross M, Tang P, Hutchins GF, Mailliard M. Spontaneous intramural duodenal hematoma in type 2B von Willebrand disease. *World J Gastroenterol* 2013; 19(41): 7205-7208 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7205.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7205>

### INTRODUCTION

Intramural duodenal hematoma is a rare occurrence of proximal gastrointestinal tract obstruction. Presentation of intramural duodenal hematoma most often occurs following blunt abdominal trauma in children, but non-traumatic cases have been linked to anticoagulant therapy, pancreatitis, bleeding disorders, malignancy, vasculitis and upper endoscopy<sup>[1-4]</sup>. We report a unique case of spontaneous intramural duodenal hematoma presenting as an intestinal obstruction associated with acute pancreatitis infection in a

patient with established von Willebrand disease, type 2B.

## CASE REPORT

A 24-year-old male was transferred to our tertiary center with a 10 h history of abrupt onset abdominal pain, nausea, and vomiting. History was notable for type 2B von Willebrand disease initially diagnosed at 12 years of age following recurrent hemorrhagic episodes (epistaxis, gingival bleeding and hemarthrosis) and diagnosis in his dizygotic sibling for a spontaneous bleeding episode. Both dizygotic siblings were genetically tested for mutations and found to have the G3946A genotype. Physical examination on admission revealed heart rate 66 beat/min, blood pressure 115/53 mmHg, temperature 36.7 °C and respiration rate 16. Patient was in moderate amount of pain. There were minor facial petechiae. Oropharynx was pink and moist and no evidence of gingival bleeding or epistaxis. There was no conjunctival injection. Neck was supple without adenopathy or thyromegaly. Heart was regular rate and rhythm without murmur. Respirations were unlabored and auscultation was clear bilaterally. Abdominal exam demonstrated hypoactive bowel sounds, epigastric tenderness, without rebound tenderness or guarding. Testicular exam revealed bilateral ecchymoses without edema or tenderness. The lower extremities noted multiple small ecchymosis in various stages of healing. There was normal range of motion in upper and lower extremities, no focal joint pain or effusions noted. Hemoglobin was 15.0 g/dL, white blood cell count 18.8 cells/mL, segmented neutrophils 85%, bands 6%, platelets were immeasurable due to clumping. Sodium 140 mmol/L, potassium 4.0 mmol/L, chloride 107 mmol/L, bicarbonate 26 mmol/L, BUN 21 mg/dL, creatinine 0.9 mg/dL, total protein 7.1 gm/dL, albumin 4.3 gm/dL, aspartate aminotransferase 33 U/L, alanine aminotransferase 34 U/L, alkaline phosphatase 46 U/L, bilirubin 2.4 mg/dL, Lipase 3828 units/L.

Computed tomography (CT) of the abdomen without contrast revealed a hyperdense mass in the second and third portions of the duodenum measuring 4.7 cm × 8.7 cm with associated retroperitoneal hemorrhage, extending from the right anterior pararenal space to the right pelvic sidewall. The duodenal mass resembled a hematoma and was abutting the pancreatic head to the left. Liver, spleen, adrenal glands and genitourinary organs were normal. The findings led to a diagnosis of intramural duodenal hematoma complicated by acute pancreatitis (Figure 1).

The patient was transfused one unit of single donor platelets and administered human antihemophilic factor/von Willebrand factor complex (Humate P<sup>®</sup>) 80 Units/kg prior to transfer from outside facility. A nasogastric (NG) tube was placed, and intravenous bolus of three liters of normal saline was given. Surgery consultation was obtained. He received factor replacement therapy (Humate P<sup>®</sup> 40 units/kg every 12 h) for the next 72 h, following an initial loading bolus.

Esophagogastroduodenoscopy and endoscopic ultrasound were delayed until day eleven of the hospitalization due to potential risk for hemorrhage from the hematoma during endoscopy. Endoscopy showed a near complete obstruction in the second portion of the duodenum with large amount of gastric fluid (Figures 2 and 3). The patient was discharged to home on day 15 of hospitalization after patient's pain receded, repeat imaging noted unchanged duodenal and retroperitoneal hematoma and resolution of gastric outlet obstruction. The patient was readmitted to the hospital the following day with worsening abdominal pain with development of nausea and vomiting. On readmission a repeat abdominal CT was performed due to concern of enlargement and extravasation of the hematoma. Imaging revealed a minute increase in size of the duodenal hematoma that measured 5.3 cm × 5.8 cm (previously 5.3 cm × 5.2 cm) in the AP and transverse dimensions and persistent retroperitoneal hematoma. After control of the patient's symptoms and his ability to tolerate a modified and limited oral diet the patient was finally discharged home on day 23 of the second hospitalization. Following discharge the patient had not experienced return of abdominal pain, nausea and vomiting. Patient was able to tolerate a full diet and total parenteral nutrition (TPN) was discontinued shortly after discharge. Abdominal CT performed at day 68 from presentation revealed decreased size of the duodenal hematoma with measurements of 2.1 cm × 2.6 cm (initially 4.7 cm × 8.7 cm).

## DISCUSSION

McLachlan described the first published report of duodenal hematoma in 1838 as a false aneurismal tumor<sup>[5]</sup>. Decades later, the first case of a non-traumatic intramural hematoma was reported by Sutherland in a child with Henoch-Schonlein purpura that presented as intussusception.

The most common clinical scenario for presentation of intramural duodenal hematoma is seen in blunt abdominal trauma in children<sup>[3,4]</sup>, but non-traumatic cases have been linked to anticoagulant therapy, pancreatitis, bleeding disorders, malignancy, vasculitis and upper endoscopy<sup>[1,3,4,6]</sup>. In adults the most common risk factor for spontaneous intramural small-bowel hematoma reported is supratherapeutic anticoagulation<sup>[7]</sup>. Other notable risk factors include bleeding disorders (hemophilia, idiopathic thrombocytopenic purpura), malignancies and its therapies (leukemia, lymphoma, myeloma, pancreatic cancer, and chemotherapy), vasculitis and pancreatitis<sup>[7,8]</sup>.

Common signs and symptoms on presentation typically include abdominal pain that ranges from vague abdominal complaints to intestinal tract obstruction with vomiting and tenderness mimicking an acute abdomen<sup>[7]</sup>. Abbas noted that most of the patients presented after having symptoms for several days<sup>[7]</sup>. Other infrequent signs and symptoms include jaundice and right upper quadrant tenderness due to bile duct obstruction second-

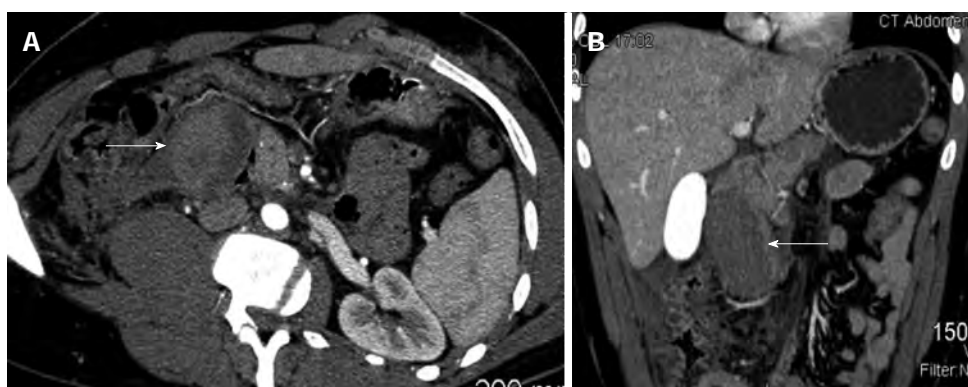


Figure 1 Axial image (A) and coronal image (B) from abdominal computed tomography with contrast depicts the intramural hematoma as it compresses the head of the pancreas and its associated hemorrhage as it extends into the retroperitoneum (arrows).

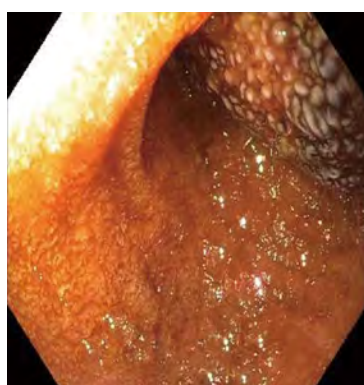


Figure 2 Esophagogastroduodenoscopy showing near complete obstruction of duodenum by intramural hematoma.



Figure 3 Endoscopic ultrasound image of intramural duodenal hematoma.

ary to compression by the hematoma<sup>[8]</sup>. In this case, the patient's elevated lipase identified pancreatitis induced by the hematoma as opposed to the case of Bellens *et al*<sup>[9]</sup> in which pancreatitis was the inciting event.

The location of the hemorrhage in spontaneous non-traumatic intraluminal hematoma is submucosal and originates from a small vessel<sup>[7]</sup>. In our case, based upon the imaging findings, the bleeding originated from the gastroduodenal artery. Extravasation of submucosal hematomas can occur into intraluminal, intra-mesenteric, and retroperitoneal sites<sup>[7]</sup>. Whereas traumatic small-bowel hematomas commonly affect the duodenum, most spontaneous small-bowel hematomas involve the jejunum<sup>[7,10]</sup>. Additionally, Abbas noted that no spontaneous intramural hematoma was less than 8 cm in length, consistent with our findings of an 8.7 cm length<sup>[7]</sup>.

In recent years, there has been an increase in detection of non-traumatic intramural small bowel hematomas in patients that are anti-coagulated. This increase has also coincided with more sensitive imaging modalities that have led to earlier detection. In general, CT is the imaging choice for diagnosis of spontaneous intramural small bowel hematoma, but as a rapid screening modality, ultrasound can be used effectively during the initial evaluation, as it is inexpensive, rapid, noninvasive and readily available in most instances<sup>[5,7]</sup>. Characteristic features

have been described include circumferential bowel wall thickening, intramural hyperdensity with Hounsfield units characteristic for blood (30-80 H), luminal narrowing and intestinal obstruction<sup>[7]</sup>. Reassessment of the status of the hematoma is generally recommended with CT in 2 wk following diagnosis and conservative management<sup>[8]</sup>.

In prior case reports, the initial treatment regimen for IDH favored medical management; if feasible in a stable patient, as spontaneous local absorption of the hematoma occurs in most circumstances<sup>[5-8]</sup>. Conservative management constituted bowel rest, continuous NG decompression, TPN, blood transfusion and correction of coagulopathy. Minimally invasive drainage through image-guided modalities is generally not recommended in consideration to the technical difficulty required to access the precarious location of the pathology as well as the risk for bowel perforation<sup>[8]</sup>. Complete resolution of the hematoma and healing of the intestine usually occurs within 2 mo after diagnosis in cases of non-extensive hematomas<sup>[5,7]</sup>. Surgical intervention is only indicated if there is significant intraluminal hemorrhage, bowel perforation or identifiable risk of ischemia<sup>[4,5,7]</sup>. Surgical evacuation of the hematoma through laparotomy or laparoscopy tends to be the best surgical treatment in these situations, specifically in instances in which the abdominal pain or obstruction did not resolve and objective findings of bowel infarction were present<sup>[11-13]</sup>. Limited case reports have detailed suc-



cessful outcomes with percutaneous aspiration and endoscopic dilation<sup>[4,14,15]</sup>. Finally, bypass surgery is reserved for patients with severe duodenal perforation and multiple comorbidities precluding resectional surgery<sup>[8]</sup>.

Our case highlights a rare but important complication of coagulopathy. Acute abdominal pain with signs and symptoms of obstruction in patients with coagulopathy should raise suspicion for spontaneous intramural small-bowel hematoma. Prompt diagnosis with CT imaging is a crucial contribution to patient treatment, because conservative medical management can be achieved in most patients. Whereas, in cases of failed conservative medical management surgical intervention is performed.

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E- Editor: Ma S



## Endoscopic treatment of efferent loop syndrome with insertion of double pigtail stent

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Author contributions: Lee WY, Moon JS were attending doctors for the patient; Lee WY performed surgical operation; Moon JS performed endoscopic procedure; Lee WY, Moon JS organized report and wrote paper.

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tient with efferent loop syndrome by implantation of a double pigtail stent. Efferent loop syndrome is a very rare postgastrectomy syndrome that can occur following Billroth-II or Roux-en-Y reconstruction. Surgical treatment is usually required. However, in this case, efferent loop obstruction was successfully resolved by the insertion of a double pigtail stent. A double pigtail stent should be considered a treatment option for relieving efferent loop obstruction if immediate surgical treatment is not required.

Lee WY, Moon JS. Endoscopic treatment of efferent loop syndrome with insertion of double pigtail stent. *World J Gastroenterol* 2013; 19(41): 7209-7212 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7209.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7209>

### Abstract

Efferent loop syndrome is a very rare postgastrectomy syndrome that can occur following Billroth-II or Roux-en-Y reconstruction. The most common loop syndrome after gastric surgery is afferent loop syndrome; however, efferent loop syndrome has been reported in rare cases. Here, we report a case of efferent loop obstruction that occurred after postoperative adhesiolysis of a small-bowel obstruction. The patient had undergone a partial gastrectomy with Billroth II anastomosis and gastric ulcer perforation 30 years prior. The efferent loop obstruction was successfully resolved by the insertion of a double pigtail stent. To the best of our knowledge, this is the first case in the literature describing the treatment of efferent loop obstruction.

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**Key words:** Efferent loop syndrome; Double pigtail stent; Postgastrectomy syndrome

**Core tip:** We report the successful treatment of a pa-

### INTRODUCTION

Efferent loop syndrome is one of two “loop syndromes” that can occur after certain types of gastric surgery. Afferent loop syndrome is commonly reported, while efferent loop syndrome is very rarely reported. The signs and symptoms of both loop syndromes may be similar and difficult to distinguish, and surgical treatment is usually required to correct these problems. This report provided the first description of the successful treatment of a patient with efferent loop obstruction with the implantation of a double pigtail stent.

### CASE REPORT

A 58-year-old man, who had undergone a previous partial gastrectomy 30 years prior, was admitted to the emergency room with severe abdominal pain and vomiting. He was resuscitated and underwent computed tomography, which revealed different sites of small-bowel obstruction in the left and middle lower abdomen. A



**Figure 1** Endoscopic finding revealed a narrowed and swollen entrance of the efferent loop.



**Figure 2** Gastrograph in study showed nearly complete obstruction of the efferent loop.

laparotomy, showed that the small bowel was markedly distended over adhesion band but not strangulated. Adhesiolysis was performed. Other abdominal findings were nonspecific. During surgery, signs of a partial gastrectomy that included a retrocolic and antiperistaltic gastrojejunostomy. Gross findings were nonspecific.

The patient recovered well and started orally; however, seven days later, he developed abdominal discomfort and experienced episodes of copious bilious vomiting at night. His abdomen remained soft and not distended. Plain abdominal radiography and laboratory tests showed no remarkable findings. The next day, the symptoms persisted, and a gastroendoscopy was performed under the suspicion of afferent loop syndrome. Copious amount of bilious fluid were found in the remnant stomach and dilatation. The lumen of the afferent loop was normal; however, the efferent loop was narrowed and edematous approximately 5 cm below the site of the gastrojejunostomy (Figure 1). The endoscope could be passed into the loop. The narrowed loop did not appear to have any abnormal mucosal lesions. A gastrografen study showed nearly complete obstruction of the efferent loop (Figure 2). The patient was treated with nasogastric tube decompression and total parenteral nutrition, but did not improve. After seven days, a follow-up endoscopy showed that the efferent loop had not changed. Endoscopic pneumatic balloon dilatation (CRE™ Balloon, Boston Scientific Co. Ltd., Ireland; 12 mm; 40 psi for 1 min, 45 psi for 1 min) over the guide-wire and under endoscopic view was immediately performed but was not effective. Subsequently, a double pigtail stent (Zimmon™ Biliary Stent, Cook Co. Ltd., Ireland; 10 Fr; 7 cm) was inserted through the efferent loop stenosis and over the guide wire using a double-channel endoscope (Olympus GIF-Type 2T240) under endoscopic view (Figure 3). Beginning the day after the procedure, the patient did not complain of abdominal discomfort or experience vomiting. The device was monitored by abdominal radiography (Figure 4A). Serial plain abdominal radiographs did not show migration of the stent to the other site. The patient subsequently recovered, and there were no further episodes of abdominal discomfort and vomiting. A repeated gastrograph in study

and gastroscopy showed a good patency and a widened loop (Figure 5). Thirteen days after procedure, the double pigtail stent was expelled with the feces (Figure 4B). The patient eventually recovered and was discharged. At a 3-mo follow-up, the patient did not have any symptoms.

## DISCUSSION

Afferent or efferent loop syndrome is a purely mechanical problem characterized by the obstruction of gastric emptying at or near the site of a gastrojejunostomy<sup>[1]</sup>. Efferent loop syndrome is a rare post gastrectomy syndrome, while afferent loop syndrome is more common<sup>[2]</sup>. The major cause of syndrome is an intestinal hernia. The more minor causes include an adhesive band and kinking because of scarring or poor reconstruction during gastric surgery<sup>[3,4]</sup>. In some cases, intussusception causes efferent loop syndrome<sup>[5]</sup>. Rarely do we experience efferent loop obstruction with mucosal prolapse-like stenosis of efferent loop due to adhesion or bowel edema.

Efferent loop syndrome usually occurs within the first few weeks following a gastric surgery. However, this syndrome can also develop years after gastric surgery<sup>[6]</sup>. The usual causes of this syndrome during the early post-operative period are anastomotic edema and kinking due to poor operative procedure. The later-occurring forms of the syndrome may be caused by anastomotic stricture, ulcer, bowel adhesion, jejuno gastric intussusception, and anastomotic cancer<sup>[7]</sup>. Rarely do we experience efferent loop syndrome caused by the adhesiolysis of a mechanical obstruction from a previous operation.

The clinical symptoms of efferent loop syndrome are characterized by abdominal cramps and copious bilious vomiting. In particular, abdominal discomfort is relieved by vomiting. This symptom is worse when the patient is supine, as in this patient who had vomiting only at nights. Patients may be dehydrated and have metabolic alkalosis if the syndrome occurs over a prolonged period of time, patients may experience paradoxical aciduria.

The treatment of efferent loop syndrome varies depending on the cause of the syndrome. Complete loop obstruction due to a mechanical cause requires surgical

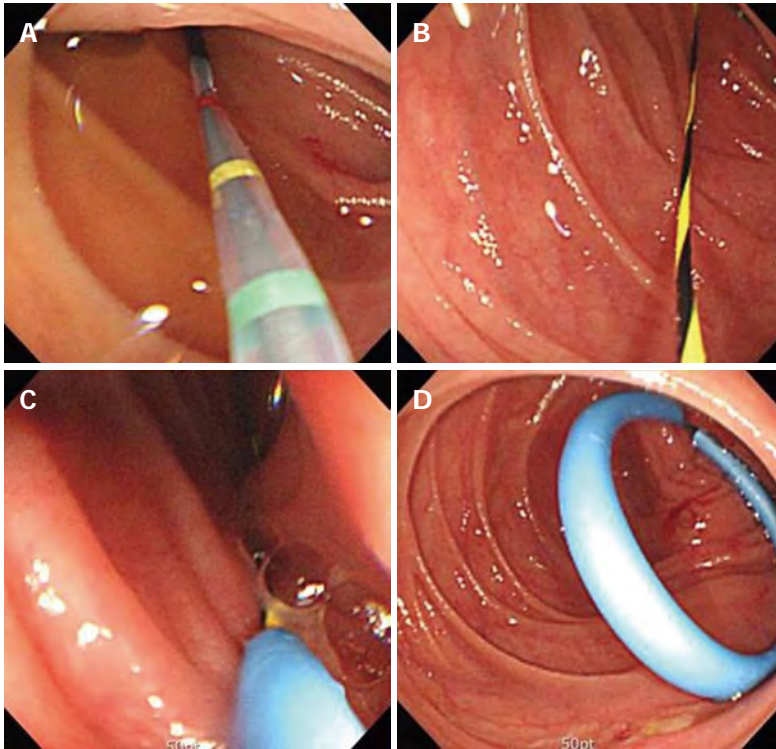


Figure 3 Endoscopic stent procedure was performed that double pigtail stent was inserted through efferent loop stenosis and over the guide wire using double-channel endoscope under endoscopic view.

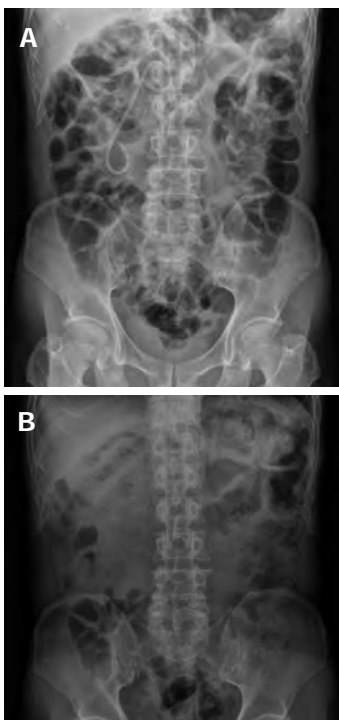


Figure 4 Plain abdominal radiography revealed double pigtail stent to efferent loop (A) and no double pigtail stent and other specific finding (B).

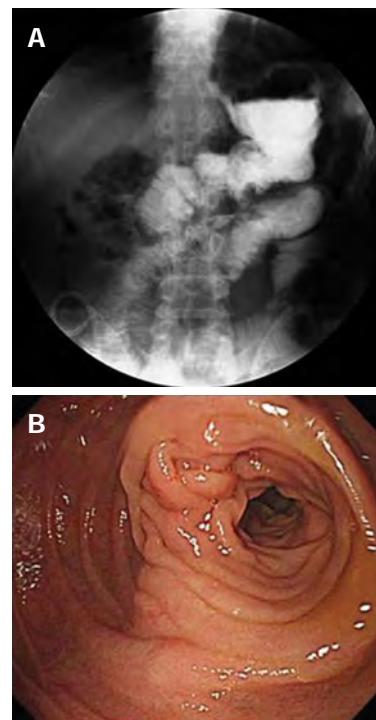


Figure 5 Follow-up gastrograin and endoscopic study showed free flow of contrast and recovery of narrowed, swollen orifice of the efferent loop.

intervention. Surgical interventions are numerous. However, if the syndrome is caused by an anastomotic ulcer or edema and adhesion, conservative treatment is indicated. Conservative treatments include nasogastric drainage,

keeping non *per os*, prescribing H2 antagonist or proton pump inhibitors, and total parenteral nutrition.

Because of recent advances in endoscopic intervention, various treatment methods have been attempted.



However, treatment has mainly been reported in afferent loop syndrome, very little documentation on the treatment of efferent loop syndrome exists in the literature. In several cases, endoscopic stent insertion was reported for the treatment of an obstruction due to tumor recurrence or peritoneal seeding. An endoscopic stent induced complication generally involve dislocation and clogging with subsequent infection. An endoscopic stent could be used in various types of gastrointestinal (GI) tract diseases, whereas a double pigtail stent is specifically used for managing biliary tract or pancreatic diseases. Pigtail stents may be inferior to straight stents in their drainage capacity, but the risk of migration of pigtail stents is lower<sup>[8]</sup>. In this case, we treated a patient with efferent loop obstruction caused by benign stricture with a double pigtail stent to prevent the dislocation of the GI stent. The patient improved following the clearing of efferent loop obstruction by the treatment. We therefore report that this case was resolved with the use of a double pigtail stent.

In conclusion, efferent loop syndrome following after a gastrectomy can be diagnosed by meticulous history-taking, physical examination, and radiologic modalities. In our opinion, a double pigtail stent should be considered a treatment option for relieving efferent loop obstruction if immediate surgical treatment is not required.

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## Fecal microbiota transplantation for severe enterocolonic fistulizing Crohn's disease

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Author contributions: Zhang FM designed and organized the study and wrote the paper; Wang HG performed the lab work; Wang M joined in the clinical work; Cui BT performed the lab work; Fan ZN and Ji GZ were the attending doctors of this group. Supported by (in part) The Public Donated Grant "Intestine Initiative"

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**Key words:** Fecal microbiota transplantation; Crohn's disease; Rescue therapy; Inflammatory bowel disease; Fistula

**Core tip:** We proposed that standardized fecal microbiota transplantation (FMT) might be a promising rescue therapy for refractory inflammatory bowel disease. This case report provided the first description of severe Crohn's disease in sustained clinical remission after FMT, and the brief protocol of patient preparation before and during FMT. Although there was only one case, the present result in our pilot clinical trial strongly supported our initial hypothesis and highlighted the attractive role of the remodeling of gut flora in host diseases.

Zhang FM, Wang HG, Wang M, Cui BT, Fan ZN, Ji GZ. Fecal microbiota transplantation for severe enterocolonic fistulizing Crohn's disease. *World J Gastroenterol* 2013; 19(41): 7213-7216 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7213.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7213>

### Abstract

The concept of fecal microbiota transplantation (FMT) has been used in traditional Chinese medicine at least since the 4<sup>th</sup> century. Evidence from recent human studies strongly supports the link between intestinal bacteria and inflammatory bowel disease. We proposed that standardized FMT might be a promising rescue therapy for refractory inflammatory bowel disease. However, there were no reports of FMT used in patients with severe Crohn's disease (CD). Here, we report the successful treatment of standardized FMT as a rescue therapy for a case of refractory CD complicated with fistula, residual Barium sulfate and formation of intra-peritoneal large inflammatory mass. As far as we know, this is the first case of severe CD treated using FMT through mid-gut.

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### INTRODUCTION

The gut microbiota is considered to constitute a "microbial organ" which plays a pivotal role in the intestinal diseases<sup>[1]</sup>. The gut metagenome sequencing showed that over 99% of the genes are bacterial<sup>[2]</sup>. Although fecal microbiota transplantation (FMT) has only recently gained popularity with its success in treating *Clostridium difficile* infection<sup>[3]</sup>, the concept of FMT for treatment of human intestinal diseases has been recorded at least for 1700 years in traditional Chinese medicine<sup>[4]</sup>. Evidence from human studies strongly supports the link between intestinal bacteria and inflammatory bowel diseases (IBD)<sup>[5-7]</sup>. IBD includes ulcerative colitis (UC) and Crohn's disease (CD). However, there have been only four publications on FMT for the treatment of IBD in 18 cases of UC<sup>[8-10]</sup>

and one case of newly diagnosed CD<sup>[10]</sup>. To date, there has been no report of FMT used as a rescue therapy in patients with severe CD. We proposed that standardized FMT might be a useful rescue therapeutic option for refractory inflammatory bowel disease.

## CASE REPORT

A 32-year-old Chinese man with known severe enterocolonic CD presented to our hospital in November 2012 because of progressive abdominal pain, bloody and purulent diarrhea and high fever of 38 °C-39.5 °C for 8 wk. He was diagnosed with CD in May 2010 when he was found three stricturing lesions and a penetrating lesion in ileum. In fact, he had gastrointestinal symptoms such as abdominal pain since 2005 and received enterectomy for presumptive appendicitis in 2007.

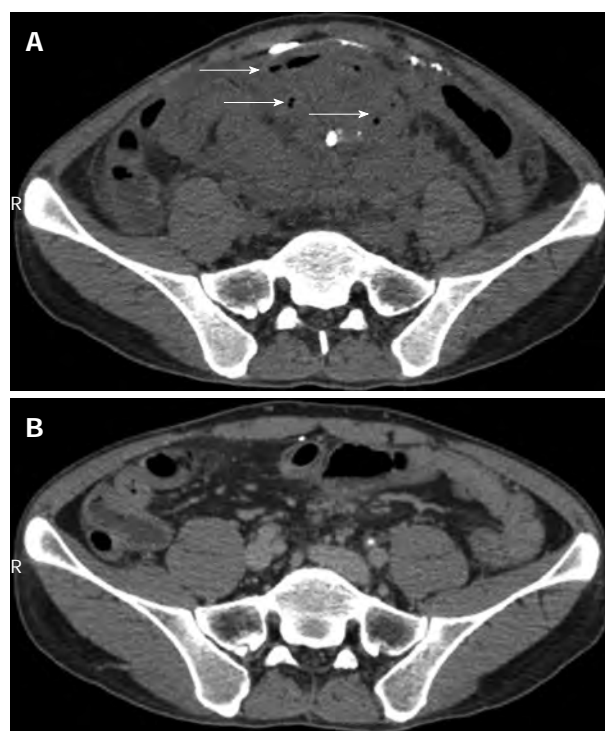
He was initially treated with intravenous prednisolone and Pentasa (mesalazine) 4.0 g daily for 6 years with frequent relapses. Colonoscopy in May 2011 revealed severe Crohn colitis with extensive ulcers throughout the entire colon. Therefore, he was treated with intravenous prednisolone and Mycophenolate Mofetil 1.0 g by mouth daily. In September 2012, many large ulcers were found in colon. He refused anti-tumour necrosis factor antibody infusion due to lack of medical insurance. He then received Mycophenolate Mofetil capsules 1.25 g daily and mesalazine 3.0 g daily. He was transferred to our center in November 2012.

On physical examination on admission, the patient showed poor general conditions. A large mass could be seen in the hypogastrium with tenderness. Laboratory tests showed leukocyte count of  $17.5 \times 10^9/L$  with 89.4% of neutrophils, erythrocyte sedimentation rate of 97 mm per hour and C-reactive protein of 141 mg/L. He had normal liver function test except album of 27.4 g/L (normal range, 35-55 g/L) and total cholesterol 2.6 mmol/L (normal range, 3.0-6.0 mmol/L).

CT scan showed an abdominal mass measuring 14 cm  $\times$  8 cm  $\times$  10 cm, enterocolic fistula and residual Barium sulfate in the mass (Figure 1A). The patient underwent barium meal examination in another hospital three months ago. Colonoscope could not pass through the severely inflamed sigmoid colon. He was given antibiotics intravenously for 10 d. However, symptoms of frequent fever, abdominal pain and abdominal mass were still the therapeutic dilemma.

He agreed to participate in a clinical trial with FMT for moderate to severe refractory CD (NCT01793831), approved by the ethics committee of our center. One week prior to FMT, Mycophenolate Mofetil capsules and Etiasa were stopped, and Salofalk (mesalazine) 3.0 g was given daily. His CD Activity Index (CDAI) score was 537.

Based on the patient's desire and the protocol of scanning tests and questionnaires made by our group (not shown in the present article), the donor was his 10-year-old healthy daughter. According to the protocol of standardized preparation, Esomeprazole Magnesium 40 mg



**Figure 1** Abdominal computerized tomography scan. A: Before FMT, formation of an intraperitoneal large inflammatory mass measuring 14 cm  $\times$  8 cm  $\times$  10 cm, arrows showing gas indicating fistula, and the scattered bright image indicating Barium sulfate in fistula; B: Four months after FMT, no mass and gas was present and only a little Barium sulfate in the mass was shown. FMT: Fecal microbiota transplantation.

by intravenous push and metoclopramide 10 mg by intramuscular injection were given one hour before endoscopic procedure. The highly purified gut flora at lab was prepared as 150 mL liquid suspension and was transplanted into mid-gut below Vater papilla<sup>[11]</sup> by a tube within the channel of gastroscope under anesthesia. The time from collection of stool to FMT procedure with endoscopy was 50 min. A week after FMT, his symptoms, such as fever, bloody purulent stool and abdominal pain, were dramatically alleviated, the size of intraperitoneal inflammatory mass became much smaller than that before FMT, and the CDAI score was reduced to 228. He had a severe cold in the whole third week after he was discharged with clinical improvement. At one month of follow-up after FMT, his CDAI score was further reduced to 143, which met the criteria of clinical remission. Three months after FMT, CDAI score was further reduced to 62, suggesting sustained clinical remission. CT scan (Figure 1B) showed resolved mass without exudation and the disappearance of the previous Barium sulfate intraperitoneally. Then, Salofalk 2.0 g daily was given. The patient was followed up for 9 mo and his CDAI score remained at 62, suggesting sustained clinical remission. Of note, he has gained his body weight by 11 kg, compared with the 50 kg as his baseline body weight before FMT. His nutrition status has also improved, evidenced by normalized album and total cholesterol 47.4 g/L (normal range, 35-55) and 4.5 mmol/L (normal range, 3.0-6.0), respectively. The key

**Table 1** Clinical parameter changes of the patient during follow-up

Parameter (normal range)	Before FMT	After FMT			
		1 wk	1 mo	3 mo	9 mo
Body weight (kg)	50	51	52	56	61
CDAI score	537	228	143	62	62
Haemoglobin (110-160, g/L)	97	113	120	142	144
CRP (0-10, mg/mL)	141	8	9	1.7	7
ESR (0-20, mm/h)	97	10	13	10	10
Album (g/L)	27.4	-	35	49.9	47.4
Total cholesterol (3.0-5.7 mmol/L)	2.6	-	2.7	4.2	4.5
Triglycerides (0.4-1.7 mmol/L)	0.5	-	0.6	2.4	2.5
HDL-C (1-2 3.1 mmol/L)	0.6	-	0.7	1.3	1.3
LDL-C (< 3.1 mmol/L)	1.8	-	1.9	2.5	2.6
IgM (6.8-14.5, g/L)	0.5	-	0.7	1.0	0.8

CDAI: Crohn's disease activity index; CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; FMT: Fecal microbiota transplantation.

clinical parameter changes are shown in Table 1.

## DISCUSSION

CD usually affects the intestine, but may occur anywhere from the mouth to the end of the rectum (anus). CD with fistula and formation of intraperitoneal large inflammatory mass has considerable morbidity associated with this complication and remains an unresolved challenge<sup>[12]</sup>. This case presented CD with fistula, residual Barium sulfate and refractory inflammation in a large mass after enterectomy and long-term use of mesalazine, prednisolone and mycophenolate mofetil. Further medications (aminosalicylic acid preparations, steroids, immunomodulators, antibiotics and biologics such as anti-tumor necrosis factor antibody) not only yielded significant side effects, but also unpredictable outcome. Long-term use of immunomodulators increased the risk of refractory inflammation. Surgery for this case does pose a challenge for a surgeon in the skill and expertise according to the recent consensus<sup>[13]</sup>.

The etiology of CD is unknown, but one dominant hypothesis is that the inflammation might result from altered or pathogenic microbiota in a genetically susceptible host<sup>[14]</sup>. We proposed that FMT might be a promising rescue therapy for refractory CD. Based on the attractive therapeutic effect<sup>[15]</sup> and less concerns in safety<sup>[16]</sup>, as well as the long history of recognition in traditional Chinese medicine<sup>[4]</sup>, standardized FMT is acceptable in treating refractory CD. As a rescue therapy in this severe CD, it is intriguing that standardized FMT was safe. The patient's sustained remission indicated that a single application of standardized FMT *via* mid-gut should be effective.

In order to prove the therapeutic role of FMT, one week before FMT, immunosuppressive agent was stopped and only Salofalk 3.0 g was continued daily. CDAI decreased

dramatically during the first week after FMT. The large mass and inflammation within the fistula subsided in 4-6 wk. The expulsion of residual Barium sulfate within 4 mo actually not only decreased the risk of refractory infection within fistula, but also played a role in improving the internal fistula.

Gut microbial communities represent one source of human genetic and metabolic diversity. Previous studies have shown that relatives of patients with CD bear the risk of this disease and have altered gut microbiome<sup>[17]</sup>. The recent reports also have shown that the gut microbiota composition in healthy persons has the age-associated changes<sup>[18]</sup>. These experimental evidences indicated that the family members of CD patients might not be the best donor of stool for FMT. However, in the present case, the clinical results demonstrated that the patient's 10-year-old daughter under healthy state was his right choice as a donor. Further studies are needed to clarify whether the ideal donor of stool for FMT can be from CD patient's relatives or family members.

We reported here the first case of severe CD using FMT through mid-gut. The single standardized FMT resulted in sustained clinical remission for more than 9 mo and the follow-up is going on. Although there was only one case, the present result in our clinical trial strongly supported our initial hypothesis and highlighted the attractive role of the remodeling of gut flora in host diseases. Our ongoing study will report more evidences of standardized FMT through mid-gut on refractory intestinal diseases in a difficult therapeutic dilemma.

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## Efficacy, effectiveness, immunogenicity - are not the same in vaccinology

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portant issues regarding immunisation in inflammatory bowel disease patients. However, in our opinion, definition of vaccine efficacy is misused. In fact this article is on vaccine immunogenicity. Here, we emphasise the differences between the definitions of efficacy, effectiveness and immunogenicity, differences that are fundamental in vaccinology.

Banaszkiewicz A, Radzikowski A. Efficacy, effectiveness, immunogenicity - are not the same in vaccinology. *World J Gastroenterol* 2013; 19(41): 7217-7218 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i41/7217.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i41.7217>

### Abstract

Manuscript of Carrera *et al* is devoted to immunization in inflammatory bowel disease (IBD) that is very important issue in gastroenterology. However, some specific definitions used in the article need clarification. Efficacy of vaccine is measured in a randomised, placebo-controlled studies, that are expensive and difficult to plan. Moreover, it is unethical to offer a placebo instead of vaccine. For all of these reasons, efficacy of vaccine is measured in IBD patients rarely. Effectiveness of vaccine is measured as an epidemiological affect from observational studies. These studies are also uncommon in IBD because it would be difficult to perform a study that assess the prevalence of one rare disease (vaccine-preventable) in patients with a chronic rare condition, such as IBD. Immunogenicity of vaccine refers to the ability of a vaccine to induce an immune response in a vaccinated individual that is, in fact, the matter of the article.

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**Key words:** Efficacy; Effectiveness; Immunogenicity; Vaccine

**Core tip:** The Carrera *et al*'s article presents some im-

### TO THE EDITOR

We read with interest the manuscript of Carrera *et al*<sup>[1]</sup>, devoted to immunisation in inflammatory bowel disease (IBD) patients. However, we would like to clarify the definitions used in the article.

The title of the article suggested that the body of the manuscript is focused on the efficacy of vaccines in IBD. Moreover, the authors defined efficacy as “percent risk reduction for clinically significant infection in a vaccinated group *vs* a control group”, but there is nothing in the article about efficacy. The entire article is about the immunogenicity of vaccines. These two words are not synonyms.

How well a vaccine works can be measured through different types of studies<sup>[2]</sup>. The measurement of a vaccine's effect in a randomised (placebo-controlled) study is referred to as efficacy. Randomised studies are expensive and are not always conducted after a recommendation for vaccination has been issued because withholding the vaccine from people recommended to receive it would place them at risk for infection, illness and possibly serious complications. The measurement of a vaccine's epidemiological effect from observational studies is referred

to as effectiveness. Apart from ethical and economic considerations, it would be difficult to perform a study that assessed the prevalence of one rare disease (vaccine-preventable) in patients with a chronic rare condition, such as IBD. The efficacy of a vaccine may indirectly predict cases of that one rare disease when the protecting level of antibodies is known from previous epidemiological studies. Immunogenicity refers to the ability of a vaccine to induce an immune response (antibody- and/or cell-mediated immunity) in a vaccinated individual. Until now, neither efficacy nor effectiveness has been assessed in IBD patients for any vaccine. All of the vaccine studies in IBD patients have instead assessed the immunogenicity of the vaccines.

Proper use of these terms is not just an academic issue, as the level of antibodies does not always predict real protection against a disease. Immunogenicity can be low, as observed in hepatitis B virus vaccine (*i.e.*, in the

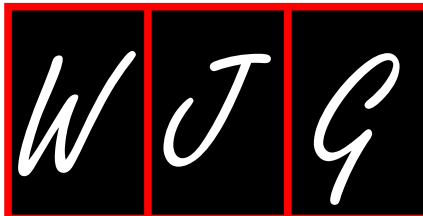
Gisbert *et al*<sup>[3]</sup> study, only 36% of IBD patients on immunosuppressive and biological therapy achieved adequate hepatitis B surface antibody levels), but hepatitis B cases are extremely rare in a vaccinated IBD population.

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*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and

safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

**Books**

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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*World J Gastroenterol* 2013 November 14; 19(42): 7219-7488



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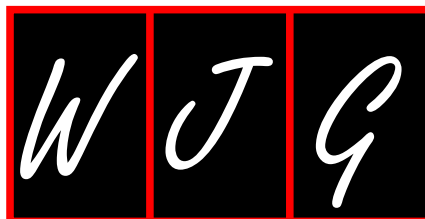
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## Understanding and treatment of chronic pancreatitis

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### CHRONIC PANCREATITIS

Chronic pancreatitis is characterized by an inflammatory process of the pancreas, which is replaced by fibrosis and progressive destruction. Clinically, the early phase is typically dominated by pain or recurrent episodes of pancreatitis and complications, whereas in the advanced phase symptoms related to exocrine and/or endocrine insufficiency are seen. Hence, the three major clinical features of chronic pancreatitis are pain, maldigestion, and diabetes. The incidence of the disease has been estimated to 2-10/100000 and seems to be increasing<sup>[1]</sup>, but there are major regional differences, and in some countries the disease is much more prevalent. It is also likely that there are many patients with, e.g., abdominal pain, diarrhea and malnutrition - without diagnostic classification - that in reality suffer from chronic pancreatitis. Apart from the illness the economic burden is also of major importance. Chronic pancreatitis has a profound impact on social life and employment patterns<sup>[2]</sup>. For society the disease in year 2000 accounted for 327000 hospitalizations, 200000 emergency room visits and 532000 physician visits costing 2.5 billion \$ in the United States<sup>[3]</sup>. Even though excess alcohol intake still is a major risk factor, recent data suggest that in some series less than half of the patients have alcoholic pathogenesis and much attention has been paid to "new" entities such as autoimmune pancreatitis<sup>[4]</sup>.

In the current issue of *World Journal of Gastroenterology*, different topics highlight experimental models of chronic pancreatitis and bridge findings from recent research to bedside<sup>[5]</sup>. The pathogenesis of pancreatitis is reviewed using the recent "MANNHEIM" classification,

### Abstract

Chronic pancreatitis is characterized by an inflammatory process of the pancreas, which is replaced by fibrosis and progressive destruction. The three major clinical features of chronic pancreatitis are pain, maldigestion, and diabetes. Chronic pancreatitis has a profound impact on social life and employment patterns. In the current issue, different topics highlight experimental models of chronic pancreatitis and bridge findings from recent research to bedside. Although the disease is still difficult to treat the current papers represent useful guidelines on how to approach chronic pancreatitis in the clinical settings with the major aim to improve the patient's suffering and quality of life.

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**Key words:** Chronic pancreatitis; Pathogenesis; Diabetes; Treatment

**Core tip:** Chronic pancreatitis has a profound impact on social life and employment patterns. In the current issue of *World Journal of Gastroenterology*, different topics highlight experimental models of chronic pancreatitis and bridge findings from recent research to bedside.

which may help us in correct diagnostics and subsequent treatment<sup>[6]</sup>. Diagnosing of chronic pancreatitis has been challenging and imaging techniques are emerging. The main development has been within magnetic resonance imaging and ultrasonography. Two review papers outlined state-of-the-art as well as arising techniques such as diffusion-weighted imaging to indirectly assess the degree of fibrosis<sup>[7]</sup>. Contrast enhanced ultrasonography and elastography are other examples of new techniques to e.g., differentiate between malignancy and benign lesions<sup>[8]</sup>. As support for imaging in diagnostics it is of major importance to assess the degree of exocrine and endocrine insufficiency. The so-called “direct or invasive methods” for assessment of exocrine insufficiency such as the Lundh test has been replaced by methods that are more suitable for screening, although imaging methods such as estimation of pancreatic juice flow during secretin stimulation may end up being a major supplement to screening methods. The pros and cons for different methods to unravel exocrine insufficiency are also outlined in the current issue<sup>[9]</sup>. Treatment of uncomplicated disease is usually conservative, with the major aim to effectively alleviate pain, maldigestion, and diabetes. Malnutrition due to the lack of enzymes results not only in weight loss, but also in specific deficits in vitamins and nutrients that are essential for normal physiological functioning. Malnutrition as complication to chronic pancreatitis is often overlooked and it is of major importance that gastroenterologists are aware of this in the differential diagnosis of patients with weight loss. The paper about nutrition highlights that depletion of nutrients on the one hand and malabsorption (with potential changes in metabolic activity) on the other shall both be considered to avoid severe complications<sup>[10]</sup>. Another frequent complication is pancreatic pain. The pain is severe and often postprandial resulting in malnutrition despite adequate enzyme replacement therapy. There has been an increasing understanding of the pathogenesis of pain in chronic pancreatitis. Hence, many patients suffering from undiagnosed extrapancreatic causes for the pain should be treated appropriately. Although the pain can be related to strictures and stones in the main pancreatic duct, new research has questioned the importance of the micro- and macrostructural pathological findings. Neurogenic pain due to destruction of the nerves may rather play a role for the pain in the majority of patients - and should be treated accordingly<sup>[11]</sup>. In the paper about pain treatment there is an update on the current treatment options and it is highly recommended that clinicians are aware of the possibilities for optimal pain relief<sup>[12]</sup>. Diabetes complicating chronic pancreatitis (type 3c) is a special entity that differs from type 1 and 2 as a variety of hormones apart from insulin are lacking - and the malabsorption also has to be taken into consideration. Despite the importance of this complication few papers have explored the pathogenesis and treatment of type 3c diabetes and this is also updated in the current issue<sup>[13]</sup>.

Finally, there are many challenges when it comes to pharmacological, endoscopical and surgical treatment of chronic pancreatitis. In the paper about pharmacology it is discussed how the influence of complications to the disease is affecting drug absorption and metabolism<sup>[14]</sup>. Surgical and endoscopic interventions are mainly reserved for complications such as pseudocysts, abscesses, and malignancies. There has been a major development of endoscopy in diagnosing complications and treatment of complications such as endosonographic transmural drainage of pseudocysts and biliary strictures and this is discussed in the paper about endoscopy<sup>[15]</sup>. Finally, surgery still has a place in treatment of chronic pancreatitis and should be considered in selected cases.

In conclusion important advances have been made in recent years with respect to our understanding of the pathogenesis of chronic pancreatitis. Although the disease is still difficult to treat the current papers represent useful guidelines on how to approach chronic pancreatitis in the clinical settings with the major aim to improve the patient's suffering and quality of life.

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Asbjørn Mohr Drewes, MD, PhD, DMSc, Professor, Series Editor

## Animal models of pancreatitis: Can it be translated to human pain study?

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**Key words:** Animal model; Pancreatitis; Visceral pain; Mechanism

**Core tip:** Choosing the right model of pancreatitis is difficult and the scientific rationale needs to be carefully considered. Furthermore, no model of pancreatitis parallels all classical symptoms and the question under investigation is of importance when choosing a model. One of the main symptoms of chronic pancreatitis is visceral pain and in order to improve the pain treatment and obtain more knowledge about the physiology behind the pancreatitis associated visceral pain, animal models of pancreatitis associated visceral pain are needed.

### Abstract

Chronic pancreatitis affects many individuals around the world, and the study of the underlying mechanisms leading to better treatment possibilities are important tasks. Therefore, animal models are needed to illustrate the basic study of pancreatitis. Recently, animal models of acute and chronic pancreatitis have been thoroughly reviewed, but few reviews address the important aspect on the translation of animal studies to human studies. It is well known that pancreatitis is associated with epigastric pain, but the understanding regarding to mechanisms and appropriate treatment of this pain is still unclear. Using animal models to study pancreatitis associated visceral pain is difficult, however, these types of models are a unique way to reveal the mechanisms behind pancreatitis associated visceral pain. In this review, the animal models of acute, chronic and un-common pancreatitis are briefly outlined and animal models related to pancreatitis associated visceral pain

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### INTRODUCTION

Pancreatitis represents a common disorder of the gastrointestinal tract. Acute pancreatitis (AP) has an incidence ranged from 4.9 to 35 per 100000 populations<sup>[1]</sup>, whereas chronic pancreatitis (CP) has an incidence from 2.4 to 4.4 per 100000 populations<sup>[2]</sup>. The etiology of this disease is complex and so far a variety of environmental factors including alcohol abuse, nicotine habits, hereditary factors, efferent duct obstructions, immunological factors and rare metabolic factors have all been described. However,

the pathophysiology of AP and CP remains poorly defined<sup>[3]</sup>. As a result appropriate therapies are still limited, and prognosis has not improved to date, which is mainly due to the lack of a satisfactory animal model of pancreatitis<sup>[4,5]</sup>.

It is well known that pancreatitis is associated with visceral pain, however, the understanding of pain signaling related to pancreatitis is poor<sup>[6]</sup>. In order to facilitate the development of new pharmaceutical treatments for AP and CP, characterization of the mediators and receptors or ion channels on the sensory nerve terminals and the pathways of the pain signaling are needed. Therefore, in this aspect, the animal models of pancreatitis are needed in parallel in order to explore the mechanism behind pancreatitis associated visceral pain, as this is difficult to study in humans.

In this review, we briefly outline the animal models of acute, chronic and un-common pancreatitis as well as animal models related to pancreatitis associated visceral pain.

## ANIMAL MODELS OF ACUTE PANCREATITIS

AP is an inflammatory condition of the pancreas characterized clinically by abdominal pain and elevated levels of pancreatic enzymes in the blood<sup>[7]</sup>. Other characteristics of AP include edema, acinar cell necrosis, hemorrhage, and severe inflammation of the pancreas. Severe AP may lead to systemic inflammatory response syndrome and multi-organ dysfunction syndrome, which account for the high mortality rates of AP<sup>[8,9]</sup>. As it is difficult to study AP in the clinic, animal studies are important in order to understand the pathogenesis of AP, however an AP model which is strictly comparable to human AP is still needed. The current animal models of AP have contributed to our knowledge of mechanisms involved in early cellular events, pathogenesis and pathophysiology of AP<sup>[10,11]</sup>. We have illustrated the summary of existing AP animal models in Table 1<sup>[12-59]</sup>. Details of different AP animal models including advantages, disadvantages and clinical relevance can be found in a recently published review<sup>[4]</sup>. From a methodological aspect, selecting the appropriate AP animal model depends on the objectives of each study as different animal models are targeted to different AP features. For developing the effective treatment for AP in the clinic, continued investigation of AP animal models are needed.

## ANIMAL MODELS OF CHRONIC PANCREATITIS

A recently published review<sup>[5]</sup> has described the most frequently used and best established models for CP in animals. The majority of the animal models are rodent models, since mice and rats are easy to handle and there is a steadily increasing number of genetic models ob-

**Table 1 Different animal models of acute pancreatitis**

Methods	Models and examples
Non-invasive	Hormone-induced
	Acute caerulein pancreatitis of rats <sup>[12]</sup> , mice <sup>[13]</sup> , dogs <sup>[14]</sup> , and syrian hamsters <sup>[15]</sup>
	Trinidadian scorpion toxin induced acute pancreatitis in dogs <sup>[16]</sup>
	Alcohol-induced: rats <sup>[17-19]</sup> , cats <sup>[20]</sup> and dogs <sup>[21]</sup>
	Immune-mediated
	Ovalbumin in rabbit <sup>[22]</sup>
	Foreign serum in mice <sup>[23]</sup> and rat <sup>[24]</sup>
	Spontaneous model of autoimmune acute pancreatitis mice <sup>[25]</sup>
	Diet-induced: Fed a choline-deficient diet containing ethionine in mice <sup>[26]</sup>
	Gene knockout: Interleukin (IL)-1 and tumour necrosis factor- $\alpha$ <sup>[27]</sup> , IL-6 <sup>[28]</sup> , IL-10 <sup>[29]</sup> , chemoattractant cytokine receptor-1 <sup>[30]</sup> , neurokinin-1 receptor <sup>[31]</sup> , intercellular adhesion molecule 1 (ICAM-1) <sup>[32]</sup> , metallothionein-1 <sup>[33]</sup> , cathepsin B <sup>[34]</sup> , mouse a2-macroglobulin and murinoglobulin <sup>[35]</sup> , complement factor C5a <sup>[36]</sup> , granulocyte-macrophage colony-stimulating factor <sup>[37]</sup> and phospholipase A2 <sup>[38]</sup>
Invasive	<i>L</i> -arginine-induced: Administration of a large dose of <i>L</i> -arginine in rats <sup>[39,40]</sup>
	Closed duodenal loop (CDL): Dog <sup>[41]</sup> and rat <sup>[42,43]</sup>
	Antegrade pancreatic duct perfusion: Cat <sup>[44]</sup> and rat <sup>[45]</sup>
	Various compounds infusion into the pancreatic duct: Rat <sup>[46]</sup> and dog <sup>[47]</sup>
	Combined intraductal glycodeoxycholic acid with intravenous caerulein: Rat <sup>[48]</sup>
	Vascular-induced
	Impairment of pancreatic circulation in dogs <sup>[49]</sup>
	To occlude pancreatic arteries in rats <sup>[50]</sup>
	Occlusion of pancreatic veins in dogs <sup>[51]</sup> and in rats <sup>[52]</sup>
	Complete but reversible ischaemia of the pancreas by occluding different arteries using microvascular clips: Rats <sup>[53]</sup> and canine <sup>[54]</sup>
	Duct ligation
	Ligating the distal bile duct at the level of the duodenum <sup>[55]</sup>
	Combined pancreatic duct ligation with the secretory stimulation, secretin in dogs <sup>[56]</sup>
	Combining duct ligation with both secretory stimulation and minimal arterial blood <sup>[57]</sup>
	Duct-ligated opossums models <sup>[58]</sup>
	Transient obstruction of the sphincter of Oddi (SO) in Australian brush tailed possums <sup>[59]</sup>

tained by gene deletion or transgenic expression of genetic variants. In the same way for animal models of AP, the models of CP can be classified into noninvasive or nonsurgical models and invasive or surgical models. Table 2 summarizes different animal models of CP<sup>[60-100]</sup>.

In the non-invasive models, repetitive caerulein injections are amongst the most widely used models. Firstly, caerulein injections are relatively easy to perform and show a high reliability and reproducibility. Secondly, other compounds mediating injury such as lipopolysaccharides or cyclosporin A can easily be added to the design. Thirdly, serial caerulein injections can be performed in transgenic or knockout animals. It is likely that there are dose and frequency dependency for caerulein. The most translational models include repetitive injections of *L*-arginine, which appears to produce CP similar to that in humans<sup>[70-72]</sup>. In this model, fibrotic tissues are progressively

Table 2 Different animal models of chronic pancreatitis

Methods	Models and examples
Non-invasive	Caerulein-induced Serial caerulein injections in mice <sup>[60]</sup> and rats <sup>[61]</sup> Combination of repetitive caerulein injections with toxins and other agents such as lipopolysaccharides <sup>[62]</sup> , cyclosporin A <sup>[63]</sup> , dibutyltin dichloride <sup>[64]</sup> and Alcohol <sup>[65-67]</sup> Intraperitoneal caerulein injections are administered in genetically transformed mice such as TRX-1 transgenic mice <sup>[68,69]</sup>
	Arginine-induced A single <i>L</i> -arginine injection in rat <sup>[70]</sup> Serial <i>L</i> -arginine injections <sup>[70-72]</sup> Alcohol feeding-induced: Lieber-DeCarli formula <sup>[73-76]</sup> Genetic models: Wistar Bonn/Kobori (WBN/Kob) rats <sup>[77-79]</sup> ; R122H transgenic mice <sup>[80]</sup> ; SPINK3-deficient (SPINK3-/-) mice <sup>[81]</sup> ; CFTR-deficient (cfrtm1UNC) mice <sup>[82]</sup> and CFTR(-/-) pigs <sup>[83]</sup> ; Kif3a-deficient mice <sup>[84]</sup> ; PERK-deficient (PERK-/-) mice <sup>[85]</sup> ; Interleukin 1-β transgenic mice <sup>[86]</sup>
Invasive	Sodium taurocholate-induced: Retrograde infusion of sodium taurocholate (NaTc) into the pancreatic duct system of the rat <sup>[87]</sup> Oleic acid-induced: Retrograde infusion of oleic acid <sup>[72,88-91]</sup> , viscous solution of zein <sup>[92]</sup> , mixture of zein-oleic acid, or viscous solution consisting of zein-oleic acid-linoleic acid <sup>[93,94]</sup> into rat pancreatic duct Congestion of pancreatic fluid flow: Combination of transient stasis of pancreatic juice flow and mild pancreatic duct injury <sup>[95]</sup> Duct ligation model Ligation of the common bile duct close to the duodenum pancreatic tissue in dogs <sup>[96]</sup> , mouse <sup>[97]</sup> and pigs <sup>[98]</sup> Incomplete pancreatic duct ligation in canine <sup>[99]</sup> Occlusion with two different tissue glues in the rat <sup>[100]</sup>

replaced with adipose tissue. Due to the high impact of alcohol consumption as a risk factor on the pathogenesis in human pancreatic diseases, alcohol has frequently been used to trigger CP in animal models<sup>[73,74]</sup>. However, it is still being considered whether a model for CP induced by alcohol alone is feasible or satisfactory. The combination of alcohol feeding with caerulein injections exacerbates the course of pancreatitis and consequently increases pancreatic fibrosis and the loss of parenchyma.

Genetic animal models of CP are suitable for different studies. It is well known that activation of trypsinogen is one of the key events in the early phase of pancreatitis, and therefore genetic abnormalities found in the trypsinogen gene and in its inhibitors might be of particular importance of which R122H transgenic mice<sup>[80]</sup> are a good example. Transgenic expression of the *R122H* mutation of murine trypsin 4 in the pancreas of mice led to progressive fibrosis and chronic inflammation of the pancreas. Repetitive inductions of experimental pancreatitis with supramaximal doses of cerulein resulted in extensive deposition of collagen in periacinar and perilobular spaces of this transgenic animal. However other genetic models might also help us to understand how CP develops<sup>[77-79,81,83-86,101]</sup>.

Invasive animal models can also be used to induce CP. As an example, retrograde infusion of sodium taurocholate (NaTc) into the pancreatic duct<sup>[46]</sup> or intraductal

infusion of NaTc<sup>[72]</sup> can generate pancreatitis, however the structure of the pancreatic tissue will return to an almost normal state after 14 d. Retrograde infusion of oleic acid<sup>[72,88-91]</sup>, viscous solution of zein<sup>[92]</sup>, a mixture of zein-oleic acid or a viscous solution consisting of zein-oleic acid-linoleic acid<sup>[93,94]</sup> into rat pancreatic duct will cause severe pancreatic atrophy with irregular fibrosis and fat replacement over a period of 6 mo. However, these models of pancreatitis appear quite distinct from CP in humans. As one factor alone is inadequate to cause persistent pancreatic injury, a combination of transient stasis of pancreatic juice flow and mild pancreatic duct injury is a well established and reliable method to generate CP in animal models<sup>[95]</sup>. It is well known that pancreatic ductal hypertension contributes to the pathogenesis of CP; therefore animal models can also be generated by complete obstruction of the pancreatic duct<sup>[96-98]</sup>, incomplete pancreatic duct ligation<sup>[99]</sup> and occlusion with different tissue glues<sup>[100]</sup>. Yamamoto *et al*<sup>[102]</sup> developed an animal model with pancreatic ductal hypertension and demonstrated that this plays an important role in the onset and development of CP in rats. However, models for CP based on duct obstruction are not common and there is only a minority of studies examining the morphological and biochemical changes of the pancreas after duct ligation<sup>[41,103,104]</sup>.

## ANIMAL MODELS OF UN-COMMON PANCREATITIS

Un-common types of pancreatitis can include autoimmune pancreatitis (AIP), hereditary pancreatitis<sup>[105]</sup>, groove pancreatitis<sup>[106]</sup>, tropical pancreatitis, pancreatitis in ectopic or heterotopic pancreatic tissue, ascaris-induced pancreatitis, pancreatitis in cystic fibrosis, pancreas divisum, annular pancreas, pancreatic cancer manifesting as AP, and duodenal villous adenoma with pancreatitis. With exception of AIP and hereditary pancreatitis, no relevant animal models were found for other un-common pancreatitis. Furthermore, hereditary pancreatitis animal models were mentioned in the genetic animal models of CP above. Therefore only animal models of AIP are briefly introduced in this section.

To date, several animal models of AIP have been described. The first model involves the adoptive transfer of amylase-specific (an antigen mainly located in acinar cells) CD4<sup>+</sup> T cells and results in pancreatitis in naive syngenic recipient animals<sup>[107]</sup>. Notably, the histological lesions of this model mimic the lobulocentric inflammatory reaction in type 1 AIP. A model developed by immunization of neonatally thymectomized mice with CA (an antigen mainly located on the pancreatic epithelium) and later transfer of CD4<sup>+</sup> lymphocytes resulted in a duct-centric pattern of pancreatitis resembling type 2 AIP<sup>[108]</sup>. In another model, NTx-NFS/sld mice spontaneously developed sialoadenitis in which α-fodrin was involved as an autoantigen, as reported in some patients with Sjogren syndrome and AIP<sup>[109]</sup>. Transforming growth factor-β



(TGF $\beta$ ) appears to be an important regulatory factor in maintaining immune homeostasis. Loss of TGF $\beta$  signaling contributes to AIP in TGF $\beta$  dominant negative mutant mice<sup>[110]</sup>.

Recently two animal models for AIP were proposed. The WBN/Kob rat model, associated with congenital decreased peripheral Tregs spontaneously develops sialoadenitis, thyroiditis, sclerosing cholangitis and tubulointerstitial nephritis<sup>[111]</sup>. Although the target antigens remain unclear, CD8<sup>+</sup> cells may be the effector cell in this rat model<sup>[112]</sup>. Another recently described animal model of AIP is the Treg-deficient NOD mouse<sup>[113]</sup>. CD28KO mice spontaneously develop AIP that closely resembles the human disease<sup>[113]</sup>. More recently, Haruta *et al.*<sup>[114]</sup> investigated the possible involvement of chronic, persistent exposure to avirulent bacteria in the pathogenesis of AIP using C57BL/6 mice.

Existing animal models for AIP have several limitations. In most models the disease is induced by adoptive transfer of autoreactive cells and/or antibodies rather than spontaneous development of the disease with identical antigen specificity. The distribution of lesions produced in animal models for AIP is also variable. This may be attributed to the diversity of target antigens, different methods of immune staining and different mouse strains. In addition, typical histopathological findings of AIP (*e.g.*, lymphoplasmacytic infiltration with fibrosis, obliterative phlebitis and GELs) are rarely observed in animal models. Thus, there is a need to develop spontaneous animal models with identical autoantigens and typical histopathological findings for AIP.

## VISCERAL PAIN IN ANIMAL MODELS OF CHRONIC PANCREATITIS

One of the main clinical symptoms of CP in humans is pain, occurring either in episodes or as a constant disabling pain<sup>[115,116]</sup>. Hence, an important goal of treatment for CP is to relieve the pain. The analgesic treatment is often inadequate as the pathophysiology behind CP as well as the mechanisms behind the accompanying pain is not yet fully understood<sup>[117]</sup>. As described in the previous sections, no single animal model displays all aspects of CP and each of the different models display histological similarities to the human condition to various degrees. In order to improve the pain treatment and obtain more knowledge about the physiology behind CP associated pain, animal models of CP associated pain are needed.

Rat models of CP where pancreatic nociception was investigated, have been established through invasive, non-invasive and spontaneous models<sup>[118,119]</sup>. In these models pancreatic pain has been shown through both mechanical and thermal stimulation of the abdomen (referred pain<sup>[120]</sup>) as well as direct electrical stimulation of the pancreas<sup>[118,121]</sup>. These models had histopathological similarities to the human disease and had progressive fibrosis and inflammation. Furthermore, the models showed correlation between nociceptive behaviour and increased

expression of nerve growth factor (NGF) in the pancreas and calcitonin gene-related peptide (CGRP), substance P (SP), proteinase-activated receptor 2 (PAR2), and brain-derived neurotrophic factor (BDNF) in thoracic dorsal root ganglion and spinal cord segments<sup>[118,122-124]</sup>. Increased expression of NGF, CGRP, SP and BDNF has also been shown in human patients with CP<sup>[125-127]</sup>.

Several animal models have investigated the mechanisms involved in pain accompanying CP. Takamido *et al.*<sup>[119]</sup> reported morphological changes of the nervous system being involved in development of CP pain. This study suggested that elongation of dorsal root ganglia axons and enlargement of intrapancreatic nerve bundles as being a possible mechanism of pain generation in CP. On a supraspinal level, findings have suggested that descending facilitation from the rostral ventromedial medulla plays an important role in persistent pain associated with CP<sup>[128]</sup>. Furthermore, recent rat experiments have suggested that spinal microglia becomes activated during CP and has an important role in initiating and maintaining chronic pain<sup>[129]</sup>.

## TRANSLATION OF PANCREATITIS-ASSOCIATED VISCERAL PAIN STUDY FROM ANIMAL TO HUMAN

It may be difficult to use animal models to study pancreatitis associated visceral pain as pain is a subjective experience. However animal models are needed to explore the molecular mechanisms behind pancreatitis associated visceral pain as this is difficult to study in humans. The molecular mechanisms behind the chronic pain associated with CP are poorly understood, but within recent years, animal experiments have suggested some mechanisms that might be involved. The transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) have been shown to be contributing factors to pain in CP<sup>[122,130,131]</sup>. It has been shown that CP is accompanied by an increased level of NGF which caused an up-regulation of TRPV1 expression and sensitivity, resulting in hyperalgesia and allodynia<sup>[122,130]</sup>. TRPA1 is important in both inflammation and pain in CP and can be sensitized through activation of PAR2<sup>[131]</sup>.

The mechanisms mentioned above could be used as targets for the development of novel therapeutics, aiming at treating the chronic pain accompanying CP. Neutralizing antibodies against neurotransmitters such as BDNF and NGF<sup>[124,130]</sup> or receptor specific antagonists<sup>[122]</sup> has proven to reverse the characteristic nociceptive behavioral changes induced by CP in several of the experimental models. Furthermore, inhibition of trypsin or inhibition of microglia activation has also abrogated the pain related behavior seen in response to CP<sup>[123,129]</sup>. All these different mechanisms of pain treatment in CP models could have a potential as targets for novel pharmacological treatment of the chronic pain associated with CP in human patients. Also established analgesic drugs such as gaba-

pentin, buprenorphine, and morphine have been tested in animal models of CP<sup>[118,121,132]</sup>, and shown to have analgesic effect. However, many of these therapeutic approaches need to be tested in humans, before their true potential analgesic treatment of CP pain in humans can be established. It is known that some of these analgesic mechanisms are species specific and specific to the different models of induced CP.

## CONCLUSION

Choosing the right model of pancreatitis is difficult and the scientific rationale needs to be carefully considered. Furthermore, no model of pancreatitis parallels all classical symptoms and the question under investigation is of importance when choosing a model. One of the main symptoms of CP is visceral pain and in order to improve the pain treatment and obtain more knowledge about the physiology behind the pancreatitis associated visceral pain, animal models of pancreatitis associated visceral pain are needed.

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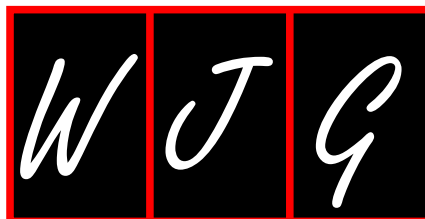


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## Pathophysiology of chronic pancreatitis

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### Abstract

Chronic pancreatitis (CP) is an inflammatory disease of the pancreas characterized by progressive fibrotic destruction of the pancreatic secretory parenchyma. Despite the heterogeneity in pathogenesis and involved risk factors, processes such as necrosis/apoptosis, inflammation or duct obstruction are involved. This fibrosing process ultimately leads to progressive loss of the lobular morphology and structure of the pancreas, deformation of the large ducts and severe changes in the arrangement and composition of the islets. These conditions lead to irreversible morphological and structural changes resulting in impairment of both exocrine and endocrine functions. The prevalence of the disease is largely dependent on culture and geography. The etiological risk-factors associated with CP are multiple and involve both genetic and environmental factors. Throughout this review the M-ANNHEIM classification system will be used, comprising a detailed description of risk factors such as: alcohol-consumption, nicotine-consumption, nutritional factors, hereditary factors, efferent duct factors, immunological factors and miscella-

neous and rare metabolic factors. Increased knowledge of the different etiological factors may encourage the use of further advanced diagnostic tools, which potentially will help clinicians to diagnose CP at an earlier stage. However, in view of the multi factorial disease and the complex clinical picture, it is not surprising that treatment of patients with CP is challenging and often unsuccessful.

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**Key words:** Chronic pancreatitis; Pathogenesis; Risk factors; Etiology

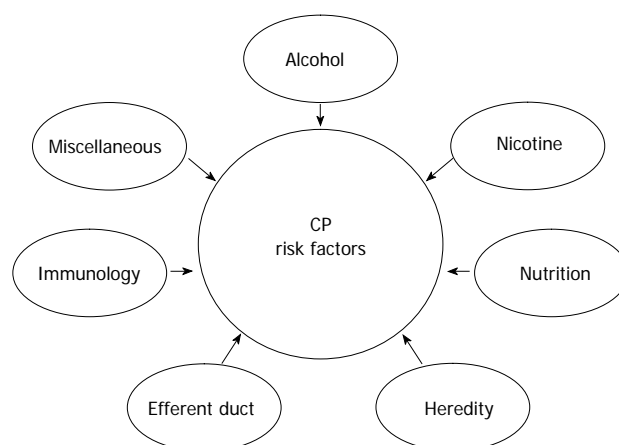
**Core tip:** The reported prevalence of chronic pancreatitis (CP) is approximately 0.5%. Etiological risk-factors associated with CP are multiple and throughout the review the M-ANNHEIM classification is used comprising environmental factors (alcohol consumption, nicotine habits and nutrition), hereditary, well characterized mutations, ductal obstruction and autoimmune factors. CP is characterized by progressive fibrotic destruction of glandular tissue, inflammation or duct obstruction, leading to irreversible functional impairment of both exocrine and endocrine functions. In view of the multi-factorial disease and the complex clinical picture, it is not surprising that treatment of patients with CP is challenging and often unsuccessful.

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### INTRODUCTION

The reported prevalence of chronic pancreatitis (CP) var-

ies due to differences in study design, diagnostic criteria, culture and geography; however in Europe and United States it is relatively rare varying between 0.2% and 0.6%<sup>[1]</sup>. The annual incidence is estimated to be approximately 7-10 per 100000<sup>[2]</sup>. The etiological risk-factors associated with CP are multiple and involve environmental factors (alcohol consumption, nicotine habits and nutrition), hereditary well characterized mutations, ductal obstruction and autoimmune factors<sup>[3]</sup>. CP is characterized by progressive fibrotic destruction of the glandular tissue. The secretory parenchyma is destroyed by processes such as necrosis/apoptosis, inflammation or duct obstruction. Increasing evidence indicates that pancreatic stellate cells (PSC) are the major mediators of fibrosis, resulting in the formation of extracellular matrix (ECM) in the interstitial spaces and in the areas where acinar cells disappear or duct cells are injured. This process ultimately leads to progressive loss of the lobular morphology and structure of the pancreas, bizarre deformation of the large ducts and severe changes in the arrangement and composition of the islets. The fibrotic destruction of the pancreatic gland is irreversible and the morphological and structural changes lead to functional impairment of both exocrine and endocrine functions, eventually leading to malnutrition and/or diabetes<sup>[3-6]</sup>. In many aspects the PSCs share many similar features to hepatic stellate cells and glomerular mesangial cells. The onset of pancreatic fibrogenesis is caused by injury which may involve interstitial mesenchymal cells, the duct cells and/or the acinar cells. Which of these elements is affected depends on the etiological risk factor. However, destruction to any one of these pancreatic tissue compartments is associated with transformation of resident fibroblasts/pancreatic stellate cells into myofibroblast-like phenotypes; a process called activation. In the activated state PSCs express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), proliferate, and secrete fibrillar collagens, including collagen I and III and fibronectin. All together this production and deposition of ECM is characteristic in chronic pancreatic fibrosis, and the PSCs likely represent the wound-healing myofibroblasts of the pancreas<sup>[7,8]</sup>. The exact pathophysiological mechanisms initiating and maintaining the development of fibrosis in the pancreas are poorly understood, but may be viewed as a progression similar to, *e.g.*, liver fibrosis<sup>[9]</sup>. Hence, the initial injury to one or all of the various tissue compartments or cell types of the pancreas, leads to cell necrosis and/or apoptosis and consequently release of cytokines/growth factors (*e.g.*, tumor growth factor b1, interleukin-8, platelet-derived growth factor and CC-chemokines), either from immigrating inflammatory cells, especially macrophages, and/or nearby preexistent epithelial or mesenchymal cells<sup>[10-13]</sup>. Thereafter damaged cells are phagocytosed by macrophages, causing release of cytokines, which in turn causes activation and proliferation of resident fibroblasts/PSC situated in the immediate surroundings of the original site of injury, which accordingly induces transformation into myofibroblast cells<sup>[7,14]</sup>. However, it has also been suggested as an alternative hypothesis, that the abovementioned progression is bypassed,



**Figure 1** The etiological risk-factors associated with chronic pancreatitis are multiple and involve both genetic and environmental factors. According to the M-ANNHEIM classification system, different synergistic risk factors are known such as: alcohol-consumption, nicotine-consumption, nutritional factors, hereditary factors, efferent duct factors, immunological factors and miscellaneous and rare metabolic factors. CP: Chronic pancreatitis.

and the initiating etiological factor (for instance, ethanol consumption) activates resident fibroblasts directly. In the final stage, myofibroblasts produce and extracellular matrix deposits replace the inflammatory infiltrate and affect the architecture and function of the remaining pancreatic tissues<sup>[4]</sup>. Subsequently, a vicious circle has started, because in order to facilitate the deposition of the newly formed ECM, myofibroblasts enhance production of specialized enzymes, such as metalloproteinase matrix metalloproteinase (MMP)-3 and MMP-9, which are able to demolish the normal pericellular ECM. These metalloproteinases are in return regulated by cytokine tumor growth factor (TGF)- $\beta$ 1s, which through autocrine inhibition enhances pancreatic fibrogenesis by reducing collagen degradation<sup>[8]</sup>. As a consequence of declined ECM production (due to withdrawal of the initiating factor), myofibroblasts may disappear either through apoptosis or retransformation into fibroblasts. Furthermore, pancreatic stellate cells express both mediators of matrix remodeling and the regulatory cytokine TGF- $\beta$ 1 that, by autocrine inhibition of MMP-3 and MMP-9, may enhance fibrogenesis by reducing collagen degradation<sup>[8]</sup>. Pancreatic stellate cells express both mediators of matrix remodeling and the regulatory cytokine TGF- $\beta$ 1 that, by autocrine inhibition of MMP-3 and MMP-9, may enhance fibrogenesis by reducing collagen degradation<sup>[8]</sup>.

To describe the heterogeneity of the underlying pathophysiology we have throughout the review used the M-ANNHEIM classification<sup>[3]</sup>, comprising detailed description of Multiple risk factors such as: alcohol-consumption, nicotine-consumption, nutritional factors, hereditary factors, efferent duct factors, immunological factors and miscellaneous including rare metabolic factors (Figure 1). Increased knowledge of the different etiological factors may encourage the use of further advanced diagnostic tools, which potentially will help clinicians to diagnose CP at an earlier stage.



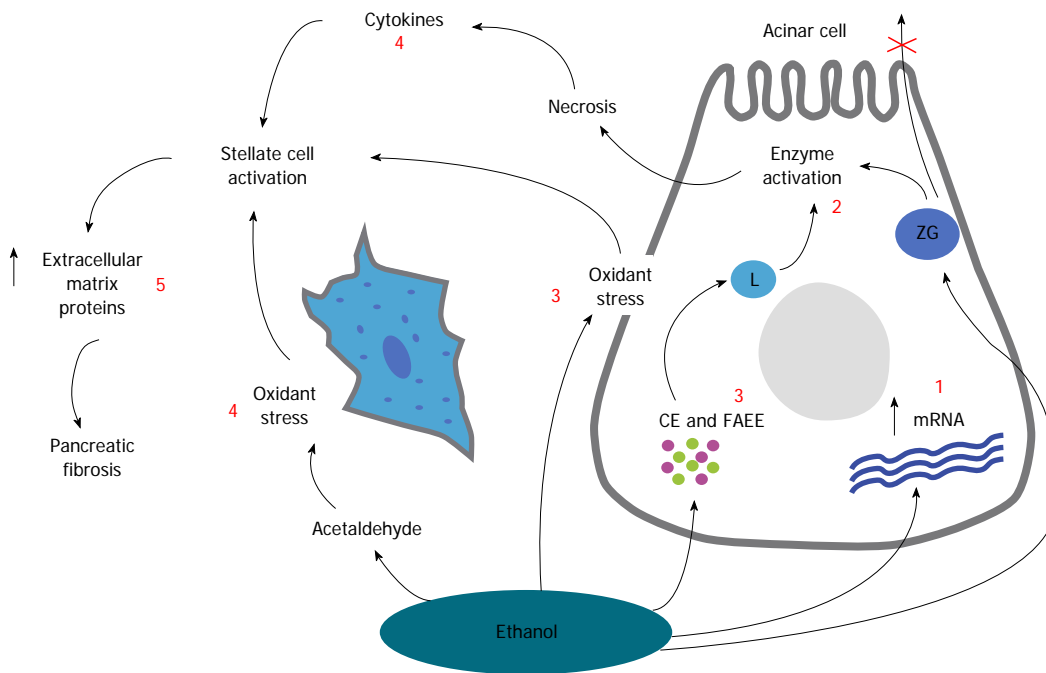


Figure 2 A schematic overview showing the overall hypothesis for the pathogenesis of alcoholic chronic pancreatitis. The effect of ethanol and its metabolites on the subcellular organelles include increased digestive and lysosomal enzyme content [due to increased synthesis (increased mRNA) and impaired secretion (1)] and destabilization of lysosomes (L) (2) and zymogen granules (ZG) [mediated by oxidant stress, cholesteryl esters (CE) and fatty acid ethyl esters (FAEE) (3)]. These changes will make the cell more sensitive to trigger factors and in the presence of appropriate trigger factors, overt acinar cell injury is initiated (alcoholic acute pancreatitis). Pancreatic stellate cells are activated by cytokines during alcohol-induced necroinflammation, or directly by ethanol *via* its metabolism to acetaldehyde and the subsequent generation of oxidant stress (4). Activated pancreatic stellate cell then increases the synthesis of extracellular matrix proteins leading to pancreatic fibrosis. Modified from Vonlaufen *et al*<sup>[84]</sup> (5).

However, in view of the multi-factorial disease and the complex clinical picture, it is not surprising that treatment of patients with CP is challenging and often unsuccessful.

According to the M-ANNHEIM classification, the following etiological risk factors are involved in the pathogenesis of chronic pancreatitis.

## ALCOHOL CONSUMPTION

A relationship between alcohol consumption and pancreatic impairment has been reported as early as 1878<sup>[15]</sup> and hence, alcohol intake has long been regarded as the primary cause of chronic pancreatitis. Nowadays, there is satisfactory indication that the pancreas has the ability to metabolize ethanol *via* both oxidative and the non-oxidative pathways<sup>[16]</sup>. The metabolites and their byproducts injure acinar cells and activate stellate cells to produce and deposit ECM (Figure 2). A study by Dufour *et al*<sup>[17]</sup> suggested that CP development is associated to the dose and duration of alcohol consumption. It was estimated that approximately 80 g of alcohol per day for a minimum of 6-12 years is required to produce symptomatic pancreatitis. However, the consumption of lesser quantities may also lead to pancreatic injury and may have an impact on the progression of the disease<sup>[18]</sup>. In order to consider the risks associated with lower consumption of alcohol, the M-ANNHEIM classification system of CP grouped alcohol consumption into patterns of moderate

(< 20 g pure ethanol per day), increased (20-80 g pure ethanol per day), or excessive (> 80 g pure ethanol per day)<sup>[3]</sup>. While alcohol consumption is doubtlessly a contributing factor in CP, it must be noted, that considerable amount of recent epidemiological studies and animal experiments suggest that alcohol alone is not sufficient to induce CP. An overview of the associations between alcohol consumption and other risk factors are listed in Table 1.

A multi-centre study from Italy reported that excessive alcohol consumption was the principal factor in only 34% of cases of CP<sup>[19]</sup> and an assessment from the United States concluded it was the main factor in 44% of CP cases<sup>[20]</sup>. Moreover, African-Americans are at particular risk of developing alcoholic CP<sup>[21]</sup>. Overall, less than 10% of heavy alcohol consumers develop alcoholic induced CP<sup>[17]</sup>. Hence, several theories have been proposed as to how alcohol might lead to CP, but no clear-cut answer exists as prolonged feeding of ethanol does not trigger the onset of CP in itself. Therefore, there are indications that alcohol sensitizes the pancreas to other external factors which interact to increase ethanol toxicity *in vivo*, such as cigarette smoking and diet<sup>[22]</sup> or genetic predisposition). Interestingly, alteration of pancreatic secretory trypsin inhibitor (*SPINK1*, Figure 3) and *CFTR* genes was present in patients with alcoholic CP and the increase of those genes was moreover associated with higher levels of alcohol consumption<sup>[23]</sup>. In this line, a recent study<sup>[24]</sup> found a genetic variant on chromosome X near the *CLDN2* gene

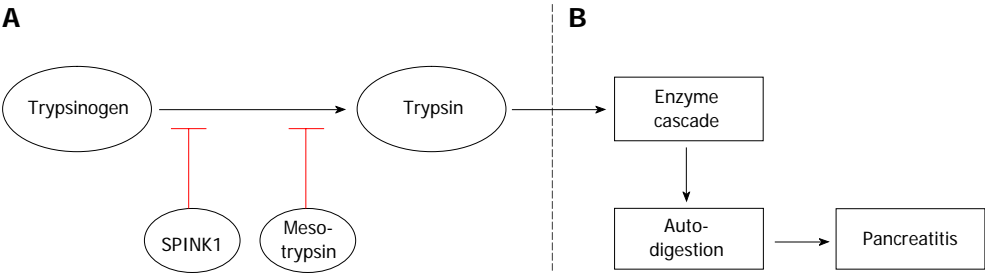


Figure 3 A scheme representing the role of digestive enzymes in normal pancreatic tissue and in the case of pancreatitis. A: In a normal pancreas, SPINK1 (first line of defense) and mesotrypsin (second line of defense) inhibit the generation of trypsin resulting from auto-activation of trypsinogen. These defense mechanisms prevent the pancreas from activating the pancreatic enzyme cascade and auto-digestion; B: If mutations are present in the SPINK1 and/or in the mesotrypsin gene, they losses their ability to inhibit the generation of trypsin resulting in activation of enzyme cascade and subsequent pancreatic auto-digestion leading to pancreatitis. SPINK1: Serine protease inhibitor, kazal type 1, gene (encodes for pancreatic secretory trypsin inhibitor).

Table 1 Association with alcoholic chronic pancreatitis

Factor	Association
Drink type	Yes: Vonlaufen <i>et al</i> <sup>[83]</sup> Nakamura <i>et al</i> <sup>[84]</sup> No: Levy <i>et al</i> <sup>[29]</sup> Wilson <i>et al</i> <sup>[85]</sup>
Drinking pattern	Yes: Lankisch <i>et al</i> <sup>[18]</sup> No: Levy <i>et al</i> <sup>[29]</sup> Wilson <i>et al</i> <sup>[85]</sup>
Diet	Yes: Levy <i>et al</i> <sup>[29]</sup> No: Wilson <i>et al</i> <sup>[85]</sup>
Tobacco	Yes: Rebours <i>et al</i> <sup>[28]</sup> Maisonneuve <i>et al</i> <sup>[27]</sup> Imoto <sup>[26]</sup> Lowenfels <sup>[86]</sup> No: Levy <sup>[29]</sup> Haber <i>et al</i> <sup>[87]</sup>
Genetics	Yes: Whitcomb <i>et al</i> <sup>[24]</sup> (PRSS1-PRSS2 and X- linked CLDN 2) Rosendahl <sup>[88]</sup> (Chymotrypsin C gene mutation) Miyasaka <i>et al</i> <sup>[89]</sup> (Cholesteryl esterlipase polymorphism) Ockenga <i>et al</i> <sup>[90]</sup> (UDP-glucuronosyl transferase) Witt <i>et al</i> <sup>[91]</sup> (SPINK1 mutations) No: Schneider <i>et al</i> <sup>[92]</sup> Perri <i>et al</i> <sup>[93]</sup> Frenzer <i>et al</i> <sup>[94]</sup> Schneider <i>et al</i> <sup>[82]</sup> Norton <i>et al</i> <sup>[95]</sup> Haber <i>et al</i> <sup>[96]</sup> Wilson <i>et al</i> <sup>[85]</sup>

PRSS1: Cationic trypsinogen gene; PRSS2: Anionic trypsinogen gene; CLDN 2: Claudin 2 gene; UDP-glucuronosyltransferase: Uridine 5'-diphospho-glucuronosyltransferase; SPINK1: Serine protease inhibitor, kazal type 1, gene (encodes for pancreatic secretory trypsin inhibitor).

that predicts which heavy drinking males were in higher risk of developing CP.

### NICOTINE CONSUMPTION

Smoking has been identified as an important risk factor for development of chronic pancreatitis. The effect of tobacco in CP was first described in 1994<sup>[25]</sup>, where heavy smokers had a significantly increased risk of developing pancreatic calcifications but no effects of alcohol were

found. Another study reported that cigarette smoking increased the risk of pancreatic calcifications in late onset idiopathic CP in a population that never drank alcohol<sup>[26]</sup>. Moreover, a study by Maisonneuve *et al*<sup>[27]</sup> showed that smoking accelerated the degenerative alcohol induced pancreatitis quantified by the presence of calcifications and diabetes. Recently, a study reported that tobacco increased the occurrence of all major complications of alcoholic CP depending on the amount of smoking (1 pack-year is calculated as the number of cigarettes per day, multiplied by the number of years of smoking divided by 20 cigarettes/pack)<sup>[28]</sup>. No differences in CP outcome were seen at nicotine consumption corresponding to 10 pack-years. At a threshold of 15 pack-years CP was diagnosed earlier (36 years *vs* 46 years) and at nicotine consumption threshold of 20 pack-years up to 76% of patients were presented with pancreatic calcifications and ductal changes<sup>[28]</sup>. Tobacco effect on CP has long been considered to merely potentiate the main pancreatic toxic role of alcohol. However, although heavy smokers tended to be heavy drinkers, these recent findings indicate that tobacco intake is an independent risk factor in CP and accelerates the course of the disease.

### NUTRITIONAL FACTORS

Only a few studies were conducted to investigate nutritional effect on CP<sup>[29-31]</sup>, finding that diets rich in fat and protein possibly have an impact on the development of CP. However, due to subjective descriptions of daily nutritional customs and determination of the retrospective body mass index make it nearly impossible to deliver a simple report of past daily nutrition in majority of patients with CP. There is a type of CP named tropical CP which has been first described in Indonesia in 1959<sup>[32]</sup>. Tropical CP is a form of non-alcoholic CP existing in tropical developing countries. There is some confusion regarding the nomenclature sine tropical CP previously was hypothesized to be linked to diets high in consumption of cassava<sup>[33]</sup>, which is a root high in carbohydrates but low in proteins and is cultivated in tropical and subtropical regions of the world. However, a study in rats which were fed cassava for up to one year failed to

produce CP<sup>[34]</sup>. Moreover, a study in humans comparing the amount of consumption of cassava did not observe a difference between healthy controls and tropical CP patients<sup>[35]</sup>, and today tropical pancreatitis is - at least partly - linked to genetic mutations<sup>[36]</sup>. Hence, tropical CP is widespread in parts of India and Africa where cassava is not a part of nutrition and tropical CP is not observed in rural West Africa where cassava intake is high<sup>[37]</sup>. Therefore, the cassava theory lacks evidence.

## HEREDITARY FACTORS

Over the last decades an association between different types of chronic pancreatitis and hereditary factors has been reported. Hereditary chronic pancreatitis is a rare form which is characterized by an early onset of recurrent attacks of severe epigastric pain usually before ten years of age<sup>[38]</sup>. Back in 1952, Comfort and Steinberg<sup>[39]</sup> first described hereditary chronic pancreatitis as an autosomal dominant disease. In 1996, the hereditary chronic pancreatitis disease gene was mapped to chromosome 7q35, which encodes the cationic trypsinogen gene (*PRSS1*), and the first mutation, *R122H*, was detected<sup>[40]</sup>. Ever since then, many others mutations have been identified (*A16V*, *D22G*, *K23R*, *N29I*, *N29T*, *R122C* and *R122H*)<sup>[41-46]</sup> and nowadays it is known that approximately 80% of hereditary chronic pancreatitis patients have a *PRSS1* mutation<sup>[47]</sup>. *R122H* is the most frequent mutation causing hereditary chronic pancreatitis, where Arginine (Arg) is substituted with Histidine (His) at residue 117 on codon 122<sup>[5]</sup>.

Trypsinogen is the inactive pro-enzyme for trypsin and cationic trypsinogen is the most abundant isoform in the human pancreatic juice (Figure 3). Trypsin becomes active when an eight-amino acid amino-terminal peptide is removed by an enteropeptidase, and the active trypsin plays a central role in pancreatic exocrine physiology as it initiates the cascade activation of other pancreatic digestive enzymes. Normally minor amounts of trypsin are activated within pancreatic acinar cells. Despite the small amount of active trypsin, an ongoing rapid inactivation takes place in order to prevent the digestion enzymes activation cascade and pancreatic auto digestion. To avoid auto digestion, two inhibitory mechanisms are present. First line of defense is the pancreatic secretory trypsin inhibitor (*PSTI*, gene name: serine protease inhibitor, kazal type 1, *SPINK1*), which inhibits up to 20 % of the trypsin activity<sup>[38,48]</sup>. If *SPINK1* fails to inhibit the trypsin activity, the trypsin-like enzymes (*e.g.*, mesotrypsin) are activated which hydrolyses trypsin and other zymogens (second line of defense)<sup>[49,50]</sup>. Hence, any mutation in *SPINK1* or the mesotrypsin gene will delay the protection, and therefore hereditary chronic pancreatitis patients are only protected against pancreatic auto digestion and progressive pancreatitis as long as the level of trypsin activity is less than *SPINK1* level.

In addition, a diagnosis of familial pancreatitis refers to pancreatitis from any cause that occurs in family with

an incidence greater than expected by chance alone, given the size of the family and incidence of pancreatitis within a defined population<sup>[51]</sup>. Familial pancreatitis may as well be caused by genetic mutations.

As mentioned above *SPINK1* encodes *PSTI* (one of the defensive mechanisms against prematurely trypsin activity within the acinar cells) and *SPINK1* mutations are thought to be a disease modifying factor rather than being disease causing factor in idiopathic chronic pancreatitis<sup>[52,53]</sup>.

Idiopathic chronic pancreatitis is defined as cases of pancreatitis within a family where no associated factor can be identified and represents 20%-30% of cases of chronic pancreatitis<sup>[54,55]</sup>. Most of the idiopathic chronic pancreatitis cases may be due to a variety of processes such as mutations in *SPINK1* and cystic fibrosis transmembrane conductance regulator (*CFTR*) gene<sup>[54-56]</sup>, or Sjögren's syndrome<sup>[57,58]</sup>.

In 1998 a relationship between the *CFTR* gene and idiopathic chronic pancreatitis was described<sup>[59,60]</sup>, but the underlying mechanisms leading to the development of chronic pancreatitis are still poorly understood. *CFTR* is identified as the cystic fibrosis gene and the link between cystic fibrosis and chronic pancreatitis is that both conditions may show abnormal sweat chloride concentrations together with pancreatic ductal obstruction caused by inspissated secretions. Furthermore, 1%-2% of patients with cystic fibrosis may suffer from recurrent pancreatitis<sup>[60,61]</sup>, however up to 85% suffer from exocrine insufficiency and even up to 93% take exogenous pancreatic supplements<sup>[62]</sup>.

There are two forms of idiopathic chronic pancreatitis: early- and late-onset pancreatitis, which both differ from alcoholic pancreatitis. Patients with early-onset develop calcification and exocrine and endocrine insufficiency more slowly than patients with late-onset disease, but they experience more severe pain<sup>[63]</sup>.

## EFFERENT DUCT FACTORS

Anatomically, the main pancreatic duct joins the common bile duct, after which both ducts perforate the medial side of the second portion of the duodenum at the major duodenal papilla. Hence any obstruction (partial or complete), compression or inflammation of the pancreatic tissue will increase the pressure within the pancreatic efferent ducts leading to ductal dilation proximal (upstream) of the stenosis and to atrophy of the acinar cells and replacement by fibrous tissue<sup>[4]</sup>. During embryogenesis, two efferent pancreatic ducts, a ventral and a dorsal duct fuse together to form one main pancreatic duct. When this fusion fails to occur, a pancreas divisum is formed, which is one of the most frequent congenital ductal anomalies. Theoretically this may cause flow problems within the pancreatic duct. However, pancreas divisum is present in up to 9% of autopsy studies and controversies exist whether presence of this abnormality is overrepresented in chronic pancreatitis in comparison to the normal pop-

ulation. Despite the rarity (5-15 cases per 100000), annular pancreas is another congenital disease which may be linked to chronic pancreatitis, as the pancreatic tissue forms a complete or partial ring around the duodenum<sup>[64]</sup>.

There are various other possible causes for a duct obstruction, but the most important and common is the narrowing and eventual occlusion of the main pancreatic duct in the head of the pancreas due to a ductal adenocarcinoma. Other causes for obstruction of the main pancreatic duct include intraductal papillary-mucinous neoplasms, cystic and endocrine neoplasms and acquired fibrous strictures. In the smaller pancreatic ducts, ductal papillary hyperplasia is a common cause of narrowing of the duct lumen. Finally, obstruction caused by a gallstones in the common bile duct (choledocholithiasis) or sphincter of Oddi dysmotility can obstruct flow and therefore cause retention of bile in the biliary tree and pancreatic juice in the pancreatic duct. The former is a frequent and well-known cause for acute pancreatitis.

## IMMUNOLOGICAL RISK FACTORS

Immunological risk factors involved in chronic pancreatitis leading to autoimmune pancreatitis (AIP) deserve increased awareness as the underlying pathogenesis is not fully elucidated. The effort to diagnose autoimmune pancreatitis is mainly focused on the treatment possibilities with *e.g.*, glucocorticosteroids and differentiating the disease from pancreatic cancer. There are several lines of evidence that immunological factors play a key role in CP. Even though lymphoplasmacytic infiltration in AIP is most pronounced in the pancreatic ducts, advanced cases also show intralobular lymphoplasmacytic infiltration, and the inflammation may also specifically attack the acinar cells. IgG antibodies to a plasminogen-binding protein homologous to the human ubiquitin-protein ligase E3 component n-recogin 2, is expressed in pancreatic acinar cells, and have been found in the sera of AIP patients<sup>[65]</sup>. In AIP patients auto-antibodies to trypsinogens are also upregulated, which is not the case in alcohol induced chronic pancreatitis patients, corresponding to loss of trypsinogen-positive acinar cells in AIP tissues<sup>[66]</sup>. Furthermore, basement membrane deposits of complement C3c, IgG4 and IgG not only around pancreatic ducts but also around acini have been demonstrated in AIP<sup>[67]</sup>.

Even though it has not yet been fully proved that there are differences in the pathogenesis, AIP is separated into two distinct types: Type 1 and type 2 AIP.

Type 1 AIP patients (most common type in East Asia) are typically older than type 2 patients, with a mean age at disease onset of 62 years<sup>[68]</sup>. Plasma levels of IgG4 are increased and in approximately 50% of the cases other organs are affected<sup>[68,69]</sup>. The key histological feature that distinguishes type 1 AIP from type 2 AIP is strong infiltration of IgG4-immunopositive plasma cells and lymphoplasmacytic infiltration<sup>[70,71]</sup>. IgG4-positive sclerosing cholangitis represents one of the main extrapancre-

atic manifestations of type 1 AIP, and hence jaundice is a common symptom<sup>[72]</sup>. Due to the multiple extrapancreatic manifestations of type 1 AIP, Kamisawa *et al.*<sup>[73]</sup> promoted the concept of AIP as part of a clinicopathological entity of IgG4-associated systemic disease, together with retroperitoneal fibrosis, sclerosing sialadenitis and sclerosing cholangitis. This systemic fibroinflammatory disease probably also includes lesions in the aorta, breast and prostate<sup>[73-76]</sup>.

Type 2 AIP patients (45% of the cases in United States and Europe) are typically younger than type 1 AIP, with a mean age of 40-48 years<sup>[68,77]</sup>. AIP is often associated with Crohn's disease and ulcerative colitis<sup>[78]</sup>. The most characteristic histological feature which distinguishes type 2 from type 1 AIP is the so-called granulocytic epithelial lesions which are a hallmark of type 2 AIP<sup>[77,79]</sup>. Besides, type 2 AIP is usually not associated with the extrapancreatic manifestations observed in type 1 AIP.

## MISCELLANEOUS

### *Tropical chronic pancreatitis*

Tropical chronic pancreatitis may be referred to an idiopathic, juvenile, non-alcoholic form of chronic pancreatitis widely prevalent in the developing countries of the tropical world. Tropical chronic pancreatitis can be sub-grouped in two entities: Tropical calcific pancreatitis and fibrocalculous pancreatic diabetes. Tropical calcific pancreatitis describes the early pre-diabetic stage of the disease and affects younger people. Tropical calcific pancreatitis is characterized by severe abdominal pain, pancreatic calcification, and signs of pancreatic dysfunction (no diabetes mellitus at the time of diagnosis). Fibrocalculous pancreatic diabetes describes the late diabetic stage of the disease where diabetes mellitus is the first major clinical sign to determine the diagnosis of fibrocalculous pancreatic diabetes. The etiology of tropical calcific pancreatitis and fibrocalculous pancreatic diabetes has shown to be related to genetic mutations in the *SPINK1* gene, but it is still unknown if environmental factors have an influence<sup>[36,80-82]</sup>.

### *Primary hypercalcemia*

It has been suggested that higher serum calcium levels may contribute to pancreatitis, as hypercalcemia may predispose the pancreatic acinar cell to abnormal, sustained calcium levels, which leads to premature pancreatic protease activation, and consequently pancreatitis.

### *Hyperparathyroidism*

Controversies exist regarding associations between primary hyperparathyroidism and acute or chronic pancreatitis. However, most studies show an increased rate of pancreatitis among patients with primary hyperparathyroidism in comparison to general hospitalized patients without the disease.

### *Hyperlipidemia*

Hyperlipidemia covers abnormally elevated levels of any



or all lipids, lipoproteins or both in the blood. Hyperlipidemia can be divided in primary and secondary subtypes. Lipid and lipoprotein abnormalities are relatively common in the general population, and are regarded as a modifiable risk factor associated with acute pancreatitis.

## CONCLUSION

Increased knowledge of different etiological factors and how they interact may encourage the use of further advanced diagnostic tools, which potentially will help clinicians to diagnose and treat CP at an earlier stage. Recent research has increased our understanding of the disease and has changed the approach to CP. However, in view of the multi-factorial disease and the complex clinical picture, it is not surprising that treatment of patients with CP is challenging and often unsuccessful.

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## Morphological and functional evaluation of chronic pancreatitis with magnetic resonance imaging

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### Abstract

Magnetic resonance imaging (MRI) techniques for assessment of morphology and function of the pancreas have been improved dramatically the recent years and MRI is very often used in diagnosing and follow-up of chronic pancreatitis (CP) patients. Standard MRI including fat-suppressed T1-weighted and T2-weighted imaging techniques reveal decreased signal and glandular atrophy of the pancreas in CP. In contrast-enhanced MRI of the pancreas in CP the pancreatic signal is usually reduced and delayed due to decreased perfusion as a result of chronic inflammation and fibrosis. Thus, morphological changes of the ductal system can be assessed by magnetic resonance cholangiopancreatography (MRCP). Furthermore, secretin-stimulated MRCP is a valuable technique to evaluate side branch pathology and the exocrine function of the pancreas and diffusion weighted imaging can be used to quantify both parenchymal fibrotic changes and the exocrine function of the pancreas. These standard and advanced MRI techniques are supplementary techniques to reveal morpho-

logical and functional changes of the pancreas in CP. Recently, spectroscopy has been used for assessment of metabolite concentrations *in-vivo* in different tissues and may have the potential to offer better tissue characterization of the pancreas. Hence, the purpose of the present review is to provide an update on standard and advanced MRI techniques of the pancreas in CP.

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**Key words:** Magnetic resonance; Chronic pancreatitis; Secretin; Diffusion weighted imaging; Exocrine pancreatic function

**Core tip:** Magnetic resonance imaging (MRI) techniques for assessment of morphology and function of the pancreas are often used in diagnosing and follow-up of chronic pancreatitis patients. The purpose of the present review is to provide an update on standard and advanced MRI techniques of the pancreas in chronic pancreatitis. In addition to standard MRI techniques, advanced MRI techniques including magnetic resonance cholangiopancreatography (MRCP), secretin-stimulated MRCP and diffusion weighted imaging can also provide important microstructural and functional information.

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### INTRODUCTION

The diagnosis and follow-up of chronic pancreatitis (CP)

patients rely on both clinical and imaging information such as the Mayo Clinic diagnostic criteria<sup>[1]</sup>. Especially, the diagnosis of CP at an early stage is a clinical challenge. The development in imaging techniques, and especially magnetic resonance imaging (MRI), has dramatically improved the information on both morphology and function of the pancreas. In the Mayo diagnostic criteria for CP, MRI is now an accepted method for assessing ductal pathology and concretions<sup>[1]</sup>.

A few decades ago, before the clinical introduction of cross-sectional imaging techniques, the imaging evaluation of CP was limited to plain radiography depicting calcifications. Traditionally, the ductal morphology has been assessed with endoscopic retrograde cholangiopancreatography (ERCP), which is based on intraductal contrast-enhancement with severity assessed using the Cambridge classification<sup>[2]</sup>. Today routine imaging modalities in the evaluation of CP typically include: Computed tomography (CT) with one or more contrast-enhancement phases, MRI with or without magnetic resonance cholangiopancreatography (MRCP) and ultrasound with a transabdominal or endoscopic approach. The advantage of MRI is the superior soft tissue visualization without radiation exposure. The main focus of imaging is typically to describe glandular atrophy and calcifications, duct pathology, pseudocysts and complications such as abscess formation and concomitant acute inflammation.

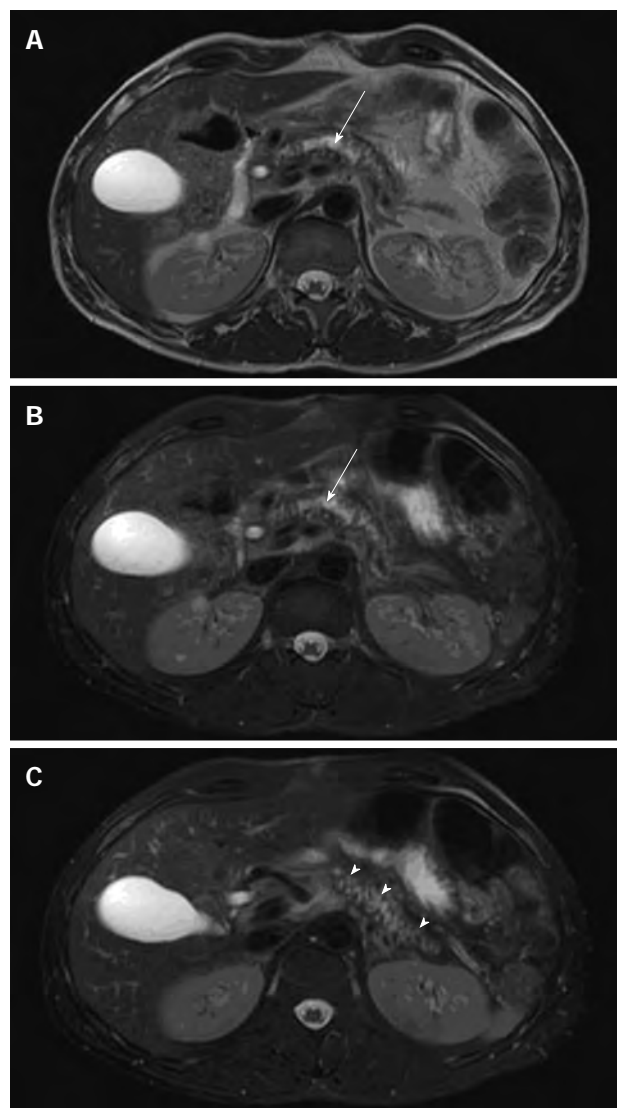
Recently, more advanced MRI methods have emerged which also provide important information on both tissue characteristics and pancreatic function.

The aim of this review is to provide an update on standard and advanced MRI techniques of the pancreas in CP.

## STANDARD MRI

Parenchymal changes can be assessed in the early stage of CP by standard MRI typically including fat-suppressed T1-weighted images, T2-weighted images and gadolinium-enhanced imaging<sup>[3-6]</sup>.

Fat-suppressed T1-weighted images show high signal intensity in normal pancreas and decreased signal intensity in CP due to loss of aqueous protein in the acini within the glandular elements of the pancreas caused by chronic inflammation and fibrosis<sup>[7-9]</sup>. Fat-suppression of T1-weighted images enhance the signal of the pancreas in relation to the surrounding retroperitoneal fat and the signal intensity can be compared to surrounding organs and tissue such as the spleen or muscles<sup>[9]</sup>. The biliary system and pancreatic ducts are enhanced on fat-suppressed T2-weighted images (Figure 1). The size of pancreas normally decreases with age but as acinar atrophy occurs more rapidly in CP, the pancreas diminishes segmentally or diffusely in the anteroposterior dimensions. The diameter can typically be assessed at the head, body and tail of the pancreas and compared with age-related normal values of the anteroposterior diameter<sup>[10]</sup>.



**Figure 1 Pancreatic morphology.** Axial T2-weighted magnetic resonance imaging views showing glandular atrophy (A), dilated irregular duct (B, arrow) and irregular side-branches (C, arrow heads) in a patient with chronic pancreatitis.

Gadolinium-enhanced imaging is used to investigate the perfusion of the pancreas during a series of contrast-enhanced images with repeated sequential scans. During the arterial phase of gadolinium infusion the normal pancreas shows pronounced enhancement due to high vascular perfusion and decreased signal during the venous phase<sup>[11]</sup>. The perfusion of the pancreas is decreased in patients with CP due to chronic inflammation and fibrosis resulting in reduced and delayed enhancement of the pancreatic signal<sup>[7,12]</sup>.

Standard MRI is often used together with MRCP to assess both parenchymal and ductal changes of the pancreas. One important limitation of MRI lies in its inability to depict small parenchymal calcifications, which can easily be evaluated with CT.

MRI also has a role in diagnosing autoimmune pancreatitis (AIP) which can be a differential diagnosis to CP<sup>[13,14]</sup>. In diffuse AIP, MRCP may show a decreased di-

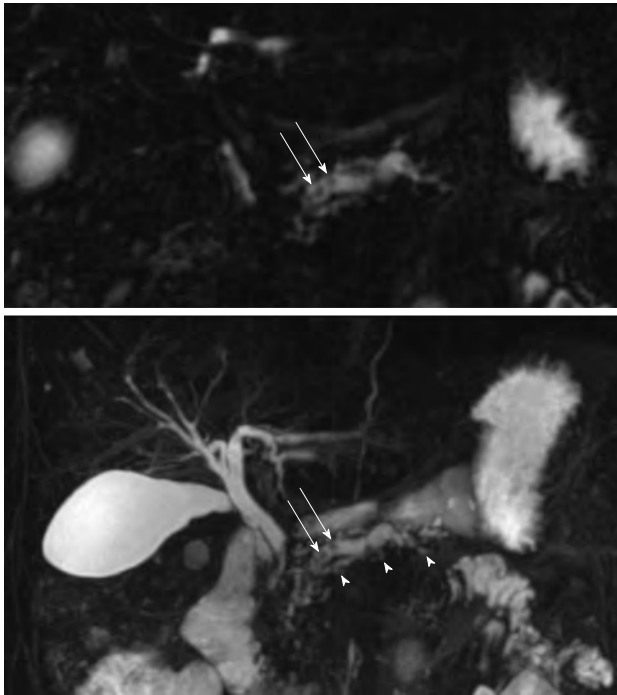


Figure 2 Magnetic resonance cholangiopancreatography. Upper figure displays a single coronal image and the lower figure a 3D view of the pancreato-biliary tree. In this chronic pancreatitis patient the dilated irregular main duct has irregular side branches (arrow heads) and contains multiple rounded filling defects (arrows).

ameter of the main pancreatic duct and can be accompanied by strictures and an irregular wall. However, in AIP the pancreas is typically with diffuse or localized enlargement with reduced signal on T1 and increased signal on T2-weighted images. A surrounding capsule with reduced signal in T2-weighted images can be seen<sup>[13,14]</sup>.

## MRCP

MRCP was first described in 1991, providing a non-invasive alternative to ERCP, which relies on the endoscopic injection of contrast fluid into the common bile duct<sup>[15]</sup>. By applying either a single-shot breath-hold technique or a free-breathing technique with respiratory triggering, MRCP can provide both 2D and 3D images<sup>[16,17]</sup>. Advances in scanner technology in the recent years allow faster image acquisition and better quality with more detailed images including 3D reconstructions. These advances benefit from a high signal-to-noise ratio. Additionally, the 3D free-breathing protocol makes it superior to 2D imaging in patients who are unable or unwilling to hold their breath for the duration of the scan<sup>[18]</sup>.

MRCP relies on heavily T2-weighted pulse sequences, benefiting from the T2-weighted differences in relaxation time between fluid-filled compartments and adjacent soft tissue. Hence, the pancreato-biliary tree is displayed as high signal intensity and the pancreatic duct is clearly visualized in the normal pancreas. Accordingly, the MRCP technique is relevant in detecting pancreatic ductal dila-

tion, small filling defects (stones and protein plugs), strictures, irregularities of the main pancreatic duct, and irregularity (sacculation and/or ectasia) of side branches<sup>[6]</sup> (Figure 2). Furthermore, pseudocysts and other ductal congenital abnormalities and normal variants (such as pancreas divisum) can be visualized<sup>[19,20]</sup>.

The Cambridge classification system of ductal dimensional changes has been modified for the MRCP technique in the following fashion: Cambridge 1 (normal pancreas): pancreatic ducts are normal; Cambridge 2 (equivocal pancreas): 1-2 side branches and main duct 2-4 mm; Cambridge 3 (mild disease):  $\geq 3$  side branches and main duct 2-4 mm; Cambridge 4 (moderate disease):  $\geq 3$  side branches and main duct  $> 4$  mm; Cambridge 5 (marked disease): as traditional Cambridge classification system<sup>[2,6]</sup>.

The acquisition of MRCP sequences following intravenous administration of secretin hormone allows a better visualization of subtle ductal changes especially in the early stage of CP<sup>[21]</sup>.

## SECRETIN-STIMULATED MRCP

The secretin hormone stimulates pancreatic duct cells to produce a large volume of watery bicarbonate-rich pancreatic juice, which is secreted into the ducts and duodenum. Typically, images are obtained before and frequently after secretin stimulation for a period. In the normal pancreas the effect starts almost immediately and peaks between 2-5 min and by 10 min the caliber of the duct should return to baseline<sup>[21]</sup>. In secretin-stimulated MRCP (s-MRCP) the ductal system including side branch pathology and filling defects are better visualized compared to traditional MRCP, and s-MRCP provides images comparable to ERCP with Cambridge classification<sup>[21-23]</sup>. Furthermore, the exocrine function can be evaluated with assessment of duodenal filling, changes in pancreatic duct caliber, change in anteroposterior diameter of the pancreas, and change in signal intensity ratio between pancreas and spleen on T1-weighted and arterial-venous enhancement ratios<sup>[10,23,24]</sup>. The s-MRCP findings in CP (reduced duodenal filling grade and reduced increase in pancreatic duct caliber) are comparable to the results of endoscopic pancreatic function testing (ePFT)<sup>[23]</sup>. Studies in CP by Manfredi *et al.*<sup>[25]</sup> and Schneider *et al.*<sup>[26]</sup> showed correlation between assessment of the pancreatic exocrine reserve by dynamic s-MRCP and exocrine function assessed by fecal elastase test and <sup>13</sup>C-mixed chain triglyceride breath test. Wathele *et al.*<sup>[27]</sup> found correlation between endoscopic aspiration-based bicarbonate test and s-MRCP findings in healthy controls. However, the use of s-MRCP has not yet been integrated as a part of the Mayo diagnostic criteria for CP and further evaluation of s-MRCP as a test for exocrine function in comparison to traditional tests is needed.

Furthermore, s-MRCP can be combined with diffusion weighted imaging (DWI) of the pancreas.



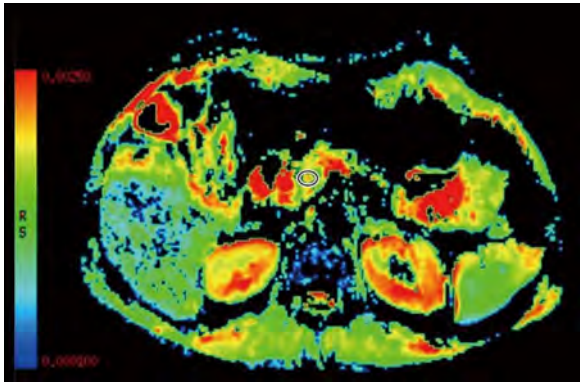


Figure 3 Diffusion weighted imaging. The apparent diffusion coefficient map of a chronic pancreatitis patient is shown with a measuring region of interest positioned in the pancreatic head to assess the degree of parenchymal fibrosis (red is high and blue low water diffusion).

DWI

DWI is an emerging technology to assess early parenchymal changes associated with CP and has shown results comparable to other existing methods such as MRCP and ePFT<sup>[9,28]</sup>. DWI assesses the random microscopic motion of water protons to obtain the apparent diffusion coefficient (ADC). The flow of water is less restricted in fluid-rich tissues, which will then be represented by a high ADC value. Water molecules interacting with cell membranes and macromolecules will be restricted thereby causing a reduction in the ADC value. A reduced amount of diffusible water is present in fibrotic tissue and in reduced pancreatic exocrine function<sup>[28]</sup>. Hence, presence of parenchymal fibrosis in CP causes diffusion restriction and results in lower ADC values<sup>[29,30]</sup> (Figure 3).

Furthermore, when used in combination with secretin stimulation, the ADC value increases both in normal pancreas and in CP as the secretion stimulation facilitates an increased mobility of water molecules and increased circulation in the pancreatic capillaries. Following secretin stimulation, the diffusion coefficients have either delayed or lower peak values in CP patients, indicating reduced exocrine function<sup>[29,31]</sup>. Also, patients in risk of CP (such as alcohol consumption, nicotine consumption, nutritional factors, hereditary factors, efferent duct obstructions, immunological factors and rare miscellaneous factors) generally exhibit a delayed peak in diffusion coefficients compared to controls<sup>[31]</sup>.

This technique can be particularly useful in patients with early stage CP where atrophy and ductal pathology are subtle. Furthermore, the technique is useful in differentiating between pancreatic cysts, inflammatory cysts and cystic neoplasms and between pancreatic adenocarcinoma and normal pancreas due to different content of cellular elements<sup>[32]</sup>. Inan *et al*<sup>[33]</sup> calculated ADC values and ADC cyst-to-pancreas ratios and found significant lower values for abscesses, hydatid cysts and neoplastic cysts compared to values of simple cysts and pseudocysts. This technique may be useful in the diagnosis of intraductal papillary mucinous neoplasms (IPMN), which

Table 1 Advantages of magnetic resonance imaging techniques

	MRI	MRCP	s-MRCP	DWI
Loss of aqueous protein	Yes	No	No	No
Glandular atrophy	Yes	No	No	No
Perfusion	Yes	No	No	No
Calcification <sup>1</sup>	No	No	No	No
Pancreatic ductal dilation	No	Yes	No	No
Filling defects	No	Yes	Yes	No
Strictures	No	Yes	Yes	No
Irregularities	No	Yes	Yes	No
Pseudocysts	Yes	Yes	Yes	No
Side branch pathology	No	Yes	Yes	No
Exocrine function	No	No	Yes	Yes
Parenchymal fibrosis	No	No	No	Yes

Advantages of the different magnetic resonance imaging (MRI) techniques in diagnosing chronic pancreatitis. <sup>1</sup>A disadvantage of MRI is the inability to detect calcifications. This can be achieved through computed tomography scans. DWI: Diffusion weighted imaging; MRCP: Magnetic resonance cholangiopancreatography; s-MRCP: Secretin-stimulated MRCP.

is often difficult since CP and IPMN may have overlapping imaging findings<sup>[13,34]</sup>. Patients with main duct IPMN can present with ductal dilatation and associated parenchymal atrophy, and patients with side-branch IPMN can present with cystic lesions often confused with pseudocysts. ADC values of the cystic lesions may be helpful in deciding the malignant potential of IPMN<sup>[34,35]</sup>.

NEW TECHNIQUES

Recently, other advanced MRI techniques have become more interesting as the development within the MR hardware and software is expanding and allows increased signal-to-noise ratios, shorter scan time and breath-hold imaging.

MR spectroscopy with non-invasive *in-vivo* assessment of metabolite concentrations has been applied in a variety of different tissues (*e.g.*, brain, prostate, breast and liver). Hence, spectroscopy of the pancreas has the potential to offer a more accurate tissue characterization. Due to methodological challenges, the pancreas has only been studied to a very limited degree with spectroscopy. Despite of this, Su *et al*<sup>[36]</sup> characterized the normal pancreas at 3T and identified metabolites such as lipid, choline and cholesterol. Cho *et al*<sup>[37]</sup> used MR spectroscopy to distinguish between patients with chronic focal pancreatitis and patients with pancreatic carcinoma and found less lipid in pancreatitis than in pancreatic carcinoma. Furthermore, other studies also detected differences between normal pancreatic tissue and carcinoma tissue with alterations in lipid, choline and fatty acids<sup>[38,39]</sup>. However, to the best of our knowledge, this technique has not yet been applied in the characterization of CP patients.

CONCLUSION

This review provides an update on standard and ad-



vanced MRI of the pancreas in CP. Table 1 summarizes the capability of the different MRI techniques to display the different aspects of pancreatic changes and dysfunction in CP. Depending on local practice, scanner configuration and radiological experience, it should be possible to construct or customize individual MR protocols including the (or some of the) techniques reviewed in this paper to get the best possible morphological and functional information in CP.

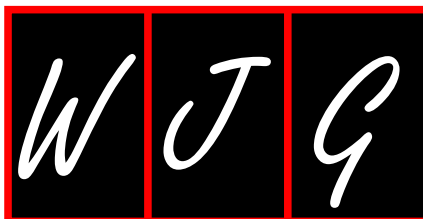
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## Ultrasonography in diagnosing chronic pancreatitis: New aspects

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### Abstract

The course and outcome is poor for most patients with pancreatic diseases. Advances in pancreatic imaging are important in the detection of pancreatic diseases at early stages. Ultrasonography as a diagnostic tool has made, virtually speaking a technical revolution in medical imaging in the new millennium. It has not only become the preferred method for first line imaging, but also, increasingly to clarify the interpretation of other imaging modalities to obtain efficient clinical decision. We review ultrasonography modalities, focusing on advanced pancreatic imaging and its potential to substantially improve diagnosis of pancreatic diseases at earlier stages. In the first section, we describe scanning techniques and examination protocols. Their consequences for image quality and the ability to obtain complete and detailed visualization of the pancreas are discussed. In the second section we outline ultrasonographic char-

acteristics of pancreatic diseases with emphasis on chronic pancreatitis. Finally, new developments in ultrasonography of the pancreas such as contrast enhanced ultrasound and elastography are enlightened.

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**Key words:** Ultrasonography; Pancreas; Chronic pancreatitis; Transabdominal ultrasound; Medical imaging technique; Contrast enhanced ultrasonography; Elastography; Strain imaging

**Core tip:** Pancreatic diseases include acute and chronic inflammatory diseases and neoplastic tumors. It is a clinical challenge to diagnose these patients at an early stage because biochemical and imaging signs may be unspecific and are only evident at an advanced stage of the disease. Advances in pancreatic imaging are important for early detection of pancreatic diseases. Ultrasonography, as a diagnostic tool, has undergone major technical improvements over the last decade. It still is the preferred method for first line imaging, as well as it is increasingly used to clarify findings by others imaging modalities to support decision making.

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### INTRODUCTION

Transabdominal ultrasonography still is the most used first line imaging modality in the diagnostic workup of abdominal diseases. Ultrasonography is, noninvasive, widely available, inexpensive, without side effects and

easy to perform on a daily basis, if needed. As a “real-time” imaging modality, transabdominal ultrasonography gives first a broad overview and then can localize the “region of interest” to perform detailed evaluation and eventually determine the cause of the disease. It can also reduce the use of computed tomography, magnetic resonance pancreatography, endoscopic ultrasonography or other diagnostic methods, which are personnel-intensive and thereby costly<sup>[1,2]</sup>.

Ultrasonography (US) of the pancreas is challenging, given its retroperitoneal location with overlying structures and relatively small size. The quality and thereby the clinical usefulness of the pancreatic ultrasound imaging has rapidly advanced along with the technological progress. Early works in the seventies and eighties describe evidence of pancreatic structural changes seen by transabdominal ultrasonography of approximately two-thirds in the diagnosed chronic pancreatitis (CP) cases<sup>[3-7]</sup>. Concerning pancreatic malignancy, most early works describe, somewhat surprisingly, pathological findings in the majority of the cases, but the distinction between malignancy and chronic pancreatitis could not be achieved<sup>[3-7]</sup>. With modern, high-end scanners, experienced physicians could achieve complete imaging of the pancreas in 90% in the nineties<sup>[6]</sup>. The current sensitivity and specificity of transabdominal ultrasonography in the diagnosis of pancreatic diseases, the ability to differentiate between acute and chronic inflammation and premalignant or malignant lesions is not yet determined. Today we have real-time, high-resolution imaging, where the spatial resolution is in line with the best image quality computed tomography (CT) or magnet resonance imaging (MRI) can offer. Furthermore, the temporal resolution far exceeds that of CT, positron emission tomography and MRI, which is particularly relevant for contrast-enhanced ultrasonography (CEUS). Moreover, the image quality and especially the amount of information are evolving along with the technological progression and introduction of new modalities such as of CEUS and elastography<sup>[8,9]</sup>.

Figure 1 shows a standard B-mode image of the pancreas, liver and surrounding vessels with a 1-5 and 12-15 MHz transducer. A clear overview of the gland and surrounding structures can be obtained and by changing the frequencies for any depth, focus on a smaller area of interest can be achieved.

We shortly review the current and forthcoming technical modalities and methods in transabdominal ultrasonography and endoscopic ultrasonography (EUS) of the pancreas with emphasis on CP. In our opinion, transabdominal ultrasonography has an important place in imaging of the pancreas, alongside CT, MRI and EUS.

## ULTRASONOGRAPHIC EXAMINATION

Ultrasonographic imaging of the pancreas can be difficult given its retroperitoneal location, variety in appearance among individuals and body habitus. Overlying bowel gas and obesity are the most frequent limitations in trans-

cutaneous scanning of the pancreas<sup>[10-12]</sup>.

Due to the food related production of intestinal gas reflecting the ultrasound beams, every examination should be performed fasting. US examination of the pancreas includes transverse, longitudinal and angled oblique scans. Successful visualization can often be achieved by manipulations with the transducer and is directly linked to the skill and persistence of the examiner. By applying graded compression by the transducer, bowel gas can be moved away, and all the portions of the pancreatic gland; head, neck, body and tail can often be visualized. Further improvement can also be obtained by drinking two glasses of water/juice, thereby using the fluid-filled stomach as an acoustic window. Other manipulations such as; changing the patient's position to stand or sit; let the patient turn from the supine position to oblique position or change/stop the breathing circle; let the patient “blow up the belly”, can also improve visualization of the pancreas<sup>[13]</sup>.

The pancreatic tail can sometimes be difficult to access in transversal scan. Figure 2 shows trans-splenic access, where the tail of the pancreas (cauda pancreatis) is scanned from the left lateral side using the spleen as acoustic window.

In a US examination of the pancreas the echotexture, the size of the gland including the main pancreatic duct (MPD), and anatomical landmarks of the pancreas should be evaluated. The echotexture in a normal pancreas is isoechoic or hyperechoic compared to the healthy liver<sup>[14]</sup>. Frequently, the echogenicity of pancreas is increasing with age. Orientational antero-posterior dimensions of the pancreas are: the head (2.5 cm), body (1.5 cm), tail (3.5 cm) and the pancreatic duct (< 2.5 mm). Fatty replacement (lipomatosis) of the pancreatic gland and decrease in size is common with increasing age but can also be found in patients with cystic fibrosis, CP, some types of diabetes and other diseases<sup>[15-17]</sup>.

The surrounding vascular, ductal and abdominal organ landmarks are the portal vein, splenic vein, confluens with the mesenteric vein, vena cava, aorta, superior mesenteric artery, common hepatic artery, splenic artery, common bile duct, duodenum, stomach and the liver.

## MODALITIES IN ULTRASONOGRAPHY

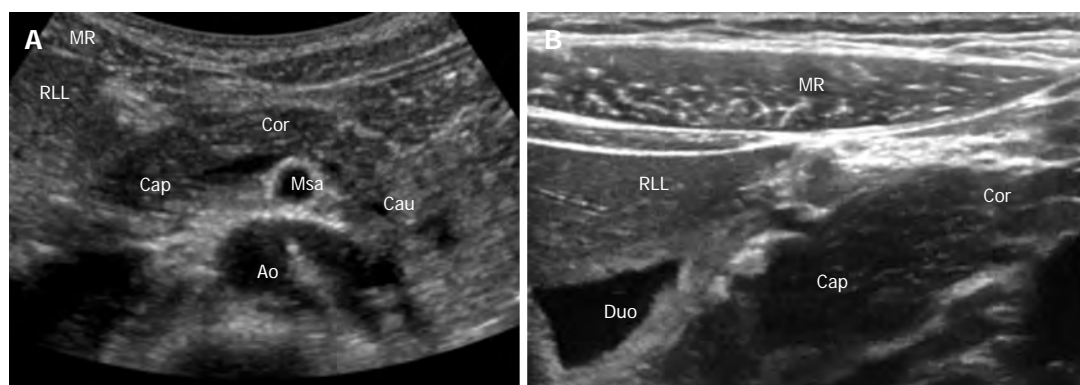
### Grayscale B-mode ultrasound

Grayscale B-mode ultrasound of the pancreas is the most used imaging modality. Complete evaluation of organ size, borders, echo structure, surrounding vessels, and pancreatic ducts can be obtained swiftly. These parameters are often sufficient to diagnose many diseases of the pancreas and patients can be managed accordingly.

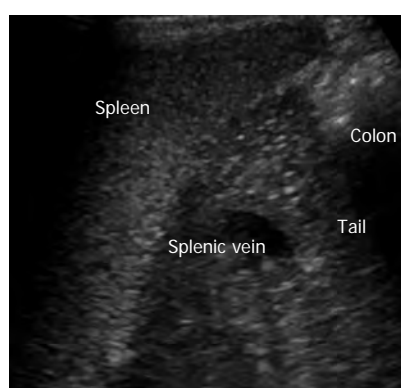
### Tissue harmonic imaging

Tissue harmonic imaging (THI) or second harmonic imaging overcomes several of the B-mode limits. By receiving harmonic overtones instead of the emitted US-frequencies, the lateral delineation is sharpened and





**Figure 1** Pancreas and the surrounding anatomical landmarks. A: B-mode image (1-5 MHz); B: B-mode image with a 12-15 MHz transducer. Details shown with high resolution. MR: Musculus rectus abdominis; RLL: Right liver lobe; Cap: Caput pancreatis; Cor: Corpus pancreatis; Cau: Cauda pancreatis; Msa: Superior mesenteric artery; Duo: Duodenum; Ao: Aorta.



**Figure 2** Left lateral side scan shows the pancreatic tail (cauda) using the spleen as acoustic window.

reverberation artifacts are reduced<sup>[18]</sup>. The image quality is improved by better discrimination between liquid and solid structures increasing the spatial and contrast resolution, making millimeter sized structures detectable. Thus, ultrasonography with THI may have better resolution than CT and MRI, in the absence of extreme obesity or a large amount of intestinal gas, which may temporarily mask the pancreas<sup>[19-21]</sup>. There are only a few disadvantages of THI; reduction of frame rate, reduced penetration depth, motion artifacts and only marginal improvement of image quality in the near sound field. This modality is normally used with CEUS, which is probably the most important application of this modality<sup>[22,23]</sup>.

### Doppler imaging

The Doppler effect in ultrasound is the change in US frequency of reflected wave from an object moving relative to the ultrasound probe, adding the option to show and record blood flow direction and velocity from vessels in ultrasound imaging.

Several modalities in conventional Doppler ultrasonography exist<sup>[24]</sup>. Combining B-mode gray scale and color-Doppler ultrasonography, overall accuracy rises substantially. By international convention, Doppler color is coded in such a way that red color expresses flow towards

the ultrasound transducer, while blue color expresses flow away from the transducer. The recent technological progress, in particular increased color-Doppler sensitivity is contributing to diagnosing and staging of pancreatic diseases. Doppler interrogation of the gland may show the outlines of the organ more precisely, since the pancreas is surrounded by vessels. The normal intrapancreatic vessels are small and difficult to show in conventional Doppler imaging. Color-Doppler shows flows in normal surrounding vessels and abnormal vascularity, such as in tumors with high vessel infiltration or tumor infiltration in vessels<sup>[25]</sup>. This method can also discriminate between cystic avascular processes without blood flow and aneurisms. Very small pancreatic calcifications which can be hard to distinguish in the irregular parenchymal pancreatic tissue in CP can be identified by the presence of twinkling artifacts. Modern high-end scanners can distinguish between inflammation (high flow) and infarction without flow<sup>[26]</sup>.

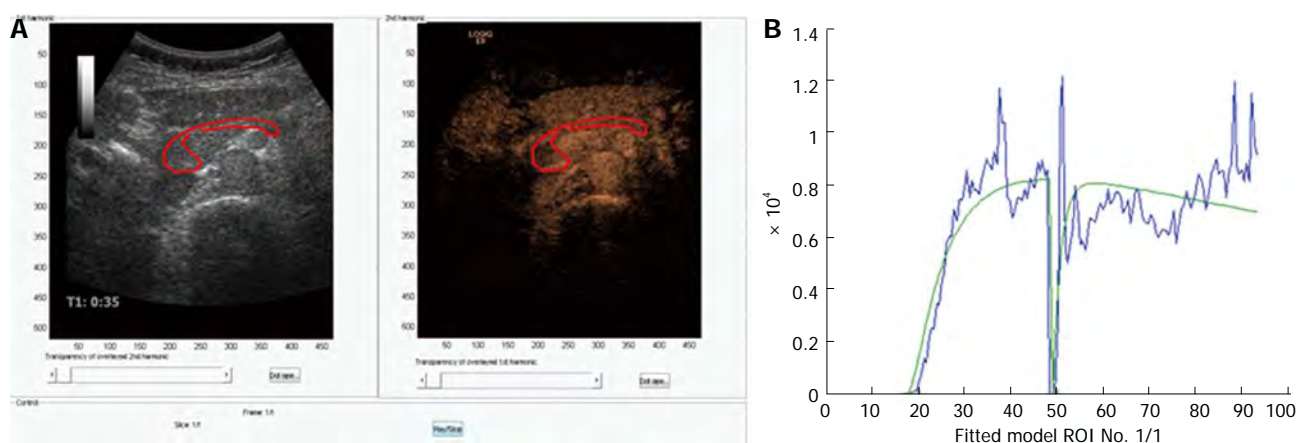
## METHODS IN ULTRASONOGRAPHY

### Panoramic imaging

In a conventional ultrasound field of view, only parts of the pancreas are visible in one image. With the panorama technique, based on real time image reconstruction the whole pancreas can be displayed in one image, allowing the creation of images similar to those in CT and MRI. However, the usefulness of these computed images regarding the evaluation of pancreatic disorders is not well documented.

### Compound imaging

Compound imaging obtains multiple coplanar images from different angles using computed beam steering. Multiple views are integrated into a single compound image with improved tissue definition, where signals from real structures are intensified and the artifacts are suppressed<sup>[14,18,19]</sup>. Organs appear smoother with clear contours. The MPD and vessels are clearly accentuated. As these images are generated from several angles, classi-



**Figure 3** Perfusion analysis of the pancreas. A: Dual view of contrast-enhanced ultrasonography examination of the pancreas in a healthy volunteer. 1.5 mL bolus of Sonovue was given as a bolus and after approximately 45 s the area of interest was exposed to high MI ultrasound bursting the bubbles in the imaging plane; B: A motion correcting analyzing software was used (DCE-US, <http://www.isibrno.cz/perfusion/>). A region of interest have been drawn including the head and body of the pancreas (unpublished data).

cal signs in transabdominal ultrasonography such as cast shadows behind pancreatic calcifications or ultrasound enhancement behind pancreatic cysts are attenuated<sup>[27]</sup>.

### Contrast-enhanced ultrasonography

The development of microbubble contrast agents enables the display of the vasculature, down to parenchymal microvasculature. With this technique enhancement patterns of lesions can be studied in real time in a similar way as contrast-enhanced CT or contrast-enhanced MRI, under full control of the operator<sup>[28-31]</sup>. Ultrasound contrast agents are confined to the blood pool where CT or MRI contrast agents are rapidly cleared into the extravascular space. As the contrast bubbles, approximately the size of a red blood cell, are gas-filled, they create strong acoustic reflectors within the fluid filled blood pool. An advantage of CEUS is the ability to study the dynamics of lesions in real time. The excellent tolerance and safety profiles allow repeated administrations in the same session if needed. The elimination of the contrast agent is by breathing, independent of liver and kidney function. Today, CEUS is increasingly incorporated in clinical use, alongside with traditional US, in liver lesions, which is by the far the most common use, but also in the kidneys, in vesico-ureteric reflux, in trauma and in the cerebral circulation<sup>[28]</sup>.

Transabdominal ultrasonography with CEUS in the pancreas has probably near the same potential, and has in some few centers been established in diagnostic routine<sup>[14,31]</sup>. The most common application is to define pancreatic lesions, in most cases already known lesions, previously seen on CT/MRI or seen in the initial US examination. Several studies show that transabdominal CEUS can be used to differentiate malignancy and CP<sup>[32-36]</sup>. The time window in a pancreatic CEUS examination is notably shorter than in the well-known liver CEUS study. This is due to the entirely arterial blood supply of the pancreas. The enhancement of the pancreas begins almost simultaneously with the aortic en-

hancement and reaches its peak between 15 and 20 s after injection of the ultrasound contrast agent. After a noticeable parenchymal enhancement in the arterial early phase, there is a washout of contrast medium with gradual loss of echogenicity during the late phase for approximately 120 s<sup>[36,37]</sup>.

New technologies may be able to discriminate between normal pancreatic perfusion and pathological in parenchymal pancreatic diseases or different tumor perfusion patterns that can be visualized using the small microbubbles of ultrasound contrast agents<sup>[38]</sup>. Time-intensity curves expressing arrival time of the microbubbles, time to peak or other parameters have so far not been able to show significant difference in focal masses in patients with CP and carcinomas<sup>[34]</sup>. The first generation analyzing software did not incorporate motion correction and that can be one of the reasons for the insensitivity of this method, since the pancreas moves in both planes, thereby moving the region of interest out of plane. New versions of more sophisticated analyzing software are under development. Figure 3 shows an example of a CEUS examination of the pancreas with a motion tracked area of interest.

In the near future, microbubbles may be an important part of the therapeutic armaments<sup>[39]</sup>. Studies have been undertaken to investigate the ability and efficacy of sonoporation in a clinical setting to increase the overall survival in patients with pancreatic adenocarcinoma. In a pilot study, numbers of treatment cycles with gemcitabine were increased from an average of 9 to 16 cycles. In two out of five patients treated, the maximum tumor diameter was temporally decreased to  $80\% \pm 5\%$  and permanently to  $70\% \pm 5\%$  of their original size, whilst the other patients showed reduced growth. This study demonstrated that it is possible to combine ultrasound, microbubbles, and chemotherapy in a clinical setting prolonging the quality of life in patients with pancreatic adenocarcinoma when comparing to chemotherapy alone. Figure 4 shows an experimental microbubble sonopora-

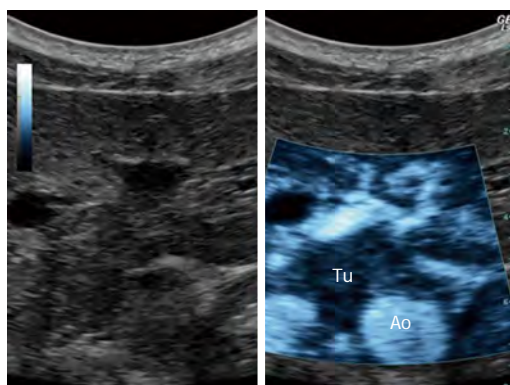


Figure 4 Ultrasound targeted treatment of pancreatic cancer using combined microbubbles and a chemotherapeutic. Left: B-mode frame. Right: The main tumor with spicules, clearly demarked, using the sonoporation settings. Tu: Tumor; Ao: Aorta.

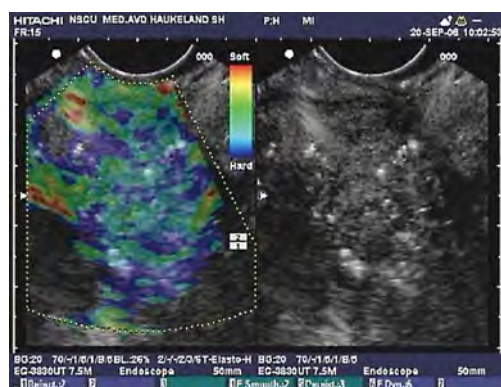


Figure 5 Endoscopic ultrasound with elastography of chronic pancreatitis. Endoscopic ultrasonography B-mode sonogram (right) and an elastogram superimposed a sonogram (left). In this image of the pancreatic head, hyperechoic foci and strands are seen in the parenchyma, as well as inhomogeneous echogenicity, which are signs of chronic pancreatitis. The elastogram shows predominantly a blue, indicating harder tissue, and green representing intermediate hardness in a honeycomb pattern over the pancreatic tissue.

tion therapy setup targeting a pancreatic adenocarcinoma, using a clinical ultrasound scanner<sup>[39]</sup>.

## ENDOSCOPIC ULTRASONOGRAPHY IN THE WORKUP OF CP

Even if transcutaneous US may image the pancreas in many cases, the limitations of obesity, reverberations and bowel gas may not always be eliminated. EUS has the advantage over trans-cutaneous US examinations that the US probe can be placed close to the organ of interest allowing the use of high frequency ultrasound (5-12.5 MHz). This may allow the imaging of small alterations in the pancreatic duct and parenchyma. The findings are usually grouped as ductal changes or parenchymal changes. The classic features include: ductal calcifications, hyperechoic duct walls, duct-caliber dilatation, visible side ducts. Parenchymal changes include: lobulation with or without honeycombing, parenchymal hyperechoic areas with or without shadowing, echogenic strands and cysts.

The problem is that some of these features also are found in healthy subjects, and increasing with age. A study compared EUS and an endoscopic pancreatic function test (ePFT) sampling the peak bicarbonate concentration after secretin stimulation. EUS showed a good correlation with fibrosis. The sensitivity of EUS and ePFT had similar sensitivity for diagnosing CP (0.84%-0.86%), but EUS was more specific (100% *vs* 67%). By combining the two methods, however, the sensitivity reached 100%<sup>[40]</sup>. The Rosemont classification is a set of EUS-based criteria for the diagnosis of CP. These criteria are based on detailed evaluation on pancreatic parenchymal and ducts. Both parenchymal and ductal features are divided in major and minor criteria<sup>[41]</sup>. Major criteria for CP are; hyperechoic foci with shadowing, lobularity with honeycombing and MPD calculi. Minor criteria for CP; cysts, dilated ducts  $\geq 3.5$  mm, irregular pancreatic duct contour, dilated side branches  $\geq 1$  mm, hyperechoic duct wall, strands, non shadowing hyperechoic foci, and lobularity with noncontiguous lobules. The Rosemont criteria, using a combination of major and/or minor criteria, categorize the patient into four groups: consistent, suggestive, indeterminate of CP or normal.

## NEW IMAGING METHODS FOR EUS IN CP-EUS ELASTOGRAPHY

Elasticity imaging of pancreatic tissue may help the examiner distinguish between harder and softer tissue. Elasticity imaging is currently an option in ultrasonography. Two main methodologies are commercially available. Strain imaging provides a qualitative strain map, frequently in a colored pattern superimposed on a B-mode echogram, visualizing the local strain as a result of endogenous movements or by an acoustic pulse. Alternatively, a shear wave method is used, providing quantitative elasticity information based on the travelling speed of shear waves. The energy for these shear waves is deposited in the tissue by an acoustic pulse. In some systems both deformation based images and local shear-wave quantification are combined.

Up to now, only strain based imaging systems have been available in combination with flexible echo-endoscopes. In Figure 5 an EUS image of a pancreas with CP is imaged with Real-Time Elastography. In Figure 6 a reactive hilar lymph node in a patient with CP is imaged as green in the strain map indicating intermediate hardness.

Several studies including two meta-analyses conclude that EUS elastography may help characterize focal pancreatic lesions as benign or malignant based on their imaged strain with an high accuracy<sup>[42-44]</sup>. In CP, the pathology is sometimes more diffusely distributed, and tissue hardness may not be limited to hypoechoic areas. Fibrosis and calcifications, if present, also increase tissue hardness in addition to local edema. In a transcutaneous shear wave study of acute pancreatitis Mateen *et al*<sup>[42]</sup> found that pancreatic tissue undergoes a cycle of increasing tissue hardness, resulting in low strain, in the acute



**Figure 6** Endoscopic ultrasonography B-mode sonogram and elastogram of lymph node in chronic pancreatitis. The sonogram (right) shows a lymph node as a hypoechoic oval shape surrounded by more echogenic tissue in the liver hilum. This lymph node approximately 18 mm × 10 mm, appeared in the liver hilum of a patient with chronic pancreatitis. On the left, the lymph node is not harder than the surrounding tissue as the predominant color hue is green. This finding is frequent in reactive lymph nodes, and may be a sign of benign etiology.

phase of inflammation which subsequently turned softer as the inflammatory reaction resolved. In a study on focal pancreatic lesions we found that hypoechoic lesions that turned out to be inflammatory in origin were significantly softer than the malignant lesions. However, these lesions were imaged at a median of 8 mo after the last inflammatory attack (range: 1.2-12 mo). It is our experience that inflammatory lesions in the pancreas may present with a wide range of tissue strain. If a high-strain area (indicating soft tissue) is found, corresponding to a hypoechoic area in a sonogram, the chance of finding malignancy is however low (abstract Havre, UEGW 2011).

## INFLAMMATORY DISEASES OF THE PANCREAS

In the following morphological and sonographic characteristics of pancreatic diseases with emphasis on CP will be outlined. Pancreatic neoplasms are also shortly described as an important differential diagnosis.

### Acute pancreatitis

Acute pancreatitis is an acute inflammatory process that may include interstitial edema, necrosis, hemorrhage of pancreatic tissue and fluid collections, depending on the severity of the inflammation. The changes can be focal enlargement or diffuse, depending on its distribution and sometimes difficult to differentiate, especially when acute pancreatitis occurs in a patient with CP. Furthermore, focal pancreatitis often occurs in the pancreatic head, as a hypoechoic mass and not easily distinguishable from a tumor or changes seen in CP. The introduction of CEUS and its ability to differentiate between avascular necrotic tissue, cysts, abscesses and normal or hypervascular inflamed tissue equates US with CT<sup>[45]</sup>. CEUS has bedside availability, better cost effectiveness and can also be used in those patients where CT contrast agents are contraindicated.



**Figure 7 Advanced chronic pancreatitis.** Classical signs in advanced chronic pancreatitis: main pancreatic duct dilatation in an atrophic organ with sharp, irregular contours, calcifications and small cysts. The pancreatic head is outlined.

licated. However, visualization of the whole gland can be a problem in very obese patients.

### *Chronic pancreatitis*

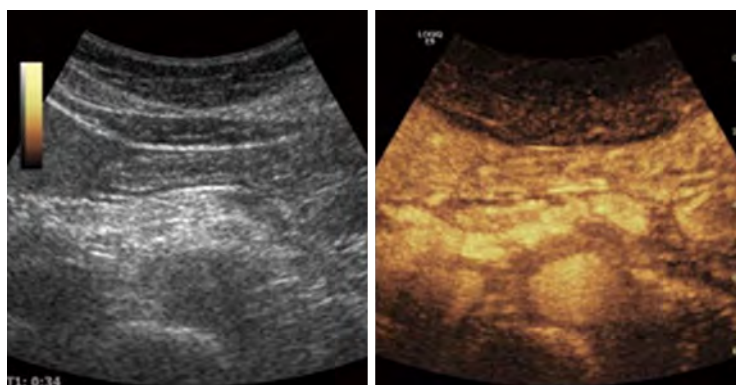
Classical diagnostic findings of CP in transabdominal ultrasonography reflects the spectrum of pancreatic disorders in CP. Ranging from progressive and irreversible morphological and functional derangement, inflammatory episodes and obstruction of structures adjacent to the pancreas<sup>[46]</sup>.

Late stage, severe CP is normally easily recognizable due to characteristic morphological changes. The presences of pancreatic or intraductal calcifications presented as hyperechoic foci are pathognomonic<sup>[47]</sup>. Caliber abnormalities such as a dilated and irregular pancreatic duct is seen with a sensitivity of approximately 70%<sup>[48]</sup>. The reported sensitivity is probably due to limited duct changes in early/mild and moderate CP, where the pancreatic duct is less than 3 mm in diameter<sup>[49]</sup>. Pseudocysts may cause benign duct obstruction (stricture) and dilatation upstream<sup>[50]</sup>. Solid or cystic lesions and malignant infiltration, especially if contiguous to the main duct, may also cause duct compression, with progressive development of obstructive CP upstream. A small atrophic gland with focal alterations and parenchymal heterogeneity is easily identified in advanced stages. Figures 7 (B-mode US) and 8 (CEUS image of the pancreas in the early arterial phase) shows typical morphological changes in advanced CP.

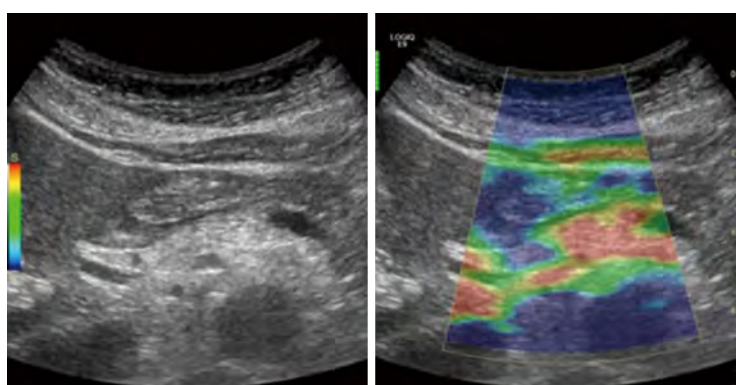
In moderate to severe CP, the pancreatic gland echo texture is inhomogeneous and rough due to coexistence of fibrotic hyperechoic and hypoechoic focal inflammation signs<sup>[49,51,52]</sup>. These findings are reported to be observed in approximately 70% of the cases, already in the late eighties<sup>[48,53,54]</sup>. Echogenicity of the pancreas is usually increased in CP due to fibrosis and fatty infiltration. This is not a specific parameter due to presence of adipose tissue in elderly and obese patients<sup>[10,53]</sup>. Increased echogenicity of duct wall can also be detected<sup>[50]</sup>. Figure 9 show an example of elastography in moderate CP.

Early stage CP is normally without or with minimal





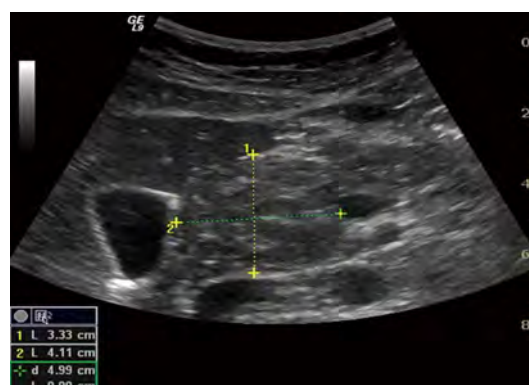
**Figure 8** An example of contrast enhanced ultrasound in advanced chronic pancreatitis. The parenchymal enhancement is clearly irregular reflecting the parenchymal heterogeneity, calcifications and focal inflammation.



**Figure 9** Elastography of the pancreas in moderate chronic pancreatitis. The colors show tissue hardness; The scale on the left defines the color code: Blue is hard, red is soft, yellow and green are intermediate. The elastogram shows predominantly soft (red) tissue with parts of green and yellow, indicating harder pancreatic tissue.



**Figure 10** Early chronic pancreatitis. Typical signs in early chronic pancreatitis: lobularity (L), stranding (S), hyperechoic foci (H) and honeycombing (Ho). Pancreatic body (Corpus). This subtle changes are usually only seen in endoscopic ultrasonography.



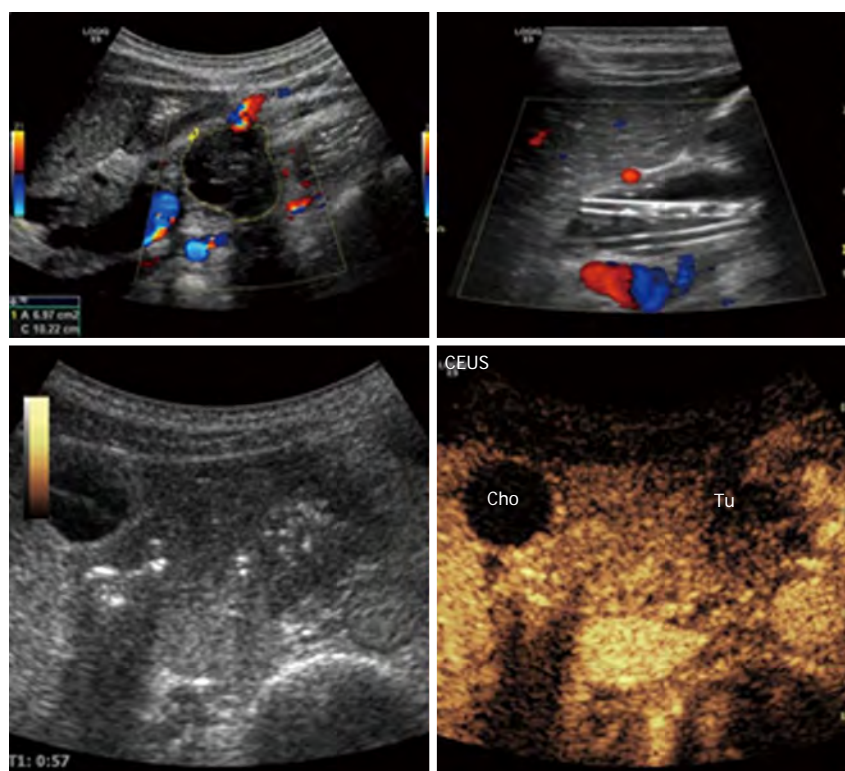
**Figure 11** Autoimmune pancreatitis. Enlarged pancreas with a tumorous formation in the head. The inflammatory lesion is isoechoic to the rest of the pancreas but still clearly visible.

morphological changes, where imaging modality falls short in diagnosing CP. Late stages signs like calcifications or dilated and irregular main pancreatic duct are diagnostic for CP, but can also be absent in severe exocrine insufficiency, depending on the type of CP<sup>[55,56]</sup>.

Cambridge classification of endoscopic retrograde pancreatography (ERP), US and CT imaging, grades the severity of CP, based on pancreatic structural changes and abnormalities of the main duct and side branches<sup>[52]</sup>. The sensitivity in diagnosing and grading the severity of CP with ultrasound alone, compared to the morphological gold standard ERP, rose to over 80% in the late eighties<sup>[53]</sup>. With the development of endosonographic

ultrasound, subtle early changes in the pancreatic echotexture could be visualized, and a new gold standard, based on morphological criteria correlating with pathologic features emerged<sup>[57,58]</sup>. Current transabdominal high-end ultrasound scanners can visualize some of these criteria, although the Rosemont classification is only for EUS images. Figure 10 shows minimal morphological changes in a patient with early CP, obtained by transabdominal US. Forthcoming studies comparing transabdominal ultrasonography findings with EUS criteria seem to be a logical step forward.

Pancreatic functional tests are still the only option to diagnose an early pancreatitis without morphological changes but with physiological insufficiency. Today, the



**Figure 12 Malignant tumor of the pancreatic body: Ductal adenocarcinoma.** The tumor is clearly visible on B-mode, left upper image. Contrast enhanced ultrasonography: The characteristics are hypoechogenicity and diffuse contours. The plastic biliary stents are clearly noticeable on B-mode, right upper image. Cho: Biliary duct (ductus choledochus); Tu: Tumor; CEUS: Contrast-enhanced ultrasonography.

short endoscopic secretin-based, pancreas function test is reasonably validated and the best available test<sup>[59,60]</sup>. This test can be combined with bedside US, to detect any morphological changes of the pancreas. Using this combined method we observed that the MPD wall had an increased echogenicity, *i.e.*, hyperechoic duct wall and changes in the diameter, short after secretin was given. Data not yet published.

### Autoimmune pancreatitis

Autoimmune pancreatitis is a benign form with autoimmune pathogenesis and a remarkable response to steroid therapy. From an imaging point of view, there are three (focal, diffuse, and combined) types of autoimmune pancreatitis where, especially the focal type can be misinterpreted as cancer<sup>[61,62]</sup>. Figure 11 shows an enlarged pancreas with a round tumorous formation, without cysts and calcification. On CEUS the affected tissue is hyperemic and shows an increased contrast-enhancement, whereas pancreatic cancer lesions are typically hypervascularized<sup>[63]</sup>. The patient avoided excessive abdominal surgery and was successfully treated with corticosteroids.

### Pseudocyst

Pseudocysts can arise as a complication of acute pancreatitis and also often occur in CP. These cysts are well-defined, fibrous-walled anechoic structures without an epithelial lining. Pseudocysts are normally easily differentiated from pancreatic cystic tumors, where especially the

premalignant and malignant forms should be identified. CEUS can classify cystic tumor's or solid nodules through evaluation of the vascularization pattern or absence of vessels in pseudocysts<sup>[36]</sup>.

### Pancreatic solid neoplasm

Differentiation between mass-forming lesions in CP and adenocarcinomas of the pancreas are a daily challenge. The ultrasonographic findings are similar with homogeneous or inhomogeneous poorly defined hypoechoic mass in both cases<sup>[64]</sup>. After the initial detection by ultrasound, the subsequent use of CEUS, with its possibility to visualize the vascular pattern will improve the diagnostic accuracy of the lesion type. A ductal adenocarcinoma is typically hypoechoic in arterial phase without clear-cut margins, with optional central necrotic parts. On the contrary, CP has usually more vascularity, with the same enhancement pattern as the surrounding pancreatic parenchyma<sup>[31,65,66]</sup>. Figure 12 shows a large hypervascularized mass with marked dilatation of the main bile duct. However, in advanced CP, due to fibrosis, inhomogeneous hypervascularization can be present, making the differential diagnosis very difficult<sup>[35,67]</sup>. Differences in vascularity seen on CEUS, often correlate well with histology. Taking in account the higher temporal and spatial resolution of ultrasound into account makes CEUS equal or even superior to CT<sup>[32,34,38]</sup>. The relationship with the surrounding arterial and venous vessels, the presence of thrombosis of the portal or splenic vein are used for local staging and

assessment of tumor resectability. The presence of liver metastases can also be evaluated during the late phase of the CEUS scanning procedure.

## CONCLUSION

Today's modern high end ultrasound scanners are still the first line modality in abdominal and pancreatic imaging. Ultrasonography is highly available, relative cheap and can be repeated on a daily basis, if needed. Ultrasound technology is developing towards smaller and really mobile scanners, implying that ultrasonography can be present in the pockets of the doctors supporting the clinical examination. With the rapid technical development, different modalities and methods, which demands computer processed images can be combined. This means highly improved image quality and diagnostic accuracy. Under good scanning conditions, ultrasonography has higher spatial resolution than CT or MRI. Furthermore, CEUS with its ability to show macro- and micro vascularity is also used as a second line modality to clarify small lesions, previously detected on CT or MRI.

Regarding transabdominal ultrasound of the pancreas, we predict that the resolution and thereby the ability to detect small morphological changes will be approaching the performance of EUS. Elastography in pancreatic imaging is now well known in EUS and studies' regarding the usefulness of this method in transabdominal ultrasonography is warranted. The ability of CEUS to visualize and quantify perfusion and thereby to characterize a given lesion or to differentiate between vascular (solid) and avascular (liquid/necrotic) components of the lesion is one of the most important progresses in pancreatic imaging today. Furthermore, the development of three-dimensional technology offers new clinical possibilities for real-time 3D volume images of the pancreas in the future.

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## Diagnosis and treatment of pancreatic exocrine insufficiency

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### Abstract

Pancreatic exocrine insufficiency is an important cause of maldigestion and a major complication in chronic pancreatitis. Normal digestion requires adequate stimulation of pancreatic secretion, sufficient production of digestive enzymes by pancreatic acinar cells, a pancreatic duct system without significant outflow obstruction and adequate mixing of the pancreatic juice with ingested food. Failure in any of these steps may result in pancreatic exocrine insufficiency, which leads to steatorrhea, weight loss and malnutrition-related complications, such as osteoporosis. Methods evaluating digestion, such as fecal fat quantification and the  $^{13}\text{C}$ -mixed triglycerides test, are the most accurate tests for pancreatic exocrine insufficiency, but the probability of the diagnosis can also be estimated based on symptoms, signs of malnutrition in blood tests, fecal elastase 1 levels and signs of morphologically severe chronic pancreatitis on imaging. Treatment for pancreatic exocrine insufficiency includes support to stop smoking and alcohol consumption, dietary consultation, enzyme replacement therapy and a structured follow-up of nutritional status and the effect of treatment. Pancreatic enzyme replacement therapy is administered in the form of enteric-coated minimicro-

spheres during meals. The dose should be in proportion to the fat content of the meal, usually 40-50000 lipase units per main meal, and half the dose is required for a snack. In cases that do not respond to initial treatment, the doses can be doubled, and proton inhibitors can be added to the treatment. This review focuses on current concepts of the diagnosis and treatment of pancreatic exocrine insufficiency.

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**Key words:** Chronic pancreatitis; Pancreatic exocrine insufficiency; Pancreatic enzyme replacement therapy

**Core tip:** This is a review on the diagnosis and treatment of pancreatic exocrine insufficiency. The review includes a discussion of the definition of pancreatic exocrine insufficiency, a pragmatic approach to its diagnosis and current concepts of indications for treatment with pancreatic enzyme replacement therapy, including measures to optimize the effect.

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### INTRODUCTION

Pancreatic exocrine insufficiency (PEI) can be defined as a reduction in pancreatic enzyme activity in the intestinal lumen to a level that is below the threshold required to maintain normal digestion. This concept is crucial for the understanding of PEI and has several important consequences for the diagnosis and treatment of this

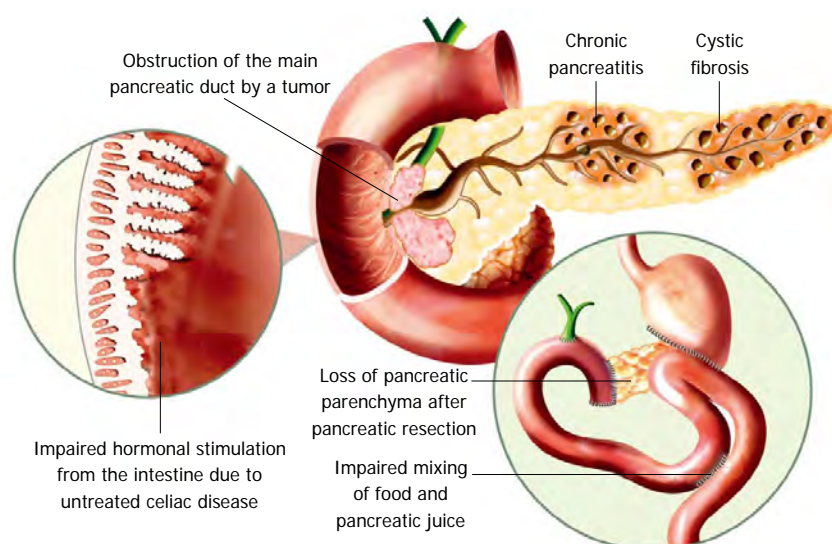


Figure 1 Different causes of pancreatic exocrine insufficiency.

condition. First, pancreatic exocrine secretion can be significantly reduced without PEI being present. In a landmark paper four decades ago, DiMagno *et al*<sup>[1]</sup> demonstrated that steatorrhea does not occur until pancreatic lipase output is reduced to 5%-10% of normal output. Hence, the demonstration of moderately reduced bicarbonate or enzyme output in sensitive tests of pancreatic secretion, such as the secretin/cholecystokinin-stimulation test, is a reliable indicator of chronic pancreatitis (CP) but does not necessarily indicate PEI. Second, any pathology, including extrapancreatic conditions, that interrupt the chain of events required for the normal digestion of ingested food by pancreatic digestive enzymes may cause PEI. Thus, “pancreatic exocrine insufficiency” is a denomination that, from a semantic point of view, is too narrow for this condition; “pancreatic maldigestion” could be an alternative and probably more correct term. Diseases of the pancreatic parenchyma, such as CP, cystic fibrosis and status post necrotizing acute pancreatitis, are the most common causes of PEI. However, PEI may also be caused by obstruction of the pancreatic duct system due to a tumor or a stricture, by reduced stimulatory capacity in the intestine secondary to untreated celiac disease<sup>[2]</sup> or Crohn’s disease, by increased intraluminal inactivation of pancreatic enzymes in Zollinger-Ellison syndrome<sup>[3]</sup> or by impaired mixing of ingested food and the pancreatic juice after upper gastrointestinal surgery<sup>[4]</sup> (Figure 1).

The pancreatic juice plays a pivotal role in the digestion and absorption of nutrients<sup>[5]</sup>. Pancreatic enzyme secretion is stimulated during the cephalic<sup>[6]</sup> and gastric<sup>[7]</sup> phases to a certain degree, but the most important stimulation occurs during the intestinal phase, when chyme enters the duodenum. The presence of fatty acids, amino acids and gastric acid in the duodenum is the most potent stimulator of exocrine pancreatic secretion<sup>[8]</sup>. Vagal and neural reflexes stimulate pancreatic secretion during the cephalic and gastric phases<sup>[6,7]</sup>. During the intestinal phase, cells in the duodenal mucosa release CCK, which stimulates the secretion of pancreatic enzymes from acinar cells<sup>[9]</sup>, and secretin, which elicits water and bicar-

bonate secretion from ductal cells<sup>[10,11]</sup>.

The pancreatic juice consists of bicarbonate and water secreted by ductal cells and several enzymes, secreted by acinar cells, with the specific capacity to digest proteins, carbohydrates and fat. In situations with reduced exocrine pancreatic function, the ability to digest fat is the determining factor that causes the most important symptoms and clinical complications because lipase, the major lipolytic enzyme of the pancreatic juice, is the pancreatic digestive enzyme with the poorest stability in the gastrointestinal lumen. The destruction of lipase is even more rapid when the pH is below 4, which is often the situation in CP, in which the buffering of gastric acid is insufficient due to low bicarbonate excretion by the pancreas<sup>[12]</sup>. Furthermore, there is minimal extrapancreatic lipolytic enzyme production, as opposed to the extrapancreatic capacity to digest carbohydrates provided by salivary amylase and intestinal oligosaccharidases or the proteolytic capacity provided by gastric pepsinogen.

PEI is one of the major complications in CP and should be considered in all CP patients. The prevalence of PEI in CP increases with disease duration, and approximately half of patients will have developed PEI by 12 years after disease onset<sup>[13]</sup>. There are no reliable estimates of the prevalence of PEI in the general population.

Patients with untreated PEI not only suffer from impaired quality of life due to steatorrhea, weight loss, abdominal discomfort and other PEI-related symptoms but are also highly likely to develop deficiencies of micronutrients and lipid-soluble vitamins<sup>[14]</sup>. These deficiencies in turn place patients at risk of malnutrition-related complications, such as osteoporosis<sup>[15,16]</sup>. Hence, an early and accurate diagnosis of PEI is of high clinical importance.

## DIAGNOSIS OF PANCREATIC EXOCRINE INSUFFICIENCY

The gold standard for the diagnosis of PEI is three-day

fecal fat quantification and determination of the coefficient of fat absorption<sup>[17]</sup>. A major drawback of fecal fat quantification is that the test is cumbersome and unpleasant for both the patient and laboratory personnel. The patient is required to keep a strict diet, with 100 g of fat per day for five days, and to collect the complete volume of feces for three days. Laboratory personnel need to handle large volumes of feces. Therefore, this test is very rarely performed in daily clinical practice and is only available at few specialized centers. Several alternative methods for the diagnosis of PEI have been proposed.

### **Diagnosis based on symptoms, blood tests and imaging**

Symptoms in patients with PEI vary, depending on the degree and etiology of PEI. The classical clinical picture is a patient presenting with foul-smelling, loose stools, weight loss, muscle wasting, and flatulence. Advanced tests of pancreatic exocrine function can usually be avoided in patients with a well-established CP diagnosis based on morphological findings and a clear clinical picture of PEI. A trial of pancreatic enzyme replacement therapy (PERT) based only on the clinical picture is recommended by several national societies when the clinical presentation is strongly suggestive of PEI<sup>[18,19]</sup>. However, only relying on symptoms may lead to both the over- and under-diagnosis of PEI. Diarrhea and weight loss may be due to conditions other than PEI, and PEI can also be present in the absence of overt steatorrhea.

In addition to explaining and treating clinical symptoms, the second rationale for the early diagnosis of PEI is to prevent complications of malnutrition. It is reasonable to assume that such malnutrition-related complications will be preceded by deficiencies of macro- or micronutrients detectable by routine blood tests. Hence, from a theoretical point of view, serum nutritional markers could be used to support the diagnosis of PEI. Deficiencies of several nutrients in blood tests have been demonstrated in CP, including apolipoproteins<sup>[20,21]</sup>, total cholesterol<sup>[22]</sup>, magnesium<sup>[22,23]</sup>, lipid-soluble vitamins<sup>[24-26]</sup>, retinol-binding protein<sup>[26]</sup>, calcium, zinc and selenium<sup>[25]</sup>, but the majority of these studies have not taken the exocrine function status of patients into consideration. Studies investigating the association between nutritional markers and PEI in CP patients have demonstrated that deficiencies of lipid-soluble vitamins are associated with an increased probability of PEI<sup>[15,26]</sup>, as opposed to B12 and folate levels, which are not associated with PEI<sup>[14,27]</sup>. The possibility of diagnosing PEI based on nutritional markers in the blood was recently studied in a cohort of 114 patients with CP, of whom 38 suffered from PEI<sup>[14]</sup>. Hemoglobin, albumin, prealbumin and retinol-binding protein levels below the lower limit of normal magnesium levels below 2.05 mg/dL; and HbA1C levels above the upper limit of normal were all significantly associated with PEI. No PEI patient in this study presented with normal values for all of these parameters. The central conclusion that can be drawn from this study is that

a normal panel of serum nutritional markers can exclude PEI with a high negative predictive value.

The probability of PEI in CP can also be estimated based on pancreatic imaging findings in the absence of more advanced tests of pancreatic function<sup>[28]</sup>. Notably, ductal changes on endoscopic retrograde pancreatography, computerized tomography (CT)<sup>[29]</sup> and endoscopic ultrasound (EUS) have been associated with decreased exocrine pancreatic function. The diagnosis of CP by EUS is based on the demonstration of several different parenchymal (hyperechoic foci, hyperechoic strands, parenchymal lobularity and cysts) and ductal (pancreatic duct dilatation, irregular pancreatic duct contour, hyperechoic pancreatic duct margin, dilated side branches and intraductal calcifications) abnormalities defined in the Rosemont classification<sup>[30]</sup>. A recent study demonstrated a clear correlation between the number of EUS criteria met and the probability of PEI. Calcifications and main pancreatic duct dilatation were independently associated with PEI in a multivariate analysis, and the probability of PEI was > 80% if these features were present<sup>[28]</sup>.

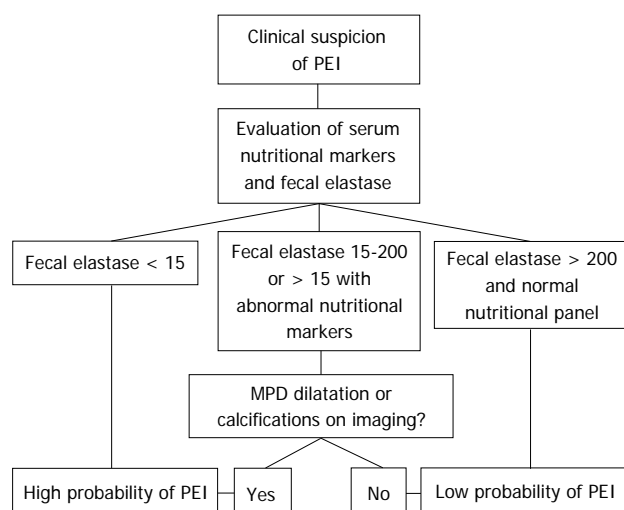
### **Fecal elastase 1**

Pancreatic elastase 1 is an enzyme of the pancreatic juice that is highly stable during passage through the gastrointestinal tract<sup>[31]</sup>. The concentration of elastase 1 can be measured in feces using a simple enzyme-linked immunosorbent assay [fecal elastase 1 (FE-1)] on a spot fecal sample<sup>[32,33]</sup>. FE-1 levels have been demonstrated to correlate with more sensitive tests of pancreatic secretion, such as the secretin test<sup>[32,34]</sup>. Low FE-1 levels have also been demonstrated to correlate with morphological tests for CP, such as endoscopic retrograde pancreatography<sup>[35]</sup> and magnetic resonance cholangiopancreatography<sup>[36]</sup>. However, as opposed to the relatively large number of studies evaluating FE-1 assessment as a test for the diagnosis of CP, studies evaluating the role of FE-1 testing in the detection of PEI in CP are scarce. Recently, Benini *et al*<sup>[37]</sup> investigated FE-1 and fecal fat in patients with CP or pancreatic resection. Three important conclusions can be drawn from this study. First, an FE-1 concentration of < 15 µg/g feces detects PEI with high sensitivity and specificity in patients with CP without prior pancreatic surgery. Second, intermediate FE-1 values (15-200 µg/g feces) are more difficult to interpret and likely warrant testing with more sensitive methods. Third, FE-1 assessment is not a reliable test for PEI in patients post-pancreatic resection. The fecal fat concentration was consistently higher in relation to FE-1 levels in operated compared with non-operated cases<sup>[37]</sup>. This finding was not unexpected; inadequate mixing of food with the pancreatic juice and other factors not related to pancreatic secretory capacity are likely to contribute to the pathogenesis of PEI after pancreatic resections.

### **<sup>13</sup>C-mixed triglycerides breath test**

The <sup>13</sup>C-mixed triglycerides (<sup>13</sup>C-MTG) breath test was introduced by Vantrappen *et al*<sup>[38]</sup>. The test directly mea-





**Figure 2** Evaluation of the probability of pancreatic exocrine insufficiency in the absence of advanced tests for maldigestion, such as fecal fat quantification and the  $^{13}\text{C}$ -mixed triglycerides breath test. PEI: Pancreatic exocrine insufficiency; MPD: Main pancreatic duct.

sure the clinically most relevant end-effect of exocrine pancreatic function: the degradation of triglycerides. This makes this test preferable to tests that measure exocrine pancreatic secretion, such as the secretin test and the FE-1 assay. For the  $^{13}\text{C}$ -MTG test, the patient ingests a small amount of  $^{13}\text{C}$ -marked triglycerides (2-octanoyl (1- $^{13}\text{C}$ )-1,3 distearoyl glycerol), together with butter on a piece of toasted bread, after an overnight fast. In the presence of normal lipase activity,  $^{13}\text{C}$ -triglycerides will be degraded in the intestinal lumen, and  $^{13}\text{C}$ -marked fatty acids will then be absorbed. These fatty acids will in turn be metabolized in the liver, and  $^{13}\text{CO}_2$  can finally be measured in exhaled air. Subjects with PEI have decreased lipase activity, which can be detected as a decreased recovery of  $^{13}\text{CO}_2$  in exhaled air. Currently, there is no general agreement on the optimal design of the test and several different protocols have been proposed<sup>[39-43]</sup>. The protocol developed by Domínguez-Muñoz *et al.*<sup>[44]</sup> has been adopted by several groups, including our institution, and this protocol is described in Table 1<sup>[44]</sup>. Values below 29% are considered as pathological, and the test detects fat maldigestion with a sensitivity of  $> 90\%$ <sup>[40]</sup>.

### Test based on analysis of pancreatic juice after secretin/erulein stimulation

Exocrine pancreatic function can be measured by so-called direct pancreatic function tests. In these tests, pancreatic secretion is stimulated by secretin and/or cerulein<sup>[45]</sup> or by the ingestion of a standard test meal<sup>[46]</sup>. After stimulation, samples of the pancreatic juice are aspirated from a tube that has been placed in the duodenum, and the concentrations of pancreatic digestive enzymes and bicarbonate are measured. A peak bicarbonate concentration in pancreatic secretion significantly

below normal values (a cut-off value of 80 mEq has been advocated by most authorities) in the secretin test has long been considered as the most sensitive test for early CP. A drawback of the direct function tests is that they require the placement of a large-bore tube in the duodenum during the complete duration of the test, which is poorly tolerated by patients. It is also important to keep in mind that a mild reduction in pancreatic exocrine function, occasionally called “exocrine pancreatic dysfunction” or “mild pancreatic exocrine insufficiency”, is not equivalent to clinically significant PEI. PEI, based on its definition, is a reduction in exocrine pancreatic function to a level that results in maldigestion. Since the introduction of highly sensitive pancreatic imaging methods, such as MRI, modern CT and endoscopic ultrasound, the need to rely on pancreatic function testing for the diagnosis of CP has diminished, and most centers have abandoned the classic secretin test.

Recently, an endoscopic direct pancreatic function test was developed, with a simplified protocol for pancreatic fluid collection through an endoscope. A good correlation between the endoscopic pancreatic function test and standard direct tests has been demonstrated<sup>[47,48]</sup>. Exocrine pancreatic secretion can also be evaluated based on the degree of duodenal filling on MRI after secretin stimulation, which has been demonstrated to correlate with a combination of the FE-1 assay, the  $^{13}\text{C}$ -MTG breath test and fecal fat testing<sup>[49]</sup> and the endoscopic pancreatic function test<sup>[50]</sup>. Further studies are needed to evaluate the role of the endoscopic function test and secretin-enhanced MRI in the diagnosis of PEI. It should be noted that all tests of pancreatic secretion share the drawback of ignoring other factors that may alter the effect of pancreatic enzymes in the intestine. Thus, tests of pancreatic secretion are irrelevant in situations in which factors other than the secretory capacity of the pancreas may contribute to PEI.

### Integrated use of methods to diagnose PEI

The optimal test for the diagnosis of PEI is a test that can detect the maldigestion of fat with high sensitivity and specificity. Fecal fat quantification and the  $^{13}\text{C}$ -MTG breath test are recommended for the accurate diagnosis of PEI because these two tests best fit this description. However, due to the limited availability of these tests and the odious nature of fecal fat quantification, there is a need for a simplified way to estimate the probability of PEI based on generally available clinical parameters. In Figure 2, a proposal for a diagnostic algorithm to estimate the probability of PEI based on routine blood tests, FE-1 assessment and standard imaging is presented. The algorithm is designed to have a high negative predictive value for PEI and integrates knowledge from recent studies<sup>[14,28,37]</sup>. However, it should be stressed that this algorithm provides only an estimation of the probability of PEI and that the algorithm has not been scientifically validated.

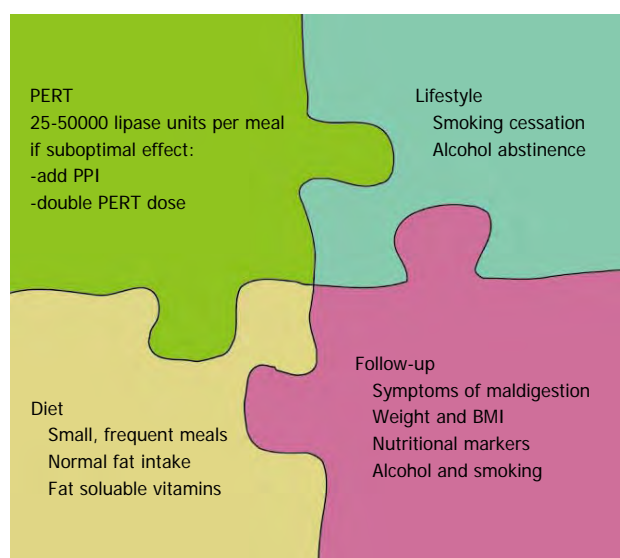


Figure 3 Fundamental aspects in the care of patients with pancreatic exocrine insufficiency. PERT: Pancreatic enzyme replacement therapy; PPI: Proton pump inhibitors.

## TREATMENT OF PANCREATIC EXOCRINE INSUFFICIENCY

Cornerstones in the treatment of PEI are PERT, support to cease smoking and alcohol consumption, consultation with a dietitian and a systematic follow-up to assure optimal treatment effect (Figure 3). The goal of this treatment concept is to normalize digestion, alleviate PEI-related symptoms and prevent malnutrition-related morbidity and mortality and disease progression.

### Diet, smoking and drinking

Historically, a low-fat diet has been recommended in PEI to reduce steatorrhea. This recommendation has been abandoned in modern dietary counseling in PEI due to the risk of aggravating PEI-related weight loss and deficiencies of lipid-soluble vitamins<sup>[18,51]</sup>. By optimization of the PERT dose and supportive treatment with PPI, most PEI patients will tolerate a normal-fat diet. Dietary consultation should include advice for sufficient caloric intake and normal fat content. Small, frequent meals are usually better tolerated than large, high-caloric meals. Deficiencies of fat-soluble vitamins are very common in PEI patients, and vitamin supplementation therapy should be given if necessary<sup>[15]</sup>. Support for alcohol abstinence should be offered to all patients with alcohol-related CP. In addition to the general health benefits of alcohol withdrawal, this withdrawal has also been demonstrated to slow the further deterioration of pancreatic exocrine function<sup>[52]</sup>. Smoking is a risk factor for pancreatic cancer, acute pancreatitis and CP<sup>[53]</sup>, and is also associated with an increased probability of reduced pancreatic exocrine function based on the endoscopic pancreatic function test in cases with CP<sup>[54]</sup>. Continued smoking has been associated with earlier development of calcifications in patients with CP<sup>[55]</sup>. Smoking cessa-

tion should be encouraged in all patients with CP with or without PEI.

### Pancreatic enzyme replacement therapy

Pancreatic digestive enzymes can be administered orally, together with meals, in patients with PEI to compensate for the lack of endogenous enzyme secretion. Modern pancreatic enzyme preparations are extracts from the porcine pancreas (pancrelipase) that are administered as enteric-coated minimicrospheres. Several different preparations are commercially available, with minor differences in particle size and pH-related release kinetics<sup>[56]</sup>. PEI with steatorrhea and/or weight loss is an undisputed indication for PERT. Several studies have demonstrated an improved coefficient of fat absorption<sup>[57-60]</sup>, decreased maldigestion-related symptoms<sup>[57,59-61]</sup> and even improved quality of life<sup>[61,62]</sup>. The need for PERT in PEI without symptoms is a matter of debate, and randomized clinical trials on this issue are lacking. Nevertheless, certain collateral evidence supports PERT, even in the absence of overt steatorrhea and weight loss: (1) A longitudinal study in patients with CP demonstrated that patients with no clinical symptoms of steatorrhea but an abnormal steatocrit who did not receive PERT lost more weight than not only CP patients with a normal steatocrit but also patients with an abnormal steatocrit and symptoms of steatorrhea who were treated with PERT<sup>[63]</sup>; (2) Laboratory signs of malnutrition have been demonstrated in a large proportion of patients with CP and asymptomatic steatorrhea who were not under treatment with PERT<sup>[64]</sup>. The clinical relevance of such deficiencies has not been specifically investigated in CP and PEI, but an increased risk of complications that are generally associated with malnutrition can be assumed<sup>[65]</sup>; and (3) Based on observational studies in CP, it is well known that malnutrition-related diseases and findings, such as osteoporosis-related fractures<sup>[16]</sup>, decreased bone mineral density<sup>[15,66,67]</sup> and deficiencies of fat-soluble vitamins<sup>[15]</sup>, are common in CP.

As evidence for nutritional deficiencies in patients with PEI with and without symptoms has increased in recent years, PERT is now increasingly regarded as a treatment for maldigestion rather than a way to suppress diarrhea in patients with CP<sup>[65]</sup>. The goal of PERT is stated to be the elimination of maldigestion in the Australasian Pancreatic Club recommendations<sup>[18]</sup>. Meanwhile, the Spanish Pancreatic Club regards any clinical or nutritional deficiency in a CP patient as an indication for PERT<sup>[68]</sup>, and the Italian Association for the Study of the Pancreas states in its guidelines that PEI is an indication for PERT<sup>[19]</sup>.

The safety and efficacy of PERT for the treatment of PEI in CP has been investigated in four randomized, double-blinded, placebo-controlled clinical trials including up to 72 patients over study periods of 1-2 wk<sup>[57-60]</sup>. Significant improvements in the coefficient of fat absorption<sup>[57-60]</sup>, the coefficient of nitrogen absorption<sup>[58-60]</sup>, stool fat content<sup>[60]</sup> and stool weight<sup>[60]</sup> have been docu-

**Table 1** The  $^{13}\text{C}$ -mixed triglycerides breath test according to Domínguez-Muñoz *et al*<sup>[44]</sup>

The patient fasts from midnight
Twenty minutes before the test, 10 mg of metoclopramide is ingested
A baseline breath sample is taken
At time 0, 250 mg of $^{13}\text{C}$ -mixed triglycerides mixed with 16 g of fat on a piece of toasted bread is ingested, together with a glass (200 mL) of water
Breath samples are taken every 15 (or 30) min for 6 h
Finally, $^{13}\text{CO}_2/^{12}\text{CO}_2$ is measured in collected breath samples by mass spectrometry or isotope-selective nondispersive infrared spectrometry

**Table 2** Pancreatic enzyme replacement therapy: How we do it

PERT is started at 50000 lipase units per main meal and 25000 lipase units per snack
The basic concepts of the pathophysiology of PEI and how PERT works are explained to the patient. It is emphasized that PERT should be taken with meals and that the dose should be adjusted to the fat content of the meal
If maldigestion persists, proton pump inhibitors can be added, and the dose is increased to 80000 lipase units per main meal and 40000 lipase units per snack
If PERT is still ineffective, despite the optimization described above, small intestinal bacterial overgrowth is considered, and the evidence for a diagnosis of PEI is revised

PEI: Pancreatic exocrine insufficiency; PERT: Pancreatic enzyme replacement therapy.

mented. Two of these trials have also reported results from open-label extension periods of 6 and 12 mo<sup>[62,69]</sup>. Continuous improvement during treatment, with a steady-state reached at week 13, was observed for most symptom variables<sup>[62]</sup>. In these clinical trials, PERT has been well tolerated, and no serious adverse events have been reported. Fibrosing colonopathy is the only serious complication that has been associated with PERT. In the vast majority of reported cases, this rare condition has been observed in patients with cystic fibrosis using high doses of PERT<sup>[70]</sup>. The pathophysiology of this condition is unknown, but factors related to cystic fibrosis disease per se, the dosing of PERT and possibly agents in the enteric coating of the pancrelipase preparations may play a role<sup>[71]</sup>.

There are no studies investigating the long-term effects of PERT on morbidity and mortality from PEI, which should be kept in mind if patients with no or minimal symptoms of PEI are considered for PERT.

### Dosing of pancreatic enzyme replacement therapy

The concept of PERT is to induce a lipolytic capacity that corresponds to the amount of ingested fat at every meal. Therefore, higher doses are necessary for large, high-fat meals, and lower doses are sufficient for snacks and lean meals. The optimal dose of PERT in CP has not been investigated systematically in clinical trials. Recommendations from different national societies range from 20-40000 lipase units per main meal, as recommended by the German Society of Digestive and Metabolic Diseases<sup>[72]</sup>, to 25-40000 lipase units per main meal, as recommended by the Australasian Pancreatic Club<sup>[18]</sup> and the Italian Association for the Study of the Pancreas<sup>[19]</sup>, to 40-50000 lipase units per mail mean, as recommended by The Spanish Pancreatic Club<sup>[68]</sup>. In general, half of the dose is recommended for snacks and minor meals. Recent randomized clinical trials have used higher doses (72000 USP units<sup>[59]</sup> and 80000 Ph.Eur.U

units<sup>[60]</sup> per main meal). It is noteworthy that even in a study using 80000 lipase units per main meal, only 26% of the patients had a normalized coefficient of fat absorption at the end of the 51-wk open-label extension of the study<sup>[62]</sup>.

There is no consensus on the definition of treatment success in PERT. If the elimination of maldigestion is accepted as the aim of the treatment, fecal fat quantification or an indirect test of maldigestion, such as the  $^{13}\text{C}$ -MTG breath test, would be the most appropriate examination for verifying treatment success. However, this approach is rarely feasible in clinical practice. Most guidelines recommend a reevaluation of symptoms and weight and a reevaluation of serum tests of malnutrition.

In fact, it is highly likely that many patients with PEI today are receiving PERT that is suboptimal. A recent study from the Netherlands has indicated that as many as 70% of patients with CP report steatorrhea-related symptoms despite PERT<sup>[73]</sup> and that persistent deficits in blood nutritional parameters despite PERT are common in PEI<sup>[15,64]</sup>. What measures can be taken to optimize the result of PERT? First, it is of utmost importance to ensure that the patient is taking the prescribed dose correctly. Capsules should be administered with meals (as opposed to before or after) for optimal effect<sup>[74]</sup>. If signs or symptoms of maldigestion persist, the PERT dose can be increased, and proton pump inhibitors can be added<sup>[44,75,76]</sup>. The rationale for adjuvant treatment with proton pump inhibitors is that bicarbonate secretion is impaired in CP, resulting in insufficient buffering of the gastric chyme when it enters the small bowel. This phenomenon may in turn compromise the effect of PERT because lipase is rapidly degraded at a low pH and because enzyme release from microspheres is pH dependent. If PERT is ineffective despite an increased dose and adjuvant treatment with PPI, the diagnosis of PEI should be revised, and possible coexisting and/or alter-



native reasons for maldigestion, such as small intestinal bacterial overgrowth, should be considered. A summary of PERT concepts in clinical practice is presented in Table 2.

In conclusion, PEI is a state of maldigestion that is the result of a reduction of pancreatic enzyme activity in the intestinal lumen to a level that is below the threshold required to maintain normal digestion. CP is the most common cause of PEI, but several other pancreatic and extrapancreatic diseases can lead to PEI. The diagnosis of PEI is best established by tests that directly measure digestion, such as fecal fat quantification or the <sup>13</sup>C-MTG breath test. If these tests are not available, clinical and biochemical signs of malnutrition; pancreatic imaging findings; and tests that measure pancreatic secretion, such as the FE-1 assay, can be used to estimate the probability of PEI. The treatment of PEI relies on the elimination of risk factors for disease progression, such as smoking and alcohol consumption; consultation with a dietitian; PERT; and a systematic follow-up of the treatment effect on nutritional status and symptoms. If required, PERT can be optimized by dose augmentation and the addition of proton pump inhibitors.

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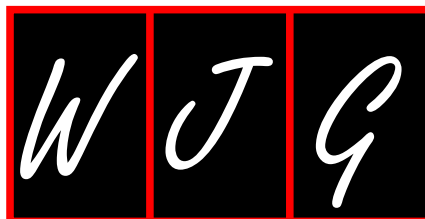


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## Nutrition in chronic pancreatitis

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### Abstract

The pancreas is a major player in nutrient digestion. In chronic pancreatitis both exocrine and endocrine insufficiency may develop leading to malnutrition over time. Maldigestion is often a late complication of chronic pancreatic and depends on the severity of the underlying disease. The severity of malnutrition is correlated with two major factors: (1) malabsorption and depletion of nutrients (*e.g.*, alcoholism and pain) causes impaired nutritional status; and (2) increased metabolic activity due to the severity of the disease. Nutritional deficiencies negatively affect outcome if they are not treated. Nutritional assessment and the clinical severity of the disease are important for planning any nutritional intervention. Good nutritional practice includes screening to identify patients at risk, followed by a thoroughly nutritional assessment and nutrition plan for risk patients. Treatment should be multidisciplinary and the mainstay of treatment is abstinence from alcohol, pain treatment, dietary modifications and pancreatic enzyme supplementation. To achieve energy-end protein requirements, oral supplementation might be beneficial.

Enteral nutrition may be used when patients do not have sufficient calorie intake as in pyloro-duodenal stenosis, inflammation or prior to surgery and can be necessary if weight loss continues. Parenteral nutrition is very seldom used in patients with chronic pancreatitis and should only be used in case of GI-tract obstruction or as a supplement to enteral nutrition.

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**Key words:** Chronic pancreatitis; Malnutrition; Nutritional risk; Malabsorption; Nutritional risk screening; Metabolism; Nutritional assessment; Nutrition therapy

**Core tip:** The pancreas is a major player in nutrient digestion and malnutrition is frequently found but is often neglected. The severity of malnutrition is correlated with malabsorption and depletion of nutrients (*e.g.*, alcoholism and pain) that causes impaired nutritional status and increased metabolic activity due to the severity of the disease. Good nutritional practice includes screening to identify patients at nutritional risk, followed by a thoroughly nutritional assessment and nutrition plan for risk patients. Treatment should be multidisciplinary and the mainstay of treatment is abstinence from alcohol, pain treatment, dietary modifications and pancreatic enzyme supplementation.

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### INTRODUCTION

Chronic pancreatitis (CP) is an inflammatory disorder that causes irreversible anatomical changes and damage, including infiltration of inflammatory cells, fibrosis and



calcification of the pancreas with destruction of the glandular structure and thereby affects normal digestion and absorption of nutrients.

Maldigestion is often a late complication of CP and depends on the severity of the underlying disease. The medium latency between onset of first symptoms and signs of maldigestion is about 8-9 years in alcoholic CP and more than 15 years in idiopathic non-alcoholic pancreatitis. Nutrient deficiencies are common in CP, driven by many risk factors including malabsorption, diabetes and, in alcoholic CP, alcoholism. However, deficiencies are frequently overlooked, leading to malnutrition<sup>[1,2]</sup>.

The aim of this article is to discuss the definition of malnutrition and good nutritional practice in patients with CP.

## DEFINITION OF MALNUTRITION AND GOOD NUTRITIONAL PRACTICE

Generally about 20%-50% of all patients in hospital are found at risk of undernutrition, dependent on definition, clinical setting and screening tool amended. A large part of these patients are at nutritional risk when admitted to hospital and in the majority of these, undernutrition develops negatively during hospital stay<sup>[3,4]</sup>.

This can be prevented if special attention is paid to nutritional care of patients. Routine identification is paramount as a first stage in a patient's care in order to identify at-risk patients, with a view to providing nutrition support if necessary<sup>[5,6]</sup>.

Good nutritional practice for the patient starts by nutrition screening. The European Society for Clinical Nutrition and Metabolism (ESPEN) Guidelines for Nutrition Screening recommend a continuity of issues to be considered in all patients admitted to hospital (Figure 1)<sup>[6]</sup>. (1) Initially on admission, a simple nutritional screening is to be done, to identify patients at actual nutritional risk; (2) Subsequently for patients at nutritional risk, a thorough nutritional assessment is to be completed; (3) This stage leads to an individual evaluation of nutritional requirements, and a plan for nutrition therapy and care; and (4) Monitoring and defining of targeted outcome should be structured in order to reconsider therapy and care-planning. Finally, communication around results of screening, assessment, plan and monitoring should be communicated to other health care professionals, when the patient is transferred, either back to the community or to another institution<sup>[7-9]</sup>.

In order to give priority to nutritional intervention for relevant patients, nutrition screening methods have been developed and validated. These screening methods regard nutritional status and acute disease. However, there is still no clear consensus agreement on a uniform definition of undernutrition or agreement for a gold standard method of screening and identification of undernutrition<sup>[10]</sup>. This also applies for patients with CP and malnutrition which has been offered numerous definitions. Simply stated, malnutrition is a sub-optimal nutrient status appearing

as a consequence of nutrient deficiencies, which change body composition and functional status. However, this definition neglects the numerous causes of malnutrition. An international guideline committee has recently proposed an aetiology-based approach that incorporates a current understanding of the inflammatory response, as seen in CP and many other patients. The committee proposed the following nomenclature for nutrition diagnosis in adults in the clinical practice setting: "Starvation-related malnutrition", for chronic starvation without inflammation *i.e.*, anorexia nervosa, "chronic disease-related malnutrition", when inflammation is chronic and of mild to moderate degree *i.e.*, CP, and "acute disease or injury-related malnutrition", *i.e.*, CP with a serious complication in the intensive ward, that is, when inflammation is acute and of severe degree<sup>[11,12]</sup>.

The causes of malnutrition in patients are thus included in its definition. As such, screening tools, which neglect to include relevant disease related parameters such as chronic or acute inflammation, may be less efficacious in identifying malnutrition risk.

Nutritional screening should be a simple and rapid process, which can be carried out by busy admitting nursing and medical staff. Most screening tools address four basic questions: recent weight loss, recent food intake, current body mass index and disease severity or some other measure of predicting risk of malnutrition. In 2003, ESPEN published guidelines for nutrition screening in the community, in the hospital and among elderly in institutions. The Nutrition Risk Screening 2002 (NRS 2002) seems to be the best validated screening tool, in terms of predictive validity, *i.e.*, that clinical outcome improves when patients identified to be at risk are treated, however the evidence is sparse<sup>[3,5,6,9]</sup>.

For adult patients in hospital it is thus suggested to use the NRS 2002 (Figure 2)<sup>[9]</sup>. A score equal to or greater than 3 generates a nutrition plan in all cases. If the patient is at risk, but metabolic or functional problems prevent a standard plan being carried out, or if there is doubt as to whether the patient is at risk, a referral should be made to an expert or expert team for a more detailed assessment.

## HOW DOES CP INFLUENCE NUTRITIONAL STATUS AND METABOLISM?

According to the definition, the severity of malnutrition is correlated with two major factors: depletion of nutrients (alcoholism and pain) and malabsorption causes impaired nutritional status and increased metabolic activity due to the inflammatory component of CP (severity of disease). Generally patients at nutritional risk have an increased number of complications and a poorer outcome, but specific studies investigating this issue in CP are however not available<sup>[2,13,14]</sup>.

A persistent alcohol intake, pain after a meal and maldigestion are the main causes of weight loss, and weight loss is strongly associated with maldigestion of fat<sup>[1,14,15]</sup>.

It is widely acknowledged that patients with CP are



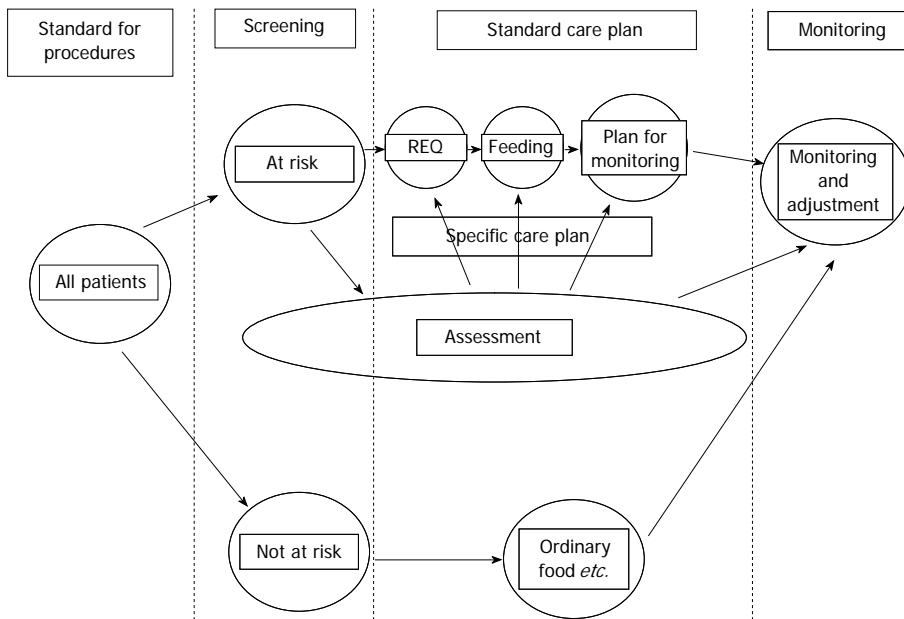


Figure 1 The nutritional care process including screening, plan and monitoring according to the European Society for Clinical Nutrition and Metabolism guideline<sup>[6]</sup>. REQ: Requirement.

often undernourished; however few studies have assessed this. In a medical rehabilitation clinic setting, 32% had a BMI < 20 kg/m<sup>2</sup>; 57% chronic diarrhea and 24% steatorrhea<sup>[2]</sup>.

Others have found that patients are malnourished prior to surgery and that the problem remains after surgery in a substantial proportion of patients with CP<sup>[16]</sup>. Furthermore, lean body mass and fat mass are found to be decreased both in patients with or without residual pancreatic function<sup>[17]</sup>. Decreased lean body mass may lead to decreased functional capacity as found in 34% of patients with moderate to severe weight loss. This also affected quality of life<sup>[18]</sup>. In this study fatigue, found in 46%, also had a major impact on quality of life. In a cohort of ambulatory patients with CP (60 patients), we found 28% at nutritional risk. These patients had a lower fat- and muscle mass and a tendency to lower handgrip strength. Furthermore, handgrip strength was associated with muscle- and fat mass<sup>[19]</sup>.

It has been found that 30%-50% of patients with CP have increased resting energy expenditure<sup>[20]</sup>. We found no difference between estimated (Harris-Benedict) and measured resting energy expenditure (indirect calorimetric) in a cohort of ambulatory CP patients. However, when adjusted for fat free mass and height, we found higher resting energy expenditure (around 20%) in patients with low BMI (< 20 kg/m<sup>2</sup>)<sup>[19]</sup>.

### Substrate metabolism during chronic pancreatitis

Daily intake of carbohydrates is about 300 grams corresponding to about half the caloric intake per day. About half of the caloric intake is carbohydrates and 30% are sucrose. Pancreatic  $\alpha$ -amylase is the only enzyme for carbohydrate digestion that is produced by the pancreas. The final digestion of sugars take place at the brushbor-

der of enterocytes where a range of disaccharidases produce the three sugars glucose, galactose and fructose that can be absorbed. In exocrine pancreatic insufficiency, carbohydrate digestion is maintained for a long time by salivary amylase and brush-border oligosaccharidases. The loss of endocrine function leads to glucose intolerance in 40%-90% of patients with severe CP. Furthermore, in 20%-30% of patients an insulin-dependent diabetes develops associated with impaired glucagon and pancreatic polypeptide regulation (discussed in other article).

Daily protein intake in the Western world lies between 70 and 100 g and gastrointestinal secretions contain further 50-60 g of proteins per day. Protein digestion is initiated by intragastric proteolytic activity and continued by intestinal brush-border peptidases. Luminal proteolytic activity is maintained even in the absence of pancreatic peptidases and azotorrhea is therefore a very late and rare symptom in CP<sup>[21]</sup>. Trypsinogen is the inactive precursor for trypsin, which is the key enzyme for activation of all proteolytic enzymes in the duodenum. Trypsinogen becomes activated by enteropeptidase that is secreted by the brush-border of duodenal enterocytes. Proelastase is produced by the pancreas and belong to the chymotrypsin-like elastase family (elastase-2A, elastase-2B, elastase-3A, and elastase-3B). The elastase-3A isoform can be measured and quantified by the faecal elastase test. About 40% of proteins are digested to free amino acids. Brush border peptidases continue the digestion of peptides longer than three amino acids. Dipeptides, tripeptides, and free amino acids are then absorbed by the enterocytes by different transport mechanisms.

In Western diet up to 40% of the daily caloric intake derives from lipids, although 30% is recommended. Lipids are insoluble in water and need to be transferred to water-soluble micelles formed by bile acids, phospho-

Step 1: Initial screening		Yes	No
1	Is BMI < 20.5 kg/m <sup>2</sup> ?		
2	Has the patient lost weight within the last 3 mo?		
3	Has the patient had a reduced dietary intake in the last week?		
4	Is the patient severely ill? ( <i>e.g.</i> , in intensive therapy)		

Yes: If the answer is 'Yes' to any question, the screening in Step 2 is performed.

No: If the answer is 'No' to all questions, the patient is re-screened at weekly intervals. If the patient *e.g.*, is scheduled for a major operation, a preventive nutritional care plan is considered to avoid the associated risk status.

Step 2: Final screening			
Impaired nutritional status		Severity of disease (approximately increase in requirements)	
Absent Score 0	Normal nutritional status A	Absent Score 0	Normal nutritional requirements
Mild Score 1	Wt loss > 5% in 3 mo or Food intake below 50-75% of normal requirement in preceding week.	Mild Score 1	Hip fracture <sup>1</sup> Chronic patients, in particular with acute complications: cirrhosis <sup>1</sup> , chronic obstructive pulmonary disease <sup>1</sup> . Chronic hemodialysis, diabetes, oncology.
Moderate Score 2	Wt loss > 5% in 2 mo or BMI 18.5-20.5 + impaired gen. condition or Food intake 25%-50% of normal requirement in preceding week	Moderate Score 2	Major abdominal surgery <sup>1</sup> Stroke <sup>1</sup> Severe pneumonia, hematologic malignancy.
Severe Score 3	Wt loss > 5% in 1 month (> 15% in 3 mo) or BMI > 18.5 + impaired general condition or Food intake 0-25% of normal requirement in preceding week	Severe Score 3	Head injury <sup>1</sup> Bone marrow transplantation Intensive care patients (APACHE >10).
Score: _____ +		Score: _____ = Total score:	
Age _____ if ≥ 70 yr: Add 1 to total score above		= Age-adjusted total score:	
Score ≥ 3: The patient is nutritionally at-risk and a nutritional care plan is initiated.			
Score < 3: Weekly rescreening of the patient. If the patient <i>e.g.</i> , is scheduled for a major operation, a preventive nutritional care plan is considered to avoid the associated risk status.			

NRS-2002 is based on an interpretation of available randomized clinical trials.

<sup>1</sup>Indicates that a trial directly supports the categorization of patients with that diagnosis. Diagnoses shown in *italics* are based on the prototypes given below. Nutritional risk is defined by the present nutritional status and risk of impairment of present status, due to increased requirements caused by stress metabolism of the clinical condition.

A nutritional care plan is indicated in all patients who are

- (1) severely undernourished (score = 3)
  - or
  - (2) severely ill (score = 3)
  - or
  - (3) moderately undernourished + mildly ill (score 2 + 1)
  - or
  - (4) mildly undernourished + moderately ill (score 1 + 2)
- Prototypes for severity of disease  
Score = 1: A patient with chronic disease, admitted to hospital due to complications. The patient is weak but out of bed regularly.

Protein requirement is increased, but can be covered by oral or supplement in most cases.

Score = 2: A patient confined to bed due to illness, *e.g.*, following major abdominal surgery. Protein requirement is substantially increased, but can be covered, although artificial feeding is required in many cases.

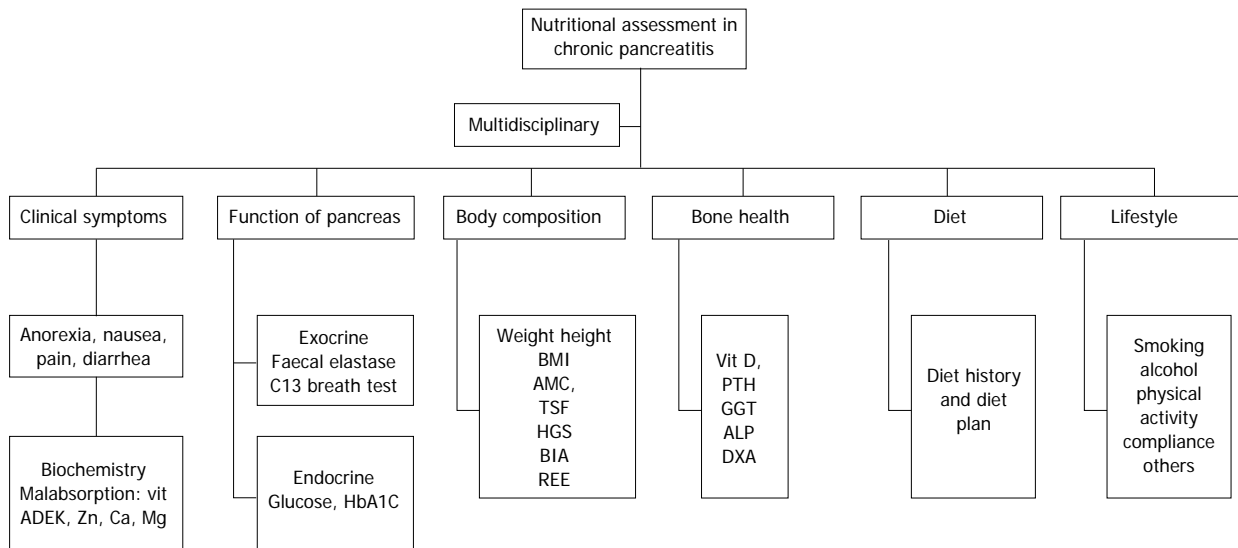
Score = 3: A patient in intensive care with assisted ventilation etc. Protein requirement is increased and cannot be covered even by artificial feeding. Protein breakdown and nitrogen loss can be significantly attenuated.

Figure 2 Nutrition risk screening 2002<sup>[9]</sup>. BMI: Body mass index.

lipids, cholesterol and other products. This facilitates hydrolysis by lipase. In the stomach lingual lipase and gastric lipase hydrolyse triglycerides to glycerol and free fatty acids. Lipases from the gastric and salivary glands have a minor role in digestion of triglycerides and cannot compensate an insufficient pancreatic fat digestion. Thus, luminal lipid digestion within the small intestine depends on the action of pancreatic lipase and cofactors such as colipase and bile acids. There are no triglyceride-digestion enzyme systems within the intestinal brush-border membrane. Consequently, lipid digestion is decreased by insufficient lipase secretion and reduced luminal bile acid concentration. Because bicarbonate secretion is also diminished in CP and postprandial intraduodenal pH may fall < 4, luminal lipase degradation occurs more rapidly than that of other enzymes due to its greater instability. Gastric lipase only partly compensates for the lack of pancreatic lipase (discussed in other article).

### Vitamin deficiency

A deficiency in vitamins A, D, E, K correlates with the severity of steatorrhoea in patients with CP, but may be caused by several different mechanisms including, suboptimal dietary intakes, increased losses, increased requirements, impaired binding of nutrients, antioxidant activity, and fat malabsorption. E vitamin deficiency may be seen more often than deficiencies of vitamin A, D, and K<sup>[22-24]</sup>. However, osteopathy (osteoporosis, osteomalacia, osteopenia) may occur in at least 25% of CP patients<sup>[22]</sup>. The adjusted hazard ratio (HR) for any fracture was 1.7 in patients with CP (95%CI: 1.6-1.8) in a recently Danish study<sup>[25]</sup>. Due to inadequate protease secretion by the pancreas, vitamin B12 deficiency can occur<sup>[26]</sup>. Zinc deficiency may be seen especially in association with diabetes<sup>[27]</sup>. Also, deficiencies in calcium, magnesium, thiamine and folic acids have been reported. The functional meaning of such deficiencies are how-



**Figure 3** Nutritional assessment in patients with chronic pancreatitis. AMC: Arm muscle circumference; TSF: Triceps skin fold; HGS: Hand grip strength; BIA: Bioimpedance; REE: Resting energy expenditure; GGT:  $\gamma$ -glutamyl transferase; ALP: Alkaline phosphatase; PTH: Parathyroid hormone; DXA: Dexa scan.

ever undetermined and in our cohort of ambulatory patients with CP we did not find any correlation between micronutrient deficiencies and handgrip strength as measured by bioimpedance (unpublished data). On the other hand we recently demonstrated that the fibrotic changes as well as atrophy and ductal-related parameters were associated with exocrine insufficiency such as reflected in vitamin D deficiency, and future studies should explore this in further details<sup>[28]</sup>.

## NUTRITIONAL ASSESSMENT

Assessment of nutrition status should include a multidisciplinary approach<sup>[14]</sup>, including assessment of clinical symptoms, exocrine and endocrine pancreatic function, body composition, bone health, dietary evaluation and lifestyle as illustrated in Figure 3.

Clinical symptoms should include routine medical history and psychological examination with special emphasis on nutrition related symptoms and risk factors (nausea, anorexia, pain, alcohol, smoking). Micronutrient status should be measured 1-2 times a year including malabsorption of fat-soluble vitamins, minerals and trace elements. Function of pancreas concerning the exocrine and endocrine part will not be discussed in this article.

Because both weight and BMI do not take body composition into account and may be misinterpreted as a result of fluid changes including ascites and edema, further investigation and assessment may be made with anthropometrics and bioimpedance measurements, both for baseline and follow-up (*i.e.*, every 3-6 mo), because even patients with a normal BMI may have a decreased muscle mass that further might decrease function and lead to a higher morbidity, *i.e.*, higher incidence of postoperative complications after surgery for CP<sup>[16]</sup>.

Anthropometric measurements as mid-arm circum-

ference estimating lean body mass and triceps skinfold estimating subcutaneous fat stores can be compared with age- and gender-specific centiles and are useful especially in patients with edema or ascites and as a long-term follow-up for nutritional status<sup>[14]</sup>.

Bioimpedance is easy, non-invasive, and relatively inexpensive, and can be performed in almost any subject because it is portable. ESPEN guidelines<sup>[29-31]</sup>, report results for fat-free mass body fat, body cell mass total body water, extracellular water and intracellular water from various studies in healthy and ill subjects. The data suggests that bioimpedance works well in healthy subjects and in patients with stable water and electrolytes balance with a validated bioimpedance equation that is appropriate with regard to age, sex and race. Clinical use of bioimpedance in subjects at extremes of BMI ranges or with abnormal hydration cannot be recommended for routine assessment of patients until further validation has proven for bioimpedance algorithm to be accurate in such conditions. Multi-frequency- and segmental-bioimpedance may have advantages over single-frequency bioimpedance in these conditions, but further validation is necessary. Longitudinal follow-up of body composition by bioimpedance is possible in subjects with BMI 16-34 kg/m<sup>2</sup> without abnormal hydration, but must be interpreted with caution.

Since muscle function correlates closely with whole body protein, body cell mass, anthropometrically measured arm muscle mass, and even with BMI, loss of weight or muscle mass invariably results in decreased muscle strength which is reflected in deteriorating function tests as well as in prominently altered muscle morphology<sup>[32]</sup>. Reduced muscle strength is in turn associated with loss of physical functionality and with negative impact on recovery of health after illness or surgery, which partly explains the high predictive power of muscle

function tests<sup>[2]</sup>. Hence, various studies have shown a close correlation between muscle strength and outcome in acute and chronic disease<sup>[32-34]</sup>. Just as measuring body composition offers a qualitative aspect of nutritional status, muscle function represents a dynamic indicator of muscle mass. Measurement of muscle function as indicator of functional as well as nutritional status has therefore gained considerable attention in the past years. Although hand grip strength correlates well with other muscle function tests such as knee extension strength or peak expiratory flow, it cannot be used as surrogate for muscle function of lower extremities when evaluating physical performance. Short term effects of nutritional therapy as *e.g.*, refeeding of acute malnutrition are seen earlier by muscle function than by changes in body composition. Long-term nutritional therapy should result in both changes of body composition and muscle function, which should be paralleled by improvements of physical status<sup>[31,35]</sup>.

Reference values for age- and gender-specific percentiles exist<sup>[36]</sup>.

Resting energy expenditure (REE): Institution of appropriate nutritional therapy necessitates accurate determination of energy requirements. Data on measured REE in CP are very limited, but has shown, that weight loss is accompanied by hypermetabolism, and that between 30% and 50% of patients with CP have increased REE<sup>[20]</sup>. It may help us predict the energy level necessary to promote weight restoration and optimize nutritional rehabilitation preventing severe medical complications such as the refeeding syndrome<sup>[37]</sup>. Unfortunately, this technology is not available in the majority of the hospitals, because it requires skilled technicians and sophisticated methodologies that are costly and difficult to apply in standard clinical settings.

Predictive formulas of REE may be used as an alternative to indirect calorimetric that may be utilized by clinicians. The most cited and used predictive formula is the Harris-Benedict equation, which includes age, stature, and body weight to estimate REE<sup>[38]</sup>. However, studies are needed to validate predictive formulas *vs* measured resting energy expenditure by indirect calorimetric.

Bone health: Despite the reported high incidence of osteopathy in CP, no disease specific guideline exists for CP. However, extrapolating from guidelines for comparable malabsorptive diseases, calcium and vitamin D supplementation as well as regular monitoring of bone health should be an integral part of the nutrition management of CP, hence biochemistry and DXA scan should be performed regularly (*i.e.*, once a year).

Diet: A detailed assessment of current and habitual dietary intakes should be made by a dietician for all patients at nutritional risk as indicated by the NRS 2002 screening tool. Nutrient intake may be measured by a 24-h recall interview or a diet history, and analyzed using specialized software providing detailed information about energy- and protein intake as well as fat and micronutrients intake. For evaluating specific food items a food fre-

quency questionnaire can be used<sup>[39]</sup>. A specific diet plan should be made and follow-up visits evaluating intake compared to recommended energy- and protein intake should be employed according to clinical monitoring practice. It has been shown, that both dietary counseling and oral supplements for CP patients at nutritional risk can improve weight, BMI and decrease fecal fat excretion<sup>[40]</sup>.

Lifestyle: To minimize nutritional risk factors in patients with CP, an effort should be made to investigate and eliminate possible individual barriers such as smoking and alcohol abuse. Furthermore, to assure patient compliance regarding medical treatment (*i.e.*, treatment with enzymes) and to evaluate pain treatment (discussed in another article). Physical activity should be encouraged alongside nutritional therapy for optimal result<sup>[32-34]</sup>.

## NUTRITIONAL MANAGEMENT

### *Nutritional treatment in chronic pancreatitis*

Maldigestion of macronutrients is the major cause of progressive nutritional and metabolic impairment in patients with CP. Nutritional interventions depend on the degree of maldigestion and the nutritional status.

The main goals for nutritional interventions are to ensure sufficient macro- and micronutrients intake, to decrease maldigestion, malabsorption and other risk factors in order to prevent or treat malnutrition.

The treatment of exocrine deficiency begins with dietary recommendations and pancreatic enzyme supplementation. About 80% of patients can be managed by a combination of analgesics, dietary recommendations and pancreatic enzyme supplements, while 10%-15% need oral nutritional supplements, 5% need enteral tube feeding and around 1% require parenteral nutrition<sup>[21,41,42]</sup>.

### *Diet recommendations*

Dietary recommendations begin with total abstinence from alcohol. In addition, an adequate number of calories should be taken. Estimation of REE [or measurement in patients with a low BMI (< 20 kg/m<sup>2</sup>)] is essential in all patients to calculate the adequate caloric intake because of risk of increased resting energy expenditure. Frequent small meals (4-8 times a day) should be given. The carbohydrate intake might be limited when an overt diabetes mellitus is present (described in more detail in other article).

A protein diet of 1.0-1.5 g/kg body weight/d is generally sufficient and well tolerated. Usually, if 30%-40% of the calories are given as fat this is well tolerated, especially when the foods are rich in vegetable fats.

If weight gain is insufficient and/or steatorrhea persists, medium chain triglycerides (MCT) can be tried to increase fat absorption. MCT are absorbed directly across the small bowel into the portal vein, even in the absence of lipase, co-lipase and bile salts. However, MCTs have low energy density and unpalatable taste, and a maximum of about 50 g/d might be given. Higher doses may be



ketogenic and are associated with side effects such as cramps, nausea and diarrhea. Fat soluble vitamins (A, D, E and K), vitamin B12 and other micronutrients should be supplemented if serum levels indicate deficiencies.

In general, a low fibre diet is recommended, because fibre may absorb enzymes and delay the absorption of nutrients. An adequate quantity of exogenous pancreatic enzymes is necessary to correct protein and lipid mal-digestion<sup>[1,43-45]</sup>. In 10%-15% of patients oral supplements can help to attenuate weight loss and delay the use of enteral tube feeding<sup>[42,46]</sup>.

The best clinical follow-up parameters for monitoring therapeutic success of dietary counseling are improvement of the patient's general condition and weight gain.

### Enteral nutrition

The cause of inadequate caloric intake in CP can be anatomical (due to pyloro-duodenal-stenosis or cyst compression) or inflammatory with acute complications (new attack of acute pancreatitis or development of fistulas). Patients suffering from serious insufficient caloric intake may benefit by oral supplements or enteral nutrition. To test if enteral nutrition is tolerated and increases nutritional status it is recommended to give the nutrition via a naso-jejunal tube. However, for long-term therapy feeding (exceeding 2-3 wk) a percutaneous endoscopic gastrostomy with a jejunal tube extension is more convenient. Continuous overnight delivery of the nutrients is suitable and entails more easily the patient's nutritional goal. From a theoretical point of view a semi-elemental diet can be recommended, but there are no studies showing improvement in the nutritional status compared to regular enteral nutritional formulas.

Owing to the fact that CP patients are frequently undernourished, nutritional support before pancreatic surgery may be beneficial. Data from patients undergoing general abdominal surgery have provided evidence that preoperative enteral or oral nutritional support improves outcome compared to undernourished patients by reducing postoperative morbidity and the length of hospital stay<sup>[47]</sup>. Thus, it should be emphasized that nutritional therapy should go alongside surgery, and that surgery for pain or any obstruction in the GI-channel should be a primary indication.

The potential to modulate the activity of the immune system by interventions with specific nutrients is termed immunonutrition. This concept is normally applied to any situation where nutritional formulas are supplemented with specific nutrients such as arginine, glutamine, omega-3 fatty acids, nucleotides and others. Meta-analysis of randomized controlled trials giving a combination of arginine and omega-3 fatty acids at home for 5-7 d before surgery reduced both postoperative infection rate and length of hospital stay in patients who underwent elective major GI surgery<sup>[48,49]</sup>. Specific trials in patients with chronic pancreatitis are not available. A recent RCT giving supplemental immunonutrition preoperatively in well-nourished and mal-nourished gastrointestinal sur-

gery patients showed, that LOS and costs were reduced especially in mal-nourished patients<sup>[50]</sup>.

Another recent study showed that enteral immunonutrition given post-operatively *vs* a standard enteral nutrition showed a reduction of infectious complications, anastomotic leak rate and LOS as well as an improved immunologic outcome<sup>[51]</sup>.

We therefore highly recommend a week with pre-operative immunonutrition to patients going to elective surgery for chronic pancreatitis. Early postoperative enteral nutrition is feasible and may additionally improve outcome after surgery.

### Parenteral nutrition

Parenteral nutrition is infrequently used in patients with chronic pancreatitis. Enteral nutrition preserves immune function and mucosal architecture and decreases the possibility for hyperglycemia while parenteral nutrition also increases the risk of catheter infections and sepsis complications. Parenteral nutrition is therefore only indicated when it is impossible to use enteral nutrition<sup>[52]</sup>. This means if the patients do not reach their requirements because gastric emptying is blocked, the patient needs gastric decompression, it is impossible to introduce a tube into the jejunum, or a complicated fistula is present. Parenteral nutrition is mainly performed over a short term period, *e.g.*, in apparent severe malnutrition prior to pancreatic surgery if enteral feeding is incomplete and may thus be used as a supplement to fulfill their requirements. There are no reported studies of patients with chronic pancreatic insufficiency who have been treated with parenteral nutrition for a long period.

### Future perspectives

Future studies evaluating a systematic approach for assessment and treatment of CP patients at nutritional risk should be made to further elucidate the nutritional course of these patients. Special emphasis should be on body composition, absorption of nutrients and metabolism, as well as measuring micronutrient deficiencies. Furthermore, nutritional interventions should be tested in randomized controlled trials with relevant clinical outcomes, *i.e.*, morbidity, quality of life, physical function and mortality.

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## Diagnosis and treatment of diabetes mellitus in chronic pancreatitis

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### Abstract

Diabetes secondary to pancreatic diseases is commonly referred to as pancreatogenic diabetes or type 3c diabetes mellitus. It is a clinically relevant condition with a prevalence of 5%-10% among all diabetic subjects in Western populations. In nearly 80% of all type 3c diabetes mellitus cases, chronic pancreatitis seems to be the underlying disease. The prevalence and clinical importance of diabetes secondary to chronic pancreatitis has certainly been underestimated and underappreciated so far. In contrast to the management of type 1 or type 2 diabetes mellitus, the endocrinopathy in type 3c is very complex. The course of the disease is complicated by additional present comorbidities such as maldigestion and concomitant qualitative malnutrition. General awareness that patients with known and/or clinically overt chronic pancreatitis will develop type 3c diabetes mellitus (up to 90% of all cases) is rather good. However, in a patient first presenting with diabetes mellitus, chronic pancreatitis as a potential causative condition is seldom considered. Thus many patients are misdiagnosed. The failure to correctly diagnose type 3 diabetes mellitus leads to a failure to implement an appropriate

medical therapy. In patients with type 3c diabetes mellitus treating exocrine pancreatic insufficiency, preventing or treating a lack of fat-soluble vitamins (especially vitamin D) and restoring impaired fat hydrolysis and incretin secretion are key-features of medical therapy.

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**Key words:** Diabetes mellitus; Chronic pancreatitis; Type 3c diabetes; Pancreatogenic diabetes; Pancreatitis

**Core tip:** Type 3c diabetes mellitus is more common than generally thought. Its prevalence is supposed to be among 5%-10% among all diabetics. Most patients with type 3c diabetes mellitus suffer from chronic pancreatitis as the underlying disease. Misclassification of these patients is very common, yet identification of these patients is very important due to some special diagnostic and therapeutic considerations in this subset of patients. Among these are *e.g.*, restoring proper fat assimilation, preventing fat-soluble vitamin deficiency and early identification of pancreatic cancer patients. Specific diagnostic criteria for type 3c diabetes mellitus are proposed within this review.

Ewald N, Hardt PD. Diagnosis and treatment of diabetes mellitus in chronic pancreatitis. *World J Gastroenterol* 2013; 19(42): 7276-7281 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7276.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7276>

### INTRODUCTION

Chronic pancreatitis is a disease characterized by pancreatic inflammatory and fibrotic injury resulting in irreversible parenchymal damage. Progressive nutrient maldigestion and disturbance of the timing and the interactions



**Table 1** Current classification of diabetes mellitus

I	Type 1 Diabetes Mellitus ( $\beta$ -cell destruction, usually leading to absolute insulin deficiency) A: Immune mediated B: Idiopathic
II	Type 2 Diabetes Mellitus (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)
III	Other Specific Types Of Diabetes Mellitus A: Genetic defects of $\beta$ -cell function B: Genetic defects in insulin action C: Diseases of the exocrine pancreas 1: Pancreatitis 2: Trauma/pancreatectomy 3: Neoplasia 4: Cystic fibrosis 5: Hemochromatosis 6: Fibrocalculous pancreatopathy 7: Others D: Endocrinopathies E: Drug- or chemical-induced F: Infections G: Uncommon forms of immune-mediated diabetes H: Other genetic syndromes sometimes associated with diabetes
IV	Gestational Diabetes Mellitus

Source: Ref. [4,5], with permission.

between nutrient digestion and absorption is observed and may lead to severe metabolic derangements. Glucose intolerance and diabetes mellitus are observed quite frequently in the course of the disease<sup>[1,2]</sup>.

Development of diabetes mellitus in chronic pancreatitis mainly occurs due to the destruction of islet cells by pancreatic inflammation. Additionally, nutrient maldigestion leads to an impaired incretin secretion and therefore to a diminished insulin release of the remaining beta-cells<sup>[3]</sup>. In contrast to the autoimmune mediated destruction of the beta-cells in type 1 diabetes mellitus, glucagon secreting alpha-cells and pancreatic polypeptide secreting pancreatic polypeptide-cells are also subject to destruction in chronic pancreatitis leading to a complex deranged metabolic situation.

Diabetes mellitus secondary to pancreatic diseases (such as chronic pancreatitis) is classified as pancreatogenic diabetes or type 3c diabetes mellitus according to the current classification of diabetes mellitus (Table 1)<sup>[4,5]</sup>. Whereas the awareness of type 1 and type 2 diabetes mellitus is rather good, type 3c diabetes mellitus, however, is a condition rarely considered in everyday practice. Yet, recent data on type 3c diabetes mellitus show that it might be more common than generally thought. Studies also suggest that this important condition might be consistently under- and misdiagnosed<sup>[6,7]</sup>.

Due to the complex pathophysiology of type 3c diabetes mellitus it bears clinical and laboratory features which are very distinct from both type 1 and type 2 diabetes mellitus. This review focuses on diagnosis and treatment of diabetes mellitus secondary to chronic pancreatitis.

## PREVALENCE OF DIABETES MELLITUS SECONDARY TO PANCREATIC DISEASES (TYPE 3C)

In contrast to type 1 and type 2 diabetes mellitus, detailed data on the prevalence of type 3c diabetes mellitus hardly exist. Some older studies estimate a rather low prevalence of about 0.5%-1.15% among all cases of diabetes mellitus in North America<sup>[8,9]</sup>. Other studies from, e.g., Southeast Asia where tropical or fibrocalculic pancreatitis is endemic, report a higher prevalence of approximately 15%-20% of all diabetes mellitus cases<sup>[10,11]</sup>.

A recent review of the currently available studies on this topic proposes a prevalence of 5%-10% for type 3c diabetes mellitus among all diabetes mellitus cases in Western populations<sup>[12]</sup>. Data are mainly based on a large retrospective study of 1868 patients at a German University Hospital, where type 3c diabetes mellitus accounted for 9.2% of all diabetics<sup>[7]</sup>. This emphasizes that previous older estimates of the prevalence of type 3c diabetes mellitus must be inaccurately low. In 78.5% of all patients with type 3c diabetes mellitus, chronic pancreatitis was identified as the underlying diseases, therefore resembling the most important causative condition<sup>[7]</sup>.

The previous underestimation of the prevalence of type 3c diabetes mellitus might partly be due to the fact that investigation of the pancreas has meanwhile been facilitated by new diagnostic procedures. Nowadays it has become much easier to detect exocrine pancreatic pathology as imaging methods of the pancreas have clearly improved and noninvasive screening methods to quantify exocrine pancreatic insufficiency are easily available.

If chronic pancreatitis accounts for nearly 80% of all type 3c diabetes mellitus cases, and if the prevalence of type 3c diabetes mellitus is expected to be approximately 5%-10% of all diabetes mellitus cases, the true prevalence of (subclinical) chronic pancreatitis in the general population seems to be far underestimated. This might especially hold true since chronic pancreatitis has previously been considered a disease of alcoholism until the discovery that it is a multifactorial disease with an impact of complex genetic genotypes, smoking, special anatomic conditions, toxic agents and autoimmunity, also<sup>[13]</sup>. Up to date quite a few autopsy studies<sup>[14-16]</sup>, endoscopic ultrasound studies<sup>[17]</sup> and exocrine pancreatic function studies<sup>[18]</sup> report a high frequency of exocrine pancreatic injury suggestive of chronic pancreatitis in the general population. This further supports the view of an underestimation of chronic (subclinical) pancreatitis in the general population.

## DIAGNOSIS OF DIABETES MELLITUS IN CHRONIC PANCREATITIS

As stated above glucose intolerance and diabetes mellitus are common in chronic pancreatitis. Diagnosing diabetes

**Table 2** Proposed diagnostic criteria for type 3c diabetes mellitus

Major criteria (must be present)
Presence of exocrine pancreatic insufficiency (monoclonal fecal elastase-1 test or direct function tests)
Pathological pancreatic imaging (endoscopic ultrasound, MRI, CT)
Absence of type 1 diabetes mellitus associated autoimmune markers
Minor criteria
Absent pancreatic polypeptide secretion
Impaired incretin secretion ( <i>e.g.</i> , GLP-1)
No excessive insulin resistance ( <i>e.g.</i> , HOMA-IR)
Impaired beta cell function ( <i>e.g.</i> , HOMA-B, C-Peptide/glucose-ratio)
Low serum levels of lipid soluble vitamins (A, D, E and K)

MRI: Magnetic resonance imaging; CT: Computed tomography; GLP-1: Glucagon-like peptide-1; HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-B: Homeostasis model assessment of beta-cell.

mellitus in a patient with known chronic pancreatitis may not be that difficult. Yet, the correct classification of type 3c diabetes mellitus is often missed and patients are commonly misclassified. In a German study only about half of the cases of type 3c diabetes mellitus were classified correctly. Type 3c diabetes mellitus patients were mostly misclassified as type 2 diabetes<sup>[7]</sup>. This might be due to the very poor awareness of this diabetes type.

However, another thing appears even more difficult: do not forget to take into account that a patient first presenting with diabetes mellitus might have a type 3c diabetes mellitus. In any case of a new diabetes mellitus manifestation we should truly use the classification criteria defined by the European Association on the Study of Diabetes (EASD) and the American Diabetes Association (ADA)<sup>[4,5]</sup> and check for type 3c diabetes mellitus. At least if a patient does not fit into the common presentation and complains about gastrointestinal symptoms the physician should be aware of the existence of type 3c and initiate further diagnostics.

### Screening for type 3c diabetes mellitus in chronic pancreatitis

Any patient with chronic pancreatitis should of course be monitored for the development of type 3c diabetes mellitus. The prevalence of diabetes mellitus among patients with an established diagnosis of chronic pancreatitis is reported to be up to 70% (in chronic calcific pancreatitis even up to 90%)<sup>[1,2]</sup>. Patients with long-standing duration of the disease, prior partial pancreatectomy, and early onset of calcific disease seem to be at higher risk for developing type 3c diabetes mellitus. There is a clear increase in the prevalence with the duration of chronic pancreatitis<sup>[19,20]</sup>.

The initial evaluation of patients with chronic pancreatitis should include fasting glucose and HbA<sub>1c</sub>. These tests should be repeated at least annually. Impairment in either one requires further evaluation. If testing suggests an impaired glucose tolerance, further evaluation by a 75 g oral glucose tolerance test is recommended<sup>[21]</sup>. A concomitant analysis of insulin and/or C-peptide levels may be help-

ful in distinguishing between type 2 and type 3c diabetes mellitus<sup>[22]</sup>.

### Distinguishing type 3c diabetes from other types

It is not always easy to diagnose and classify a patient with type 3c diabetes mellitus correctly. Long-standing type 1 and type 2 diabetes mellitus patients are associated with exocrine pancreatic failure<sup>[23]</sup> and patients with diabetes mellitus are at a higher risk for developing acute and/or chronic pancreatitis anyway<sup>[24,25]</sup>. Patients with previous episodes of pancreatitis may also develop type 1 or type 2 diabetes independently of their exocrine pancreatic disease. In order to classify patients with type 3c diabetes mellitus correctly, commonly accepted diagnosis criteria should be established.

In distinguishing between the different diabetes types the presence of islet cell antibodies is consistent with type 1 diabetes mellitus, and the presence of clinical or biochemical evidence of insulin resistance is associated with type 2 diabetes mellitus. Due to the lack of commonly accepted diagnostic criteria up to date, we propose the following criteria for diagnosing type 3c diabetes mellitus (Table 2).

The evaluation of pancreatic polypeptide response to insulin-induced hypoglycemia, secretin-infusion or a mixed nutrient ingestion might be of additional diagnostic interest as discussed elsewhere<sup>[21]</sup>. An absent pancreatic polypeptide response is able to distinguish between type 3c diabetes mellitus from early type 1 and may also distinguish type 3c from type 2, which is characterized by elevated pancreatic polypeptide levels<sup>[26-28]</sup>. Routinely testing of incretin secretion or pancreatic polypeptide response in everyday practice, however, does not seem feasible.

## TREATMENT OF DIABETES MELLITUS SECONDARY TO CHRONIC PANCREATITIS

### Managing hyperglycemia

The derangement in glucose metabolism in type 3c diabetes mellitus ranges from a mild impairment to a severe form characterized by frequent episodes of hypoglycemia, commonly referred to as brittle diabetes<sup>[9]</sup>. In type 3c diabetes mellitus, blood glucose control may be unstable due to the loss of glucagon response to hypoglycemia, carbohydrate malabsorption and/or inconsistent eating patterns due to concomitant pain and/or nausea or chronic alcohol abuse. Thus it is generally reported that type 3c diabetes mellitus is difficult to control, although there are only very few studies in this field<sup>[29,30]</sup>. Astonishingly, all large clinical trials, including Diabetes Control and Complications Trial<sup>[31]</sup> and United Kingdom Prospective Diabetes Study<sup>[32]</sup> specifically excluded patients with type 3c diabetes mellitus.

Currently, there are no generally accepted guidelines

regarding treatment pathways for type 3c diabetes mellitus. Yet, a first step was taken at Pancreas Fest 2012<sup>[21]</sup>. The pharmacological agents typically used for the treatment of type 3c diabetes mellitus are the same as for type 2 diabetes mellitus. The ADA and the EASD recommend metformin as the first-line oral therapy for type 2 diabetes mellitus<sup>[33]</sup>. Therefore many type 3c diabetes mellitus patients are initially treated with metformin as a drug of first choice. If hyperglycemia is rather mild and concomitant insulin resistance is additionally diagnosed or suspected, therapy with metformin may be a good choice in the absence of contraindications. However, metformin treatment might not be tolerated by a majority of patients since its main side effects include nausea, abdominal complaints, diarrhea and weight reduction. A patient with chronic pancreatitis will probably not tolerate these symptoms. Since metformin therapy proves capable of reducing the risk of pancreatic cancer by as much as 70%, however, its anti-diabetic and anti-neoplastic effects may be beneficial in patients with type 3c diabetes mellitus due to chronic pancreatitis<sup>[34]</sup>. This holds especially true since chronic pancreatitis and diabetes mellitus are both well accepted risk factors for the development of pancreatic cancer<sup>[35-37]</sup>.

Incretin based therapies [*e.g.*, glucagon-like peptide-1, (GLP-1)-analogues, dipeptidyl peptidase (DPP)-IV-inhibitors] also enhance insulin secretion. Yet, GLP-1-analogues as well as DPP-IV-inhibitors are both associated with a higher risk of pancreatitis and are reported to have a high frequency of prominent gastrointestinal side effects (*e.g.*, nausea, delayed gastric emptying, weight loss)<sup>[38]</sup>. Therefore their use should best be avoided at present time until their safety is confirmed. A better and probably safer way to positively influence the incretin system might be a proper supplementation with pancreatic enzymes in these patients as discussed below.

In early type 3c diabetes mellitus, oral therapy with insulin segretagogues (sulfonylurea and glinides) may also be considered, thiazolidines should be avoided due to prominent side effects (*e.g.*, bone fractures, fluid retention, congestive heart disease).

Chronic pancreatitis, however, must be seen as a progressive disorder and many patients will eventually require insulin therapy. Patients should then be treated using general insulin dosing guidelines as established for type 1 diabetes mellitus. In patients with severe malnutrition insulin therapy is commonly used as a therapy of first choice. This is due to the desired anabolic effects of insulin in this special subset of patients.

Insulin pump therapy may also be considered for patients who experience a brittle form of diabetes mellitus despite being sufficiently motivated.

As it is in the other diabetes types, initial treatment should include all efforts to correct lifestyle factors which contribute to hyperglycemia and the risk of pancreatic malignancy (*e.g.*, abstinence from alcohol and smoking cessation, weight loss in overweight subjects, physical exercise and dietary modifications).

### Managing exocrine pancreatic insufficiency

Many patients with chronic pancreatitis manifest some degree of fat malabsorption, regardless of the presence of symptoms. In patients with type 3c diabetes mellitus exocrine pancreatic insufficiency is nearly ubiquitous present. Since clinically overt steatorrhea is usually not observed until over 90% of exocrine pancreatic function have vanished, exocrine pancreatic insufficiency and maldigestion might remain undetected. However, the relevant maldigestion, which is present in the majority of patients with chronic pancreatitis, may cause qualitative malnutrition. This is especially important concerning the absorption of fat-soluble vitamins (A, D, E and K).

Very recent studies show a vitamin D deficiency in > 90% of patients with chronic pancreatitis<sup>[39,40]</sup>. Additionally a significant correlation of exocrine pancreatic insufficiency and osteoporosis and/or alterations in bone metabolism can be observed<sup>[41,42]</sup>.

Further considering the possible role of vitamin D deficiency in the pathogenesis of type 1 diabetes mellitus and the association of low vitamin D levels and poor glycemic control in observational studies<sup>[43,44]</sup>, qualitative malnutrition of vitamin D in patients with type 3c diabetes mellitus seems of clinical importance. Measuring serum-25-hydroxyvitamin D levels and supplementing deficient patients might thus be beneficial.

The incretin system may play another crucial role in the metabolic control of type 3c diabetes mellitus. The regulation of the beta-cell mass and the physiological incretin secretion are directly dependent on normal exocrine pancreatic function and fat hydrolysis. Chronic pancreatitis and exocrine dysfunction have been associated with a functional impairment of the incretin system. Impaired GLP-1 secretion, however, can be normalized by pancreatic enzyme supplementation as previously described<sup>[3,45,46]</sup>.

Adequate oral pancreatic enzyme replacement therefore seems very important in type 3c diabetes mellitus. Besides helping to control symptoms of steatorrhea, it also seems capable of preventing qualitative malnutrition and metabolic complications.

### CONCLUSION

Type 3c diabetes mellitus is a clinically important disease with a prevalence of 5%-10% among all patients with diabetes mellitus. The prevalence and clinical importance of this condition has been underestimated and underappreciated in the past.

Most patients with type 3c diabetes mellitus suffer from chronic pancreatitis as the underlying disease. The prevalence of (subclinical) chronic pancreatitis might also been underestimated as some studies suggest. Recognizing a diabetic state in patients with known chronic pancreatitis is obligatory. Patients should undergo screening tests in order to detect hyperglycemia early. Fasting glucose, HbA<sub>1c</sub> and 75 g oral glucose tolerance testing are appropriate diagnostic tools. When diagnosing diabetes



mellitus in patients with chronic pancreatitis, physicians should be aware of the existence of type 3c diabetes mellitus and should classify this condition correctly as pancreatogenic diabetes or type 3c diabetes mellitus.

To identify a (subclinical) chronic pancreatitis as the underlying condition of patients with the established diagnosis of diabetes mellitus certainly is the greater challenge in everyday practice. This is due to the fact that most physicians are not aware of type 3c diabetes mellitus and (subclinical chronic) pancreatitis does not necessarily present in a clinically impressive manner. A patient with unspecific gastrointestinal complaints and diabetes mellitus should therefore always prompt further diagnostics with regard to type 3c diabetes mellitus.

Identifying patients with type 3c diabetes is important since the endocrinopathy in type 3c diabetes is very complex and complicated by additional present comorbidities such as maldigestion and concomitant qualitative malnutrition. Specific diagnostic criteria are proposed above (Table 1). The failure to correctly diagnose type 3c diabetes mellitus leads to failure to implement an appropriate medical therapy. It is mandatory to treat pancreatic exocrine insufficiency in these patients even if clear clinical symptoms such as steatorrhea or gastrointestinal complaints are missing. Adequate pancreatic enzyme supplementation therapy might for once help preventing a lack of fat-soluble vitamins (especially vitamin D). Additionally it might exert beneficial effects on the impaired incretin release in patients with chronic pancreatitis. Furthermore one has to realize that type 3c diabetes mellitus due to chronic pancreatitis might be referred to as a premalignant condition since both diseases are well accepted risk factors for the development of pancreatic cancer.

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## Pain and chronic pancreatitis: A complex interplay of multiple mechanisms

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resembles that seen in neuropathic and chronic pain disorders. However, pain due to *e.g.*, complications to the disease and adverse effects to treatment must not be overlooked as an additional source of pain. This review outlines the current theories on pain generation in chronic pancreatitis which is crucial in order to understand the complexity and limitations of current therapeutic approaches. Furthermore, it may also serve as an inspiration for further research and development of methods that can evaluate the relative contribution and interplay of different pain mechanisms in the individual patients, before they are subjected to more or less empirical treatment.

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**Key words:** Chronic pancreatitis; Abdominal pain; Pain mechanisms; Neuropathy; Sensitization

### Abstract

Despite multiple theories on the pathogenesis of pain in chronic pancreatitis, no uniform and consistently successful treatment strategy exists and abdominal pain still remains the dominating symptom for most patients and a major challenge for clinicians. Traditional theories focussed on a mechanical cause of pain related to anatomical changes and evidence of increased ductal and interstitial pressures. These observations form the basis for surgical and endoscopic drainage procedures, but the outcome is variable and often unsatisfactory. This underscores the fact that other factors must contribute to pathogenesis of pain, and has shifted the focus towards a more complex neurobiological understanding of pain generation. Amongst other explanations for pain, experimental and human studies have provided evidence that pain perception at the peripheral level and central pain processing of the nociceptive information is altered in patients with chronic pancreatitis, and

**Core tip:** Pain management in chronic pancreatitis often remains unsatisfactory. An overview of the current theories on pain generation in chronic pancreatitis is crucial in order to understand the complexity and limitations of current therapeutic approaches. Also, optimal treatment will only be achieved on the basis of a better understanding of these mechanisms. Furthermore, this review may serve as an inspiration for future research and development of methods that can determine the relative contribution of different mechanisms to the "collective" abdominal pain, before patients are subjected to more or less empirical treatment.

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## INTRODUCTION

Abdominal pain is the most significant symptom and a major clinical challenge in chronic pancreatitis (CP). It is present in up to 90% of the patients and the primary cause of hospitalization<sup>[1]</sup>. Pancreatic pain is characteristically described as a constant, severe, dull, epigastric pain that often radiates to the back and typically worsens after high-fat meals. However, many different pain patterns have been described, ranging from no pain to recurrent episodes of pain and pain free intervals, to constant pain with clusters of severe exacerbations<sup>[2,3]</sup>. In the often cited Zürich series, pain was reported to decrease over time, coinciding with the occurrence of exocrine insufficiency, which led to the “burn-out” hypothesis of pain in CP<sup>[3,4]</sup>. However, evidence against this hypothesis was subsequently provided in two large prospective studies<sup>[5,6]</sup>, where no association between the duration of CP and the quality or frequency of pain was found. Today the “burn-out” hypothesis is regarded obsolete by most clinicians, and even though a few patients may experience spontaneously pain relief, pain in the majority of patients is unpredictable and has a substantial impact on physical and mental quality of life, employment and health care expenses<sup>[6-8]</sup>.

The etiology of pain in CP is increasingly better understood and likely involves multiple mechanisms. Traditional theories focused on the role of local pathology within or in close proximity to the pancreas, as the main generator of pain, but since the late 1990's there has been a shift towards a neurobiological understanding of pain in CP. Accordingly, there is solid histological and neurophysiological evidence for an abnormal pain processing in many of these patients<sup>[1,9]</sup>. Various mechanisms responsible for this altered pain processing have been proposed, among them sensitization of the peripheral and central nerves, reorganization of the cerebral cortex and alterations in pain control systems. In addition, local complications (such as pancreatic pseudocysts and duodenal and/or bile duct obstruction) and adverse effects to treatment also contribute to the complex symptomatology in many patients and is an often a neglected problem in the clinical settings. Also, it has been shown, that the clinical pain pattern varies with the etiology of CP, and hypothesized that different pathogenic mechanisms of pain are associated with or predominates depending on etiology<sup>[2,4]</sup>, but so far it is not possible to correlate pain mechanism and clinical pain pattern to the etiology of CP uniformly.

The aim of this review is to highlight current theories regarding the pain mechanisms in CP. This is important in order to understand the complexity of this debilitating disease and why pain management is often insufficient. Also, optimal treatment will only be achieved on the basis of a better understanding of the mechanisms underlying pain in CP<sup>[10]</sup>. There is a variety of potential pain mechanisms that needs to be taken into consideration (Figure 1), but in this review we present for simplicity

a schematic division of the pain mechanisms into five main categories as an easy-to-remember overview (Table 1).

## “PLUMBING” PROBLEMS

The mainstay of pain treatment in CP has for many years focused on the pancreatic gland based on the assumption, that pain is generated by increased pressure in the pancreatic duct or in the pancreatic parenchyma<sup>[11]</sup>. This mechanistic understanding of pain, termed the “the plumbing theory”, has been the most widely accepted theory regarding the cause of pain and it is the theoretical background of most interventions including surgical and endoscopic drainage procedures<sup>[12]</sup>.

### *Pancreatic duct hypertension and pain*

A direct relationship between pancreatic duct hypertension and pain was reported for the first time in a case report by White *et al.*<sup>[13]</sup>. A patient undergoing open necrosectomy following acute necrotizing pancreatitis had a drainage catheter placed in a fistula tract communicating with the pancreatic duct. He reproducibly developed pain after saline infusion when the ductal pressure exceeded 25 mmHg<sup>[13]</sup>. Inspired by this finding, subsequent studies attempted to compare ductal pressure measurements in CP patients and other patient groups. Many of these studies were flawed by inappropriate comparison of intraoperative pressure measurements in CP patients to endoscopic pressure measurement in controls. Accordingly, only a few studies have compared ductal pressure using the same technique in patients and controls. Based on intraoperative measurement of pancreatic duct pressure, significantly increased pressures were evident in patients with CP undergoing a surgical pancreatic drainage procedure compared to patients undergoing surgery for gastric cancer<sup>[14]</sup>. Moreover, in a study comparing endoscopic manometry of the pancreatic duct, CP patients had evidence of ductal hypertension compared to controls<sup>[15]</sup>. Other studies based on endoscopic manometry have, however, not documented ductal hypertension in CP, and the only study comparing CP patients by the presence or absence of pain demonstrated no difference in pressure levels<sup>[16-20]</sup>. In addition, the pathological mechanism by which increased ductal pressure causes pain in CP is not clear and, taken together, the link between ductal hypertension and pain in CP remains hypothetical.

### *Pancreatic parenchymal hypertension and pain*

Measurement of intrapancreatic pressure was pioneered by Ebbelhøj *et al.*<sup>[21]</sup>, who developed a technique based on a needle probe inserted directly into the pancreatic parenchyma. Measurements were employed directly intraoperatively or by using a percutaneous procedure guided by ultrasonography<sup>[21]</sup>. In 39 patients with CP, they found that the intrapancreatic pressure was higher in patients

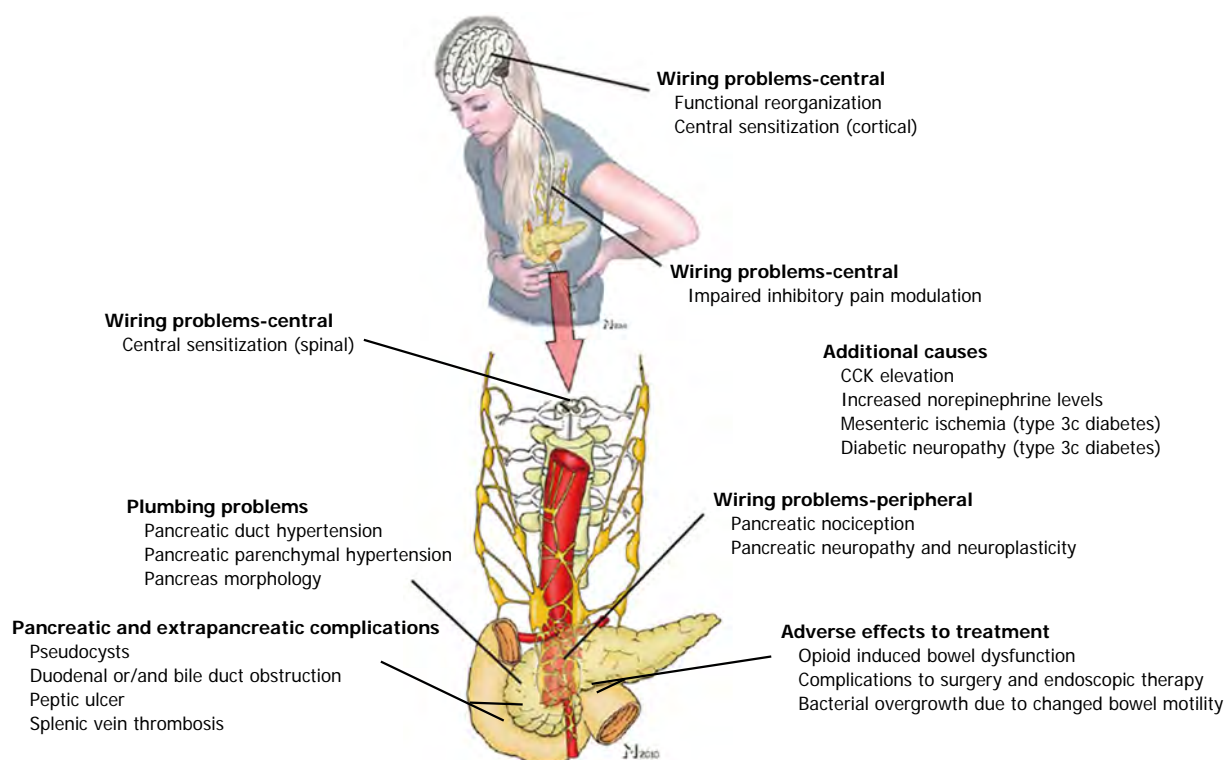


Figure 1 Pain mechanisms in chronic pancreatitis. The source of pain in chronic pancreatitis is complex and almost certainly multifactorial, and the relative contribution of different factors can be difficult to determine in the individual patient. See manuscript for further details. Modified from Demir *et al*<sup>[87]</sup>. CCK: Cholecystokinin.

with pain than in patients without pain, and this intrapancreatic hypertension was reversed following surgical drainage<sup>[22]</sup>. At one-year follow-up, patients with recurrent pain had a rebound of intrapancreatic hypertension, while patients who remained pain free continued to have normal intrapancreatic pressures<sup>[23]</sup>. Although intriguing, these findings were not reproduced by a subsequent study in which no relation between intrapancreatic pressure and pain was found<sup>[24]</sup>.

The pathophysiological link between intrapancreatic hypertension and pain has been explained as a “compartment-like syndrome” induced by fibrosis of the pancreatic parenchyma and peripancreatic capsule<sup>[2]</sup>. During secretory stimulation in an animal model of CP, increased interstitial pressures, diminished blood flow and ensuing tissue acidosis were documented<sup>[25]</sup>. The latter was possibly secondary to ischemia, thus mimicking the pathophysiology underlying muscular compartment syndrome. Although no pain data were collected in this experiment, it is plausible that acidosis may have caused pain by activation of the transient receptor potential vanilloid-1, which is a nociceptor found to be up regulated in CP and other conditions characterized by neurogenic inflammation<sup>[26,27]</sup> (described further below). However, it must be highlighted that these findings have never been reproduced in a human study and needs confirmation.

### Pancreas morphology and pain

In clinical practice, measurement of ductal or intrapancreatic pressure is not routinely performed and most

decisions regarding surgery or endotherapy to relieve pain rely on morphological abnormalities of the pancreas<sup>[2,11,28]</sup>. However, the relationship between ductal or intrapancreatic pressure and pancreatic morphology is ambiguous<sup>[29]</sup>. Furthermore, in a very recent study by Frokjaer *et al*<sup>[30]</sup> magnetic resonance cholangiopancreatography including diffusion weighted imaging was obtained in 23 patients with painful CP and 17 controls. Compared to traditional imaging techniques (*i.e.*, endoscopic retrograde cholangiopancreatography, magnetic resonance cholangiopancreatography, computed tomography, and endoscopic ultrasound), which mainly focus on atrophy and ductal pathology, this modality also provides a measure of fibrotic changes in the pancreatic gland<sup>[31]</sup>. Interestingly, the authors found no association between degree of pathological imaging (fibrosis, atrophy, and ductal pathology) and pain. However, pancreatic atrophy and ductal pathology were associated with diabetes (*i.e.*, more severe atrophy in patients with high levels of glycated hemoglobin), and low levels of phosphate and hemoglobin.

Taken together, pathological pancreatic morphology does not necessarily reflect the burden of pain in CP. Nowhere is this observation made more obvious, than by the patient who undergoes total pancreatectomy for painful CP and still continues to suffer from pain<sup>[11]</sup>.

### “WIRING” PROBLEMS

Lesions to intrapancreatic nerves and their impact on the pathogenesis of pain in CP have been the focus of



**Table 1 Pain mechanisms in chronic pancreatitis**

Main category	Proposed mechanism/source	Ref.
Plumbing problems	Pancreatic duct hypertension	[14,16,20]
	Pancreatic parenchymal hypertension	[21-23]
Wiring problems	Pancreas morphology	[29,30]
	Peripheral nociception	[35-37]
	Pancreatic neuropathy	[41,42,44]
	Central mechanisms of pain	[50,52,53,56]
Pancreatic and extrapancreatic complications	Pseudocysts	[62,64]
	Duodenal obstruction	[66]
	Bile duct obstruction	[66,67]
	Peptic ulcer	[71,72]
	Splenic vein thrombosis	[75,78]
Adverse effects to treatment	Opioid induced bowel dysfunction	[79]
	Complications to surgery and endoscopic therapy	[12]
	Bacterial overgrowth due to changed bowel motility	[81]
Additional causes	CCK elevation	[82]
	Increased norepinephrine levels	[85]
	Diabetic neuropathy (type 3c diabetes)	[86]
	Mesenteric ischemia <sup>1</sup>	

<sup>1</sup>Complications to and/or possible concomitant diseases. CCK: Cholecystokinin.

a substantial amount of research past 25 years. The International Association for the Study of Pain recently changed its definition of neuropathic pain to “pain caused by a lesion or disease of the somatosensory system”<sup>[32]</sup>. In the context of pain and CP there is emerging histological and neurophysiological evidence of such lesions to peripheral nerves in the pancreatic gland and coincident aberrant central pain processing. Three aspects of the neural basis of pain in CP are usually considered: Peripheral nociception, pancreatic neuropathy and central mechanisms of pain.

### Peripheral nociception

A nociceptor is a sensory nerve cell capable of integrating and transducing nociceptive stimuli into pain signals<sup>[33]</sup>. Nociception begins with the primary afferent nerves, whose cell bodies lies in the dorsal root ganglia next to the spinal cord. They have two branches-one terminating in the target tissue and one that ends in the dorsal horn of the spinal cord. The peripheral nerve endings sense tissue injury or pain stimuli *via* a variety of receptors, which respond to physical or chemical factors in their surroundings<sup>[12]</sup>. Depending on the excitability of the neural membrane, the stimulus leads to an action potential, which travels to the spinal end of the nerves in the dorsal horn. Here it triggers the release of neurotransmitters, which cross the synapse and act on secondary neurons and transmit the stimulus to the brain through diverse pathways, ultimately resulting in the sensation of pain.

Currently, it is unknown what actually activates the intrapancreatic nociceptors in humans. However, increased expression of the transient receptor potential vanilloid-1 has been demonstrated in an animal model of CP<sup>[34]</sup>, as well as in humans, although no correlation to pain was seen<sup>[35]</sup>. Furthermore, studies on nociceptive activation markers, *i.e.*, neurotransmitters secreted by

activated afferents, especially the large family of neurotrophic factors, are gaining increasing attention. Among others, both nerve growth factor and brain-derived neurotrophic factor have been shown to be upregulated in CP patients<sup>[36,37]</sup>, as well as many other proinflammatory cytokines, and in some cases this upregulation has been associated with increased pain intensity and/or frequency (for a review see<sup>[2]</sup>).

These changes render the nociceptors more sensitive to further stimulation, so that there can be a reduction in the threshold for activation, an increase in the response to a given stimulus, or the appearance of spontaneous activity<sup>[38,39]</sup>. This sensitization, called peripheral sensitization, results in an increased barrage of pain signals to the spinal cord<sup>[40]</sup>.

Taken together, several articles have demonstrated upregulation signaling molecules involved in inflammation and pronociceptive mediators, but also neurotrophic factors in CP. The trophic nature of the nociceptive activation markers further suggests a link between this altered nociception and the neuropathy of CP described below.

### Pancreatic neuropathy

Increased neural density and hypertrophy, sprouting and neuritis of the intrapancreatic nerves, as well as activation of glia and immune cells have also been reported in pancreatic tissue from CP patients<sup>[41-43]</sup>. These changes, collectively known as neuropathy, have been strongly associated with clinical pain scores, and thus suggests to be an important factor in the pathogenesis of pain in CP<sup>[41,42,44]</sup>. Autonomic innervation, especially sympathetic, is also decreased<sup>[43]</sup>, and has been correlated with abdominal pain.

Overall, substantial evidence supports the notion that pancreatic neuropathy leads to a remodeling of the intrapancreatic innervation, a concept known as pancreatic

neuroplasticity, which is likely an important factor in the pathogenesis of pain in CP.

### Central mechanisms of pain

A sustained and increased peripheral nociceptive drive may result in an increased responsiveness of central pain transmitting neurons. This phenomenon is known as central sensitization, and refers to an increased synaptic efficiency established in sensory neurons in the dorsal horn of the spinal cord (and/or at supraspinal sites), following intense peripheral noxious stimuli, tissue injury, or nerve damage<sup>[45,46]</sup>. The end result is pain, which is no longer coupled to the presence, intensity, or duration of noxious peripheral stimuli<sup>[47]</sup>. Clinically these changes manifests as allodynia (a painful response to stimuli not normally painful), hyperalgesia (increased sensitivity to painful stimuli) and secondary hyperalgesia (a receptive field expansion that enables input from non-injured tissue to produce pain)<sup>[45]</sup>.

Several studies have reported findings compatible with central sensitization in CP. Patterns of referred pain can be examined to assess central sensitization, as viscera-somatic convergence between peripheral nerves occurs at the spinal cord and higher levels of the central nervous system. Hence, visceral sensitivity of the upper gastrointestinal organs (sharing spinal innervation with the pancreatic gland) serves as a proxy of spinal sensitization<sup>[48,49]</sup>. In one study, increased areas of referred pain to electrical stimulation of the esophagus, stomach, and duodenum was reported in CP patients compared to controls<sup>[50]</sup>. Other studies reported decreased pain thresholds to visceral stimulation of the rectosigmoid as well as somatic stimulation of muscle and bone<sup>[51,52]</sup>. Taken together, these findings characterize a generalized hyperalgesic state of the pain system and likely mirrors widespread sensitization of the central nervous system as seen in many other chronic pain disorders<sup>[45]</sup>.

From the spinal cord, visceral afferents are projected to cortical and subcortical structures of the brain. Experimental pain studies, based on somatic stimulation of the epigastric skin area (sharing spinal segmental innervation with the pancreatic gland) as well as visceral stimulation of the upper and lower gut with concomitant recording of evoked brain potentials and brain source localization, have indicated that chronic pain and hyperalgesia is associated with functional reorganization of the cerebral cortex<sup>[50,53-55]</sup>. Hence, compared to healthy controls CP patients show reorganization of the brain areas involved in visceral pain processing including the insula, secondary somatosensory cortex and cingulate cortex similar to what is seen in phantom pain<sup>[53]</sup>. Interestingly, insular reorganization was associated with clinical pain intensity. In addition to reorganization of the brain areas involved in visceral pain processing, the excitability of these neural networks is abnormal with evidence of impaired habituation to noxious stimuli, possibly reflecting a neuronal hyperexcitability (*i.e.*, cortical sensitization)<sup>[55]</sup>.

The structural correlate of functional cortical reor-

ganization and hyperexcitability is found in studies based on advanced magnetic resonance imaging (MRI). In one study using diffusion weighted MRI, microstructural changes in the insular and frontal brain areas was associated with clinical pain intensity and functional scores<sup>[56]</sup>. Patients with a constant pain pattern demonstrated the most severe microstructural abnormalities compared to patients with an attack-wise pain pattern. This translates well to the clinic where patients with constant pain was recently reported to have the most reduced quality of life and functioning<sup>[6]</sup>. In another MRI study based on cortical volumetry, brain areas involved in visceral pain processing was shown to have a reduced thickness<sup>[57]</sup>. This finding suggests a central neurodegenerative response to severe and sustained pain.

The pain system has several inherent mechanisms whereby inflowing pain signals can be modulated. In particular, modulation of spinal pain transmission from the brainstem and higher cortical areas has been subject to increased interest during the last decades. Descending pain modulation where the brain can exert a downstream gating of the incoming spinal activity can lead to either an increase in the spinal transmission of pain impulses (facilitation) or a decrease in transmission (inhibition). The balance between these states ultimately determines the quality and strength of the pain signals perceived by the brain. Alterations in the state of descending modulation from inhibition towards facilitation have been implicated in the transition of acute into chronic and neuropathic pain. In the context of pain and CP, impaired descending inhibitory pain modulation has been reported in studies based on experimental human pain models<sup>[52,58]</sup>. In addition, brainstem facilitation has been reported to maintain pancreatic pain in an animal model of CP<sup>[59]</sup>.

Taken together, several lines of evidence indicate that central pain processing is abnormal in CP. However, it is difficult to determine whether these central abnormalities are maintained by a sustained nociceptive drive from the pancreatic gland or whether they have become independent of peripheral input. In favor of the latter, a recent pilot project documented generalized hyperalgesia (a clinical measurable proxy of central sensitization) to be associated with failure of thoracoscopic splanchnic denervation<sup>[60]</sup>. The authors concluded that in patients with hyperalgesia and failure to denervation, the disease has advanced and the generation of pain becomes self-perpetuating and independent of the initial peripheral nociceptive drive. However, these findings need confirmation in larger and longitudinal studies.

## PANCREATIC AND EXTRAPANCREATIC COMPLICATIONS

Although questions still remain, “plumbing” and “wiring” problems are probably important generators of pain in CP. Nevertheless, pain due to complications to

the disease is also likely to contribute, and should not be overlooked, when evaluating the origin of abdominal pain in CP patients.

### Pseudocysts

Pancreatic pseudocysts are relatively common in CP with estimated incidence rates of 20%-40%<sup>[61,62]</sup>, however, there is a lack of precise data based on long term follow-up. Yet, due to the chronic nature of the disease, CP patients are at high risk of developing pseudocyst in the course of their disease<sup>[63]</sup>. The exact pathogenesis of pseudocyst formation in CP is not known, but it has been proposed, that blockage of the major branch of the main pancreatic duct, and ongoing pancreatic secretion proximal to the obstruction leads to a saccular dilatation of the duct. This is then filled with pancreatic juice resulting in a pseudocyst<sup>[62]</sup>. The range of symptoms is wide-from asymptomatic to severe abdominal pain dependent on etiology, localization, and size<sup>[64,65]</sup>.

### Duodenal and bile duct obstruction

Advanced CP is characterized by replacement of the pancreatic parenchyma with fibrous tissue. Due to the close anatomical relationship of the common bile duct and the second part of the duodenum with the head of the pancreas, fibrosis can exert extrinsic pressure on these structures. The end result is mechanical obstruction of the common bile duct and duodenum<sup>[66,67]</sup>.

The clinical presentation of duodenal and bile duct obstruction is variable and can be asymptomatic<sup>[66,68]</sup>. Postprandial abdominal pain, early satiety and nausea are the most common symptoms of duodenal obstruction, while pain and abnormal liver function tests are suggestive of bile duct stenosis and cholangitis<sup>[69]</sup>. The mechanisms underlying such "obstructive pain" are unclear and one study concluded that bile duct obstruction without cholangitis is not the cause of pain in patients with CP<sup>[70]</sup>.

### Peptic ulcer

Upper abdominal pain is a prominent symptom in patients with peptic ulcer. Previous studies have demonstrated, that the prevalence of duodenal ulcer is high in patients with CP (ranges from 3.6% to 37.5%)<sup>[5,71,72]</sup>. The reason for the higher prevalence is unclear and many hypotheses have been suggested, including higher prevalence of *Helicobacter pylori* infection<sup>[71]</sup>, increased gastric acid secretion<sup>[73,74]</sup>, and insufficient pancreatic exocrine function which reduces bicarbonate secretion and decreases duodenal pH<sup>[72]</sup>. Furthermore, changes in gastric and intestinal blood flow due to previous acute pancreatitis may also be of importance.

As peptic ulcer can be asymptomatic, the high prevalence may be a result of a "detection bias" due to intensive search for the "origin" of epigastric pain in a selected group of patients. Patients with CP are more likely to undergo an upper gastrointestinal endoscopy, and therefore ulcers with atypical or no symptoms may

be included, causing a higher prevalence than in the background population<sup>[72]</sup>.

Under all circumstances, peptic ulcer may contribute to or be the reason for abdominal pain in CP patients and therefore diagnostic upper gastrointestinal endoscopy should be considered on wide indications.

### Splenic vein thrombosis

The splenic vein runs along the posterior surface of the pancreas and may be affected by inflammation in the pancreatic gland, leading to thrombosis and abdominal pain. Because most patients are asymptomatic the incidence of splenic vein thrombosis in CP is not known<sup>[75]</sup>, but incidence from 4%<sup>[76]</sup> to 45%<sup>[77]</sup> have been reported. Only few studies address the issue, but in one of the largest studies on splenic vein thrombosis in CP, 266 patients were investigated prospectively for a mean time of 8.2 years<sup>[78]</sup>. Splenic vein thrombosis was found in 22 patients (8.3%), but was only symptomatic in two patients. Hence, the role of splenic vein thrombosis in the pathogenesis of pain in CP is still questionable.

## ADVERSE EFFECTS TO TREATMENT

In many CP patients, strong opioids are often necessary to relieve pain. Unfortunately, opioids have the potential to produce substantial gastrointestinal adverse effects, including constipation, reflux, nausea and abdominal pain-a phenomenon known as opioid-induced bowel dysfunction<sup>[79]</sup>. In a study on the prevalence of gastrointestinal symptoms in patients treated with opioids for non-cancerous diseases, chronic abdominal pain was reported in 58% of the patients<sup>[80]</sup>. Hence, opioid consumption may confuse the clinical picture in CP and worsen or contribute significantly to abdominal pain. Medications that change bowel motility may also contribute to the bacterial overgrowth that is reported in up to 40% of the patients<sup>[81]</sup>. This may manifest as abdominal distension and pain.

In addition to side effects to medical therapy, complications to surgical endoscopic therapy may cause pain in a number of patients. These may include post-operative adhesions, ductal and parenchymal trauma, pancreatic and bile duct strictures, and complications to pancreatic stenting-all of which may result in abdominal pain. However, the relative contribution to abdominal pain due to surgical complications in CP is difficult to assess, and as far as the authors are aware of, no studies have examined this.

## ADDITIONAL CAUSES FOR PAIN

The above mentioned theories and mechanisms of pain are considered the most common and well-founded by the authors, but other rarer, less well examined causes, and complications to concomitant and comorbid diseases may also play in role in pain generation. A thorough evaluation of all these plausible causes is beyond the

scope of this review, but the reader is referred Table 1 for an overview.

However, elevated cholecystokinin (CCK) levels and increased sympathetic activity deserves to be mentioned separately as an additional cause of pain in CP. It has been shown, that CCK levels are elevated threefold in some early pancreatitis patients compared to controls<sup>[82]</sup>. This may generate pain by increasing the pressure in the pancreatic duct, but also through direct activation of nociceptive pathways in the central nervous system<sup>[83]</sup>. In one placebo-controlled, multicentre trial including 207 patients with chronic pancreatitis, an oral CCK receptor antagonist significantly decreased pain over placebo, although the placebo response was 30%<sup>[84]</sup>. However, further research into CCK antagonists has been hampered by concerns about induction of exocrine insufficiency and gallstones<sup>[11]</sup>.

Although limited by a small number of patients, Buscher *et al.*<sup>[85]</sup> showed significantly lower pain tolerance thresholds to pressure pain in dermatome T10 (pancreatic dermatome) in CP patients with increased norepinephrine levels compared to CP patients with normal norepinephrine levels, suggesting that sympathetic activity may play a role in these patients pain processing.

## CONCLUSION

Intense abdominal pain is a dominant feature of CP and it is associated with poor mental and physical quality of life. Basic studies of pancreatic nerves and experimental human pain research have provided evidence that pain processing is abnormal in these patients and in many cases resembles that seen in neuropathic and chronic pain disorders. This neurobiological view of pain is somewhat in opposition to the traditional view of pain etiology in CP, where pain was assumed to arise from pathology in or in close proximity to the pancreatic gland. However, these theories are not mutually exclusive, and aspects of both may contribute in the generation and perpetuation of pain. In addition, adverse effects and complications to medical and interventional therapies may account for a substantial morbidity in many patients and should be considered as an additional source of pain. Therefore, it is important to consider the different mechanisms, when evaluating the origin of pain in CP patients (Figure 1), and it is plausible that the “collective” abdominal pain is a result of a complex interplay of several mechanisms. This novel and multifaceted understanding of pain etiology in CP requires a paradigm shift in pain management. Hence, modern mechanism based pain treatments taking into account especially altered pain processing are likely to increasingly replace traditional invasive therapies. In addition, the improved understanding of pain etiology will likely pave the way for new treatment modalities.

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## Pharmacological pain management in chronic pancreatitis

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### Abstract

Intense abdominal pain is a prominent feature of chronic pancreatitis and its treatment remains a major clinical challenge. Basic studies of pancreatic nerves and experimental human pain research have provided evidence that pain processing is abnormal in these patients and in many cases resembles that seen in neuropathic and chronic pain disorders. An important ultimate outcome of such aberrant pain processing is that once the disease has advanced and the pathophysiological processes are firmly established, the

generation of pain can become self-perpetuating and independent of the initial peripheral nociceptive drive. Consequently, the management of pain by traditional methods based on nociceptive deafferentation (*e.g.*, surgery and visceral nerve blockade) becomes difficult and often ineffective. This novel and improved understanding of pain aetiology requires a paradigm shift in pain management of chronic pancreatitis. Modern mechanism based pain treatments taking into account altered pain processing are likely to increasingly replace invasive therapies targeting the nociceptive source, which should be reserved for special and carefully selected cases. In this review, we offer an overview of the current available pharmacological options for pain management in chronic pancreatitis. In addition, future options for pain management are discussed with special emphasis on personalized pain medicine and multidisciplinary.

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**Key words:** Chronic pancreatitis; Pain; Treatment; Pharmacology; Analgesics; Adjuvant analgesics

**Core tip:** Pharmacological pain management in chronic pancreatitis is complicated and requires a multidisciplinary approach. Identification of risk factors associated with disease progression and evaluation of extra pancreatic causes of pain and complications is essential in all patients. Analgesics are typically titrated according to the World Health Organization ladder principle, but in some situations a top-down approach may be useful to control pain and avoid sensitization of central pain pathways. Adjuvant analgesics and combinations of drugs should be considered at an early stage. Non-encapsulated enzyme therapy, somatostatin-analogues and antioxidants can be considered as supplements to conventional analgesics in special situations.

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## INTRODUCTION

Chronic pancreatitis (CP) remains a major source of morbidity in the Northern Europe, with an annual incidence of approximately 10/100000 inhabitants<sup>[1]</sup>. It is a disease characterised by progressive destruction of the pancreatic gland and is typically characterised by severe abdominal pain. As the disease evolves, significant impairment of exocrine as well as endocrine functions also become evident<sup>[2,3]</sup>. The aetiological risk-factors associated with CP are multiple and involve both genetic and environmental factors. In the Northern Europe, excessive alcohol consumption is the leading cause of CP, although genetic susceptibility is also recognised as playing an increasing role<sup>[1,4]</sup>.

From the perspective of the patients (and their doctors) pain is the most significant symptom in CP, and most patients develop chronic pain during the course of their disease. The classic description of the pain is that of a constant, severe, dull ache in the mid-epigastrium, which often radiates to the back. It is typically worsened by high-fat foods, and pain attacks may last for days. However, just as the disease has different causes and morphological expressions, this classic pain pattern is not universal, and the location, character and quality of pain can be quite variable<sup>[2]</sup>. Furthermore, pain has been associated with malnutrition, narcotic addiction, physical and emotional disability, and major socioeconomic problems. Consequently, the clinical evaluation of pain is often blurred by addiction to alcohol and narcotic analgesics as well as by the personality disorders underlying these dependencies<sup>[3,5]</sup>. In view of this complex clinical presentation, it is not surprising that treatment of pain in patients with CP is challenging and often unsuccessful<sup>[6]</sup>.

The aim of this review is to summarise current available pharmacological therapies for pain in CP. In addition, future options for pain management are discussed with special emphasis on personalized pain medicine and multidisciplinary.

## OVERVIEW OF PAIN MECHANISMS IN CP

A detailed overview of the complex pain mechanisms underlying pain in CP is beyond the scope of this review and provided elsewhere in this issue of the journal. It is important to emphasise that none of the current acknowledged theories are mutually exclusive, and it is most likely that several pain mechanisms act in concert to cause pain in the individual patient.

Historically, the focus of pain treatment has been on

the pancreatic gland as a nociceptive source, based on the assumption that pain is generated by local pathology within or in close proximity to the pancreas. This mechanistic understanding of pain, has for many years, been the most widely accepted theory regarding the origin of pain in CP<sup>[7]</sup>. However, there is no direct relationship between the presence of pancreatic pathology such as duct dilation, pancreatic duct stones, pancreatic duct strictures, etc. and abdominal pain in CP pain patients<sup>[8-11]</sup>. Furthermore, the experimental evidence supporting this theory is sparse and findings have been conflicting<sup>[12]</sup>.

On the contrary, many current theories of the pathophysiology of CP postulate that in a high number of cases, repeated episodes of inflammation and pancreatic injury drive the process within the gland towards irreversible injury and are associated with damage to the pancreatic nerves<sup>[7,13,14]</sup>. Key in this theory is the recognition that the resulting ongoing and aggressive nociceptive input is likely associated with altered function of the pain processing system, particularly at the central level<sup>[15-18]</sup>. An important ultimate outcome of such aberrant pain processing is that once the disease has advanced and the pathophysiological processes are firmly established, the generation of pain can become self-perpetuating and independent of the initial peripheral nociceptive drive<sup>[19,20]</sup>. Consequently, the management of pain by traditional methods based on nociceptive deafferentation (*e.g.*, surgery and visceral nerve blockade) becomes difficult and ineffective<sup>[20]</sup>. This novel and improved understanding of pain aetiology requires a paradigm shift in pain management of CP. Hence, modern mechanism based pain treatments taking into account altered pain processing are likely to increasingly replace invasive therapies targeting the nociceptive source, which should be reserved for special and carefully selected cases.

## RISK-FACTOR MODIFICATION AND PROPHYLAXIS

The risk-factors associated with CP can be classified according to the MANNHEIM risk-factor classification system<sup>[4]</sup>. In this system, the multiple (M) risk factors underlying CP are categorised into six major subcategories of alcohol consumption (A), nicotine consumption (N), nutritional factors (N), hereditary factors (H), efferent pancreatic duct factors (E), immunological factors (I), and various rare miscellaneous and metabolic (M) factors. The rationale for modifying these risk-factors is to reduce recurrent injury to the pancreas. Hence, with repeated episodes of acute inflammation triggered by one or more risk factors, the inflammatory environment within the pancreas shifts towards chronic inflammation, with subsequent activation of pancreatic stellate cells, fibrinogenesis, and irreversible pancreatic damage<sup>[21]</sup>. Although not well established for all risk-factors (see below), it seems likely that prevention of recurrent pancreatitis attacks, clinical or sub-clinical, by risk-factor modification, will translate into a slowing of disease progression, less exo-

**Table 1 Recommended risk factor modifications in chronic pancreatitis according to the MANNHEIM criteria**

Risk factor	Treatment	Comments
Alcohol	Alcohol cessation	Decrease disease progression and may have beneficial effects on pain
Nicotine	Smoking cessation	Decrease disease progression and may have beneficial effects on pain
Nutritional	No specific recommendations	No prospective data
Hereditary	Endoscopic surveillance	Currently no formal evidence, a prospective trial has been initiated
	Pancreatectomy with autolog stem cell transplantation	Preferred strategy in some United States centers
Efferent duct	Endoscopy or surgical interventions	The benefit of intervention is controversial
Immunological	Steroid treatment	Treatment of autoimmune pancreatitis follows guidelines provided in <i>e.g.</i> , Ref. 32
Metabolic	Lipid lowering therapy, parathyroidectomy, <i>etc.</i>	Consider referral to an endocrinologist

**Table 2 Treatment of extrapancreatic causes of pain in chronic pancreatitis**

	Treatment	Comments
Peptic ulcer	Proton pump inhibitor +/- eradication of <i>H. pylori</i>	Avoid NSAIDs in CP Patients
Pseudocysts	Endoscopic drainage, transcutaneous drainage or surgical drainage	Preferred treatment dependent on pseudocyst localization and morphology
Duodenal obstruction	Endoscopic dilation or surgical therapy	Endoscopic dilation preferred as first line therapy
Bile duct obstruction	Covered metal stent or plastic stent	Controversial, one study found no relationship between bile duct obstruction and pain

NSAID: Nonsteroidal anti-inflammatory drugs; CP: Chronic pancreatitis; *H. pylori*: *Helicobacter pylori*.

crine and endocrine insufficiency and most importantly decreased abdominal pain. In Table 1 recommended risk factor modifications are summarized.

In patients with an alcoholic aetiology of CP, there is evidence to support that cessation of alcohol may have beneficial effects on disease progression and pain<sup>[22,23]</sup>. Furthermore, there is increasing evidence that tobacco use is also an important and independent risk factor for CP and that cigarette smoking accelerates progression of alcoholic CP<sup>[24,25]</sup>. Hence, tobacco cessation is highly recommended in these patients, although the association with pain relief has yet to be determined.

Data on the association between nutritional factors and CP are sparse. The consumption of a diet rich in fat and protein was associated with the development of CP in a case-control study<sup>[26]</sup>. However, retrospective descriptions of daily nutritional habits are difficult and such data may be subject to recall bias. Thus, it is difficult to provide a simple description of past daily nutrition in the majority of patients with CP and these findings needs to be confirmed in a prospective trial before specific recommendations can be made.

In patients with CP following gallstone pancreatitis, prevention of recurrent choledocholithiasis is crucial and reduces further damage to the pancreas<sup>[27]</sup>. In this situation cholecystectomy is recommended for patients suitable for surgery<sup>[28]</sup>. Also, patients with recurrent pancreatitis and efferent duct abnormalities such as pancreas divisum may benefit from endoscopic therapy or surgery to decrease the risk of recurrent pancreatitis and progression to CP<sup>[29]</sup>. However, data on this subject are limited and the optimal treatment of this specific entity is still a subject of controversy.

No specific treatment exists to modify the disease progression in hereditary CP. These patients have a sig-

nificantly increased risk of pancreatic cancer and surveillance or even total pancreatectomy with autologous islet-cell transplantation is recommended in some centers<sup>[30]</sup>. Patients with autoimmune pancreatitis comprise a special subset of patients with a potentially curable form of pancreatitis. Management of these patients is beyond the scope of this review and the reader is referred to reference<sup>[31]</sup>.

In CP due to metabolic abnormalities such as hypertriglyceridaemia, maintenance of triglycerides within the normal range would be expected to reduce the chance of repeated pancreatitis attacks and thus progression to CP<sup>[32]</sup>. Also, patients with hypercalcaemia induced pancreatitis due to hyperparathyroidism should be managed appropriately and - if necessary - referred to an endocrinologist.

## TREATMENT OF EXTRA-PANCREATIC CAUSES OF PAIN

In addition to risk factor modifications, extra-pancreatic causes of pain should be thoroughly investigated and treated (Table 2). Peptic ulcers are reported to have an increased prevalence in CP. This is possibly explained by changes in blood flow to the mucosa following attacks of acute pancreatitis as well as deterioration of pancreatic exocrine function resulting in a reduction of bicarbonate concentration and hence acidification of the milieu in the duodenal lumen. Also, increased gastric acid secretion and an increased prevalence of *Helicobacter pylori* in CP have been associated with the increased prevalence of peptic ulcers<sup>[33]</sup>. Another important source of pain in CP is pseudocysts, which should be investigated by an appropriate radiological work-up and treated accordingly<sup>[34]</sup>. Some patients may have pain as a consequence of obstruction of adjacent viscera (duodenum or common

**Table 3** Current available pharmacological treatments for pain in chronic pancreatitis

Pain mechanism	Treatment option(s)	Comments	Ref.
Raised levels of CCK	Pancreatic enzyme replacement therapy	Only non-enteric coated enzymes have proven effective	[57-65]
	Somatostatin-analogues	Conflicting results, prolonged release formulations may be of value	[67,68]
Pancreatic inflammation and oxidative stress	Antioxidants	Conflicting results, probably most valuable in tropical calcifying CP	[71,72]
Central sensitisation	Antidepressants (TCA, SSRI, SNRI)	Expert opinion, no clinical data (Ref.)	[2]
	Gabapentinoids (Gabapentin/Pregabalin)	Modest effect on pain in a randomised placebo controlled trial (Pregabalin)	[42]
	Ketamine	Reverses hyperalgesia in an experimental pain study	[54]
Analgesics	Tramadol <i>vs</i> morphine	No difference in pain relief in a randomised controlled trial, fewer side effects on tramadol treatment	[35]
	Fentanyl <i>vs</i> Morphine	No difference in pain relief in a randomised controlled trial	[41]
	Oxycodone <i>vs</i> Morphine	Oxycodone superior to morphine on experimental pain measures	[39]
	ADL 10-0101:KOR agonist	KOR agonist superior to morphine on experimental and clinical pain measures. Limited number of patients ( <i>n</i> = 6)	[40]

CCK: Cholecystokinin; CP: Chronic pancreatitis; TCA: Tricyclic antidepressant; SSRI: Selective serotonin reuptake inhibitor; SNRI: Serotonin norepinephrine reuptake inhibitor; KOR: Kappa opioid receptor.

bile duct)<sup>[35]</sup>. However, the mechanisms underlying such “obstructive pain” remain unclear and in the case of bile duct obstruction there is evidence to the contrary<sup>[36]</sup>.

## ANALGESICS

The standard guideline for analgesic therapy in CP patients follows the principles of the “pain relief ladder” provided by the World Health Organization (WHO)<sup>[37]</sup>. This principle is based on the serial introduction of drugs with increasing analgesic potency, titrated until pain relief is obtained. However, in patients with a severe and debilitating pain pattern, a more aggressive approach using opioids combined with adjuvant analgesics as first line therapy (*i.e.*, a top-down approach), is useful to control pain and prevent sensitization of central pain pathways. An overview of the current available pharmacological therapies used to treat pain in CP is reported in Table 3.

Paracetamol is usually the preferred drug in level I analgesia due to its limited side effects. It has analgesic and antipyretic activity that work through central and peripheral non-opioid mechanisms, which have not yet been fully characterised<sup>[38]</sup>. Nonsteroidal anti-inflammatory drugs (NSAIDs) are particularly useful for treating musculoskeletal pain and are in general less favourable for visceral pain because of their toxicity to the GI tract<sup>[39]</sup>. Consequently, we recommend avoiding NSAIDs for painful CP.

Codeine is a weak opioid in level II analgesia, but is still associated with the same spectrum of opioid-related side effects seen for stronger opioids, *e.g.*, constipation, nausea, dyspepsia amongst other symptoms involved in opioid-induced bowel dysfunction<sup>[40]</sup>. Tramadol possesses both a weak opioid agonist activity along with an effect on noradrenaline and serotonin uptake in the spinal cord. It has been shown to be more potent than codeine and may be considered as a halfway house between level II and level III analgesics. Tramadol was also shown to be more efficacious than morphine in patients with CP, with fewer gastrointestinal side effects for the same level of

analgesia<sup>[41]</sup>.

Strong opioids, such as morphine, mainly exert their analgesic effects in the central nervous system, although it is now well known that opioid receptors are synthesised in the dorsal root ganglia and transported towards both central and peripheral nerve terminals<sup>[42]</sup>. Several opioid receptors exist, including the  $\mu$ -receptor,  $\delta$ -receptor and the  $\kappa$ -receptor<sup>[43]</sup>. Most clinically available opioids have their primary activity at the  $\mu$ -receptor and have been used widely to treat pain in CP patients<sup>[6]</sup>. However, animal studies have suggested that activation of the  $\kappa$ -receptor may be more efficacious for attenuation of gastrointestinal pain<sup>[44]</sup>. In keeping with these findings, oxycodone (an opioid targeting the  $\mu$ -,  $\delta$ - and  $\kappa$ -receptor) was shown to attenuate experimental visceral pain better than morphine in CP patients<sup>[45]</sup>. Also, in a pilot study including six CP patients with chronic abdominal pain, infusion of a peripherally restricted  $\kappa$ -receptor agonist (ADL 10-0101) - but not placebo - reduced clinical and experimental pain scores<sup>[46]</sup>. These findings were not replicated in patients with pain due to pancreatic cancer<sup>[47]</sup>, but this may relate to the confounders associated with clinical studies on opioids<sup>[48]</sup>. Taken together, these findings may suggest differentiated effects of opioids for pain management in CP patients. However, it must be emphasized that data from well-designed clinical studies with long-term follow-up are not yet available.

Opioids used in the outpatient clinic can be administered either orally (*i.e.*, tablets) or transdermally (*i.e.*, patch formulation). In an open label randomized crossover trial, transdermal fentanyl plaster was compared to sustained release morphine tablets in an equipotent dosage regime<sup>[49]</sup>. No significant differences were found for pain control, patients' preference or quality of life, while 44% of patients treated with fentanyl plaster reported skin side effects. Taken together with the increased costs of patch formulation, the authors concluded that transdermal administration of opioids cannot be recommended as first line opioid therapy for CP, but should be reserved to patients having trouble with tablet ingestion<sup>[49]</sup>.

As discussed above, CP patients may be suffering from hyperalgesia due to sensitization of the central nervous system<sup>[14,19]</sup>. In general, opioids are not very effective in treating established central sensitization and may even cause hyperalgesia themselves (*i.e.*, opioid induced hyperalgesia)<sup>[50]</sup>. Furthermore, opioid induced bowel dysfunction is a common problem in clinical practice and typically manifests as abdominal discomfort or even diffuse abdominal pain<sup>[40]</sup>. Taken together, opioid based therapies often become ineffective and associated with gastrointestinal side effects in the context of advanced CP and hence other treatments are highly warranted.

### Adjuvant analgesics

Adjuvant analgesics are a heterogeneous group of drugs initially developed for indications other than pain. However, many have proven effective in painful conditions, which has now been widely recognised as a separate therapeutic indication. Adjuvant analgesics modify the nociceptive processes through several modes of action, including anxiolytic effects (benzodiazepines, alpha-2-delta ligands), antidepressive effects (antidepressants), and anti-hyperalgesic effects (antidepressants, alpha-2-delta ligands). Although they have been widely used to treat pain associated with CP, only the alpha-2-delta ligand pregabalin has been studied in the context of painful CP<sup>[2,51]</sup>. Hence, in a placebo controlled double blinded randomized trial, we recently demonstrated the efficacy of pregabalin as an adjuvant analgesic for pain in CP. We found that CP patients treated with pregabalin escalated to a maximal dose of 600 mg *bid* had a significant reduction in self-reported pain scores compared to placebo. Furthermore, the percentage of patients with much or very much improved health status score was higher in the pregabalin group compared to the placebo group. The side effects were relatively few and of mild to moderate severity; with a “drunk feeling” being the most prevalent side effect (35% of patients) and typically showing a ceiling effect after one or two weeks of treatment<sup>[51]</sup>.

The analgesic mechanisms of action underlying pregabalin analgesia are not completely understood, and it probably exerts a range of effects on pain transmission<sup>[52,53]</sup>. *In vitro* studies indicate that pregabalin binds selectively to the alpha-2-delta subunit of voltage-dependent calcium channels, thereby blocking the influx of calcium into pre-synaptic nerve terminals. This reduces release of excitatory neurotransmitters, including glutamate, noradrenalin and substance P, and dampens pain transmission<sup>[54,55]</sup>. These findings translate well to experimental pain studies in CP, where antinociceptive effects of pregabalin on electrical evoked pain from the gut and skin were observed, compatible with a reduction of central sensitization<sup>[56,57]</sup>.

### Ketamine

Introduced in 1965 as an anesthetic, today ketamine is used not only for anesthesia, but also as a potent analgesic in acute and chronic pain as well as an antihyperalgesic

used to reduce central sensitization in various chronic pain conditions. It is a noncompetitive *N*-Methyl-*D*-aspartate (NMDA) receptor antagonist, but it also exerts its analgesic effects through other mechanisms including opioid receptor activation<sup>[58]</sup>. Sensitization of the central nervous system has been documented in several studies of painful CP and is believed to play a prominent role in pain generation in this entity<sup>[15,16]</sup>. One of the best-characterized mechanisms in the early phase of central sensitization is activation of the NMDA receptors<sup>[59]</sup>. Multiple studies have consistently produced positive results regarding the use of ketamine in chronic pain patients with central sensitization and hyperalgesia and it thus comprises an interesting remedy to revert reduce central sensitization and its associated hyperalgesia in CP<sup>[60]</sup>. This was supported by a double-blinded crossover trial designed to evaluate the effect of ketamine infusion on hyperalgesia associated with CP<sup>[61]</sup>. Infusion of ketamine temporarily reversed pressure pain hyperalgesia and the underlying sensitized state of the pain system. However, only short-term effects were evaluated and no effect was seen on clinical endpoints. Hence, the use of ketamine for pain in CP is still in its infancy and prospective clinical trials are warranted to establish its role in the management of painful CP.

## SUPPLEMENTARY THERAPIES

### Pancreatic enzyme therapy

Pancreatic enzyme therapy for pain control in CP has been the subject of several randomized trials and meta-analyses (Table 3). The proposed mechanism of action is the ability to degrade cholecystokinin (CCK) releasing factor in the duodenum and thereby lower CCK<sup>[2]</sup>. An elevated level of CCK have been reported in CP patients and may generate pain by increasing the pressure in the pancreatic duct (CCK-A), but also through direct activation of nociceptive pathways in the central nervous system (CCK-B)<sup>[62,63]</sup>. Only non-enteric coated formulations have duodenal protease activity and studies using this type of enzymes have documented improvement in pain<sup>[64,65]</sup>. In contrast, most studies using enteric coated preparations (which are not active in the duodenum and hence cannot degrade CCK-releasing factor) have not shown any improvement on pain measures<sup>[66-69]</sup>. One study, however, showed pain relief of enteric coated enzymes during acid inhibition, but this study used a measurement of pain that included symptoms of malabsorption (bloating, gas or cramping), rather than more traditional pain measures<sup>[70]</sup>. A meta-analysis combining all studies found no effect of enzymes on pain relief in CP<sup>[71]</sup>. Nevertheless, combining the two types of enzyme formulations in a meta-analysis is probably not appropriate given the proposed mechanism of action<sup>[72]</sup>.

### Somastotatin-analogues

Somastotatin-analogue inhibits pancreatic secretion by blocking CCK and secretin release and also by a direct



inhibitory effect on acinar cells<sup>[73]</sup>. As discussed above, these effects may alleviate pain through reduction of pancreatic ductal pressure and by lowering the central effects of CCK. There are conflicting data about the efficacy of somatostatin-analogues for pain in CP. While early pilot series of octreotide showed an effect on pain control, this effect could not be confirmed in a double-blind cross-over study enrolling 10 CP patients treated with octreotide (100 µg *tid*) or placebo for 3 d<sup>[74]</sup>. Although pancreatic secretion measured by fecal chymotrypsin was reduced by octreotide, no differences were seen in pain control or analgesic use. This study has been criticized for its relatively short follow-up and limited wash out period (48 h). Also, four patients had evidence of concretions in the pancreatic duct, which may have compromised the effect of octreotide. In a later pilot study, a long-acting version of octreotide (Octreotide LAR) administered once monthly, was compared to conventional subcutaneous octreotide treatment administered three times daily. Although not significant, there was a trend toward improved pain control for octreotide LAR<sup>[75]</sup>. These results, however, have never been subject to a formal placebo controlled trial and the role of octreotide treatment for painful CP has so far not been satisfactorily documented. Taken together with the numerous side effects and their cost, a general use of somatostatin-analogues for pain in CP cannot be recommended<sup>[76]</sup>.

### Antioxidants

The use of antioxidants for pain control in CP was presented two decades ago, but never gained widely clinical popularity. The proposed analgesic mechanism of action underlying this therapy is an anti-inflammatory and blocking effect on free radicals<sup>[77]</sup>. Propelled by an Indian randomized placebo controlled trial, antioxidant therapy recently had a rebirth for pain management in CP. In this trial, six months antioxidant therapy was associated with significant and prolonged pain relief compared to placebo<sup>[78]</sup>. However, these findings were not reproduced by a subsequent study from North America<sup>[79]</sup>. A possible explanation for this dichotomy may be that the patients included in the two trials were different. While the Indian study mostly included patients with trophic calcifying pancreatitis and malnutrition (and hence deficiency in antioxidants), the American study included a more elderly population who had alcohol as the leading etiology of CP and a normal nutritional condition. Hence, the efficacy of antioxidant therapy may be related to the etiology of CP and its associated malnutrition<sup>[80]</sup>. This idea was supported by a subgroup analysis of the patients with alcohol etiology in the Indian trial, who, in agreement with the American study, demonstrated no benefit of antioxidants<sup>[78]</sup>. Taken together, the evidence is not sufficient to recommend antioxidant therapy be used routinely for the typical Western CP patient with alcoholic pancreatitis.

### Other treatments

In addition to the abovementioned treatment options, various other pharmacological principles have been used

to treat pain associated with CP, including leukotriene antagonism and stimulation with secretin<sup>[81,82]</sup>. However, none of these treatments have documented any effect on pain and are regarded obsolete by most experts.

## INDIVIDUALISED PAIN THERAPY AND FUTURE ANALGESICS FOR PAIN IN CHRONIC PANCREATITIS

A major problem in pain medicine is the lack of knowledge about which treatment suits a specific patient. In a recent study, we tested the ability of quantitative sensory testing to predict the analgesic effect of pregabalin and placebo in patients with CP<sup>[83]</sup>. Pregabalin effect was associated with pretreatment sensitivity to electric tetanic stimulation of the upper abdominal area (sharing spinal segmental innervation with the pancreatic gland). Hence, patients expressing lower pain thresholds in the “pancreatic viscerotome” were more likely to benefit from pregabalin treatment compared to patients with normal sensitivity<sup>[83]</sup>. These findings suggest sensitization of spinal neurons in the segment innervated by pancreatic visceral afferents to be an important predictor of pregabalin efficacy in patients with painful CP. Interestingly, this method may be used to tailor pain medication based on patient's individual sensory profile and thus comprises a significant step towards personalized pain medicine.

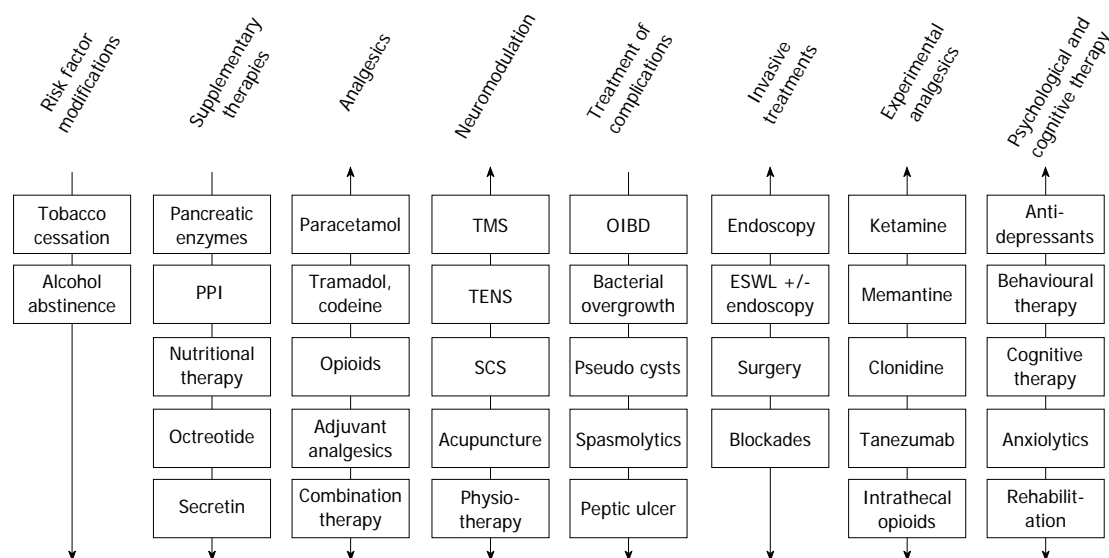
The novel and improved understanding of pain mechanisms in CP may pave the way for new treatments. Analgesics specifically targeting neural or humoral mediators of pain, such as nerve growth factor (NGF) and transient receptor potential vanilloid-1 antagonists, are currently being tested in clinical trials and hold promise for the future, although these drugs have yet to be tested in patients with CP<sup>[84,85]</sup>. Recently, a NGF-antagonist (Tanezumab) was shown to relieve pain in patients with knee pain due to gonarthrosis<sup>[84]</sup>. As NGF has been shown to be up-regulated in CP patients and is known to play a pivotal role in the process of peripheral sensitization, NGF-antagonism may be effective for pain relief in CP patients<sup>[86]</sup>.

### Multidisciplinary pain treatment

As discussed above the mechanisms underlying pain in CP are highly variable in the individual patient. Consequently, there is no single approach that is effective for all patients and choosing the right algorithm for pain treatment is highly depending of the pathogenesis of pain in the individual situation. Hence, a successful management of pain requires a multidisciplinary approach as illustrated in Figure 1. In addition, establishing a stable doctor-patient relationship is an important factor for a successful treatment outcome<sup>[80]</sup>.

## CONCLUSION

Intense abdominal pain is the most prominent feature of CP and its treatment remains a major clinical challenge.



**Figure 1** An illustration of the multidisciplinary approach recommended for managing pain in chronic pancreatitis. The mechanisms underlying pain in chronic pancreatitis (CP) are highly variable in the individual patient and there is no single approach that is effective for all patients. Hence, choosing the right algorithm for the pain treatment is highly depending of the pathogenesis of pain in the individual situation. Some treatments follow a typical step-up approach as indicated by the unidirectional arrows. Other treatments follow either a step-up or a top-down approach depending on the specific situation as illustrated by the bidirectional arrows. The latter is seen for example for analgesic therapies, where weak analgesics may be appropriate to control pain in one situation. On the other hand a more aggressive approach, using opioids combined with adjuvant analgesics as first line therapy (*i.e.*, top-down), is useful in patients with a more aggressive and debilitating pain pattern to control pain and prevent sensitization of central pain pathways. Often combination therapies of *e.g.*, opioids or adjuvant analgesics are used. Surgery, endoscopic therapies *etc.* are included in the figure for completeness, although these therapies are beyond the scope of this review. PPI: Proton pump inhibitor; TENS: Transcutaneous electrical nerve stimulation; SCS: Spinal cord stimulation; OIBD: Opioid-induced bowel dysfunction; TMS: Transcranial magnetic stimulation; ESWL: Extracorporeal shock wave lithotripsy.

Medical management requires a multidisciplinary approach including identification of risk factors associated with disease progression and appropriate modification. A systematic evaluation of extra pancreatic causes of pain and complications followed by appropriate treatments is essential in all patients. Analgesics are typically titrated according to the WHO ladder principle, but in some situations a top-down approach may be useful to control pain and avoid sensitization of central pain pathways. Also, adjuvant analgesics should be considered at an early stage and combinations of drugs are often used. Non-encapsulated enzyme therapy, somatostatin-analogues and antioxidants can be considered as supplements to conventional analgesics in special situations. An improved understanding of pain mechanisms in CP will undoubtedly pave the way for new treatments and future strategies should be based on modern mechanism based and personalized pain treatment.

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## Pharmacological challenges in chronic pancreatitis

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### Abstract

Drug absorption in patients with chronic pancreatitis might be affected by the pathophysiology of the disease. The exocrine pancreatic insufficiency is associated with changes in gastrointestinal intraluminal pH, motility disorder, bacterial overgrowth and changed pancreatic gland secretion. Together these factors can result in malabsorption and may also affect the efficacy of pharmacological intervention. The lifestyle of chronic pancreatitis patients may also contribute to gastrointestinal changes. Many patients limit their food intake because of the pain caused by eating and in some cases food intake is more or less substituted with alcohol, tobacco and coffee. Alcohol and drug interaction are known to influence the pharmacokinetics by altering either drug absorption or by affecting liver metabolism. Since patients suffering from chronic pancreatitis experience severe pain, opioids are often prescribed as pain treatment. Opioids have intrinsic effects on gastrointestinal motility and hence can modify the absorption

of other drugs taken at the same time. Furthermore, the increased fluid absorption caused by opioids will decrease water available for drug dissolution and may hereby affect absorption of the drug. As stated above many factors can influence drug absorption and metabolism in patients with chronic pancreatitis. The factors may not have clinical relevance, but may explain inter-individual variations in responses to a given drug, in patients with chronic pancreatitis.

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**Key words:** Pharmacology; Absorption; Metabolism; Chronic pancreatitis; Treatment

**Core tip:** In patients with chronic pancreatitis several pathophysiological factors can account for malabsorption and may also affect the efficacy of pharmacological intervention by reduced drug absorption. For example it can be speculated that changes in gastrointestinal intraluminal pH, motility disorder, bacterial overgrowth and changed pancreatic gland secretion may contribute. The lifestyle of chronic pancreatitis patients may also be a factor to gastrointestinal changes. The factors may not have clinical relevance, but may explain inter-individual variations in responses to a given drug, in patients with chronic pancreatitis.

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### INTRODUCTION

Chronic pancreatitis is a persistent inflammation of the pancreas that results in irreversible morphological

changes and impairment of both exocrine and endocrine functions<sup>[1]</sup>. The etiology of chronic pancreatitis is multifactorial and the risk factors include alcohol and nicotine consumption, hereditary factors, efferent duct obstructions, immunological factors or rare metabolic disorders<sup>[2]</sup>. It is well known that pancreatitis patients suffer from malabsorption<sup>[3-5]</sup> and it could be hypothesized that this would also affect drug absorption.

Drug absorption in patients with gastrointestinal disorders can be influenced by alterations in several factors. For example; gastric and intestinal motility, changes in the mucosal surface area available for drug absorption, and altered physical and chemical properties of the intestinal luminal content. These properties are usually changed in combination and the degree of each factor impact is dependent on the duration and severity of the disease<sup>[6]</sup>. Despite this, most of the data about bioavailability of orally administered drugs are obtained from healthy individuals.

The knowledge about drug absorption in patients with chronic pancreatitis is limited. It has been demonstrated that the pharmacokinetic profile of pregabalin was not extensively affected by chronic pancreatitis<sup>[7]</sup>. However, inter-individual variations were found and several factors affecting drug pharmacokinetic profiles in patients with chronic pancreatitis may be relevant to consider.

This aim of this review was therefore to evaluate different factors which could possibly affect drug absorption in chronic pancreatitis patients leading to pharmacological challenges in this patient group. Moreover, suggestions on how to diminish the impact of these factors will be provided.

## GASTROINTESTINAL PHYSIOLOGICAL CHANGES THAT MAY AFFECT DRUG EFFECTS

Two primary factors influencing bioavailability of orally administered medications is the amount of drug absorbed, and metabolism by the liver. Hence, any factor influencing the gastrointestinal tract can alter drug absorption, such as gastric pH, regional blood flow, mucosal surface area and gut motility<sup>[8]</sup>.

### Drug absorption

Chronic pancreatitis is a clinical condition in which exocrine pancreatic insufficiency occur leading to secondary maldigestion. Several causes of exocrine pancreatic insufficiency may be associated with changes in gastrointestinal physiology such as: (1) changes in gastrointestinal intraluminal pH; (2) motility disorders; (3) bacterial overgrowth; and (4) pancreatic secretion.

Together these factors can result in malabsorption and may also affect the efficacy of pharmacological treatment in exocrine pancreatic insufficiency<sup>[8]</sup>. The main clinical manifestation of exocrine pancreatic insufficiency

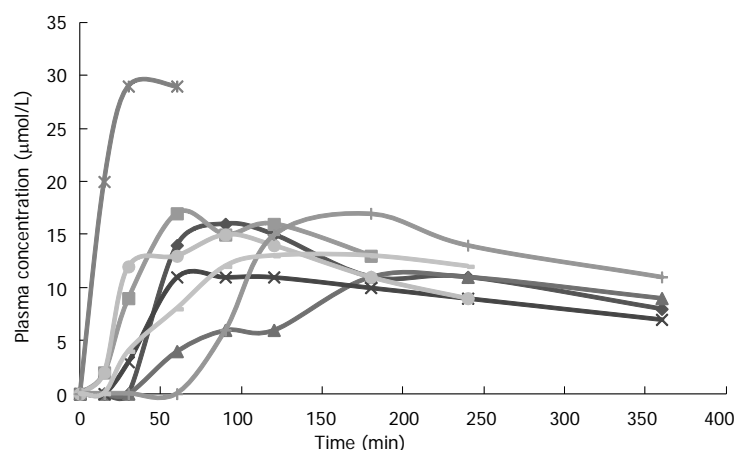
is fat malabsorption, identified as steatorrhea<sup>[4]</sup>. Steatorrhea leads to deficit of fat-soluble vitamins (A, D, E and K) with consequent clinical manifestations<sup>[4]</sup>. Moreover, fat malabsorption may affect absorption of lipophilic drug formulations and as such deserves particular attention<sup>[6]</sup>.

Changes in intraluminal pH: Low intraluminal pH in the upper small intestine might be a factor in the pathogenesis of fecal loss of bile acids in pancreatic insufficiency<sup>[4]</sup>. The drug's ability to cross membranes is determined by the environmental pH and the acid dissociation constant (pKa). Therefore, decreased intraluminal pH may affect drug absorption. However, this may be of variable importance, as changes in intraluminal pH might be negligible in some cases and more pronounced in others.

**Motility disorders:** The rate of gastric emptying and intestinal transit is abnormal in patients with chronic pancreatitis and this may affect the efficacy of treatments<sup>[8]</sup>. Gastric emptying can be accelerated due to diarrhea<sup>[4]</sup> or decreased by, *e.g.*, opioids. Because most drugs are absorbed through the small intestines, delayed gastric emptying will prolong the time to peak concentration and delay the onset of the action of a drug<sup>[9]</sup>. Clinicians should be aware that delayed gastric emptying can delay the onset of action of a medication if administered orally<sup>[9]</sup>. On the other hand accelerated gastric emptying will have the opposite effect. Taken together gastric emptying is likely to be a rate limiting step in drug absorption unless normal absorption (prolonged release) is slow<sup>[10]</sup>.

**Bacterial overgrowth:** One mechanism which has been hypothesized between maldigestion and intestinal alterations relates to bacterial overgrowth in the small intestine. It has been speculated that lack of coordination between motor activity and peak of secretory activity in the gastrointestinal tract may reduce the effectiveness of the "housekeeper" function and thereby contribute to the intestinal bacterial overgrowth often observed in patients with chronic pancreatitis<sup>[11]</sup>. Bacterial overgrowth might either contribute to diarrhea or account for the persistence of diarrhea<sup>[4]</sup>. Moreover, bacterial overgrowth might give rise to bile acid malabsorption and changes in intestinal permeability<sup>[4,12]</sup>. Bacterial overgrowth may interfere with the normal intestinal environment and lead to atrophic mucosa with structural abnormalities which can decrease drug absorption<sup>[13]</sup>.

**Pancreatic gland secretion:** The pancreatic gland normally secretes more than 2 L of juice per day (high protein content) with enzymes able to digest lipids, proteins and carbohydrates. Patients with exocrine pancreatic insufficiency exhibit decreased pancreatic bicarbonate secretion resulting in reduction in duodenal pH postprandial, leading to inactivation of orally administered exogenous enzymes<sup>[6]</sup>. In the same line acidic inactivation of pancreatic enzymes is considered a significant reason for



**Figure 1** Absorption of pregabalin in eight female patients with chronic pancreatitis. Curves illustrate the variance in absorption of pregabalin after 75 mg pregabalin (oral capsule). Especially one patient varied from other subjects by having increased plasma concentration of pregabalin. Not a single cause could explain this outlier, but it could be a combination of several factors suggested.

failure of drug therapy<sup>[8]</sup>.

A combination of the above mentioned factors may affect drug absorption in patients with chronic pancreatitis. Figure 1 illustrates the variance in absorption of pregabalin in a group of women with chronic pancreatitis.

### Drug elimination

To our knowledge only one study investigated hepatic drug metabolism in patients with chronic pancreatitis and found reduced drug elimination capacity in this group of patients<sup>[14]</sup>. The results were most probably explained by the patients' general state, with a fairly overt malnutrition, although theoretically, a subclinical, probably alcohol-induced, liver affection could not be ruled out<sup>[14]</sup>. Interestingly, the study results yielded the need for caution upon administration of drugs that are biotransformed in the liver.

Thus, to optimize the efficacy of pharmaceutical treatment, the management of exocrine pancreatic insufficiency should ideally be individually tailored to account for both the underlying cause and any associated disturbance in gastrointestinal physiology.

### Lifestyle

The gastrointestinal physiological changes in chronic pancreatitis can be further affected by patients' lifestyle. Many patients limit their food intake because of the pain caused by eating and in some cases food intake is more or less substituted with tobacco and coffee<sup>[15]</sup>. Due to malabsorption and lifestyle factors, chronic pancreatitis patients are likely to have a lower body mass index than those not suffering from this disease<sup>[15]</sup>. Thus, body mass index is an easy accessible but important factor to consider in dose decision in these patients, as drug disposition might be affected. This is especially important when drugs with a narrow therapeutic index, such as, *e.g.*, warfarin and digoxin are prescribed<sup>[14]</sup>.

Furthermore, decreased food intake, could be an important factor in several cases of chronic pancreatitis as food intake will affect some of the physiological processes, *e.g.*, changes in pH, reduced gastric emptying time, increased gall secretion, increased motility, increased gastrointestinal and liver blood flow. Most of these pro-

cesses are already affected by the pancreatitis and hence these changes could possibly affect drug bioavailability. Consequently, reduced food intake in itself will worsen gastrointestinal physiological processes.

Insufficient food intake due to nausea, anorexia or alcoholism may also be of some significance<sup>[16]</sup>. Alcohol abuse is a well known etiological factor (alcoholic chronic pancreatitis) and the lifestyle of the alcoholic chronic pancreatitis patient group is in general characterized by excessive alcohol consumption and smoking. Together, these factors can be further accompanied by insufficient food intake and will eventually also lead to malnutrition.

Alcohol can be used as a central nervous system depressant to relieve pain and concerns about the disease. Therefore many patients with chronic pancreatitis continue their alcohol consumption throughout disease progression. In relation to alcohol induced chronic pancreatitis it is relevant to consider how the lifestyle with regard to alcohol- and alcohol related dietary habits may provide pharmacologically challenges. The most frequent pharmacological interaction is the combination of alcohol with other depressors of the central nervous system<sup>[17]</sup>. Moreover, alcohol drug interactions can influence the pharmacokinetics of a drug by altering the drug absorption or by affecting the liver metabolism. The effect will vary with, *e.g.*, the amount of alcohol consumed, the nature of the drug, the dosage and how the drug is administered. There are various interactions between alcohol and drugs. Antihistamines, analgesics and antidepressants are examples of drugs which may interact with alcohol.

The liver is the primary site of drug metabolism and the cytochrome P450 mixed-function oxidase enzyme system, is primarily responsible for this process. Alcohol inhibits the oxidation of drugs by cytochrome P450 isoenzyme (CYP2E1). In contrast, chronic alcohol consumption will cause induction of the CYP2E1 leading to increased drug metabolism. Previously, concerns have been raised regarding interactions of paracetamol and alcohol. However, paracetamol interaction is only a problem when ingestion of alcohol is suddenly stopped. Consequently, the hepatic glutathione is unable to detoxify which leads to irreversible hepatic damage. Therefore, in chronic alco-



**Table 1 Pathophysiological and lifestyle related effects on drug absorption**

Pathophysiology	Effect on drug absorption
Low intraluminal pH	The drugs ability to cross the luminal wall
Motility disorder	Delayed drug effect
Bacterial overgrowth	Decreased absorption due to diarrhea or structural abnormalities
Pancreatic secretion	Lack of enzymes leads to inactivation of pro-drugs
Steatorrhea	Problems with absorption of lipophilic drugs
Low body mass index	Increased plasma concentration

holic patients the consumption of alcohol should not be suspended on prescribing paracetamol<sup>[17]</sup>. Additionally, extensive alcohol consumption may result in gastric mucosal injury and hereby further affect drug absorption<sup>[8]</sup>.

Smoking may increase hepatic drug metabolism to a significant extent<sup>[14]</sup> and may in addition result in induction of the cytochrome oxidases<sup>[18]</sup>. It has also been demonstrated that body mass index is associated with cigarette smoking in chronic alcoholic-associated pancreatitis patients; the more cigarettes smoked per day, the lower the mean body mass index<sup>[19]</sup>. This is in line with the hypothesis that alcohol consumption and smoking can cause insufficient food intake and hereby affecting drug absorptions.

Abdominal pain is usually the symptom that causes patients with chronic pancreatitis to seek medical attention. In parallel, they can have a history of alcoholic abuse making opioids, with their associated abuse potential together with other side effects as, for example, bowel dysfunction, less suitable for these patients and other treatment regimens should be considered<sup>[7]</sup>. Recently it was demonstrated that pregabalin was superior to placebo for attenuation of experimental visceral pain in chronic pancreatitis patients<sup>[20]</sup>. Therefore, pregabalin may be used to treat pain in patients with chronic pancreatitis when there is a conflict or concern with abuse.

A summary of pathophysiological effects on drug absorption is given in Table 1.

## MANAGEMENT IN CHRONIC PANCREATITIS

The physiological processes affected by chronic pancreatitis are widespread. This section will focus on how to treat the pathophysiology of chronic pancreatitis in order to reduce the impact of factors possibly affecting drug absorption.

### Pharmacological management

**Enzyme therapy:** The main goal with enzyme treatment is to achieve optimal enzyme activity in the duodenum<sup>[21]</sup> and hereby improve the nutritional status, preventing weight loss, vitamin deficiencies and exocrine pancreatic insufficiency related symptoms, resulting in steatorrhea. The most widely used enzyme preparation is porcine

pancreatin. The preparation contains a mixture of protease, lipase and amylase<sup>[22]</sup>. Löhr *et al.*<sup>[23]</sup> analyzed the effectiveness of different preparations and concluded that overall pancreatin preparation replacements must contain high lipase activity. Lipase of porcine pancreatin is destroyed by protease and acids, thus it is necessary to protect the pancreatin against the influence of gastric acids. Another factor that is of great importance is the particle size and the rate of which the porcine pancreatin is released into the duodenum. The best particle size is assumed to be a diameter of  $\leq 2$  mm, since these particles leaves the stomach at the same time as solid food. The enzymes should be released within 30 min<sup>[24]</sup>. Pancreatic enzyme supplements improve fat absorption<sup>[25]</sup>, and hence reduces steatorrhea<sup>[26]</sup> and this may have beneficial effects on drug absorption. In contrast high-dose enzyme replacement therapy with or without gastric acid suppression may cause additional challenges related to drug absorption and interactions if additional drug therapy is required<sup>[6]</sup>. Thus, enzyme treatment can either enhance or complicate drug absorption in different aspects and this should be considered in the pharmacological management of clinical symptoms.

### Endoscopic therapy or surgical management

More invasive treatment is recommended for patients with pancreatic duct stones and pancreatic obstruction in whom standard medical therapy is not sufficient. The goals of endoscopy and surgery are to decompress ducts, dilate stricture with stent placement and preserve pancreatic tissue and adjacent organs<sup>[27,28]</sup>. Endoscopic therapy should be the first-line option because it is less invasive than surgery<sup>[29]</sup>. Surgery should be the first-line option in patients in whom endoscopic therapy failed or those with pancreatic mass with suspicion of malignancy<sup>[29]</sup>. It has been assumed that invasive procedures designed to improve drainage of the main pancreatic duct, will result in decreased pain. It could therefore be hypothesized that pain attenuation can improve the lifestyle and hereby indirectly affecting drug absorption. There is consensus that endoscopical and surgical management have both benefits and harms<sup>[26]</sup> and it has been suggested that management may include medical, endoscopic and surgical approaches with the interaction between various specialties, calling for a concerted multidisciplinary approach<sup>[28]</sup>. Moreover, as surgery has a low rate of success with attendant morbidity, mortality and slow recovery rates, it is not considered an option for optimizing absorption of drug in the pharmacological management.

### Lifestyle changes

Patients with chronic pancreatitis are often advised to eat small meals with low-fat content ( $< 20$  g of fat) in an attempt to decrease the need of pancreatic secretion<sup>[30]</sup>. Low-fat diets decreases the amount of overall fat presented to the intestine for digestion and absorption, and may be helpful alleviating steatorrhea<sup>[26]</sup> and hereby could lead to better conditions for some drug absorptions.

However, a systematic review of benefits and harms of low-fat diet in chronic pancreatitis found no studies of sufficient quality to confirm the effect of low-fat diet<sup>[31]</sup>. Thus, if people are given pancreatic enzyme supplements, they are usually advised to maintain a normal diet, as there is no need to lower fat intake alongside enzyme supplementation<sup>[31]</sup>.

Overeating is dissuaded, instead smaller meals on a more frequent basis is preferred. Frequent meals will also benefit gastric motility and result in more normal gastrointestinal conditions, and hereby improve drug absorption. The restriction in fat intake should be monitored by a dietitian who follows the total caloric intake and the diet should compensate the loss in caloric intake by carbohydrate-enriched diet. On a carbohydrate-enriched diet 65%-70% of the total daily energy intake should derive from carbohydrate. Hereby, it is possible for the patient to gain weight, which again will be beneficial in several ways, *e.g.*, in relation to drug absorption, where body weight and body composition is directly related to drug distribution volumes.

In general alcohol consumption and smoking should be diminished or avoided to reduce the impact on the pharmacokinetic profiles. Total alcohol abstinence is only recommended for patients whose chronic pancreatitis is derived from alcohol abuse.

## ADDITIONAL GASTROINTESTINAL CHANGES CAUSED BY OPIOIDS

Patients with painful chronic pancreatitis are often treated with opioids which lead to diverse issues and alterations in the gastrointestinal tract. Opioids have intrinsic effects on gastrointestinal motility and can modify the absorption of other drugs taken at the same time<sup>[10]</sup>. It has been demonstrated that, *e.g.*, tramadol is an effective oral opioid analgesic for reducing pain in people with chronic pancreatitis, but it is also associated with gastrointestinal adverse effect<sup>[26]</sup>. Opioids will affect opioid receptors in the enteric nervous system and will cause changes in motility, sphincter function and secretion which affect absorption leading to opioid induced bowel dysfunction<sup>[32]</sup>.

Opioid induced decreased motility occurs throughout the entire gastrointestinal tract. In the circular muscle in the small and large intestine, opioids induce increased resting contractile tone and decreases tonic inhibition of the muscle tone, which leads to increased tone in the circular muscle layer. This is accompanied by occasional occurrence of high-amplitude, non-propulsive phasic contractions enhanced by rhythmic contractions and associated changes in smooth muscle electrical activity. These motility abnormalities result in decreased propulsive forward peristalsis, and increased segmental contraction which in clinical settings manifests as constipation, abdominal cramps and gut spasm<sup>[33-35]</sup>. An additional consequence of this peristaltic disruption is stasis of luminal contents, which leads to increased passive fluid absorption<sup>[36]</sup>. Furthermore, intestinal fluid secretion is inhibited

by opioids directly *via* the enteric nervous system. In the sympathetic nervous system opioids also increase activity and thereby decrease the secretion. Serotonin and nor-adrenaline as terminal transmitters seems to dominate the local effect<sup>[37,38]</sup>. An overall decreased gut secretion of intestinal fluids takes place and together leads to harder and dryer stools<sup>[36-38]</sup>. Therefore, patients treated with opioids might have even more pronounced decreased motility which will complicate drug absorption further. Furthermore, as solid drug forms must dissolve before absorption can occur; dissolution rate determines availability of the drug for absorption. The increased fluid absorption caused by opioid effects will decrease water available for drug dissolution and may hereby affect absorption. If dissolution is slower than absorption it becomes the rate-limiting step.

## CONCLUSION

Several factors might affect drug absorption and metabolism in patients with chronic pancreatitis: gastric pH, regional blood flow, mucosal surface area and gut motility. The impact of these factors on drug absorption may be reduced by treating the pathophysiology of chronic pancreatitis. Treatment can be pharmacological management, enzyme therapy, endoscopic therapy or surgical management. Moreover, as gastrointestinal physiological changes in chronic pancreatitis can be affected by patients' lifestyle, lifestyle changes may lead to more optimal drug absorption. However, issues raised in this review may not have clinical relevance, but could explain part of the variation observed in drug effects in this patient group.

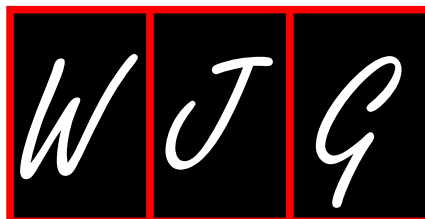
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## Endoscopic management of complications of chronic pancreatitis

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### Abstract

Pseudocysts and biliary obstructions will affect approximately one third of patients with chronic pancreatitis (CP). For CP-related, uncomplicated, pancreatic pseudocysts (PPC), endoscopy is the first-choice therapeutic option. Recent advances have focused on endosonography-guided PPC transmural drainage, which tends to replace the conventional, duodenoscope-based coma immediately approach. Ancillary material is being tested to facilitate the endosonography-guided procedure. In this review, the most adequate techniques depending on PPC characteristics are presented along with supporting evidence. For CP-related biliary obstructions, endoscopy and surgery are valid therapeutic options. Patient co-morbidities (*e.g.*, portal cavernoma) and expected patient compliance to repeat endoscopic procedures are important factors when selecting the most adapted option. Malignancy should be reasonably ruled out before embarking on the endoscopic treatment of presumed CP-related biliary strictures. In endoscopy,

the gold standard technique consists of placing simultaneous, multiple, side-by-side, plastic stents for a one-year period. Fully covered self-expandable metal stents are challenging this method and have provided 50% mid-term success.

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**Key words:** Biliary stricture; Chronic pancreatitis; Pseudocyst; Endoscopic retrograde cholangio-pancreatography; Endoscopic ultrasonography; Stent

**Core tip:** Endoscopy is the first-choice treatment of pancreatic pseudocysts. The transduodenal route may be preferable over the transgastric route. Two transmural double pigtail stents should be left for at least 2 mo. In the case of a disconnected pancreatic tail, secretin-enhanced magnetic resonance pancreatography should be obtained to decide about stent removal. Biliary strictures should be thoroughly investigated to rule out malignancy. To this aim, improved methods of biliary sampling have become available. Even with multiple biliary stents, potentially fatal cholangitis is frequent in the absence of regular stent revision. Fully covered self-expandable metal stents have provided 50% mid-term success.

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### INTRODUCTION

Common local complications of chronic pancreatitis (CP) include pancreatic pseudocysts (PPCs) and bili-



ary obstructions. These two complications develop in patients during the course of CP at a rate of 20%-40% for PPCs and 3%-23% for biliary obstructions<sup>[1,2]</sup>. PPCs consist of a collection of pancreatic juice enclosed by a wall of fibrous granulation tissue, which may arise as a consequence of acute pancreatitis, pancreatic trauma or CP<sup>[3]</sup>. Biliary obstruction may be caused by fibrosis, compression by a PPC or cancer. The present review covers the full spectrum of endoscopic management of local complications of CP; it is not an analysis of specific studies and it does not encompass the management of uncomplicated CP, which has recently been reviewed elsewhere<sup>[4]</sup>.

## SEARCHES

Searches for relevant articles were conducted in Medline through PubMed on May 2013, without time limits, using the following search terms: “pancreatitis, chronic”[MeSH Terms] OR (“pancreatitis”[All Fields] AND “chronic”[All Fields]) OR “chronic pancreatitis”[All Fields] OR (“chronic”[All Fields] AND “pancreatitis”[All Fields]) AND pseudocyst[All Fields] AND (“endoscopy”[MeSH Terms] OR “endoscopy”[All Fields]) OR “pancreatitis, chronic”[MeSH Terms] OR (“pancreatitis”[All Fields] AND “chronic”[All Fields]) OR “chronic pancreatitis”[All Fields] OR (“chronic”[All Fields] AND “pancreatitis”[All Fields]) AND pseudocysts[All Fields] AND (“surgery”[Subheading] OR “surgery”[All Fields] OR “surgical procedures, operative”[MeSH Terms] OR (“surgical”[All Fields] AND “procedures”[All Fields] AND “operative”[All Fields]) OR “operative surgical procedures”[All Fields] OR “surgery”[All Fields] OR “general surgery”[MeSH Terms] OR (“general”[All Fields] AND “surgery”[All Fields]) OR “general surgery”[All Fields]); “pancreatitis, chronic”[MeSH Terms] OR (“pancreatitis”[All Fields] AND “chronic”[All Fields]) OR “chronic pancreatitis”[All Fields] OR (“chronic”[All Fields] AND “pancreatitis”[All Fields]) AND biliary[All Fields] AND (“endoscopy”[MeSH Terms] OR “endoscopy”[All Fields]). Articles written in English were selected for complete review on the basis of the abstract. Additional papers were identified by manually checking the reference lists of the articles selected for review.

## PANCREATIC PSEUDOCYSTS

### *Differential diagnosis of pseudocyst-cystic neoplasm*

Pseudocysts are the most frequent pancreatic fluid collections. The differential diagnosis between PPCs and cystic neoplasms or, less frequently, necrotized tumors, may be difficult in patients who present for the first time with a pancreatic fluid collection. Amongst the various cystic neoplasms that may affect the pancreas, mucinous cystic neoplasms and intraductal papillary mucinous neoplasms harbor a malignant potential-many of which require surgical resection<sup>[5]</sup>. As only a few of the available tests provide a high degree of certainty, a diagnosis is usually

made by analyzing a set of data, including demographic data and clinical history<sup>[6]</sup>, cross-sectional imaging<sup>[7]</sup>, and endosonography-guided sampling of the fluid content and of the wall of the lesion<sup>[8,9]</sup>. Research has recently focused on the identification of new biomarkers and on *in vivo* confocal microscopic examination of the cyst wall through a needle inserted under endosonographic guidance<sup>[10,11]</sup>.

### *Indications for treatment*

Widely accepted indications for PPC treatment include the presence of symptoms such as abdominal pain, gastric outlet obstruction, early satiety, weight loss, jaundice, and infected or enlarging PPC<sup>[12]</sup>. Some authors also recommend treating PPCs in asymptomatic patients to prevent potential PPC-related complications, although these occur only in a minority of patients<sup>[13,14]</sup>. Other such debated indications include compression of major vessels, intracystic hemorrhage, pancreaticopleural fistula, and PPCs with a diameter greater than 5 cm without any regression after more than 6 wk and a cyst wall thickness larger than 5 mm<sup>[15]</sup>. In patients with CP, PPCs rarely resolve spontaneously, particularly if their diameter is greater than 4 cm or if they have developed outside of the pancreas<sup>[16]</sup>.

### *Results: Choosing endoscopic vs surgical treatment*

Endoscopic drainage is recommended as a first-line treatment of accessible uncomplicated PPCs because it provides significantly better results compared to surgery in terms of cost, duration of hospital stay and quality of life up to three months post-procedure, as demonstrated in a small randomized controlled trial (RCT)<sup>[17]</sup>. Reviews of non-comparative historical series of endoscopic and of surgical treatments of PPCs have reported similar results for both modalities in terms of morbidity, with 13% for endoscopic treatments and 16% for surgical treatments. A PPC recurrence rate during long-term follow-up has also been reported of 11% *vs* 10%, respectively, for endoscopic and surgical treatments. An advantage was found in favor of the endoscopic method in terms of mortality (0.2% *vs* 2.5%)<sup>[15,18]</sup>.

Some, but not all, authors have reported that endoscopic PPC drainage yielded higher success rates in the setting of CP *vs* acute pancreatitis. For example, Baron *et al*<sup>[19]</sup> reported resolution of 92% of chronic pseudocysts *vs* 74% of acute pseudocysts in a series of 138 patients while Hookey *et al*<sup>[20]</sup> reported resolution of 94% of chronic pseudocysts *vs* 92% of acute pseudocysts in a series of 116 patients.

A first-line surgical approach is usually adopted if necrosis has not yet liquefied and if treatment cannot be delayed. Endoscopy carries a lower success rate and higher morbidity rate in such instances; the reader is referred to a recent review for the comparison of currently available techniques in this particular indication<sup>[21]</sup>. Pancreatic necrosectomy requires expert endoscopic skill, dedication and adequate patient selection.

### Endoscopic technique

Access route: A direct communication between the PPC and the main pancreatic duct (MPD) may be demonstrated in 40%-66% of all PPCs<sup>[22]</sup>. Such a communication allows drainage of the PPC *via* a stent inserted into the PPC through the papilla ("transpapillary drainage") as opposed to a stent being inserted into the PPC through the digestive wall ("transmural drainage").

No RCT has compared the transpapillary *vs* the transmural drainage route but, in nonrandomized comparative studies, procedure-related morbidity was lower with the transpapillary route (2% *vs* 15%) and long-term success was similar<sup>[20,22,23]</sup>. The transpapillary route is usually reserved for relatively small (diameter < 5 cm) PPCs located in the head or the body of the pancreas.

### "Conventional" endoscopic-guided vs endosonography-guided technique

Endosonography-guided PPC drainage tends to replace the "conventional" endoscopic approach that uses a duodenoscope or, in some cases, a gastroscope. A recent meta-analysis found that the single demonstrated advantage of the endosonography-guided technique is the possibility to drain non-bulging PPCs<sup>[24]</sup>, which represent approximately half of all PPCs<sup>[22]</sup>. The most important limitations of the endosonography-guided technique reside in the thinner diameter of the working channel of the echoendoscope and in the lower maneuverability of the elevator. While the "conventional" approach is relatively standardized, new material is constantly being tested to make endosonography-guided PPC drainage a single-step, reliable procedure. One of the most recent devices allows puncturing, dilating the puncture tract and inserting two guidewires into the PPC without any device exchange. The device is made of a catheter with two balloons, one to anchor it to inside the PPC and the other one to dilate the puncture tract<sup>[25]</sup>.

### Transgastric vs transduodenal transmural route

Some PPCs may be accessed through either the gastric or the duodenal wall. In such cases, the transduodenal route may be preferable as long-term success has been reported more frequently with the transduodenal *vs* the transgastric route (83% *vs* 64%); procedure-related morbidity was 10% with both routes<sup>[26]</sup>. The difference in long-term success may be related to the longer durability of cystoduodenal compared with cystogastric fistulas (the latter ones typically close a few days after stent removal).

### Number and type of stents

Two double pigtail stents are usually inserted for transmural drainage; a naso-cystic catheter may be left in place to rinse the PPC cavity with saline if debris is present. In a large retrospective series, the insertion of a single *vs* multiple stent was independently associated with the failure of endoscopic PPC drainage, defined as severe procedure-related complication or need for another treatment modality<sup>[27]</sup>. In that series, straight stents were used

and they were associated with frequent bleeding (7% of patients, with surgery required in two thirds of them) and stent migration.

### Stenting duration

Enterocystic transmural stents should not be retrieved before PPC resolution and not before at least 2 mo of stenting. This recommendation is mostly based on a RCT that allocated 28 patients (including 15 with CP) who had PPC resolution after transmural drainage to either stent maintenance or early stent retrieval; in the latter allocation group, stent retrieval was performed at a median of 2 mo post stent insertion<sup>[28]</sup>. PPC recurrence was more frequent in the early stent retrieval group (38% *vs* 0%) and, in another, retrospective, series, a stenting duration of 6 wk or less was independently associated with the failure of endoscopic PPC drainage<sup>[27]</sup>.

### Procedure-related complications

Reported figures largely vary from center to center with average morbidity rates of 13% and average mortality rates of 0.3%<sup>[15,29]</sup>. Major complications include hemorrhage, perforation and infection. Most of these can be managed by non-operative means, including endoscopic coagulation, arterial embolization, repeat endoscopic drainage in the case of secondary infection and antibiotics in the case of retroperitoneal perforation. The following measures may help in preventing procedure-related complications:

**Secondary infection:** Although no data on the efficacy of antibiotic prophylaxis for endoscopic PPC drainage are available, antibiotic administration has been recommended immediately before transmural or transpapillary PPC drainage<sup>[30]</sup>. The decision whether to continue antibiotics or not after the procedure should be based on drainage adequacy and on the presence or absence of necrosis<sup>[12]</sup>.

**Bleeding:** Severe bleeding usually arises from dilated arteries or veins. Pseudoaneurysms of the splenic artery may develop in the vicinity of PPCs. Imaging preceding the endoscopic drainage of PPCs should look for pseudoaneurysms and, in the case that one is discovered, have its prophylactic embolization discussed if the transmural route is elected. Extrahepatic portal hypertension develops during the course of CP in 15% or more of patients. It is frequently associated with PPC as well as leading to higher morbidity in patients who undergo pancreatic surgery<sup>[31]</sup>. The endosonography-guided technique of PPC drainage has been recommended in such patients although it has not been demonstrated to decrease the risk of bleeding<sup>[32]</sup>.

### In the case of infected PPC, should the strategy be different?

Primary infection is a rare complication of CP-related PPCs; secondary infection following stent occlusion or

endoscopic attempt at draining pancreatic necrosis is more frequent<sup>[33]</sup>.

Infected PPCs present a thick content that may not drain adequately through one or two thin plastic stents. Traditionally, in such cases, more large-bore stents are inserted together with a nasocystic catheter that is used for PPC irrigation. These additional interventions have resulted in similarly high success rates in patients with infected PPCs as compared with those who present uncomplicated PPC<sup>[20,34]</sup>. As inserting multiple stents plus a nasocystic catheter requires time and may be technically challenging, fully covered self-expandable metal stents (FCSEMSs) seem to be a promising alternative for draining PPCs with a thick content. In a series of 20 patients with an infected PPC that was drained by endosonography-guided FCSEMS transmural insertion alone, clinical success was achieved in 17 patients<sup>[35]</sup>. The authors suggested that using FCSEMSs rather than plastic stents plus nasocystic drains in patients with infected PPCs may decrease the number of endoscopic procedures, increase the final success rate, and reduce the time required for PPC resolution. FCSEMSs specifically designed for PPC drainage have become available from various manufacturers; they present a short length, a large lumen, and a diabolo shape aimed at preventing stent migration<sup>[25,36]</sup>.

#### ***In the case of complete MPD rupture, should the strategy be different?***

If complete MPD rupture occurs, the disconnected pancreatic tail may keep secreting pancreatic juice that, in the absence of effective drainage, will lead to prolonged fluid accumulation. Bridging of complete MPD ruptures should be attempted and a combination of transmural PPC drainage plus a transpapillary stent bridging the MPD rupture should be considered<sup>[37,38]</sup>. The stent should be left in place for a long duration, at least as long as secretin-enhanced magnetic resonance pancreatography demonstrates juice outflow from the disconnected pancreatic tail<sup>[39]</sup>.

## **BILIARY STRICTURES**

### ***Differential diagnosis***

It is of paramount importance to reasonably rule out malignancy before embarking on the endoscopic treatment of presumed CP-related biliary strictures, as such a treatment usually lasts for one year and the course of pancreatic cancer is rapid. Particular attention should be paid to patients who present risk factors for pancreatic cancer; these include patients over 50 years of age, female gender, white race, or an absence of pancreatic calcifications and presence of exocrine insufficiency<sup>[40,41]</sup>. Patients with hereditary pancreatitis present a very high risk of pancreatic cancer.

The accuracy of standard CT scanning and of endosonography for disclosing pancreatic cancer is limited in patients with CP<sup>[42,43]</sup>. Endosonography, supplemented by fine needle aspiration (FNA) plus biliary endoluminal

sampling and assessment of malignancy biomarkers, is part of the standard work-up of a biliary stricture detected in the setting of CP. Other examination modalities such as probe-based endoluminal real-time microscopy are investigational. It should be kept in mind that endosonography-guided FNA is less accurate in the presence than in the absence of CP<sup>[41,44]</sup>, although this decrease in accuracy has been suggested to be confined to a subset of patients who present with obstructive jaundice and a biliary stent<sup>[45]</sup>. Furthermore, in the community, the accuracy of endosonography-guided FNA for diagnosing pancreatic cancer is likely to be much lower than the 90% figure that is widely reported in the literature (latter reports originate from tertiary centers and use per-protocol analysis)<sup>[46,47]</sup>. The technical details of the sampling procedure and of the sample processing are extremely important to reach a high diagnostic accuracy; they have recently been reviewed elsewhere for endosonography-guided FNA and for endoluminal biliary sampling<sup>[48-50]</sup>. Recent improvements in the field of endoluminal biliary sampling include the development of more effective sampling devices and the use of rapid on-site examination for smears as well as for tissue biopsies<sup>[48,51,52]</sup>.

### ***Indications for treatment***

Generally accepted indications for the treatment of CP-related biliary strictures include symptoms such as secondary biliary cirrhosis, biliary stones, progression of biliary stricture, and asymptomatic elevation of serum alkaline phosphatase (greater than 2 or 3 times the upper limit of normal values) or of serum bilirubin or both for longer than one month.

### ***Results: Choosing endoscopic vs surgical treatment***

Guidelines recently issued by the European Society of Gastrointestinal Endoscopy propose that the choice between endoscopic and surgical treatment should rely on local expertise, loco-regional or systemic patient comorbidities and expected patient compliance with repeat endoscopic procedures<sup>[53]</sup>. No strong recommendation could be made about the choice between the endoscopic and the surgical approach to CP-related biliary strictures due to the lack of comparative studies. In conditions different from CP, two comparative nonrandomized studies that included 143 patients with biliary strictures related to a traumatism have found that long-term success was similar (77%-83%) with the endoscopic and the surgical approaches<sup>[54,55]</sup>. However, the endoscopic techniques used in these studies are not current anymore and the endoscopic treatment is more effective in post-traumatic compared with CP-related biliary strictures<sup>[29]</sup>. Another study has compared the endoscopic *vs* the surgical drainage of CP-related biliary strictures; however, surgery was performed in only 6 patients, of whom five also had a pancreatic resection<sup>[56]</sup>.

Patient complications such as portal cavernoma or cirrhosis are often decisive factors in the selection of the endoscopic *vs* the surgical modality. Other factors that

may influence this selection include the expected patient compliance with endoscopic stent exchanges and, less importantly, the presence or absence of pancreatic calcifications. In a retrospective series of 14 patients, only two patients presented for elective stent exchanges scheduled at 3-mo intervals<sup>[57]</sup>. Most patients were admitted with biliary infection due to stent occlusion after the scheduled stent exchange date. Another series that included 29 patients treated with multiple, side-by-side, plastic biliary stents reported the occurrence of at least 20 episodes of cholangitis (in this latter series, stents were exchanged when symptoms of clogging developed). The mean interval between stent exchanges was 6 mo in patients who were alive at the end of follow-up as compared to 22 mo ( $P < 0.05$ ) in the three patients who died during follow-up (two of them from cholangitis)<sup>[58]</sup>. The presence of pancreatic calcifications has been associated with long-term failure of single plastic biliary stenting<sup>[59]</sup> but this factor may be less relevant if simultaneous multiple, side-by-side, plastic stents are used<sup>[60]</sup>.

### Endoscopic technique

**Plastic stents:** If the endoscopic treatment modality is selected, temporary placement of simultaneous multiple, side-by-side, plastic stents is the gold standard amongst the various techniques available. A single nonrandomized series has compared long-term results after temporary placement of single *vs* multiple simultaneous plastic stents; clinical success was reported in 24% *vs* 92% of patients, respectively<sup>[60]</sup>.

From a practical point of view, amongst plastic biliary stents, polyethylene models are recommended because they allow obstruction relief more frequently than Teflon models<sup>[61]</sup>, and the exchange of plastic stents with an increasing number of stents is usually scheduled at 3-mo intervals for a total stenting duration of 12 mo<sup>[58,60,62]</sup>. It has recently been suggested that with multiple, side-by-side, plastic stents, the interval between stent exchanges could be extended<sup>[63]</sup>. However, as mentioned above, special care should be taken regarding the generally poor compliance and poor physical status of patients with alcoholic CP. It is recommended to implement a recall system to care for patients who do not turn up for stent exchanges at scheduled dates.

**Self-expandable metal stents:** “Definitive” insertion of self-expandable metal stents (SEMSs) (*i.e.*, with no intended SEMS removal) for benign biliary strictures has almost been abandoned due to the development of biliary epithelial hyperplasia that leads to late biliary obstruction<sup>[64]</sup>. Recent studies of the endoscopic treatment of CP-related biliary strictures have focused on the temporary placement of covered SEMS with a shift of interest from partially covered to fully covered SEMS designs<sup>[65,66]</sup>. Spontaneous SEMS migration has been the main drawback with FCSEMSs; new stent designs aiming to prevent migration include the adjunction of anchoring fins, the positioning of the stent covering on the internal

side of the SEMS and a flared-ends design. Such SEMSs remain investigational for the treatment of benign biliary strictures.

If FCSEMSs are used to treat benign biliary strictures, a stenting duration  $> 90$  d is recommended as this was independently associated with stricture resolution in a multicenter trial that included 133 patients with benign biliary strictures, 44 of these being CP-related<sup>[67]</sup>. In this trial, stricture resolution at the time of stent removal was reported in 26 (59%, intention-to-treat analysis) patients with CP. Other studies that included more than 10 patients followed up for at least one year after FCSEMS removal showed a success rate of approximately 50%: (1) Perri *et al*<sup>[66]</sup> inserted a Niti-S stent with either a straight or a flared-ends design in 17 patients who had previously received a single plastic stent. Two years following FCSEMS removal, 56% of patients had presented no stricture relapse and had normal liver function tests. The flared-ends design partially prevented FCSEMS migration while all straight FCSEMSs migrated; and (2) Poley *et al*<sup>[68]</sup> inserted a Hanaro prototype FCSEMS in 13 patients who had previously received a single plastic stent; success was reported in 6 (43%) of them.

FCSEMSs currently are the most promising alternative to multiple, side-by-side, plastic biliary stents. Despite their main advantages, *i.e.*, a reduced number of endoscopy procedures and a lower incidence of stent obstruction, FCSEMSs need improvements in their design as well as additional large multicenter trials before they can possibly be recommended as a first-line option for the endoscopic treatment of CP-related biliary strictures.

## OTHER COMPLICATIONS

Other complications of CP include splenic vein thrombosis, pancreatic adenocarcinoma, pancreatic ascites and pleural effusion.

Splenic vein thrombosis is present in approximately 12% of patients with CP and it is usually asymptomatic but may cause bleeding in 7% of patients<sup>[69]</sup>. In the case of bleeding, endoscopic variceal obturation using *N*-butylcyanoacrylate is effective in achieving hemostasis, and a splenectomy is an effective way to prevent recurrent bleeding<sup>[70]</sup>.

For the treatment of pancreatic adenocarcinoma, the endosonography-guided delivery of various cytotoxic agents is a rapidly evolving field that remains investigational<sup>[71]</sup>.

Pancreatic ascites and pleural effusion are rare complications of CP; they may or may not be associated with PPC and they present a high morbidity. The aim of endoscopic therapy in such patients is to insert a stent to bridge the MPD rupture that is responsible for pancreatic juice leakage; high success rates have been reported<sup>[72]</sup>.

## CONCLUSION

Progress has recently been made in the field of the endo-



scopic treatment of CP-related complications.

With regard to uncomplicated PPCs, endoscopic drainage has been shown to be feasible in almost all cases and to be superior to surgical drainage. Techniques associated with the best clinical outcome have been identified. With regard to CP-related biliary obstructions, improvements in the design of FCSEMSs are challenging the standard technique and have the potential to improve patient acceptability.

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## Multimodal treatment of hepatocellular carcinoma on cirrhosis: An update

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### Abstract

Hepatocellular carcinoma (HCC) is the most frequent primary liver tumor, and overall, it is one of the most frequent cancers. The association of HCC with chronic liver disease, and cirrhosis in particular, is well known, making treatment complex and challenging. The treatment of HCC must take into account the presence and stage of chronic liver disease, with the aim of preserving hepatic function that is often already impaired, the stage of HCC and the clinical condition of the patient. The different treatment options include surgical resection, transplantation, local ablation, chemoembolization, radioembolization and molecular targeted therapies; these treatments can be combined in various ways to achieve different goals. Ideally, liver transplantation is best treatment for early stage HCC on cirrhosis because it removes both the tumor and the chronic disease that produced it; however, the application of this powerful tool is limited by the scarcity of donors. Downstaging and bridging are different strategies for the management of HCC patients who will undergo liver transplantation. Several professionals, including

gastroenterologists, radiologists and surgeons, are involved in the choice of the most appropriate treatment for a single case, and a multidisciplinary approach is necessary to optimize the outcome. The purpose of this review is to provide a comprehensive description of the current treatment options for patients with HCC by analyzing the advantages, disadvantages and rationale for their use.

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**Key words:** Hepatocellular carcinoma; Multimodal treatment; Locoregional treatments; Molecular targeted therapies; Liver resection; Liver transplantation

**Core tip:** Hepatocellular carcinoma (HCC) occurs frequently, and its association with cirrhosis makes treatment complex and challenging. The treatment of HCC must take into account the presence and stage of chronic liver disease with the aim of preserving hepatic function that is often already impaired. The different treatment options include surgical resection, transplantation, local ablation, chemoembolization, radioembolization and molecular targeted therapies. Downstaging and bridging are different strategies for the management of HCC patients who will undergo liver transplantation. The purpose of this review is to provide a comprehensive description of the current treatment options for patients with HCC.

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### INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for nearly



90% of the primary liver tumors and is currently the third leading cause of cancer death worldwide. Approximately 500000 new cases of HCC are diagnosed worldwide each year, with a peak incidence observed in countries in which the hepatitis B virus (HBV) is endemic, such as Southeast Asia and sub-Saharan Africa<sup>[1]</sup>. As the number of carriers of chronic liver disease increases, HCC has become a major public health issue.

The main risk factor for HCC is the presence of chronic liver disease, particularly when the disease has already resulted in liver cirrhosis. The constant process of destruction and repair within the parenchyma that is associated with cirrhosis increases hepatocyte metabolism and amplifies the risk of mutations in a multistep progression from hyperplastic nodule to early HCC and finally to moderately/poorly differentiated HCC.

Hepatitis B and C viruses (HBV and HCV) are known to have oncogenic potential, and the risk of HCC in HBV and HCV carriers is increased independently of the presence of cirrhosis<sup>[2,3]</sup>. Malignant transformation of hepatocytes in the infected liver could be caused by chronic inflammation and the oxidative DNA damage that leads to genetic and epigenetic changes. There is also evidence that proteins encoded by HBV and HCV may have a direct role in hepatocarcinogenesis; in fact, proteins encoded in the genome of each of these viruses have been linked to alterations in hepatocyte physiology and hepatocellular signal transduction<sup>[4]</sup>. Other causes of chronic liver diseases, such as alcohol abuse, non-alcoholic steatohepatitis (NASH), hemochromatosis,  $\alpha$ 1-antitrypsin deficiency, autoimmune disease and Wilson disease are associated with a higher risk of developing HCC and require close patient monitoring. In Western countries, obesity and metabolic syndromes associated with type II diabetes, which are strictly related with NASH, are now emerging as new potential predisposing factors for HCC<sup>[1]</sup>.

Among patients with cirrhosis, the cumulative 5-year risk of developing HCC ranges from 5% to 30%, depending on the presence and stage of underlying liver disease, ethnicity, age, sex and duration of the exposure to primary hepatotropic viruses<sup>[1,5]</sup>.

The main peculiarity of HCC is that the treatment of the tumor must take into account the presence and stage of chronic liver disease, with the aim of preserving hepatic function that is often already impaired. A key step in the choice of therapy is therefore the correct assessment of the functional reserve of the liver, which is often more important than the staging of the tumor itself.

Several options are available for the treatment of HCC, and these can often be combined; the choice of treatment and the timing of its administration therefore must be balanced accurately.

The aim of the present review is to provide an update than can be useful in clinical practice for determining the most appropriate treatment for HCC patients.

## HCC SCREENING

The achievement of a curative treatment for HCC de-

pends on the detection of the tumor at an early stage. Once the population at risk is identified, screening of HCC is based on ultrasonography and measurement of serum alpha-fetoprotein (s-AFP) levels.

It is recommended that patients with advanced liver fibrosis (F3) or established cirrhosis undergo a liver ultrasound (US) and serum measurements every 6 mo<sup>[6]</sup>. In these patients, ultrasound has a sensitivity between 58% and 89% and a specificity of 90%<sup>[7]</sup>. The sensitivity of s-AFP measurements is lower and ranges between 25% and 65% when values above 20 ng/mL are considered positive<sup>[1]</sup>. The sensitivity and specificity of s-AFP measurement for the detection of HCC increase proportionally with higher blood s-AFP levels, particularly when the level is above 400 ng/mL.

Once the presence of HCC is confirmed, the s-AFP level can be correlated with tumor stage, particularly with the size and multifocality of the tumor and the presence of microvascular invasion. Overall, a screening strategy that combines abdominal ultrasonography and measurement of s-AFP every 6 mo in patients with cirrhosis can reduce HCC mortality by approximately 40%<sup>[8,9]</sup>.

## HCC DIAGNOSIS

The cirrhotic liver often displays multiple non-neoplastic nodules (regenerative nodules) that result from the chronic inflammatory process. These nodules must be differentiated from HCC, and the diagnostic strategy varies depending on the size of the nodule detected on ultrasound. At present, the most widely used diagnostic algorithm for the diagnosis of HCC is that proposed by the American Association for the Study of Liver Diseases (AASLD)<sup>[10]</sup> (Figure 1, modified by Forner *et al.*<sup>[11]</sup>).

The nature of nodules with a diameter of less than 1 cm cannot be precisely defined during ultrasound, and a follow-up control after 3-4 mo is required. Nodules detected at US with a diameter greater than 10 mm must be further investigated with contrast-enhanced triphasic or quadriphasic computed tomography (CT) imaging or magnetic resonance (MR) imaging. The diagnosis of HCC is based on an arterial hypervascular phase (wash-in) followed by disappearance of the contrast in the venous phase (wash-out)<sup>[12]</sup>.

Recent reports have demonstrated that MR has a higher sensitivity compared with CT<sup>[13]</sup>; however, if the data from the first imaging procedure are not conclusive, confirmation using a different technique is recommended. In cases in which the diagnosis is uncertain, a s-AFP level > 400 ng/mL has a high positive predictive value<sup>[1]</sup>. Histological confirmation through percutaneous liver biopsy should be restricted to those nodules with features on MR or CT that are not typical enough to allow a diagnosis<sup>[14]</sup>. In fact, the histological diagnosis of HCC is complex, requires a great degree of expertise and relies on the assumption that the core of the nodule has been effectively sampled by the small needle that is used for the percutaneous biopsy. The sensitivity and specificity reported for nodules less than 20 mm in diameter is

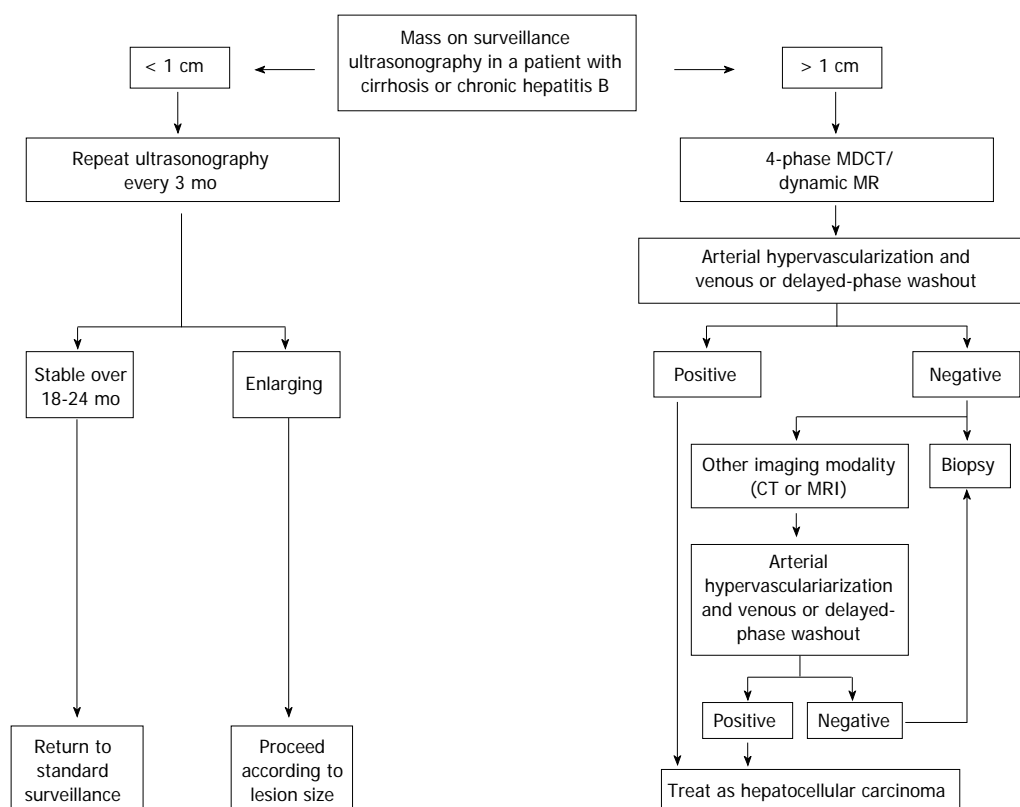


Figure 1 Diagnostic algorithm for hepatocellular carcinoma. Modified by Forner *et al*<sup>[11]</sup>. MDCT: Multidetector computed tomography; MR: Magnetic resonance; CT: Computed tomography; MRI: Magnetic resonance imaging.

60%<sup>[15]</sup>. A risk of tumor seeding in the path of the puncture has been reported in approximately 2.5% of the cases with a median time of development of 17 mo<sup>[16]</sup>.

### HCC staging

The severity of chronic liver disease is usually classified according to the Child-Pugh and model for end-stage liver disease (MELD) scores, and the tumor is staged with the TNM system; in the setting of liver transplantation, the indication for liver replacement is based mainly on the Milan criteria<sup>[17]</sup>. Another staging system that has gained acceptance is the Barcelona Clinic Liver Cancer (BCLC) score, the advantage of which is that it takes into account the characteristics of the tumor as well as the liver function and the general conditions of the patient<sup>[8]</sup> (Figure 2). The BCLC system was developed after a retrospective analysis of several cohort studies on patients with HCC at different stages. This system identifies patients with early HCC who may benefit from curative therapies (stage 0 and A), those at intermediate (stage B) or advanced (stage C) stages who may benefit from palliative treatments and those with a very poor life expectancy (stage D). BCLC is the most commonly used staging system in Europe, and it has been approved by the European Association for the Study of the Liver (EASL) and the AASLD.

### Multidisciplinary management of HCC

Given the complexity of the clinical scenario, the deci-

sion on the most appropriate treatment for a patient with HCC should be made by a multidisciplinary team that includes a hepatologist, hepatobiliary surgeon, transplant surgeon, radiologist and pathologist<sup>[18]</sup>. No single treatment strategy can be applied to all patients, and treatment should be individualized.

In the management of HCC, attention must be focused on the presence and degree of the underlying chronic liver disease at first observation, which will influence the choice of the treatment<sup>[19]</sup>.

Liver resection can be offered to patients with well-preserved liver function; however, the amount of parenchyma that can be removed in carriers of chronic liver disease is inferior to that which is considered as the safety limit in a normal liver (a “future remnant liver” that is  $\geq 30\%$  of the hepatic volume is generally considered acceptable)<sup>[19]</sup>. Despite a normal liver function, the regenerative potential of a liver that harbors a chronic disease can be surprisingly low.

In cases of impaired liver function, non-surgical procedures or liver transplantation (LT) can be offered; in this setting, the number, size and location of the nodules determine the choice of treatment.

Surgical resection, transplantation and ablation are the treatments that offer the highest rates of complete response and are therefore considered as curative<sup>[10]</sup>. There are no randomized trials comparing the efficacy of these three approaches, and all evidence is based on the rate of cure reported in different series.

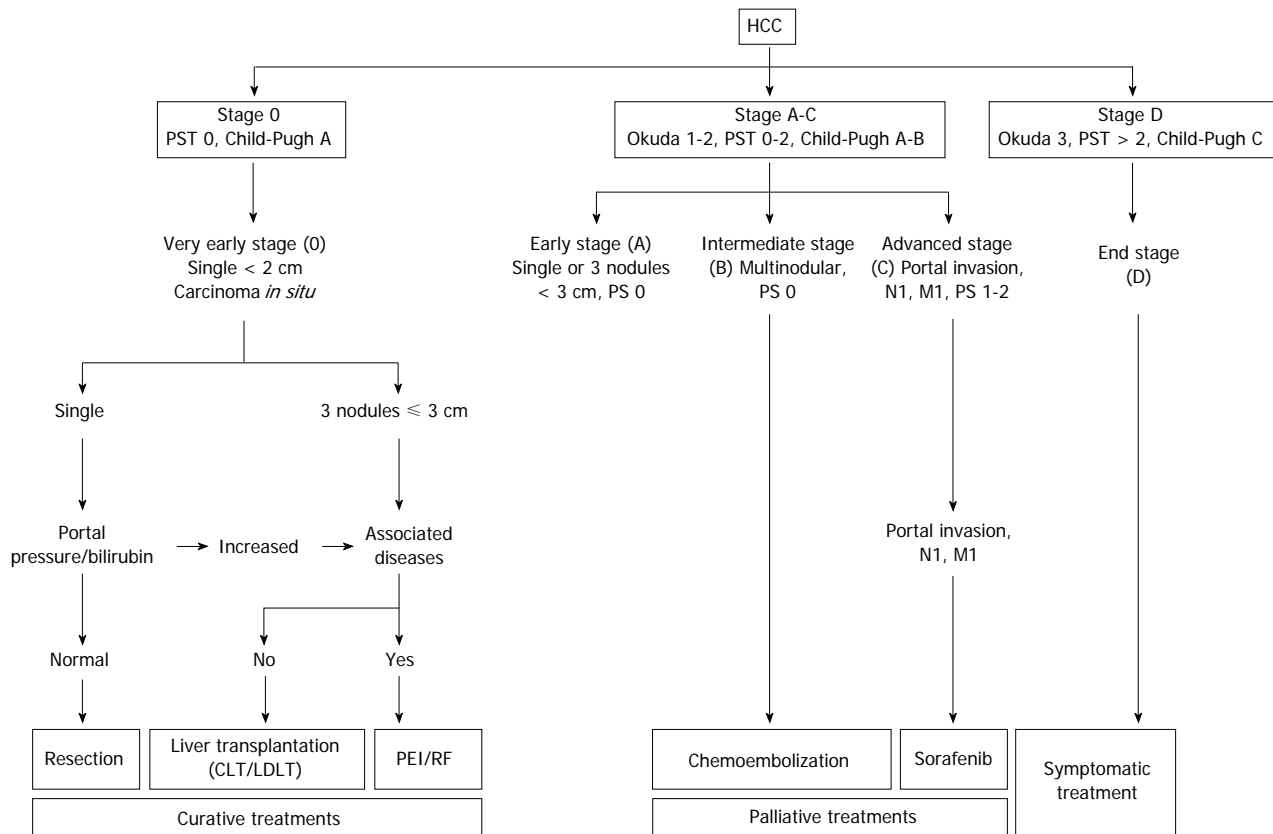


Figure 2 The Barcelona Clinic Liver Cancer staging system and treatment allocation. Copyright © 2010, American Association for the Study of Liver Diseases. CLT: Cadaveric liver transplantation; HCC: Hepatocellular carcinoma; LDLT: Living donor liver transplantation; PEI: Percutaneous ethanol injection; RF: Radiofrequency; PST: Performance status.

Transarterial chemoembolization (TACE) is the least invasive approach; however, it cannot be considered curative.

Ideally, LT is best treatment for early stage HCC on cirrhosis because it removes both the tumor and the chronic disease that has produced it. However, the application of this powerful tool is limited by the scarcity of donors, which results in strict patient selection criteria to optimize the results.

Candidates for LT for whom a long waiting time (> 6 mo) is predicted may be offered resection, local ablation or transarterial chemoembolization as a 'bridge' to transplantation to minimize the risk of tumor progression while they are on the waiting list<sup>[20]</sup>. The same procedures can also be utilized in attempts downstage tumors that are beyond the eligibility criteria for LT at the time of diagnosis.

Yu *et al*<sup>[21]</sup> performed a study on HCC patients who exceeded the University of California San Francisco (UCSF) criteria for LT; these patients were downstaged to fit the UCSF criteria using locoregional therapy and finally underwent LT. Patients who were successfully downstaged prior to transplantation had tumor-free and overall survival rates similar to those observed in patients who met the criteria from the beginning.

A 5-year survival comparable to that of "within criteria" HCC patients can be achieved using LT after successful downstaging. Successful downstaging should include tumor size, number of viable tumors and s-αFP

concentrations before and after downstaging. Then, a minimum observation period of 3 mo is recommended before considering LT<sup>[22]</sup>.

If a patient's hepatic function allows it, liver resection can be offered prior to future transplantation by pursuing two different strategies: first, resection can be used as the primary therapy with LT offered as a rescue therapy should the patient develop tumor recurrence or postoperative liver failure (salvage transplantation); second, resection can be performed on patients with a high risk of tumor progression while awaiting transplantation (bridge to transplantation)<sup>[23,24]</sup>.

## SURGICAL RESECTION

When performed in specialized centers, hepatic resection (HR) can be highly effective, with 5-year overall survival rates well above 50% in the major series<sup>[25]</sup>. Resection is the recommended treatment for patients without advanced fibrosis as long as an R0 resection can be performed with a low risk of postoperative liver failure<sup>[14]</sup>.

The elements that must be taken into account when considering resection of the cirrhotic liver are the Child-Pugh and MELD scores of the underlying liver disease, the degree of portal hypertension and the extension of the parenchymal excision required to obtain a free resection margin.

The aim of HR is to obtain radical resection with

limited surgical morbidity; to achieve this goal, patient selection is crucial. For the last several decades, the selection of candidates for resection has been based on Child-Pugh classification. However, Child-Pugh classification is far from accurate for predicting postoperative liver failure; in fact, some Child-Pugh A patients already have liver functional impairment with an increased bilirubin concentration, clinically significant portal hypertension or even minor fluid retention necessitating diuretic treatment<sup>[26]</sup>. The further investigation of hepatic functional reserve tests, such as the aminopyrine breath test or clearance of indocyanine green (ICG), has been proposed; however, their predictive value remains poorly validated. In Japan, the ICG retention rate is utilized to identify the best candidates for resection<sup>[27]</sup>, whereas portal pressure and bilirubin are the variables used in Europe and the United States<sup>[10]</sup>. Recently, a preoperative MELD score  $\geq 10$  was associated with a higher incidence (40%) of postoperative liver failure<sup>[28]</sup>.

Markers of portal hypertension including a porto-caval gradient  $> 10$  mmHg, the presence of esophageal varices, splenomegaly and a platelet count lower than  $1 \times 10^{11}/L$  are predictors of postoperative morbidity and mortality<sup>[29]</sup>. In patients without relevant portal hypertension and normal concentrations of bilirubin, the 5-year survival is 70%, whereas this value is 50% for individuals with portal hypertension and is even lower when both these risk factors are present<sup>[30,31]</sup>.

By integrating all of these factors, HR can be safely performed on patients with Child-Pugh class A chronic liver disease, a MELD score  $\leq 10$ , a platelet count  $> 100,000/mm^3$  and a porto-caval gradient  $< 10$  mmHg. These factors dramatically limit the potential number of candidates, and overall, less than 30% of patients are candidates for HR<sup>[29]</sup>.

After HR, the 5-year survival for cirrhotic patients with HCC ranges from 30% to 50%, whereas the operative mortality ranges from 3% to 8%<sup>[29]</sup>. The severity of cirrhosis, size of the tumor, number of tumors, presence of vascular tumor invasion and presence of satellite nodules are well-established prognostic factors for recurrence and survival<sup>[27,32,33]</sup>. Late recurrence is mainly due to the carcinogenic effect of underlying chronic liver disease<sup>[34]</sup>.

Absolute contraindications to HR are the presence of extrahepatic metastases or neoplastic invasion of the main portal trunk. Neoplastic portal vein thrombosis is a poor prognostic factor; however, in highly selected cases, hemi-hepatectomy can be feasible, particularly when thrombosis of a main branch of the portal vein has led to hypertrophy of the contralateral hemiliver.

When compared with open surgery, laparoscopy in cirrhotic patients could have the advantage of avoiding the interruption of collateral abdominal veins that are present as a result of portal hypertension. Several studies have indeed demonstrated the benefits of the laparoscopic approach in terms reduced bleeding and lower postoperative morbidity and mortality<sup>[35,36]</sup>.

## LIVER TRANSPLANTATION

HCC is the only solid cancer that can be treated with transplantation. Transplantation is the best curative option for patients with decompensated (Child-Pugh B or C) cirrhosis; however, due to the shortage of donors, it can only be offered to a limited number of patients. Candidates for LT are patients with tumors that have favorable pathological features and therefore a low likelihood of recurrence.

The most widely adopted criteria for selecting HCC carriers for transplantation are the Milan criteria. According to these criteria, LT can be considered only for patients with a single tumor  $< 5$  cm in diameter or for patients with up to 3 tumors  $< 3$  cm without macrovascular invasion<sup>[17]</sup>. A recent systematic review of 90 studies that followed 17780 patients over a 15-year period identified the Milan criteria as an independent prognostic factor of outcome after LT<sup>[37]</sup>.

Despite recent discussions concerning the restrictive nature of the Milan criteria, these criteria have been adopted by the vast majority of transplant centers. The results of LT in HCC within the Milan criteria are outstanding, with 5-year survival approaching 80%, which is similar to that observed in patients transplanted for benign diseases<sup>[30,38,39]</sup>. Outside the Milan criteria, survival is significantly reduced, which is likely due to an increased prevalence of variables associated with risk of recurrence, such as microvascular invasion, in tumors at more advanced stages<sup>[40]</sup>.

Neoadjuvant therapy through TACE can occasionally downstage tumors that were outside the Milan criteria at the time of diagnosis; in these cases, the results of LT are similar to those achieved when the criteria were met at first observation<sup>[41]</sup>.

The time spent on the waiting list is a key factor that must be considered when assessing the results of LT in HCC patients; it depends on the availability of donors in a given area and on the system used to prioritize organ allocation. It was demonstrated that 15%-20% of patients with HCC initially within the Milan criteria experience tumor progression until they dropped out from the waiting list; this highlights the need to analyze the results of LT using an intention to treat approach<sup>[42]</sup>. Although treatments aimed at delaying tumor progression, such as ablation and transarterial chemoembolization, are widely used, their efficacy is unproven<sup>[43]</sup>. Live donation is a valid strategy for extending the donor pool; however, its applicability is reduced because of societal constraints, scarcity of appropriate donors and the possible morbidity and mortality of donors<sup>[44]</sup>.

Currently, organs are allocated worldwide based on MELD score; however, HCC patients with low MELD scores are given extra points to shorten their waiting time and avoid tumor progression. This policy is questioned by some authors because it might be disadvantageous for patients without HCC<sup>[45]</sup>.



## LOCOREGIONAL TREATMENTS

Local ablation techniques have been developed for patients with surgical contraindications and can be performed either through a percutaneous approach or, less commonly, through laparoscopy. These maneuvers include percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), microwave ablation, cryoablation, laser-induced thermotherapy, high-intensity focused ultrasound and irreversible electroporation<sup>[46]</sup>.

The first percutaneous treatment was PEI, which induces coagulative necrosis of the lesion as a result of cellular dehydration, protein denaturation and chemical occlusion of small tumor vessels due to the effects of the injected absolute alcohol. Ablation techniques such as RFA, microwave ablation, and laser ablation utilize high temperatures; conversely, cryoablation causes direct tumor freezing<sup>[47]</sup>.

The evaluation of responses to locoregional treatments and molecular-targeted therapies of HCC is currently based on modified RECIST (mRECIST) criteria, which measure the diameter of the viable tumor component of target lesions<sup>[48]</sup>.

### Radiofrequency ablation

RFA is the technique of choice for local destruction of liver tumors. RFA induces coagulative necrosis of the tumor with safety margins around the lesion and is the most commonly used local ablative technique. RFA has largely replaced PEI because it produces better results in terms of recurrence-free survival and requires fewer treatment sessions<sup>[49]</sup>. RFA can be performed percutaneously under imaging guidance (ultrasound, CT or MRI) or during surgery guided by intraoperative US. The advantage of RF in the treatment of HCC in cirrhotic patients is that it allows selective destruction of the tumor, sparing the surrounding parenchyma, and can be easily repeated in case of recurrence. Complete ablation of lesions smaller than 2 cm is possible in more than 90% of cases<sup>[50]</sup>.

There are several major limits to the use of RF: (1) complete necrosis is rarely observed when the tumor diameter is > 3 cm or when the tumor is adjacent to a major blood vessel due to the cooling effect of the blood flow; (2) it is difficult to reach some areas of the liver parenchyma percutaneously (*e.g.*, segment 1); (3) subcapsular lesions can undergo rupture in the peritoneum; (4) bladder injury can occur when lesions in segments IVb-5 are treated; and (5) targeting the lesion can be difficult under ultrasound guidance in livers with multinodular cirrhosis.

Taking these limitations into account, the benefit of RF in the treatment of HCC has been well demonstrated, with overall 5-year survival rates between 33% and 55% in selected series<sup>[51]</sup>. The effectiveness of RFA has led to the proposal of this technique as an alternative to HR.

In the only randomized prospective trial with balanced groups of patients comparing HR to RFA for HCC < 3 cm in patients with cirrhosis, no difference was

observed in terms of overall and disease-free survival, whereas RF was associated with lower perioperative morbidity (4.2% *vs* 55.5%,  $P < 0.05$ ) and mortality (0% *vs* 1.1%)<sup>[52]</sup>. However, in a retrospective comparative study of Child-Pugh class A patients, surgery was significantly more effective for patients with single tumors > 3 cm in diameter, with an overall 3-year survival of 66% after surgery (*vs* 37% after RFA,  $P = 0.004$ ) and a 3-year disease-free survival of 44% (*vs* 19%,  $P = 0.001$ )<sup>[53]</sup>. This observation was confirmed by subsequent studies performed by different groups<sup>[54-56]</sup>. A recent systematic review and meta-analysis of 12 controlled trials showed a not notable difference in the short-term effectiveness of RFA and HR in the treatment of early-stage hepatocellular carcinoma meeting Milan criteria, but the long-term efficacy of HR was better than that of RFA<sup>[57]</sup>. However, HR was associated with more complications and a longer hospital stay.

Rather than competing techniques, RFA and HR are effective therapeutic options that can be chosen based on the severity of chronic liver disease as well as the size and location of the tumor.

### Transarterial chemoembolization

Transarterial chemoembolization (TACE) is the most commonly used initial treatment for unresectable HCC<sup>[58]</sup> and is also the first-line therapy for downstaging tumors that exceed the criteria for transplantation or to avoid tumor progression in patients awaiting LT. TACE may also be considered as a neoadjuvant treatment that can be utilized before HR or RF ablation to reduce tumor volume and possibly target satellite micrometastases<sup>[59]</sup>.

The rationale behind TACE use is the well-characterized angiogenic activity of HCC that results in hypervascular arterial feeding. This technique depends on the intra-arterial infusion of a cytotoxic chemotherapeutic agent emulsified with Lipiodol followed by embolization of the feeding vessels through a trans-arterial catheter<sup>[60]</sup>. TACE is a well-established treatment for HCC in cirrhotic patients, and its efficacy for improving survival compared with the other supporting treatments has been demonstrated<sup>[61]</sup>.

The maximum and sustained retention of the chemotherapeutic agent is used as a measure of the success of TACE; thus, embolic microspheres are employed that have the ability to sequester chemotherapeutic agents. The concentration of these microspheres is increased within the tumor, and their contents are subsequently released in a controlled manner over a 1-wk period, which reduces the systemic toxicity to a minimum<sup>[62]</sup>.

Doxorubicin-eluting beads (DEB) are another trans-arterial liver-directed therapy. The use of an eluting bead can be considered an improvement over conventional TACE. DEB are preformed, deformable microspheres that are loaded with doxorubicin (up to 150 mg per treatment). The pharmacokinetic profile of DEB significantly differs from that of conventional TACE; in particular, the peak drug concentration in the serum is lower for DEB-

TACE compared with conventional TACE. An objective response rate of 70% to 80% according to the EASL criteria has been achieved<sup>[63]</sup>. One- and 3-year survival rates of 89.9% and 66.3%, respectively, have been reported in a heterogeneous cohort of patients with BCLC (stages A to C) treated with DEB-TACE<sup>[64]</sup>.

Absolute contraindications for TACE are decompensated cirrhosis (Child-Pugh B  $\geq$  8, including jaundice, clinical encephalopathy and refractory ascites), extensive tumor with massive replacement of both lobes in their entirety, severely reduced portal vein flow (portal vein occlusion or hepatofugal blood flow), and a creatinine clearance  $< 30$  mL/min<sup>[65]</sup>.

### Microwave ablation

Microwave ablation (MWA) is a potentially curative ablation procedure that has been proven to be safe in both percutaneous and intraoperative settings<sup>[66]</sup>. MWA can be utilized in patients with advanced liver disease and HCC, provides a more predictable ablation compared with RFA and requires fewer treatment sessions<sup>[21,67,68]</sup>.

Microwave ablation creates an electromagnetic field in the tissues surrounding the ablation antenna with an extension of several centimeter, without flow of electrical current. Tissue within the MWA field heats rapidly to temperatures over 100 °C without the detrimental effects of tissue impedance, allowing a more rapid and consistent ablation<sup>[10]</sup>. Microwave ablation carries the risk of more severe injury to adjacent structures due to differences in energy delivery when compared with RFA; therefore, the operative approach is preferred over the percutaneous approach because it allows liver mobilization and protection of adjacent organs<sup>[66,69,70]</sup>.

### Radioembolization

Radioembolization is a newer hepatic transarterial technique that employs radioactive substances such as Iodine-131-labeled Lipiodol<sup>[71]</sup> or microspheres containing Yttrium-90<sup>[72]</sup>. This technique has been shown to be feasible and safe for the treatment of HCC in cirrhotic patients<sup>[73,74]</sup>. Microspheres are delivered to the tumor area for selective production of high energy and low penetration radiation. Radioembolization can be safely performed in patients with portal vein thrombosis due to the minimally embolic effect of 90Y microspheres<sup>[75]</sup>. The reported rate of complete tumor necrosis is 90% for patients with HCC  $< 3$  cm<sup>[76]</sup>, whereas the rate of complete necrosis after TACE varies widely in the literature, from 15% to 70%<sup>[77]</sup>.

### Radiation therapy

Radiation therapy is generally not considered an option in HCC consensus documents or national guidelines, primarily because of the lack of level 1 evidence<sup>[78]</sup>. However, experience with conformal radiation therapy (RT), intensity modulated RT, stereotactic body RT and particle therapy is rapidly increasing. RT should be considered as a treatment option in patients unsuitable for other established local therapies<sup>[78]</sup>. RT has also been used as

a bridge to liver transplant and can be safely combined with locoregional therapies such as TACE<sup>[78,79]</sup>.

## SYSTEMIC TREATMENTS

Systemic treatment of HCC in cirrhotic patients is not effective due to the poor chemosensitivity of the tumor, and the impairment of hepatic function significantly increases the toxicity of chemotherapy.

Because the demonstrated benefits of systemic chemotherapy and hormonal treatments are lacking<sup>[80,81]</sup>, molecular targeted therapies have recently been developed. Sorafenib, an inhibitor of multi-kinase, has antiproliferative and antiangiogenic activity, delays tumor progression and is currently the only agent with proven efficacy for the treatment of patients with advanced HCC<sup>[11,82-84]</sup>. This multitargeted tyrosine kinase inhibitor is a small molecule that inhibits vascular endothelial growth factor receptor, platelet-derived growth factor receptor, B-Raf, Fms-related tyrosine kinase and c-kit<sup>[85,86]</sup>.

The use of sorafenib is currently recommended for patients with preserved liver function and advanced HCC who are not suitable for HR or LT and have failed to respond to locoregional treatments<sup>[87]</sup>. It is recommended that the treatment be continued until progression of the tumor is demonstrated. The main side effects associated with the use of sorafenib are diarrhea and hand-foot skin reaction; other possible side effects include anorexia, nausea, vomiting, weight loss, hoarseness of voice, asthenia and hypertension<sup>[52,88]</sup>. These effects can occasionally require dose reduction or treatment discontinuation. The potential benefit of sorafenib as adjuvant treatment after LT has not been demonstrated.

On the basis of a large randomized phase III study, the Sorafenib HCC Assessment Randomized Protocol<sup>[83]</sup>, Sorafenib has been approved by the United States Food and Drug Administration for the treatment of patients with advanced HCC.

Studies are ongoing that aim to identify the best responders to Sorafenib; c-Jun N-terminal kinase (JNK) activity was positively correlated with the CD133 expression level and inversely correlated with the therapeutic response to Sorafenib. Accordingly, JNK activity may be considered as a new predictive biomarker for response to Sorafenib treatment<sup>[89]</sup>.

To date, there is no second-line treatment for patients who are intolerant to Sorafenib or experience tumor progression while undergoing treatment.

## COMBINED TREATMENTS

A combination of the different therapeutic approaches mentioned thus far is often required to address the different clinical peculiarities of HCC patients. For example, chemoembolization or RFA can be performed before radical surgery with curative intent (HR or LT) to allow effective tumor downstaging or reduce tumor growth. Different authors have proposed HR as a first-line ther-

apy in patients who are candidates for LT<sup>[90]</sup>; however, the possible effect of previous surgery on the technical complexity of the liver transplantation procedure is a matter of debate<sup>[90,91]</sup>. One argument in favor of hepatic resection prior to LT is that histological analysis of the tumor can provide useful information regarding its oncological behavior: features such as the presence of a tumor capsule, the degree of differentiation or the presence of micro-vascular invasion can be precisely assessed in the surgical specimen and are well-known prognostic factors. However, there is no consensus among different authors regarding the influence that histological parameters should have on the therapeutic strategy; some authors suggest that LT should be contraindicated for patients with tumors that display poor prognostic histological criteria, whereas others recommend transplant priority in the presence of the same risk factors<sup>[23,92]</sup>.

## CONCLUSION

The treatment of HCC in cirrhotic patients has changed significantly over the past several decades and has become a major clinical issue. Patients with HCC on cirrhosis can benefit from several effective treatments that will improve their survival; however, the choice of the most appropriate options depends on several factors, namely: (1) severity of the underlying chronic liver disease; (2) stage of the tumor assessed *via* imaging; (3) histological features of the tumor (when available); (4) availability of an active transplant program; (5) availability of a hepatobiliary surgical unit; and (6) availability of an experienced interventional radiology service.

Some of these variables may account for the different attitudes that are often observed. To ensure that the most effective treatment can be offered for a given case, a multidisciplinary approach is warranted, and professionals skilled in the administration of the different treatment types should be available. The increase in the incidence of HCC justifies the development of services related to the management of this tumor.

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## Hepatobiliary manifestations in inflammatory bowel disease: The gut, the drugs and the liver

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### Abstract

Abnormal liver biochemical tests are present in up to 30% of patients with inflammatory bowel disease (IBD), and therefore become a diagnostic challenge. Liver and biliary tract diseases are common extraintestinal manifestations for both Crohn's disease and ulcerative colitis (UC), and typically do not correlate with intestinal activity. Primary sclerosing cholangitis (PSC) is the most common hepatobiliary manifestation of IBD, and is more prevalent in UC. Approximately 5% of patients with UC develop PSC, with the prevalence reaching up to 90%. Cholangiocarcinoma and colon cancer risks are increased in these patients. Less common disorders include autoimmune hepatitis/PSC overlap syndrome, IgG4-associated cholangiopathy, primary biliary cirrhosis, hepatic amyloidosis, granulomatous hepatitis, cholelithiasis, portal vein thrombosis, liver abscess, and non-alcoholic fatty liver disease. Hepatitis B reactivation during immunosuppressive therapy is a major concern, with screening and vaccination being recommended in

serologically negative cases for patients with IBD. Re-activation prophylaxis with entecavir or tenofovir for 6 to 12 mo after the end of immunosuppressive therapy is mandatory in patients showing as hepatitis B surface antigen (HBsAg) positive, independently from viral load. HBsAg negative and anti-HBc positive patients, with or without anti-HBs, should be closely monitored, measuring alanine aminotransferase and hepatitis B virus DNA within 12 mo after the end of therapy, and should be treated if the viral load increases. On the other hand, immunosuppressive therapy does not seem to promote reactivation of hepatitis C, and hepatitis C antiviral treatment does not influence IBD natural history either. Most of the drugs used for IBD treatment may induce hepatotoxicity, although the incidence of serious adverse events is low. Abnormalities in liver biochemical tests associated with aminosalicylates are uncommon and are usually not clinically relevant. Methotrexate-related hepatotoxicity has been described in 14% of patients with IBD, in a dose-dependent manner. Liver biopsy is not routinely recommended. Biologics-related hepatotoxicity is rare, but has been shown most frequently in patients treated with infliximab. Thiopurines have been associated with veno-occlusive disease, regenerative nodular hyperplasia, and liver peliosis. Routine liver biochemical tests are recommended, especially during the first month of treatment. All these conditions should be considered in IBD patients with clinical or biochemical features suggestive of hepatobiliary involvement. Diagnosis and management of these disorders usually involve hepatologists and gastroenterologists due to its complexity.

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**Key words:** Inflammatory bowel disease; Hepatobiliary disorders; Extraintestinal manifestations; Primary sclerosing cholangitis; Drug-induced liver injury; Hepatotoxicity; Hepatitis B; Hepatitis C

**Core tip:** Hepatobiliary disorders are common extraintestinal



testinal manifestations of inflammatory bowel disease (IBD) that become a diagnostic challenge for the gastroenterologist. In this review, we have summarized the main diseases involving the hepatobiliary system in IBD and secondary liver toxicity to IBD treatment. This review also highlights the impact of immunosuppressive and anti-tumor necrosis factor treatment in hepatitis B and C, as well as its prophylaxis and treatment, according to current clinical practice guidelines.

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## INTRODUCTION

Hepatobiliary diseases are relatively common in inflammatory bowel disease (IBD) and therefore become a diagnostic challenge. Liver and biliary tract disorders are typical extraintestinal manifestations in both Crohn's disease (CD) and ulcerative colitis (UC). In patients receiving immunosuppressive therapy, including biologics, the risk of hepatitis B reactivation is high, so patients undergoing this therapy should be screened for hepatitis B surface antigen (HBsAg) and anti-HBc prior to starting the treatment. Most of the drugs used for IBD treatment have also been associated with hepatotoxicity. All these conditions should be ruled out in IBD patients with clinical or biochemical features suggestive of liver involvement, as summarized in this review.

Bibliographic searches were performed in the MEDLINE electronic database up to February 2013 using the Medical Subject Headings terms: ("inflammatory bowel disease" OR "Crohn's disease" OR "ulcerative colitis") AND ("liver" OR "biliary tract" OR "primary sclerosing cholangitis" OR "hepatobiliary disorders" OR "small-duct PSC" OR "PSC/AIH overlap syndrome" OR "IgG4-associated cholangitis" OR "primary biliary cirrhosis" OR "hepatic amyloidosis" OR "granulomatous hepatitis" OR "cholelithiasis" OR "portal vein thrombosis" OR "liver abscess" OR "non-alcoholic fatty liver disease" OR "viral hepatitis" OR "hepatitis B" OR "hepatitis C" OR "drug-induced liver injury" OR "drug-induced hepatitis" OR "hepatotoxicity").

## HEPATOBIILIARY DISORDERS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

Hepatobiliary manifestations constitute some of the most common extraintestinal manifestations of IBD. They typically adopt an independent course irrespective of intestinal activity and are present in both UC and CD.

### Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a chronic fibro-sclerotic disorder of the intrahepatic and extrahepatic biliary tree, and is the most common hepatobiliary manifestation of IBD. The association of PSC and IBD was described for first time in 1965<sup>[1]</sup>.

**Epidemiology:** Approximately 70%-80% of patients with PSC have concomitant IBD and about 1.4%-7.5% of patients with IBD will develop PSC<sup>[2]</sup>; however the course of IBD is not related to PSC. It is more prevalent in males, in UC, and in young and middle-aged patients<sup>[3]</sup>. UC has been reported in 25 to 90 percent of patients with PSC<sup>[4]</sup>. In a Spanish multicenter study, based on a survey, UC was present in 44%<sup>[5]</sup>. Nevertheless, the real prevalence of UC in PSC is up to 90% when rectal and sigmoid biopsies are routinely obtained<sup>[6]</sup>.

PSC is typically characterized by progressive inflammation, obliterative fibrosis, and destruction of intra- and extrahepatic bile ducts, leading to end-stage liver disease and portal hypertension<sup>[7]</sup>. Patients with PSC may also develop complications such as cholestasis-associated manifestations, biliary stricture, cholangitis, cholelithiasis, cholangiocarcinoma, and colon cancer. The diagnosis of PSC is usually previous to IBD, but PSC may be diagnosed over time, after a proctocolectomy in UC patients<sup>[8]</sup>.

**Etiology:** The etiology of PSC remains unclear. Genetic, immunological, and environmental factors seem to contribute to its pathogenesis. First-degree relatives of patients with PSC show an increased risk of PSC and UC, supporting a genetic predisposition to these conditions<sup>[9]</sup>. Multiple genetic factors associated with susceptibility have been described, like HLA-B8, HLA-DRB1\*0301 (DR3), HLADRB3\*0101 (DRw52a), and HLA-DRB1\*0401 (DR4)<sup>[10,11]</sup>. In addition, three UC susceptibility loci have been associated with PSC, harboring the presumed candidate genes REL, IL2, and CARD9<sup>[12]</sup>. An autoimmune mechanism has been suggested, since both are immune-mediated disorders, and are also associated with other autoimmune diseases. Several autoantibodies may be present, such as antinuclear antibodies (ANA) in 24%-53%, smooth muscle antibodies (SMA) in 13%-20%, and anti-perinuclear cytoplasmic antibodies (pANCA) in 65%-88% of patients<sup>[13-15]</sup>. Other autoantibodies, including anticardiolipin, thyroperoxidase, and rheumatoid factor may be present, but show uncertain clinical significance. In one study, 97% of cases with PSC were positive for, at least, one autoantibody, while 81% were positive for three or more<sup>[16]</sup>. An inflammatory response to chronic or recurrent bacterial infection into the portal circulation or ischemic damage to the bile ducts has also been postulated<sup>[17]</sup>. Therefore, the most plausible theory involves the exposure of genetically predisposed individuals to an environmental agent that provokes an anomalous immune response, leading to disease development.

IBD in PSC patients has a distinct behavior, as it shows a higher incidence of rectal sparing, backwash



ileitis, extensive colitis, pouchitis after ileal pouch anal anastomosis, colon dysplasia, colon cancer, and poorer prognosis<sup>[18-21]</sup>. Patients with UC and PSC usually have a lower grade of colon inflammation and a milder course, compared to patients without PSC<sup>[22]</sup>. In addition, severe progressive PSC requiring liver transplantation appears to reduce histological activity and the need for colectomy in UC<sup>[23,24]</sup>.

**Diagnosis:** Most patients with PSC are asymptomatic at diagnosis. This disease should be considered in patients with IBD and abnormal liver biochemical tests, where a marked elevation of serum alkaline phosphatase is commonly found<sup>[25]</sup>. In symptomatic patients, fatigue and pruritus are common. Other features include abdominal pain, jaundice, and weight loss. Cholangitis occurs in 10%-15% of patients during the course of the disease. Biochemical tests usually show a cholestatic pattern. Aminotransferases levels are typically lower than 300 IU/L. Additional biochemical parameters are hypergamma-globulinemia (30% of cases), increased serum IgM levels (40%-50%), and p-ANCA (30%-80%). Serum albumin levels later decrease during the course of the disease, and the presence of hypoalbuminemia earlier may indicate active IBD.

Diagnosis is established by the demonstration of diffuse, multifocal strictures and dilations in the intra- and extrahepatic bile ducts. In 41% of cases, the gallbladder and cystic duct may also be involved<sup>[26]</sup>. In the early stages of the disease, superficial ulcerations of the bile ducts may be the only manifestation found. Endoscopic retrograde cholangiopancreatography (ERCP) is considered the gold standard technique for PSC diagnosis. It can be both diagnostic and therapeutic, and also may be useful in the early diagnosis of cholangiocarcinoma. Magnetic resonance cholangiography (MRCP) is a non-invasive alternative with high sensitivity and specificity, and without the risks related to the technique<sup>[27]</sup>. Liver biopsy is only recommended in cases of clinical suspicion of small-duct PSC, as it is rarely diagnostic of PSC<sup>[28]</sup>. The most specific histologic finding in PSC is fibrous obliteration of small bile ducts, with periductal concentric fibrosis in an "onion skin" pattern. Other abnormalities are non-specific and similar to those in primary biliary cirrhosis. Liver biopsy is helpful for staging the disease and determining prognosis. Ludwig described 4 stages of PSC based on morphologic features<sup>[29]</sup>.

**Prognosis:** PSC is a progressive disease that, ultimately, results in portal hypertension, cirrhosis, and hepatic failure. The median survival time without liver transplantation is approximately 12 years. Survival is significantly worse in symptomatic patients at the time of diagnosis<sup>[30]</sup>. Coexisting IBD may also be related to a poorer prognosis, as it has been associated with a younger age at diagnosis, the development of malignant complications, dysplasia and/or colon cancer<sup>[31,32]</sup>. Patients with PSC usually develop complications of end-stage liver disease

with portal hypertension, such as varices, ascites, and hepatic encephalopathy. The Mayo Risk Score based on age, serum bilirubin, albumin, aspartate aminotransferase, and the presence of variceal bleeding, has been used to assess disease progression and prognosis<sup>[33]</sup>. Other complications include steatorrhea and fat-soluble vitamin deficiency, secondary to chronic cholestasis, amyloidosis secondary to amyloid A protein deposition in tissues due to a progressive inflammatory process<sup>[34]</sup>, dominant biliary strictures, cholangiocarcinoma, and colon cancer. The risk for cholangiocarcinoma is significantly increased in PSC and its development remains unpredictable. The annual incidence has been estimated as 1.5%<sup>[35]</sup>. Risk factors include the presence of IBD, cirrhosis, variceal bleeding, a dominant stricture in the bile duct, and alcohol intake<sup>[36]</sup>. Worsening jaundice, weight loss, and abdominal discomfort are suspicion symptoms. Diagnosis may be difficult, as imaging techniques and brush cytology show a lack of sensitivity for early detection. However, ERCP and cytology of bile duct strictures is highly specific<sup>[37]</sup>. Prognosis is devastating, with a survival rate of 10% two years after diagnosis<sup>[38]</sup> and a recurrence rate in the transplanted liver of about 20%-25%<sup>[39]</sup>. Patients with PSC have an increased risk for gallbladder cancer, pancreatic cancer and, in cirrhotic patients, hepatocellular carcinoma. A higher risk of colorectal dysplasia/cancer has also been described among UC patients with PSC<sup>[21]</sup>, even after liver transplantation<sup>[40]</sup>. The severity and the duration of PSC have not been significantly associated with the risk of colon cancer<sup>[41]</sup>. In patients with ileal pouch-anal anastomosis, the risk for dysplasia persists after colectomy<sup>[42]</sup>. Therefore, surveillance for colorectal cancer should be strongly recommended in PSC patients with UC<sup>[43]</sup>.

**Treatment:** Treatment of PSC associated with UC does not differ from PSC without IBD. As no pharmacologic therapy has proven effective for PSC, treatment goals are the control of symptoms and the management of complications. Ursodeoxycholic acid (UDCA) has been shown as effective in liver function improvement based on biochemical tests but it had no effect on liver histology, liver transplant-free survival, requirements for liver transplantation, development of cholangiocarcinoma, or incidence of death<sup>[44,45]</sup>. In a meta-analysis, UDCA does not appear to decrease either the risk of adenomas or colon cancer<sup>[46]</sup>. Immunosuppressants, chelators, and steroids have been used without any benefit.

Liver transplantation is the only therapy that can change the inevitable outcome. The appropriate moment for liver transplantation can be difficult to determine, as patients with advanced disease may not show signs of liver failure. Survival rates after hepatic transplant at 5 and 10 years are 85% and 70%, respectively<sup>[47]</sup>. However, in 20%-25% of cases, PSC recurs in the transplanted liver<sup>[39]</sup>.

Endoscopic management of PSC is indicated in cases of cholangitis, exacerbate jaundice, or suspicion of cholangiocarcinoma. Endoscopic dilation of dominant

strictures, with or without stenting, has been shown to alleviate cholestasis and to improve laboratory test results, although it does not prevent disease progression<sup>[48]</sup>.

### **Small-duct PSC**

Small-duct PSC is characterized by laboratory and histological findings similar to PSC but with normal cholangiogram. The presence of coexisting IBD is required for the diagnosis of this entity<sup>[49]</sup>. In a large multicenter study, 80% of patients with small-duct PSC had concurrent IBD (78% UC and 21% CD)<sup>[50]</sup>. Progression of small-duct PSC to PSC was observed in 12%-23% of cases. Small-duct PSC has been associated with a better long-term prognosis as compared with large-duct PSC. Cholangiocarcinoma has not been previously described. Some patients may require liver transplantation for end-stage liver disease, and the disease may recur after liver transplantation. In IBD patients with cholestatic liver function tests altered and a normal cholangiogram by ERCP/MRCP, a biopsy is recommended to rule out small-duct PSC, after excluding other hepatobiliary disorders.

**AIH/PSC overlap syndrome:** AIH/PSC overlap syndrome has been described in patients with IBD, especially UC<sup>[51]</sup>. The diagnosis is suspected when features of AIH and PSC are present in the same patient, requiring a definitive diagnosis of AIH based on the International Autoimmune Hepatitis Group Criteria, which includes demographic, histologic, and laboratory markers<sup>[52]</sup>. Diagnosis, treatment, and prognosis of the overlap syndrome are controversial and so standardized diagnostic criteria are needed<sup>[53,54]</sup>. Previous studies have reported cases that initially presented with laboratory markers and histologic features of either AIH or both diseases with a normal cholangiography, only to develop pathologic characteristics of PSC during the follow-up<sup>[52,55,56]</sup>. Intrahepatic and extrahepatic bile ducts may be affected. Conventional corticosteroid therapy, alone or in conjunction with UDCA (13-15 mg/kg daily), has been variably effective, and cyclosporine, mycophenolate mofetil, and budesonide have been beneficial in selected patients. The cholestatic features that influence the prognosis of autoimmune hepatitis must be defined and incorporated into the definition of the syndrome<sup>[57]</sup>.

### **IgG4-associated cholangiopathy**

IgG4-associated cholangiopathy (IAC) is a biliary disease of unknown immunopathogenesis. Indistinguishable from PSC according to cholangiographic characteristics, it shows distinct histological findings. It is one of a variety of IgG4-related systemic disease and has been described in patients with concurrent UC<sup>[58]</sup>. Clinical diagnostic criteria for IgG4-related disease require systemic organ involvement, elevated serum IgG4 levels ( $\geq 135$  mg/dL), and histopathological findings<sup>[59]</sup>. IgG4 levels have also been reported in 9%-36% of patients with PSC, although these levels are usually lower than in patients with IAC<sup>[60,61]</sup>. The identification of IgG4 plasma

cell infiltrating the bile duct and other organs is decisive in reaching the diagnosis<sup>[58,59]</sup>. Clinically, patients with IAC are older at diagnosis compared to patients with PSC. Obstructive jaundice can be the first symptom, whereas it is rarely present in PSC<sup>[62]</sup>. Steroids are the first-choice therapy of IAC, as they result in the resolution of jaundice, improve liver laboratory parameters, and reduce serum IgG4 levels and the reversal of strictures on cholangiogram<sup>[63]</sup>. Azathioprine (AZA) should be considered alongside those with proximal and intrahepatic stenosis, and those that relapse during and/or after corticosteroid therapy<sup>[64]</sup>.

### **Primary biliary cirrhosis**

Primary biliary cirrhosis (PBC) frequently accompanies various autoimmune diseases including Sjögren syndrome, chronic thyroiditis, and rheumatoid arthritis, but rarely IBD<sup>[65]</sup>. There are a few reported cases of both diseases in the literature<sup>[66,67]</sup>. The clinical presentation varies from typical PBC; affecting males more frequently, being diagnosed at younger age and at earlier stages of PBC, and usually associated with previously diagnosed mild left-side UC. Although the pathogenesis of this disease has not yet been clarified, environmental and genetic factors are considered important in the susceptibility to both diseases.

### **Hepatic amyloidosis**

Secondary amyloidosis is an unusual complication of IBD, more frequent in CD than in UC (0.9% *vs* 0.07%)<sup>[68]</sup>. Chronic activity in the bowel contributes to amyloid deposition in the vasculatures and sinusoids of almost any organ, including the liver. It may present as asymptomatic hepatomegaly and is more common in men with colonic diseases. Treatment is based on controlling gut inflammation, thereby decreasing the release of the acute phase reactant serum amyloid A<sup>[69]</sup>. In some cases, colchicine can be effective.

### **Granulomatous hepatitis**

Granulomatous hepatitis is another rare complication of CD, which is characterized by granulomas on the liver biopsy. The main manifestation is an increase in cholestatic enzymes such as alkaline phosphatase. Granulomatous hepatitis is often secondary to different medications, including sulfasalazine<sup>[70]</sup>. Other causes are malignancies, infections, or CD metastasis<sup>[71]</sup>. Corticosteroids and immunosuppressive drugs have been used in its treatment.

### **Cholelithiasis**

It has been estimated that patients with CD have a doubled risk for gallstones comparing to IBD-free controls, while UC is not associated with an increased risk<sup>[72]</sup>. The incidence of gallstones is raised in patients with Crohn's ileitis or ileal resection, ranging from 13% to 34%<sup>[73]</sup>. Risk factors associated with its development are CD location at diagnosis, surgery, and extent of ileal resection. Other factors include the age of the patient, frequency

of clinical recurrences, length of hospital stay, and the use of total parenteral nutrition. The pathophysiology of cholelithiasis in CD is not well defined. Abnormal malabsorption of bile acids that interfere with enterohepatic circulation has been proposed. Moreover, reduced gallbladder motility has been described in CD and increased gallstone cholesterol concentrations have been identified in patients with ileoanal anastomosis<sup>[74]</sup>.

### Portal vein thrombosis

IBD is associated with an increased risk of vascular complications, such as arterial and venous thromboembolisms, which are considered extraintestinal manifestations. Portal vein thrombosis is a rare but potentially life-threatening complication, with an incidence in IBD patients higher than that of the general population. In a Mayo Clinic study, portal/mesenteric vein thrombosis was reported in 1.3% of IBD cases, with a mortality of 50%<sup>[75]</sup>. Recent abdominal surgery, younger age, and female gender are associated with a higher incidence of portal vein thrombosis<sup>[76]</sup>. The factors involved in this pathogenesis are diverse. Acquired prothrombotic factors can be identified, such as inflammation, immobilization, extent of colon disease, surgery, central catheters, corticosteroids, and smoking<sup>[77,78]</sup>. Furthermore, patients with IBD have increased platelet counts, factor V and VIII levels, and fibrinogen, along with decreased antithrombin III levels. Anticoagulants, such as low-molecular-weight heparin and warfarin, are mainstays of therapy, even in the setting of gastrointestinal bleeding<sup>[73]</sup>. In the presence of a congenital hypercoagulable state, lifelong systemic anticoagulation should be considered, although in other prothrombotic conditions, a six-month course provides adequate coverage<sup>[79]</sup>.

### Liver abscess

The association between liver abscesses and IBD is uncommon<sup>[80,81]</sup>, but hepatic abscesses can be an initial manifestation of CD<sup>[82]</sup>. The mechanism of abscess development may be related to direct extension of intra-abdominal abscesses or due to portal pyemia, secondary to an increase in intestinal mucosa permeability. Among associated risk factors, intra-abdominal abscesses, fistulizing disease, malnutrition, and treatment with steroids and metronidazole have been reported.

### Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome with a histologic spectrum ranging from benign steatosis to non-alcoholic steatohepatitis (NASH)<sup>[83]</sup>. Steatosis is described in up to 50% of abnormal liver biopsies in IBD patients and has been related to colitis severity. It was presumed secondary to severe illness, with malnutrition, hypoproteinemia, and corticosteroids primarily responsible<sup>[84]</sup>. On the other hand, NAFLD occurs in 8.2% of the IBD population, which is much lower than the frequency reported in the United States general population (33.6%). Those patients who

developed NAFLD tended to be older, and developing IBD at an older age normally requires small bowel surgery<sup>[85]</sup>. It has been reported that IBD patients develop NAFLD with fewer metabolic risk factors than non-IBD NAFLD patients. In multivariate analysis, hypertension (OR = 3.5), obesity (OR = 2.1), small bowel surgeries (OR = 3.7), and use of steroids at the time of imaging (OR = 3.7) were independent factors associated with NAFLD. NAFLD is also less common among patients who received antibodies against tumor necrosis factor alpha (anti-TNF- $\alpha$ ) therapy.

## VIRAL HEPATITIS AND IBD

Chronic hepatitis B and C are two common diseases. The WHO estimates that 350 million people in the world suffer from chronic hepatitis B, and more than 200 million from hepatitis C<sup>[86,87]</sup>. Hepatitis B infection is transmitted during delivery or early childhood. Hepatitis C is a blood-borne disease spread mainly *via* blood product transfusion and misuse of illegal drugs. Around 20% of patients with chronic hepatitis B show cirrhosis progression and 5% are at risk of developing hepatocellular carcinoma.

Chronic hepatitis B infection is a dynamic process, owing to the interaction between the hepatitis B virus and the host immune system. Its natural history is covered by five different phases<sup>[88]</sup> (Table 1): (1) Immunotolerant phase: characterized by positive hepatitis B e antigen (HBeAg), higher HBVDNA titre, and normal or near normal ALT levels. Liver biopsy at this phase shows mild or no inflammatory lesions and scarce non-progressive fibrosis; (2) Immunoclearance phase: positive HBeAg, lower HBVDNA, and raised aminotransferase levels. Liver histology shows necroinflammatory activity together with fibrosis progression. Spontaneous HBeAg clearance and anti-HBe seroconversion could occur at this phase; (3) HBsAg inactive carrier: characterized by negative HBeAg, positive anti-HBe, residual viremia (HBVDNA lower than 2000 IU/mL), and ALT levels under the normal limit. Liver histology shows minimal or no lesions; (4) Chronic hepatitis B: HBeAg negative could appear after unsuccessful seroconversion. HBVDNA remains quantifiable and ALT levels are fluctuant. Fibrosis progression is common; and (5) Resolved infection: characterized by loss of HBsAg and could be a stable phase, with negative HBsAg and non-detectable HBVDNA with normal ALT levels and excellent prognosis.

Chronic hepatitis C is a progressive liver disease that could evolve to cirrhosis. Risk factors associated with fibrosis progression are alcohol consumption, HIV co-infection, and adult age at infection.

Chronic hepatitis B patients showing HBVDNA > 2000 IU/mL and at least moderate necroinflammatory activity or fibrosis in a liver biopsy should be treated with entecavir or tenofovir and, in selected cases, with peginterferon  $\alpha$ -2a. In patients with chronic hepatitis C and positive HCV RNA, antiviral treatment should be started. In genotype 1, protease inhibitor-based triple therapy is

**Table 1** Phases of chronic hepatitis B infection

Phase	Characteristics	HBVDNA	ALT	Liver histology
Immune tolerant	HBeAg positive	> 2000000 UI/mL	Normal	Normal or mild inflammation
Immune active	HBeAg positive	> 200000 UI/mL	Elevated	Chronic hepatitis Active cirrhosis
Inactive carrier	HBeAg negative Anti-HBe positive	< 2000 UI/mL	Normal	Normal or mild inflammation Mild fibrosis Inactive cirrhosis
HBeAg negative chronic hepatitis	HBeAg negative Anti-HBe positive	> 20000 UI/mL	Elevated (fluctuating)	Chronic hepatitis Active cirrhosis
Remission	HBsAg negative Anti-HBe positive	Indetectable in serum and detectable in liver	Normal	Normal Mild fibrosis Inactive cirrhosis

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen.

the first choice and, in non-1 genotype, standard peginterferon plus ribavirin.

Viral reactivation during immunosuppressive therapy is a major concern in viral hepatitis B and C. Viral reactivation is defined by an increase of 1 log in viral load or re-appearance of the virus after previous clearance. A flare of ALT is common and in some cases may develop into acute liver failure.

Hepatitis B reactivation depends on two main factors: (1) type of immunosuppressive drug used, and (2) hepatitis B phase prior to treatment. In a recent meta-analysis including 14 studies and 485 HBsAg-positive patients undergoing chemotherapy, one in three patients developed hepatitis and the mortality rate reached 7%. Positive HBeAg and detectable HBVDNA, together with steroids or rituximab in hematologic neoplasms, were independent factors associated with the risk of developing reactivation<sup>[89]</sup>. In patients with HBsAg loss, reactivation risk was around 3%-10% and mainly associated with the combination of steroids and rituximab in hematologic neoplasms<sup>[90]</sup>.

Hepatitis B reactivation prophylaxis is recommended from one week before chemotherapy to 12 mo after cessation of this therapy. Lamivudine has shown to decrease mortality rate<sup>[89]</sup>. However, it has been associated with an increased risk of developing resistant variants, so drugs with a higher genetic barrier, like entecavir or tenofovir<sup>[91]</sup>, are recommended if immunosuppressors need to be used for more than one year.

Hepatitis C reactivation management is controversial, due to both its frequency and clinical manifestations remaining unclear. Hepatitis has been reported in 11% of cases and reactivation in 36% of the few patients with HCV RNA evaluated before and after chemotherapy, mainly in those treated with rituximab for hematologic neoplasms<sup>[92]</sup>. Interferon-based therapy is not recommended for hepatitis C reactivation prophylaxis. Further combination of direct antiviral drugs could be useful in avoiding hepatitis C reactivation in this setting.

As immunosuppressive drugs, like AZA, methotrexate (MTX), and anti-tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), are being used more frequently in IBD, concerns about viral reactivation are increasing.

### Hepatitis B and IBD

At the beginning of this century, hepatitis B infection prevalence was slightly higher in patients with IBD than in the general population, which was mainly related to increased surgical procedures and blood transfusions<sup>[93,94]</sup>. However, recent studies in France and Spain did not confirm these data. Prevalence of hepatitis B in IBD has been estimated to be similar to the general population as a consequence of several health system measures, like overall vaccination, safe transfusions, and surgical procedures. Prevalence of anti-HBc was 7.1% in CD and 8% in UC in a Spanish population<sup>[95,96]</sup>.

Hepatitis B reactivation is a major health problem. Patients undergoing immunosuppressive therapy are at risk of developing hepatitis B reactivation. Acute liver failure requiring orthotopic liver transplantation has been reported in a patient under AZA and steroid treatment<sup>[97]</sup>. TNF $\alpha$  plays a role in hepatitis B virus replication, so anti-TNF $\alpha$  drugs could promote hepatitis B reactivation<sup>[98]</sup>. Infliximab, together with AZA or steroids, have been implicated in hepatitis B reactivation in seven cases from several series<sup>[94,99-102]</sup>. No cases have been reported in patients receiving adalimumab or certolizumab pegol. Nevertheless, hepatitis B reactivation seems to be a class-effect, so more cases should be expected with the continuing use of these new drugs. In patients with HBsAg loss reactivation is infrequent, but one case treated with infliximab has been reported<sup>[103]</sup>. A collaborative multicenter and retrospective Spanish study (REPENTINA) showed a reactivation rate of 36% (9/25) in patients with positive HBsAg; six out of nine developed liver failure, three underwent liver transplantation, and one died. No HBsAg-negative patients developed hepatitis B virus reactivation. In this study, the key factor for reactivation was the combination of two or more immunosuppressive drugs, independently of agent type. The absence of reactivation was associated with the use of only one drug for a short period of time<sup>[104]</sup>.

Clinical practice guidelines from AEEH, EASL, and ECCO revised this topic and recommended prophylaxis of hepatitis B reactivation in patients with IBD receiving immunosuppressive agents<sup>[104-106]</sup>. HBsAg, anti-HBc, and anti-HBs should be tested before immunosuppressive



therapy with two goals: avoid possible fatal complications and use antiviral drugs to control hepatitis B virus replication: (1) HBV serologically-negative patients should receive vaccination. Vaccination rates in IBD are variable. In Spain, only 56% of young people showed anti-HBs antibodies<sup>[95]</sup>. Vaccine response was around 46% using double doses in patients with IBD receiving anti-TNF drugs. However, AZA seems not to influence vaccine response. In non-responders a new complete vaccination course should be recommended. Hepatitis B screening is highly recommended immediately after the diagnosis of IBD, and vaccination is indicated in serologically-negative patients<sup>[107]</sup>; (2) Patients showing HBsAg positive and HBVDNA > 2000 IU/mL should be treated with tenofovir or entecavir as chronic hepatitis B patients; (3) Patients showing HBsAg positive and HBVDNA < 2000 IU/mL or undetectable levels, and patients with HBsAg negative and HBVDNA positive should be treated with tenofovir or entecavir for 6 to 12 mo after the end of immunosuppressive therapy. ALT levels and HBVDNA titre should be monitored every three months during treatment; and (4) Patients showing HBsAg negative and anti-HBc positive with or without anti-HBs should be closely monitored every 1 to 3 mo, measuring ALT and HBVDNA until 6 to 12 mo after the end of therapy. For patients with an increase in viral load, entecavir or tenofovir therapy should be immediately started.

### Hepatitis C and IBD

The prevalence of hepatitis C infection was increased in IBD patients younger than 50-year-old in comparison with the general population in studies of the last decade of the twentieth century<sup>[94]</sup>. However, recent studies have demonstrated a similar prevalence to the general population both in Spain (2.3% in CD and 1.3% in UC) and France (0.79% in CD and 1.59% in UC)<sup>[96,97]</sup>.

The impact of immunosuppressive therapy for IBD on hepatitis C remains controversial. Steroids could promote viral replication, as demonstrated after liver transplantation. However, steroids have been used to treat hepatitis C without success or adverse events. ALT flares have been reported after stopping steroid therapy in patients with IBD<sup>[108]</sup>. Steroid therapy should be avoided in patients with hepatitis C, and patients should be closely monitored after any withdrawal or tapering process. Immunosuppressive drugs like AZA, MTX, cyclosporine, and mycophenolate mofetil have been largely used in the liver transplantation setting, showing a slight antiviral activity against hepatitis C<sup>[109-111]</sup>. MTX did not affect hepatitis C disease in patients with arthritis<sup>[112]</sup>. Therefore, immunosuppressive therapy with these drugs seems to be safe. Indeed, in a Spanish study, 16% (8/51) of patients with chronic hepatitis C and IBD receiving immunosuppressive therapy developed non-severe liver dysfunction, seven related to steroids, and one case with AZA<sup>[104]</sup>.

TNF $\alpha$  plays a major role in the pathogenesis of chronic hepatitis C and associated metabolic abnormalities. TNF $\alpha$  is crucial in hepatitis C-induced insulin resistance, but could also modulate interferon response.

Increased TNF $\alpha$  levels have been associated with impaired sustained virological response (SVR)<sup>[113]</sup>. Thus, TNF $\alpha$  inhibition could be more beneficial than harmful in the management of chronic hepatitis C. Etanercept improved SVR in patients with chronic hepatitis C receiving peginterferon plus ribavirin<sup>[114]</sup>. Patients suffering from rheumatoid arthritis and hepatitis C treated with etanercept or infliximab did not show any changes in transaminases level or viral load<sup>[115]</sup>. Although etanercept is not effective on IBD, a class-effect should be expected, and the use of anti-TNF $\alpha$  could be safe in hepatitis C associated with IBD.

CD is characterized by a Th1 response. Interferon seems to play an immunomodulatory activity-enhancing Th1 response, and can cause outbreak of CD<sup>[116]</sup>. On the other hand, some authors have suggested that interferon could be safely used in patients with IBD, due to a lack of negative impact on the gut<sup>[117,118]</sup>. In a prospective study including 11 patients with IBD and hepatitis C, peginterferon plus ribavirin achieved SVR in a similar way to non-IBD patients. During treatment six patients developed gastrointestinal symptoms that required optimization of immunosuppressive agents without impacting antiviral treatment<sup>[119]</sup>. Several case reports showed a beneficial effect of interferon-alpha and beta on patients with UC<sup>[120,121]</sup>. A systematic review, including three prospective studies, demonstrated no effect of interferon-alpha on UC<sup>[122]</sup>, although new cases or exacerbation of UC have been seen in patients treated with interferon-alpha<sup>[123,124]</sup>. Higher doses of interferon used in hepatitis C, in comparison with lower doses in UC trials, could partially explain this discrepancy. Finally, 10 patients with CD and 10 with UC in remission or showing mild bowel activity underwent antiviral therapy for hepatitis C. No patient developed reactivation of IBD during treatment or the 12 mo of follow-up<sup>[125]</sup>, confirming data from a recent review concluding that interferon does not impair IBD course<sup>[126]</sup>.

In summary, hepatitis C antiviral treatment has no influence on IBD natural history, and immunosuppressive therapy for IBD does not promote reactivation of hepatitis C. Currently, no therapeutic option for reactivation prophylaxis nor vaccination are available for hepatitis C, but in the near future interferon-free regimen combining protease, polymerase, and NS5A inhibitors could be useful in the management of hepatitis C in IBD.

## DRUG-INDUCED LIVER INJURY IN IBD

Approximately 30% of patients with IBD show abnormalities in liver biochemical tests during the course of the disease. Most of the drugs used in IBD have potential hepatotoxicity<sup>[127]</sup> (Table 2).

### Aminosalicylates: Sulfasalazine and mesalazine

Sulfasalazine, an association between sulfapyridine and 5-aminosalicylate (5-ASA), was the first aminosalicylate used in the treatment of IBD. This drug has been replaced in the last two decades by mesalazine or 5-ASA,

**Table 2 Drug induced hepatobiliary manifestations in inflammatory bowel disease**

Manifestation	Drug
Drug induced hepatitis	Azathioprine
	6-mercaptopurine
	Methotrexate
	Cyclosporine
	Infliximab
Reactivation of hepatitis B	Anti-TNF therapy
	Corticosteroids
Drug induced pancreatitis	Azathioprine
	6-mercaptopurine
	Methotrexate
Hepatosplenic T-cell lymphoma	Combination of anti-TNF and immunosuppressive therapy

TNF: Tumor necrosis factor.

due to its adverse effects. Mesalazine is indicated for the induction and maintenance of the clinical remission in patients with UC with mild-moderate activity. The efficacy of this drug in CD remains controversial.

The anti-inflammatory effect of aminosalicylates is unclear. Synthesis inhibition of prostaglandins and leukotrienes (which show antioxidant and immunomodulatory activity) are well known. Aminosalicylates are safe drugs, and rarely lead to severe adverse effects such as bone marrow aplasia, pancreatitis, nephropathy, or hepatotoxicity. Altered liver function tests (cytolysis or cholestasis) may be detected during treatment with them. These abnormalities usually have no clinical relevance, although hepatotoxicity induced by acute hypersensitivity and acute liver failure has been described. In clinical trials, abnormalities in liver biochemical tests have been observed in 2% of UC patients treated with mesalazine<sup>[128]</sup>. The United Kingdom's Committee on Safety of Medicines observed that, between 1991 and 1998, the incidence of toxic hepatitis was 3.2 and 6 cases per million of prescriptions for mesalazine and sulfasalazine, respectively, and the presence of rheumatoid arthritis was a stronger risk factor than IBD<sup>[129]</sup>. Therefore, given the low risk of hepatotoxicity, a close monitoring of liver biochemical tests is not necessary in patients treated with aminosalicylates.

### Methotrexate

Methotrexate (MTX) has both anti-proliferative and immunosuppressive activities, impairing DNA synthesis (*via* inhibition of dihydrofolate reductase) as well as decreasing the production of proinflammatory cytokines and inducing lymphocyte apoptosis, respectively. The main indication in IBD is maintenance of clinical remission in steroid-dependent CD patients, after adverse effects or lack of efficacy of thiopurines. MTX efficacy in UC is controversial. On the other hand, MTX is contraindicated in pregnancy. Regarding adverse effects, myelosuppression and hepatotoxicity are dose-dependent. These effects were documented in up to 25% of patients with rheumatoid psoriatic arthritis, highlighting the association

with obesity, alcoholism, diabetes, previous abnormalities in biochemical liver tests and, especially, an accumulated dose higher than 15 g, as risk factors. Currently, liver fibrosis and cirrhosis are less frequent, probably due to close monitoring of liver parameters, proper selection of patients, and simultaneous treatment with folic acid, which decreases MTX-related adverse effects. Sabeni *et al.*<sup>[130]</sup>, in Italy, detected hepatotoxicity in 14.3% of patients with IBD treated with MTX during a mean follow-up of 26 mo. Te *et al.*<sup>[131]</sup>, in the United States, carried out a study in 20 IBD-patients treated with MTX with a mean follow-up of 131 mo and accumulated doses of 2.6 g. Liver fibrosis in biopsies was detected in one patient; the rest of the patients showed mild histological changes only. No association between abnormalities in liver biochemical tests and liver histology was found<sup>[131]</sup>. Regular liver laboratory studies are recommended in patients treated with MTX<sup>[132]</sup>. Nowadays, liver biopsy is not recommended routinely during MTX treatment<sup>[133]</sup>. However, it should be performed in cases of persistent alteration of transaminases (especially if they do not decrease after reducing the drug dose) and in patients with high accumulated doses, together with other risk factors. On the other hand, transient elastography (Fibroscan®) is emerging as diagnostic method of liver fibrosis in these patients. Treatment needs to be discontinued in cases of liver fibrosis or cirrhosis<sup>[134]</sup>.

### Anti-TNFα: Infliximab and adalimumab

Infliximab and adalimumab are monoclonal antibodies against TNFα, indicated in several rheumatologic, dermatologic, and gastrointestinal diseases. The main indication is the induction and maintenance of clinical remission in steroid-resistant or steroid-dependent CD and UC patients without response to immunosuppressive therapy. Early on, they are recommended in CD associated with risk factors or in the presence of severe perianal disease. Biological agents inhibit TNFα, preventing the release of proinflammatory cytokines, leukocyte migration, expression of endothelial molecules, fibroblast proliferation, and prostaglandin synthesis.

The main adverse effects of these drugs are opportunistic infections, hepatitis B virus reactivation, lymphoproliferative diseases, neurological diseases, and autoimmune diseases, such as lupus-like syndromes. Severe hepatotoxicity is a very rare condition in biological therapy, being most frequent in patients treated with infliximab. It is difficult to determine cause-effect associations between liver damage and these drugs in some cases due to confounding factors, like the presence of other drugs and other concomitant diseases. The drug label of infliximab (Remicade®) showed that, in clinical trials, ALT raised more than three times in 4.9% of patients with CD and in 2.5% of patients with UC, without clinical relevance. In contrast, serum ALT levels were less altered in the placebo group. On the other hand, the drug label of adalimumab (Humira®) indicates that serum ALT levels were similar in patients with IBD and a placebo.

According to infliximab indications, jaundice has been an uncommon finding, as well as infectious hepatitis, with liver failure being a very rare condition<sup>[135]</sup>. The Food and Drug Administration considers infliximab a hepatotoxic drug<sup>[136]</sup>. Recently, hepatotoxicity by these drugs has been evaluated in the United States (2003-2011), where only 34 cases were found, confirming the peculiarity of this adverse effect. Most cases (76%) were related to infliximab, showing a hepatocellular or cholestatic pattern with autoimmune characteristics, and improving after discontinuation of the drug<sup>[137]</sup>. Cross hepatotoxicity has not been documented in anti-TNF agents. In fact, in cases of infliximab-induced hepatotoxicity, adalimumab has been shown to be safe<sup>[138]</sup>.

### Thiopurines (azathioprine and 6-mercaptopurine)

AZA and its metabolite, 6-mercaptopurine (MP), are the immunosuppressive agents most commonly used in IBD. They are purine analogues, which interfere in nucleic acid synthesis and inhibit the proliferation of B and T lymphocytes, although the most relevant action is the apoptotic activation of T lymphocytes. The main indication of these drugs in CD and UC is the maintenance of clinical remission, preventing the use of steroids.

The active metabolites of AZA and MP are the 6-thioguanine nucleotides. In the liver, AZA is modified to MP, which is metabolized by xanthine oxidase and thiopurine methyltransferase (TPMT) in 6-thiouric acid and 6-methylmercaptopurine, resulting ultimately in 6-thioguanine nucleotides by hypoxanthine phosphoribosyltransferase. The decreased activity of TPMT facilitates the increasing in 6-thioguanine nucleotide levels, which are related to adverse effects. In fact, the efficacy of AZA and MP is limited owing to their adverse effects, which are responsible for treatment discontinuations in up to 15% of patients. Adverse effects are classified as dose-independent, dose-dependent, or idiosyncratic (which appears during the first two weeks of treatment). Regarding dose-independent adverse effects, the most common are allergic reactions (fever, exanthema, myalgias, and arthralgias) and acute pancreatitis. Among dose-dependent adverse effects, gastrointestinal intolerance and myelotoxicity are present in 2%-5% of patients. In retrospective studies, hepatotoxicity affected 3% of patients with an annual incidence of 1.4%, while these results are higher in prospective studies (10%)<sup>[139]</sup>. AZA and MP are able to damage the vascular endothelium, especially sinusoids and terminal veins, promoting veno-occlusive disease, regenerative nodular hyperplasia, and liver peliosis. These complications could be detected between 3 mo and 3 years after the beginning of treatment, and generate portal hypertension<sup>[140,141]</sup>. In general, mechanisms for AZA and MP hepatotoxicity remain unclear. It is thought that the main reason is the intracellular accumulation of 6-thioguanine nucleotides due to the decreased activity of TPMT.

It is recommended to determine levels of TPMT before the beginning of treatment with AZA or MP and

routinely perform liver biochemical tests, especially during the first months of treatment, to detect myelotoxicity and/or hepatotoxicity. Mild abnormalities in liver parameters, without clinical relevance, allow the continuation of treatment at a lower dose. However, jaundice or persistent alterations in spite of reduced dose require an immediate stop to treatment<sup>[142]</sup>.

## CONCLUSION

Hepatobiliary disorders are common extraintestinal manifestations of IBD, and PSC represents the most prevalent disease among them. Abnormal liver biochemical tests are present in up to 30 percent of patients with IBD and emerge as a diagnostic challenge. Drug-induced hepatotoxicity should always be rule out, as most IBD treatments have been associated with liver toxicity, although the incidence of serious complications is low. Hepatitis B screening and vaccination is recommended in patients with IBD. Reactivation prophylaxis with entecavir or tenofovir is mandatory in patients under immunosuppressive therapy showing HBsAg positive, independently from viral load. HBsAg negative and anti-HBc positive patients, with or without anti-HBs, should be closely monitored, and treated if the viral load increase. Diagnosis complexity often requires a joint gastroenterologist and hepatologist approach.

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## Physiological and molecular biochemical mechanisms of bile formation

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### Abstract

This review considers the physiological and molecular biochemical mechanisms of bile formation. The composition of bile and structure of a bile canaliculus, biosynthesis and conjugation of bile acids, bile phospholipids, formation of bile micellar structures, and enterohepatic circulation of bile acids are described. In general, the review focuses on the molecular physiology of the transporting systems of the hepatocyte sinusoidal and apical membranes. Knowledge of physiological and biochemical basis of bile formation has implications for understanding the mechanisms of development of pathological processes, associated with diseases of the liver and biliary tract.

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**Key words:** Bile acids; Bile phospholipids; Bile micelle structures; Bile salt transporters

**Core tip:** Over the past 50 years, significant progress has been made in understanding the mechanisms of bile formation and secretion. This became possible due to the advances of fundamental investigations in cell

### INTRODUCTION

Bile is a lipid-rich hepatic secretion that is necessary for elimination of cholesterol and xenobiotics from the body and for dispersion and efficient absorption of digested dietary lipid in the upper small intestine<sup>[1]</sup>. Biliary function is a vital function of the liver, which results from the sequential vectorial transport of endogenous and exogenous substrates through three compartments: the vascular space, cellular space and biliary space. Canalicular bile is produced by polarized hepatocytes that hold transporters in their basolateral (sinusoidal) and apical (canalicular) plasma membrane<sup>[2]</sup>. The biliary function is responsible for the homeostasis of lipid metabolism, in particular cholesterol metabolism, elimination of toxic endo- and xenobiotics such as bilirubin, lipid bacteria products (endotoxin), and several inflammatory mediators<sup>[3]</sup>. Bile coming into the canaliculi is modified by cholangiocytes through secretion and absorption. The main determinant of bile formation is an osmotic filtration process resulting from active transport of bile acids and other osmotic solutes<sup>[3]</sup>. Pavlov *et al*<sup>[4]</sup> established the basic mechanisms of bile secretion, its entry into the duodenum, and the role of bile in digestion. Bile is essential for the intestinal digestion and absorption of nutrients. Almost half a century had passed before the

experimentally founded ideas on the mechanisms of bile formation appeared. Later, they were clarified and in the late 1970s took shape as a number of hypotheses of the mechanisms of bile formation. Recent insights into the cellular and molecular mechanisms that control the function and regulation of hepatobiliary transport have led to a greater understanding of the physiological significance of bile secretion. Individual carriers for bile acids and other organic anions in the liver and intestine have now been obtained from several species. In addition, complex networks of signals that regulate key enzymes and membrane transporters located in cells that participate in the metabolism or transport of biliary constituents are being unraveled<sup>[5]</sup>. Most of the membrane transporters ensuring bile formation have now been identified. The expression of these membrane transporters is regulated through transcriptional and post-transductional mechanisms. Transcriptional regulation is under the control of nuclear receptors activated by ligands such as bile acids, which act as endogenous steroids synthesized from cholesterol in hepatocytes<sup>[3]</sup>.

Bile is an iso-osmotic electrolytic fluid that is formed in the liver and is a product of its secretory function. Bile is primarily secreted by hepatocytes (*i.e.*, canalicular bile) and subsequently delivered to the intrahepatic bile ducts, where it is modified by cholangiocytes (*i.e.*, ductal bile). Bile secretion by liver parenchymal cells is the result of vectorial transcellular transport of solutes and involves the coordinated action of transport proteins at the basolateral (sinusoidal) and apical (canalicular) membranes of the hepatocyte. A complex network of signals controls uptake and efflux transporters on a long- and short-term timescale, including regulation at the level of gene transcription, protein translation and maturation, covalent modification, and dynamic localization of transporter proteins, as well as substrate availability<sup>[6]</sup>.

## COMPOSITION OF BILE AND STRUCTURE OF A BILE CANALICULUS

Bile contains almost all body components: proteins, lipids, carbohydrates, vitamins, mineral salts, and trace elements. The greater part of the bile proteins consists of globulins, and the lesser part comprises albumins. Phospholipids, cholesterol and its esters, neutral fats, and fatty acids rank high among bile lipids. Lecithins (phosphatidylcholines, PCs) are the major representatives of bile phospholipids. They are synthesized in the liver from the same components as plasma PCs; however, they differ from the latter in the higher content of palmitic acid<sup>[7]</sup>. The human bile concentration of free fatty acids and  $\alpha$ -monoglycerides is small. Human cystic bile shows small quantities of diglycerides and is virtually free of triglycerides.

The electrolyte content of bile is similar to that of plasma. The major cations are sodium, potassium, and calcium; the anions are chloride and bicarbonate. The bile content of sodium is about 10 times higher than that of

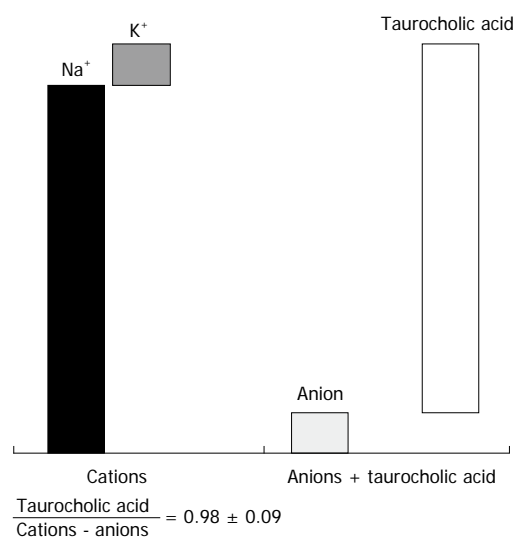


Figure 1 The bile content of anions, cations and taurocholic acid.

potassium. Excretion of sodium, potassium, and calcium into the bile is closely related to the rate of metabolic processes in the liver and depends on its functional state and on the content of salts in the body. The bile concentration of anions is 5-15 times smaller than that of cations.

A deficit of anions is compensated for by taurocholate (Figure 1). Bile contains a considerable quantity of phosphorus, magnesium, iodine, iron, and copper. The relative proportions of the major bile components are distributed in the following order: bile acids (67%), phospholipids (22%), proteins (4.5%), cholesterol (4%), and bilirubin (0.3%). Among the bile acids, the primary bile acids, cholic and chenodeoxycholic acids in a ratio of 1:1, account for about 50%. These are followed by the secondary bile acids, deoxycholic and lithocholic acids, as well as ursodeoxycholic and sulfolithocholic acids in the decreasing order.

The use of color cathode-luminescence scanning electron microscopy (CCL SEM) has made it possible to study the qualitative composition of dehydrated human bile and its components in the native state. CCL SEM provides video information about the structural pattern of bile and its chemical composition. Loginov *et al*<sup>[8]</sup> were the first to show the earlier unknown phenomenon of cathode autoluminescence of non-conjugated bilirubin, unesterified cholesterol, high-molecular-weight protein, and other organic compounds. The pictures (Figure 2) show that the amorphous powder mass of bilirubin is red-emitting (Figure 2A), crystal cholesterol luminesces blue (Figure 2B), and the green coloration tinged with red is predominant in the sample of protein (Figure 2C). Graphically, all three samples have chromaticity histograms in the form close to the Gaussian curve.

Examination of the dehydrated samples of normal bile by CCL SEM has ascertained the tree-type structure of precipitates (Figure 2D). The qualitative assessment of the samples suggests their balanced content of three ma-

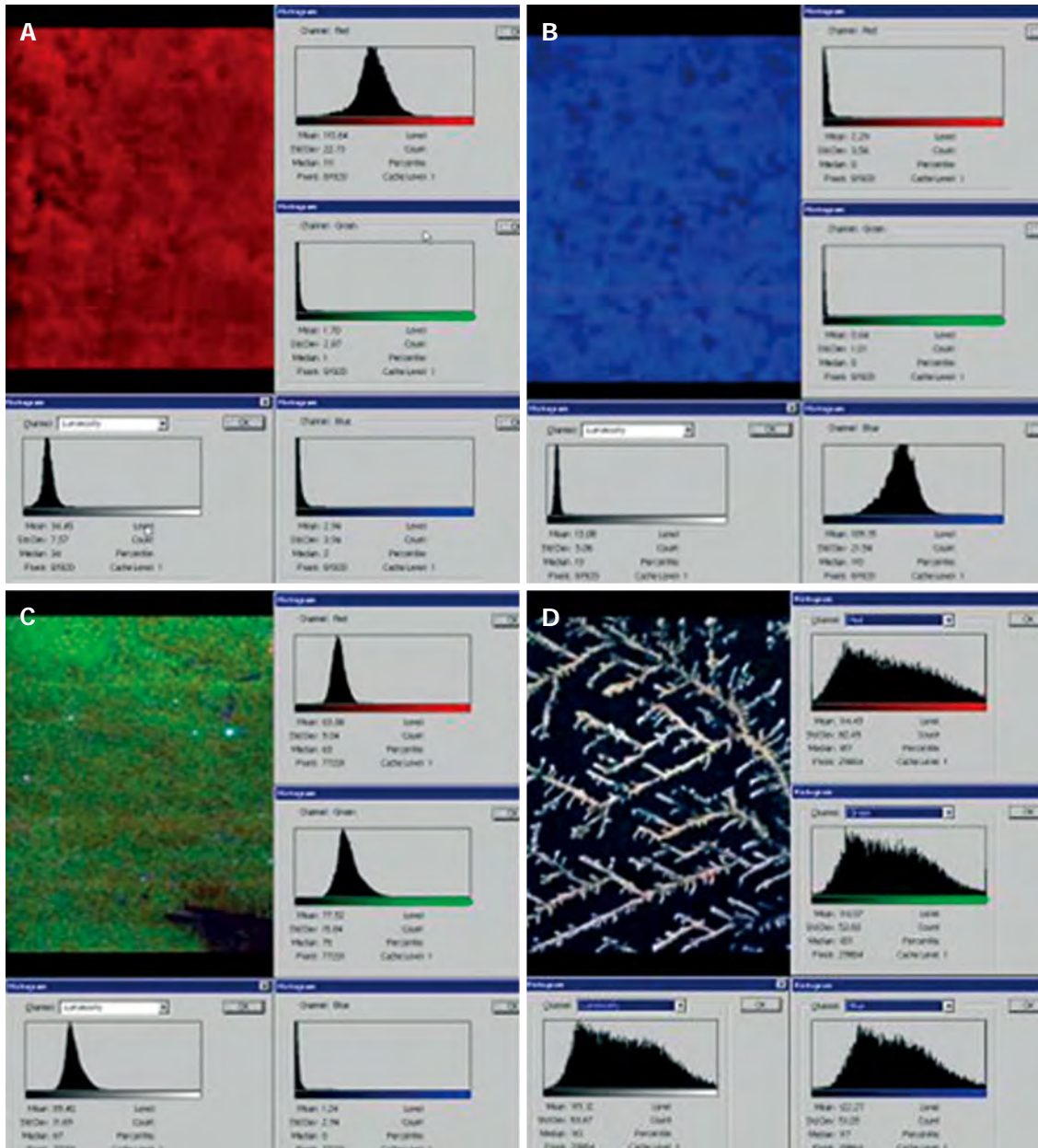


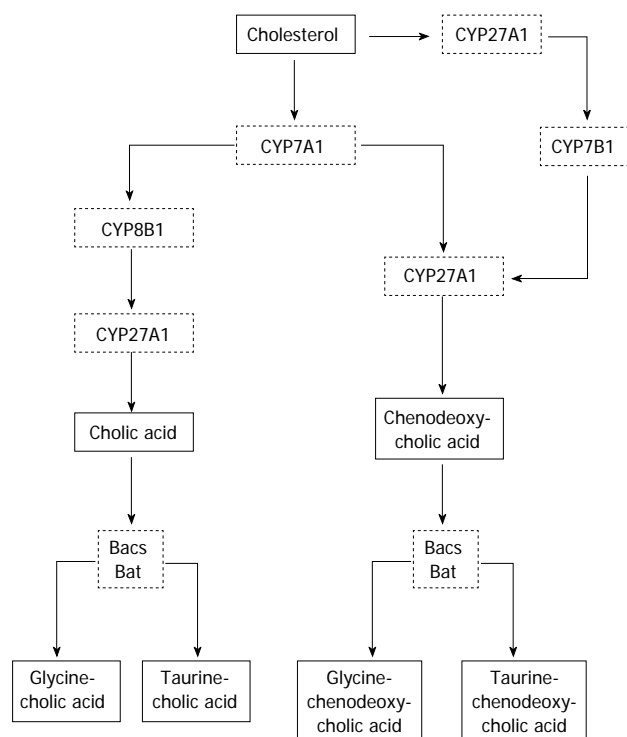
Figure 2 Color cathode-luminescence scanning electron microscopy images. Color cathode-luminescence scanning electron microscopy (CCL SEM) micro images of unconjugated bilirubin (A), unesterified cholesterol (B), high-molecular-weight protein (C), and dehydrated sample of normal human cystic bile (D).

for bile components: bilirubin, cholesterol, and protein. Hepatocytes are the major epithelial cells in the liver, and are polarized. The polarized surfaces of hepatocytes consist of a basolateral domain facing the circulation and an apical domain that forms the bile canaliculus; the smallest branch of the biliary tree<sup>[9]</sup>. A major function of the liver is biliary secretion, which requires hepatocyte polarization. The mechanisms controlling hepatocyte polarization are only partially understood<sup>[10]</sup>. Structurally, they include cytoskeletal, tight junction and intracellular trafficking components. Recently, it was discovered that the major mammalian bile acid, taurocholate, accelerated polarity in primary rat hepatocytes. Taurocholate increases cellular cAMP and signals through an exchange protein activated by cAMP (EPAC)-Rap1-MEK-liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK) pathway for its

polarity effect. Fu *et al*<sup>[9]</sup> have discussed possible mechanisms for how taurocholate affects different cell polarity factors, particularly AMPK, and thereby regulates events that generate polarity.

Bile production starts in the intercellular bile canaliculi. The bile canaliculus lumen is formed by the external hemileaflet of the apical part of the plasma membrane of the adjacent hepatocytes and the tight junctions located at the point of contact of the hepatocytes. The hepatocyte tight junctions separate the bile canaliculus lumen from the hepatic blood system<sup>[11]</sup>. Tight junctions contain proteins, including occludin, claudin and zona occludens (ZO)-1 protein. The integrity of tight junctions depends on the presence of protein ZO-1 on the inner surface of the plasma membrane. The impaired integrity of the tight junctions is accompanied by regurgitation of





**Figure 3** Bile acid synthesis. CYP7B1: 7 $\alpha$ -hydroxylase; CYP7A1: Enzyme cholesterol-7 $\alpha$ -hydroxylase; CYP8B1: Sterol 12  $\alpha$ -hydroxylase; CYP27A1: Mitochondrial sterol 27 hydroxylase.

canalicular bile into the sinusoids. Three individual domains, such as sinusoidal, lateral, and canalicular parts of the membrane, are arbitrarily identified in the hepatocyte plasma membrane. The differences in the lipid and protein composition of the three domains of the hepatocyte plasma membrane determine its functional polarity. The sinusoidal membrane is enriched with receptors, enzymes, and transport proteins. The lateral membrane of the hepatocyte is involved in intercellular interaction, but it contains no transport systems. Transport systems for bile acids, organic anions and cations, as well as the enzymes  $\gamma$ -glutamyltransferase,  $Mg^{2+}$ -ATPase, and alkaline phosphatase are located on the canalicular membrane.  $Cl/HCO_3^-$  exchangers and selective  $Cl$  channels are also identified<sup>[12]</sup>. Whether there are transport systems for glutathione and its conjugates in the apical membrane is still debated<sup>[13]</sup>. The release of glutathione S-conjugates from cells is an ATP-dependent process mediated by integral membrane glycoproteins<sup>[14]</sup>.

The hepatocyte cytoplasm around the bile canaliculus contains cytoskeletal structures: microtubules, microfilaments, and intermediate filaments<sup>[15]</sup>. The microtubules take part in the mediated vesicular transport, secretion of lipids. The microfilaments determine canalicular contractility and motor activity. The intermediate filaments form a network between the plasma membranes, nucleus, intracellular organelles, and other cytoskeletal structures. The canaliculus is the least branch of a biliary tree and has a diameter of approximately 1  $\mu m$ .

## BIOSYNTHESIS OF BILE ACIDS

In 1848 Strecker discovered bile acids<sup>[16,17]</sup>. In chemical structure, they belong to a group of steroids and are cholan acid derivatives. Bile acids are the end metabolic product of cholesterol and one of the most important routes of its elimination from the body<sup>[18-20]</sup>. Bile acid synthesis occurs through two pathways: the classic (neutral) pathway or the alternative (acidic) pathway<sup>[21,22]</sup>. The liver is the only organ that has all 14 enzymes required for *de novo* synthesis of two primary bile acids in humans, cholic acid (CA; 3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxy-cholan acid) and chenodeoxycholic acid (CDCA; 3 $\alpha$ , 7 $\alpha$ -dihydroxy-cholan acid)<sup>[23]</sup>.

The biosynthetic pathway includes a number of successive enzymatic conversions associated with the oxidation of cholesterol in the smooth endoplasmic reticulum and the shortening of its side chain in mitochondria. The production of bile acids involves restoration of a double bond in cholesterol, C-3 inversion to give rise to a 3 $\alpha$ -OH-group, further  $\alpha$ -hydroxylation of either only a 7-carbon atom or 7- and 12-carbon atoms, as well as  $\beta$ -oxidation of the side chain of cholesterol. Cytochrome P450 is involved in all oxidative reactions. The classic bile acid biosynthetic pathway is initiated by enzyme cholesterol-7 $\alpha$ -hydroxylase (CYP7A1) (Figure 3)<sup>[20]</sup>. The enzyme CYP7A1 of smooth endoplasmic reticulum is a limiting link in the synthesis of bile acids<sup>[20]</sup>. The activity of this enzyme is regulated by the quantity of intestinally absorbed bile acids, other than cholesterol<sup>[20,24]</sup>. Numerous studies have demonstrated that bile acids, steroid hormones, inflammatory cytokines, insulin, and growth factors inhibit CYP7A1 transcription through the 5'-upstream region of the promoter<sup>[25-30]</sup>. Sterol 12  $\alpha$ -hydroxylase (CYP8B1) is required for synthesis of CA. Mitochondrial sterol 27 hydroxylase (CYP27A1) catalyzes sterol side chain oxidation, after which cleavage of a three-carbon unit in the peroxisomes leads to formation of a C24 bile acid. Cholesterol is converted to two primary bile acids in human liver, CA and CDCA. Key regulated enzymes, CYP7A1, CYP8B1, CYP27A1, and 7 $\alpha$ -hydroxylase (CYP7B1) expressed, in the pathways are indicated. CYP7A1 initiates the classic (neutral) bile acid biosynthetic pathway in the liver. CYP27A1 initiates the alternative (acidic) pathway in the liver and macrophages. CA and CDCA are conjugated to glycine (G) and taurine (T). Bile acid: CoA synthase (BACS) and bile acid: amino acid transferase (BAT) are two key enzymes involved in amino conjugation of bile acids<sup>[20]</sup>.

An alternative (acidic) pathway is initiated by CYP27A1, which in addition to the liver is expressed in macrophages and most other tissues, and may contribute significantly to total bile acid synthesis (Figure 3). Other minor pathways initiated by 25-hydroxylase in the liver and 24-hydroxylase in the brain also may contribute to bile acid synthesis. A nonspecific CYP7B1 expressed in all tissues is involved in the generation of oxidized metabolites (oxysterols), which may be transported to the



liver and converted to CDCA<sup>[20]</sup>. Farnesoid X receptor (FXR) plays a central role in the regulation of bile acid synthesis, excretion, and transport<sup>[31,32]</sup>. FXR inhibits *CYP7A1*<sup>[33]</sup>, *CYP8B1*<sup>[34]</sup>, and *CYP27A1*<sup>[35]</sup> transcription. It has been reported that peroxisome proliferator activated receptor (PPAR) $\alpha$  plays a role in the regulation of bile acid synthesis<sup>[36]</sup>. Bile acids induce *PPAR $\alpha$*  transcription *via* induction of FXR<sup>[37]</sup>.

Di- and tri-hydroxycholanolic primary bile acids are produced from cholesterol during biosynthesis of bile acids in humans. Under normal conditions, 200-600 mg primary bile acids is formed daily<sup>[20,38]</sup>. Bile acid synthesis increases in the morning regardless of food intake<sup>[39]</sup>. In humans, bile acid synthesis exhibits a diurnal rhythm with two peaks around 15:00 and 21:00 h<sup>[20]</sup>. Bile acids lost in the feces (0.2-0.6 g/d) are replenished by *de novo* synthesis in the liver to maintain a constant bile acid pool. Hepatic conversion of cholesterol to bile acid balances fecal bile acid excretion and this process represents a major route for elimination of cholesterol from the body<sup>[40,41]</sup>.

Bile acids are physiological detergents that generate bile flow and facilitate intestinal absorption and transport of lipids, nutrients and vitamins<sup>[20]</sup>. Bile acids, end products of the pathway for cholesterol elimination, are required for dietary lipid and fat-soluble vitamin absorption and maintain the balance between cholesterol synthesis in the liver and cholesterol excretion<sup>[21]</sup>.

## CONJUGATION OF BILE ACIDS

Under physiological conditions, free bile acids are not frequently found in bile. Newly synthesized bile acids conjugate with glycine or taurine, by giving rise to bile salts: (chenodeoxy-)cholyglycine or (chenodeoxy-)cholytaurine (Figure 3). The physiological significance of bile acids conjugation is that their salts are more polar compounds than free bile acids. Conjugation of bile acids to glycine or taurine decreases bile acid toxicity and increases their solubility that benefits secretion into bile. Conjugated bile acids have a smaller critical concentration for micellar formation<sup>[42]</sup>. The taurine conjugates of bile acids are more polar compounds than their glycine conjugates<sup>[43]</sup>. Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in humans and plays an important role in several essential biological processes such as bile acid conjugation, maintenance of calcium homeostasis, osmoregulation and membrane stabilization<sup>[44]</sup>. Taurine is efficient at reducing plasma and liver cholesterol concentrations. The cholesterol-lowering effect of taurine is involved in the regulatory mechanism of cholesterol and bile acid homeostasis that is mediated by *CYP7A1*, which has become a biomarker for cholesterol metabolism and is itself also regulated by several factors and nuclear receptors<sup>[45]</sup>.

The C-24 conjugation of free cholanolic acids with glycine or taurine is accomplished by hepatocyte acyl-transferases. The reaction proceeds in two steps with the participation of ATP and in the presence of  $Mg^{2+}$ . BACS and BAT are involved in amino acid conjugation of bile

acids<sup>[20]</sup>. FXR stimulates bile acid conjugation by inducing expression of genes encoding BACS and BAT, which also are induced by hepatocyte nuclear factor (HNF) 4 $\alpha$ <sup>[46]</sup>. Thus, FXR and HNF4 $\alpha$  may coordinately regulate bile acid synthesis and conjugation.

The normal human ratio of the glycine to taurine conjugates of bile acids is 3:1. The ratio of the glycine/taurine conjugates may be altered under the influence of alimentary and hormonal factors, in some liver diseases. The presence of unconjugated bile acids in the bile most frequently is a result of hepatic disease.

In the intestine, glyco- and tauro-conjugated CA and CDCA are deconjugated, and 7 $\alpha$ -dehydroxylase activity in bacterial flora removes a 7 $\alpha$ -hydroxy group to form secondary bile acids deoxycholic acid (DCA; 3 $\alpha$ ,12 $\alpha$ -dihydroxy) and lithocholic acid (LCA; 3 $\alpha$ -monohydroxy), respectively<sup>[20]</sup>.

Bile acids may be also exposed to glucuronidation or sulfation, which results in a reduction of their toxic properties and promotes their urinary and fecal excretion<sup>[43,47-49]</sup>. A small amount of bile acids circulated to the liver is sulfoconjugation at the 3-hydroxy position by sulfotransferase (SULT2A1) and rapidly secreted into bile. Sulfation is the major pathway for detoxification of extremely hydrophobic bile acids in humans<sup>[50]</sup>. Details of bile acid chemistry, biology, physiology, and synthesis have been reviewed recently<sup>[51,52]</sup>.

## BILE PHOSPHOLIPIDS

Phospholipids are a heterogeneous group of substances. They contain fatty acids with a varying length of carbon chain. Phospholipids are an important component of cell membranes, plasma, and bile. In all mammalian species, including humans, bile phospholipids are represented solely by a mixture of PC (lecithin) molecules. The fatty acids of bile PC molecules greatly differ from those of lecithin of plasma and liver tissue. Depending on fatty acid residues at C1 and C2 of the glycerine molecule that is part of PC, there are a variety of lecithins. Human bile predominantly contains 1-palmitoyl, 2-linoleyl, and 1-palmitoyl, 2-oleoyl PCs. This lecithin subpool represents nearly 5% of the total pool of hepatic PCs. The lecithins destined for bile secretion have been shown to be apparently completely synthesized *de novo*<sup>[53]</sup>. Selection of certain types of PC seems to occur during bile formation<sup>[7]</sup>. Bile salts and primarily PC are the main organic solutes in bile, and play a crucial role in cholesterol and dietary lipid solubilization. Bile salts and phospholipids in concert increase the biliary solubility of cholesterol by > 1 million-fold, thereby permitting entry of hepatocellular cholesterol into bile<sup>[54]</sup>. Hepatic PC biosynthesis is likely to be regulated by bile salts. Biliary lipid secretion is driven by bile salts, the primary metabolites of cholesterol. There are multiple data showing that the biliary secretion of PC (as cholesterol secretion) depends on that of bile acids<sup>[55,56]</sup>. It has also been shown that the secretion of bile phospholipids is not only associated with that of bile acids, but is regulated by the amount and type of bile

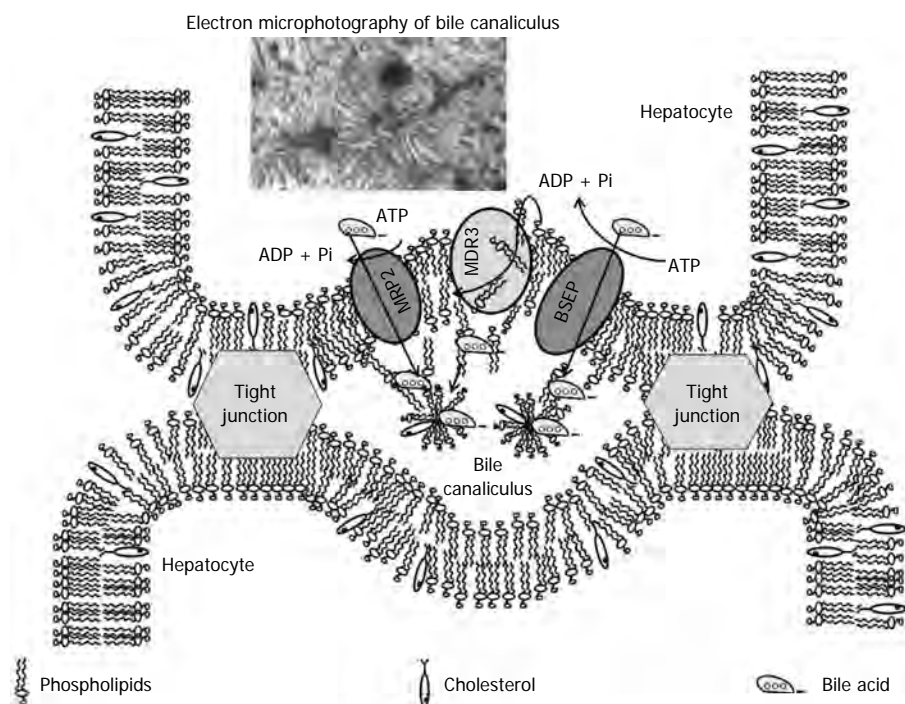


Figure 4 Proposed model for lipid secretion into bile<sup>[58]</sup>. BSEP: Bile salt export pump; MDR3: Multidrug-resistance protein 3; MRP2: Multidrug-resistance-associated protein 2; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; Pi: Inorganic phosphate.

salts passing through the hepatocytes<sup>[7,57]</sup>.

The use of electron microscopy has demonstrated that phospholipids are secreted as vesicles into bile, and that vesicle secretion is almost wholly dependent upon MDR3 P-glycoprotein function<sup>[58]</sup>. Biliary-specific PC molecules are recruited initially from intracellular sources, predominantly the endoplasmic reticulum<sup>[59]</sup>. A proposed mechanism for PC delivery to the canalicular plasma membrane involves monomeric transfer *via* binding to the cytosolic PC transfer protein (PC-TP)<sup>[60]</sup>; a process that is stimulated by low concentrations of bile salts typical of the hepatocytic cytosol. Alternatively, biliary lipid may arrive at the canalicular membrane *via* intracellular vesicle transport<sup>[1]</sup>.

Biliary PC molecules are translocated from the endoplasmic to the exoplasmic hemileaflet by the action of a transmembrane translocator<sup>[61]</sup>.

MDR3 P-glycoprotein is thought to translocate PCs from the internal to the external hemileaflet of the hepatocyte canalicular plasma membrane; possibly generating microdomains of the more fluid biliary-type PCs. Luminal bile salts, secreted as monomers through the action of bile salt transporters (cBATs), interact preferentially with these microdomains to promote vesiculation of the membrane external hemileaflet; possibly involving non-lamellar phase transitions. Cholesterol, which can freely flip between the internal and external hemileaflets, may be released into bile either by diffusing laterally into nascent vesicles, or by bile-salt-mediated transfer through the aqueous phase. The model is based on data from the study<sup>[59]</sup> and published work<sup>[1,62,63]</sup>.

Crawford *et al*<sup>[58]</sup> proposed the concept that biliary PC

molecules are flipped from the internal to the external hemileaflet of the hepatocyte canalicular membrane by the action of MDR3 P-glycoprotein, followed by release of PC vesicles from the external hemileaflet into bile (Figure 4). Formation and detachment of unilamellar vesicles from the canalicular membrane is presumably mediated by the detergent action of luminal bile salts.

Vesiculation of the external hemileaflet provides an explanation of how luminal bile salts can extract large quantities of phospholipid on the basis of their detergent action, without disrupting the integrity of the detergent-resistant canalicular plasma membrane. This remarkable mechanism for selective secretion of membrane phospholipids appears to be uniquely adapted to the detergent environment of the bile canaliculus.

## BILE MICELLES

Bile lecithins, bile acid salts, and cholesterol are amphiphilic molecules (Figure 5). In this connection, in the water medium, such as bile, these compounds cannot exist in the monomolecular form and they generate micellar or lamellar structures.

According to the inclusions into the lipid complex, simple micelles (PC + cholesterol), up to 3 nm in size, mixed (PC + cholesterol + bile acids), 3-6 nm in diameter, and vesicles (PC + cholesterol + bile acids), 25-130 nm in diameter, are identified (Figure 6).

They can all contain amphiphilic proteins on their surface. Lipid molecular inclusions in the micelles of bile acids and the formation of mixed micelles are the main form of interaction of bile acids and lipids in the bile.

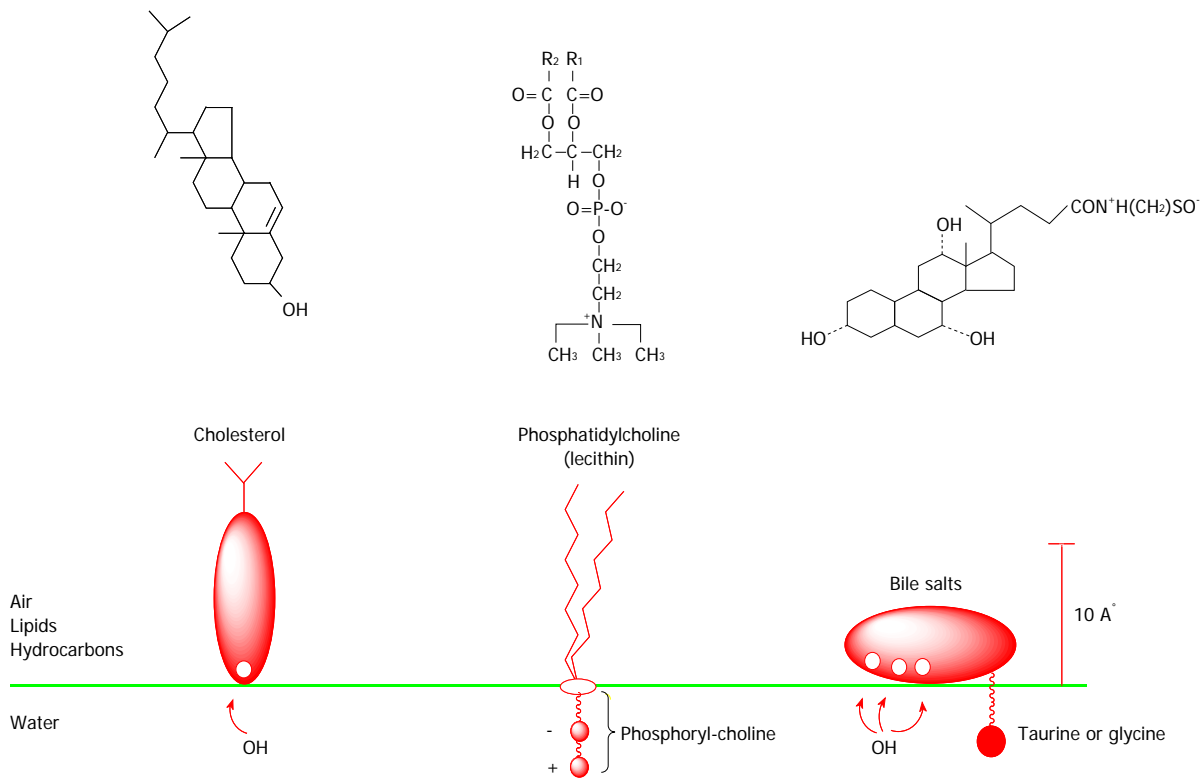


Figure 5 Chemical formulas and simple images of cholesterol, lecithins, and bile salts on a boundary of two phases-water: lipids (air, hydrocarbons).

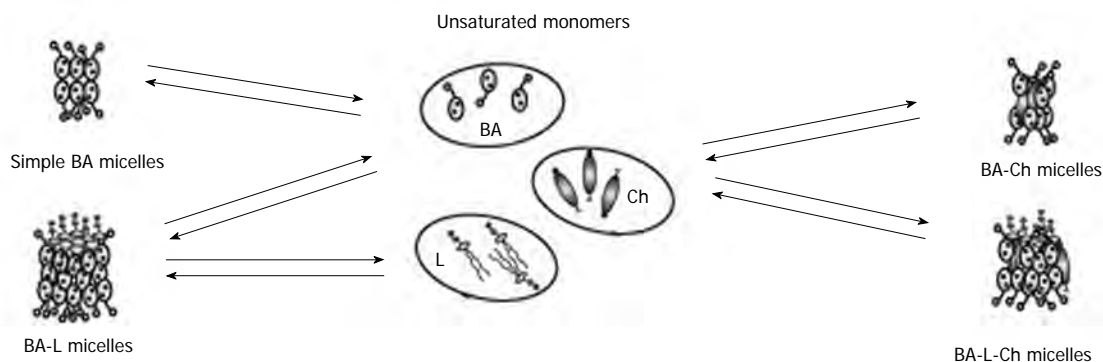


Figure 6 Formation of micelles structures in the system containing bile acids, lecithin, and cholesterol. BA: Bile acids; Ch: Cholesterol; L: Lecithin.

By forming mixed micelles, bile acids along with lecithin provide cholesterol solubilization. Bile salts as detergents form micelles when the critical concentration of micellar formation (CCM) is achieved. CCM depends on the type of bile salts and their hydrophobicity. Based on their hydrophobic properties, bile acids may be arranged in the following order: deoxycholate > chenodeoxycholate > cholate > ursodeoxycholate<sup>[64]</sup>. Hydrophobic, but not hydrophilic, bile acids are efficacious endogenous ligands of the nuclear receptors FXR (NR1H4), pregnane X receptor (PXR; NR1I2), and vitamin D receptor (VDR; NR1I1) that play critical roles in the regulation of bile acid synthesis and metabolism<sup>[20]</sup>.

A mixture of bile acids, lecithin, and cholesterol at certain molecular ratios is able to form lamellar liquid-crystalline structures<sup>[64]</sup>. Vesicles are monolamellar glob-

ules that consist of phospholipids and bile acids. At a certain concentration (about 0.1 mmol/L), phospholipids as amphiphiles have been found to produce enclosed globules, in which the polar heads are directed outward, and the nonpolar "tails" are inside. This particle is capable of forming many substances, including protein, and cholesterol, by providing solubilization of the latter by bile acids. Vesiculation is largely determined by the hydrophilic-hydrophobic balance of bile acids and the content of cholesterol<sup>[65]</sup>. The proportion of bile-mixed micelles and vesicles depends on the concentration and composition of bile acids. The most important feature of phospholipid masses is their ability to change their consistency from crystalline gel to liquid-crystalline state depending on environmental conditions. The liquid-crystalline states are typified by the structural order peculiar to true crystals

with the preserved mobility of the molecules observed in solutions. The fusion of monolamellar vesicles may give rise to multilamellar vesicles. The vesicles are a rather stable formation and together with micelles play a pivotal role in the solubilization and transport of cholesterol into the bile<sup>[24]</sup>.

Lipid vesicles 60-80 nm in diameter have been documented repeatedly by electron microscopy and quasielastic light scattering of freshly secreted hepatic bile<sup>[1,58]</sup>. Microscope laser light scattering of bile canaliculi in isolated rat hepatocyte couplets also detects intraluminal vesicles of similar size; the numbers of which increase upon cellular incubation with bile salts<sup>[66]</sup>.

Acid-dependent and acid-independent bile fractions are identified<sup>[67]</sup>. The former is associated with the synthesis and active transport of bile acids in the hepatocytes. The volume of the resultant bile is linear with the concentration of bile acids, caused by the osmotic effect of bile acid anions, and Sperbet<sup>[68]</sup> first argued it in 1959. The bile composition greatly varies in the bile canaliculi due to the increased constant volume of water and bicarbonates irrespective of the secretion of bile acids. This acid-independent bile fraction is regulated by an active  $\text{Na}^+$  transport. Its physiological role is to dilute and excrete an acid-dependent fraction<sup>[69]</sup>. Further bile formation occurs in the bile ducts because a fluid enriched with bicarbonates and chlorides is produced in the latter. This process is under the control of secretin (80%) and other gastrointestinal hormones. The volume of the water secreted in the ducts depends to greater degree on the active transport of bicarbonates<sup>[70]</sup>.

## ENTEROHEPATIC CIRCULATION OF BILE ACIDS

The bile acids synthesized in the hepatocyte participate in the human body, in the so-called enterohepatic circulation. Conjugated bile acids are secreted from the hepatocytes into the bile canaliculus by canalicular bile salt export pump (BSEP, ABCB11). Some bile acids secreted in the bile duct are reabsorbed in the cholangiocytes and recycled back to hepatocytes (the cholangiohepatic shunt)<sup>[20]</sup>. The bulk of bile acids secreted are stored in the gallbladder. After each meal, gallbladder contraction empties bile acids into the intestinal tract. Bile acids are mixed up with food masses (together with other digestive secretions), and take an active part in the processes of fat and fat-soluble vitamins metabolism and absorption.

Fatty acids and monoglycerides formed from neutral fats with participation of bile acids and of lipases in the upper parts of the small intestine are absorbed by enterocytes as lipid-biliary complexes. These complexes disintegrate in the enterocytes. Released monoglycerides and fatty acids are used by the cells as energy and building materials or are transported to the basolateral membrane of the enterocytes and enter the portal venous system. Bile acids may return to the intestine and continue to participate in metabolism and absorption of fat and fat-

soluble vitamins. As bile acids are moving through the intestine they may participate 4-6 times in the transportation of monoglycerides and fatty acids in enterocytes.

In the small intestine, bile acids are absorbed by both passive and active mechanisms<sup>[71]</sup>. Although passive absorption occurs down the length of the intestine, active absorption of bile acids is restricted to the ileum<sup>[72,73]</sup>. Passing through the intestinal tract, some bile acids are reabsorbed in the upper intestine by passive diffusion, but most bile acids (95%) are reabsorbed in the distal ileum by apical sodium-dependent bile acid transporter (ASBT) located in the brush border membrane<sup>[71]</sup>. In humans and all other vertebrates examined to date, the ileal epithelium has developed an efficient transport system for the active reclamation of bile acids<sup>[51]</sup>. Approximately 95% of the bile acids secreted into bile are derived from the recirculating pool<sup>[71]</sup>. Bile acids are transdiffused across the enterocytes to the basolateral membrane where the organic solute transporter and heterodimer (OST/OST) effluxes bile acids into the portal blood circulation<sup>[74,75]</sup>, transports them to the sinusoids, where they are taken up by  $\text{Na}^+$ -dependent taurocholate cotransport peptide (NTCP) into hepatocytes<sup>[20]</sup>. The bulk of bile acids are absorbed by hepatocytes into the liver, and again excreted into the bile. Later, this enterohepatic cycle recurs. Hepatocytes are known to secrete as much as 90% of the bile acids that have returned into the cells during enterohepatic circulation, and about 10% of the newly synthesized bile acids<sup>[20]</sup>. A bile acid pool of about 3 g is recycled 4-12 times a day<sup>[71]</sup>. At a fundamental level, the bile acid enterohepatic circulation can be viewed as a series of storage chambers (gallbladder and small intestine), valves (sphincter of Oddi and ileocecal valve), mechanical pumps (canaliculi, biliary tract, and small intestine), and chemical pumps (hepatocyte and enterocyte transporters)<sup>[71]</sup>.

The enterohepatic circulation of bile acids serves as an important physiological route for recycling bile acids and absorption of nutrients, as well as regulation of whole-body lipid metabolism<sup>[20]</sup>. Enterohepatic circulation ensures a continuous supply of bile acids to be used repeatedly for lipid absorption during the digestion of a single meal or multiple meals throughout the day<sup>[76]</sup>. Efficient intestinal reabsorption and hepatic extraction of bile acids also enables effective recycling and conservation that largely restricts these potentially cytotoxic detergents to the intestinal and hepatobiliary compartments.

FXR plays a key role in the control of enterohepatic circulation of bile acids. FXR induces the expression of BSEP in the canalicular membrane, which is the driving force for bile formation. FXR inhibits *Ntcp* transcription by SHP-dependent inhibition of retinoid X receptor/retinoic acid receptor induction of *Ntcp*<sup>[77]</sup>. Thus, FXR plays a critical role in the coordination of bile acid synthesis, biliary bile acid secretion, intestinal bile acid reabsorption and secretion, and bile acid uptake into hepatocytes.

Hepatocytes hold a central position in the enterohepatic circulation of bile acids, with which their highest (as compared to other cells) content of cholanics acids is as-



sociated.

### **Molecular mechanisms of hepatocyte bile secretion**

Hepatocyte secretion of bile is the passive transhepato-cellular filtration of water and electrolytes ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ ) from blood into the bile canalicular cavity, which is caused by the osmotic gradient between the bile and blood, which results from active transport of organic and inorganic anions from blood and hepatocytes to the bile canaliculi. The ionic transport systems that govern ionic and electrical gradients onto the membrane surface ( $\text{Na}^+/\text{K}^+$ ,  $\text{Na}^+/\text{H}^+$ ,  $\text{Na}^+/\text{HCO}_3^-$ ,  $\text{Cl}^-/\text{HCO}_3^-$ , and  $\text{Na}^+/\text{Ca}^{2+}$ ) are the basis for the mechanism of targeted bile secretion<sup>[78]</sup>. The transport of bile salts, organic anions and cations, bilirubin and other substances from the portal blood into the biliary system is accomplished through the action of an array of transporter proteins in the hepatocytes<sup>[79]</sup>.

The hepatocytes are polar cells in which the transport of osmotically active bile components has a strict direction from the sinusoidal (basal) surface to its canalicular (apical) part. There is evidence on the importance of taurocholic acid in the directed transport of substances in the liver cells<sup>[44]</sup>. This vectorial trans-hepatocellular movement of bile acids is a remarkably concentrative transport process that is driven by a distinct set of primary (ATP-dependent), secondary ( $\text{Na}^+$  gradient-dependent), and tertiary ( $\text{OH}^-$  or  $\text{HCO}_3^-$ -dependent anion exchange) transport systems at the sinusoidal and canalicular plasma membranes<sup>[80,81]</sup>. Bile secretion is a highly regulated process. To maintain this process, liver cells must transport bile acids efficiently from the portal blood into bile. ATP binding cassette (ABC) transporters<sup>[57]</sup> mediate transport of bile salts. Expression of these transporters is regulated in a coordinate fashion by a set of nuclear hormone receptors explaining the old observation of coupling between bile salt secretion and biliary lipid secretion. Over the past two decades, there has been significant progress toward identifying the individual membrane transporters and unraveling their complex regulation<sup>[74]</sup>. The hepatocytes are polarized cells that express differential transport systems in their plasma membrane domains. Molecular cloning has identified most of these transport proteins, and their transport properties characterized by functional studies. The NTCP, the bile salt export pump (BSEP), the ASBT, and the organic solute transporter OST $\alpha$ -OST $\beta$ , the major bile acid transporters that control the fate of bile acids, through either absorption and enterohepatic cycling or excretion and elimination from the body.

These transporters play a key role in the vectorial transfer of solutes and water from sinusoidal blood into the bile, thus contributing to bile formation and the biliary excretion of various xenobiotics. In the liver and intestine, transporters play a critical role in maintaining the enterohepatic circulation and bile acid homeostasis<sup>[71,82]</sup>.

Bile acids determine the secretion of an acid-dependent bile fraction. The transport of bile acids from the sinusoidal space to the bile canaliculus comprises their penetration across the sinusoidal and canalicular

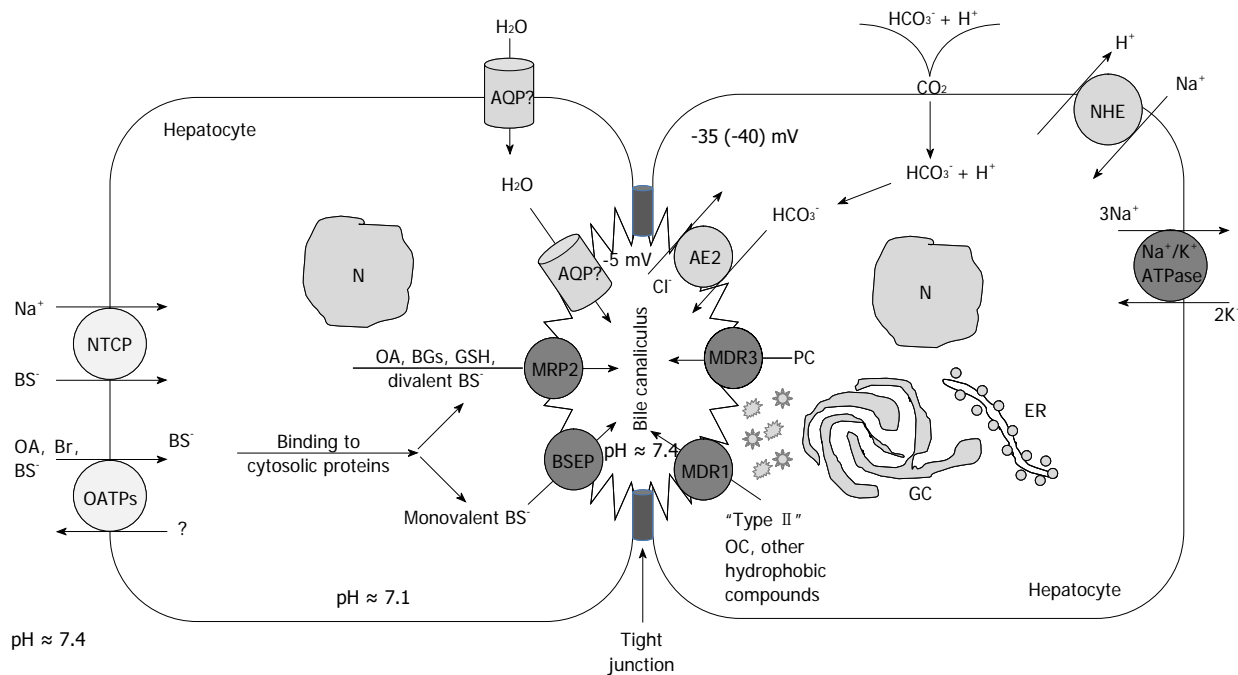
membranes and movement inside the hepatocytes. The molecular and metabolic processes occurring on the hepatocyte sinusoidal membrane involve: (1) hepatocyte absorption of bile acids and proteins from blood; and (2) transmembrane transfer of inorganic ions. The principal intracellular molecular and metabolic processes determining the mechanisms of hepatocyte bile secretion are: (1) biosynthesis and conjugation of bile acids; (2) hydroxylation and conjugation of bile acids entering the hepatocytes from the blood; and (3) transfer of bile acids and proteins from the perisinusoidal to pericanalicular area of the hepatocytes.

The molecular mechanisms of hepatocyte canalicular membrane function ensure the transmembrane transfer of bile acids, proteins, inorganic and organic cations and anions into the bile canalicular cavity.

**Molecular physiology of the transporting systems of the hepatocytic sinusoidal membrane:** The specific feature of transport of bile acids from blood to the hepatocyte across the sinusoidal membrane is to overcome the high gradient of their concentration and electrical potential. Hepatocytes absorb bile acids from the blood by the bile acid carriers, which are integral hepatocyte sinusoidal membrane proteins<sup>[78]</sup> (Figure 7).

Liver sinusoids possess a specific architecture that allows passage of organic compounds bound to albumin through endothelial fenestrae into the space of Disse, from where they can be taken up by the sinusoidal transport systems of the hepatocytes<sup>[83,84]</sup>.

The bulk (about 85%) of the bile acids are present in plasma as a complex of plasma proteins (mainly, albumin)-bile acid, therefore, it has been shown that albumin performs its inherent transport function and contributes to the interaction of bile acids with specific sinusoidal membrane receptors in the mechanisms of hepatocyte absorption of bile acids. The bile acids in sinusoidal blood are efficiently taken up by hepatocytes from Disse's space despite being highly albumin bound, due to the existence in the basolateral membrane of transporters<sup>[85,86]</sup>. The powerful techniques of molecular biology have enabled gene cloning of the transporters involved in biliary secretion and the enterohepatic circulation of bile acids. This has permitted elucidation of their function as well as their regulation by nuclear receptors<sup>[87]</sup>. The enterohepatic circulation results from efficient ileal absorption, and is highly regulated at two sites. In the hepatocytes, biosynthesis of bile acids is regulated by negative feedback by the nuclear receptor FXR as well as by cytokines and by a peptide (FGF-19) liberated by bile acids from the ileal enterocytes. In the ileal enterocytes, bile acid reclamation is regulated by negative feedback by FXR and other nuclear receptors. BSEP mediates uphill canalicular bile acid secretion<sup>[87]</sup>. The plasma membranes of hepatocytes have been found to contain the proteins that selectively bind bile acids<sup>[87]</sup>. This uptake is carried out against an electrochemical gradient, is saturable<sup>[88]</sup>, and depends on the structure of the bile acids. It is more



**Figure 7** Mechanisms of the transport of bile acids, water, and electrolytes through the hepatocyte (transcellular pathway) and intercellular space (paracellular pathway). Localization and function of sinusoidal and canalicular hepatocellular transporters. The  $\text{Na}^+$ -dependent sinusoidal uptake of bile salts (BS) is mediated by  $\text{Na}^+$ -dependent taurocholate cotransport peptide. The  $\text{Na}^+$ -independent hepatic uptake of organic anions (OA), BSs and type II organic cations (OC) is mediated by members of the OATP family. Sinusoidal uptake of type I OC is mediated by OCT1. Transport across the canalicular membrane is driven mainly by ATP-dependent export pumps. MDR1 mediates canalicular excretion of amphiphilic type II OC and other hydrophobic compounds. MDR3 functions as a PC flippase. BSEP mediates apical excretion of BSs. MRP2 transports non-bile-salt organic anions, such as bilirubin glucuronides (BGs), GSH, and sulfated/glucuronated bile salts. Canalicular transport of  $\text{HCO}_3^-$  is mediated by the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE2. AQP9 and AQP8 are involved in the transport of water across the sinusoidal and the canalicular membrane, respectively. The nature of the water channels in human liver has been characterized<sup>[84]</sup>. GC: Golgi apparatus (complex); ER: Endoplasmic reticulum; N: Nucleus.

efficient for trihydroxy- than for dihydroxy-bile acids and for conjugated more than for unconjugated bile acids<sup>[89]</sup>. Transporters on the basolateral membrane, which faces the space of Disse, are responsible for the uptake of bile salts and organic anions<sup>[79]</sup>. The liver cells have an active transport system to transfer bile acids from the blood to the hepatocytes. Basolateral uptake transporters can be divided into  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -independent systems. The NTCP, the main  $\text{Na}^+$ -dependent bile acids transporter<sup>[90-92]</sup> is only expressed in the basolateral membrane<sup>[93]</sup>. NTCP, (gene name SLC10A1) is the founding member of the SLC10 family of solute carrier proteins, which includes two bile acid carriers (SLC10A1/NTCP and SLC10A2/ASBT), one steroid sulfate transporter (SLC10A6/SOAT), and four orphan carriers (SLC10A3, SLC10A4, SLC10A5, SLC10A7)<sup>[94-96]</sup>.  $\text{Na}^+$ -dependent uptake involves co-transport of solutes with  $\text{Na}^+$ . Moreover, the driving force is the energy of ATP generated by  $\text{Na}^+, \text{K}^+$ -ATPase. This enzyme is located on the sinusoidal and lateral membranes of hepatocytes<sup>[97,98]</sup> and undetectable on the canalicular (apical) part<sup>[99,100]</sup>. The active transport of bile acids ensures their continuous hepatocyte pumping from blood to bile, thus causing their low level in the blood flowing from the liver and in plasma as a whole. The properties of NTCP satisfy all the functional criteria for hepatocyte  $\text{Na}^+$ -coupled bile acid uptake<sup>[71,94]</sup>. The major physiological substrates of NTCP include all the major glycine- and taurine-conjugated bile ac-

ids<sup>[93,101-104]</sup>. Depending on the structure of the bile acid and NTCP, unconjugated bile acids are moderate or weak substrates<sup>[102,105,106]</sup> and sulfated bile acids appear to be only weakly transported<sup>[107]</sup>. The high level of NTCP expression at the sinusoidal membrane of hepatocytes and high affinity of NTCP for conjugated bile acids promote efficient extraction of bile acids from portal blood. Thus, NTCP functions to maintain the enterohepatic circulation of bile acids and maintain plasma concentrations at a minimum.

The uptake of bile acids by  $\text{Na}^+$ -independent mechanisms seems to be mediated by less-specific transporters, known as organic anion-transporting polypeptides (OATPs), which exchange these molecules for other anions, such as  $\text{HCO}_3^-$ , glutathione (GSH) or even other bile acids<sup>[89,90,108]</sup>.

Four OATPs have been cloned and characterized from human liver: OATP1A2 (SLC10A2/SLC21A3; formerly, OATP-A), OATP1B1 (SLC21A6; formerly, OATP-C or LST-1), OATP1B3 (SLC21A8; formerly, OATP-8) and OATP2B1 (SLC21A9; formerly, OATP-B). These transporters may take up bile acids (mainly unconjugated forms), endogenous OA- (thyroid hormones, monoconjugated bilirubin) and xenobiotic compounds (e.g., toxins, drugs and food components)<sup>[109]</sup>. In contrast to NTCP, members of the OATP family expressed on the hepatocyte sinusoidal membrane, such as Oatp1a1 (Slc10a1), efficiently transport unconjugated or sulfated

bile acids, suggesting that these carriers participate in the hepatic uptake of those bile acid species *in vivo*<sup>[89,102,110]</sup>. The heterodimeric protein OST $\alpha$ /OST $\beta$  is expressed at the basal membrane of hepatocytes and cholangiocytes<sup>[111]</sup>. This is a sodium-independent bile acid transporter that may play a role in bile acid efflux from hepatocytes toward blood when these compounds are accumulated under cholestatic conditions. Moreover, in cholangiocytes, in addition to playing a similar role, this transporter may also be involved in the cholehepatic shunting of bile acids.

Hepatocellular uptake of organic cations is mediated by two separate transport systems, which depends on the substrate molecular size<sup>[112]</sup>. Thus, small (type I) organic cations are taken up by the organic cation transporter, OCT1/Oct1 (SLC22A1/Slc22a1), which is electrogenic in nature. In contrast, human OATP-A mediates the uptake of bulky (type II) organic cations. In addition to conjugated and unconjugated bile salts, Oatps/OATPs accept other cholephilic compounds, including glucuronidated (and maybe unconjugated) bilirubin, exogenous organic anions (*e.g.*, sulfobromophthalein), leukotrienes, estrogen-conjugates (*e.g.*, estrone-3-sulfate or estradiol-17- $\beta$ -d-glucuronide), thyroid hormones, mycotoxins, and numerous xenobiotics<sup>[92,113-115]</sup>.

**Molecular physiology of the intracellular processes underlying the hepatocyte secretion of bile:** In order to explain the transit of bile acids from the sinusoidal membrane to the pericanalicular region, two different, not mutually exclusive, mechanisms have been proposed: (1) simple diffusion of bile acids bound to intracellular proteins<sup>[116]</sup>; and (2) vesicular transport of bile acids driven by cytoskeleton contractile activity<sup>[117,118]</sup>. Two arguments have been raised against the role of the second mechanism. One is that hepatic transit of labeled bile acids is too fast<sup>[118]</sup>. The second is that the baseline secretion of bile acids is not modified by microtubular disruption<sup>[117]</sup>. However, the overload of bile acids intensifies the vesicular trafficking from the Golgi complex to the pericanalicular zone<sup>[119]</sup>, and under these circumstances the alteration in the functional integrity of the cytoskeleton results in impaired bile acid secretion<sup>[120]</sup> and subsequent cholestasis<sup>[121]</sup>.

The quantity of ABC transporters in the apical membrane is regulated by the amount of biliary components available for secretion<sup>[122,123]</sup>. The regulated intracellular vesicular traffic of canalicular ABC transporters<sup>[123,124]</sup> is crucial for normal bile secretion. BSEP (formerly SPGE, a sister of P-glycoprotein) is the main, if not the only, canalicular bile acid transporter<sup>[125]</sup>, and it is also located in subcanalicular vesicles that may act as an intracellular pool. It is therefore probable that the impaired secretion of bile acids observed in overloaded conditions is an indirect result of the distortion of the increased vesicular traffic of transporters to the canalicular membrane<sup>[120]</sup>. These and other studies<sup>[126,127]</sup> have established the actual role of vesicular trafficking in hepatocytes, and have

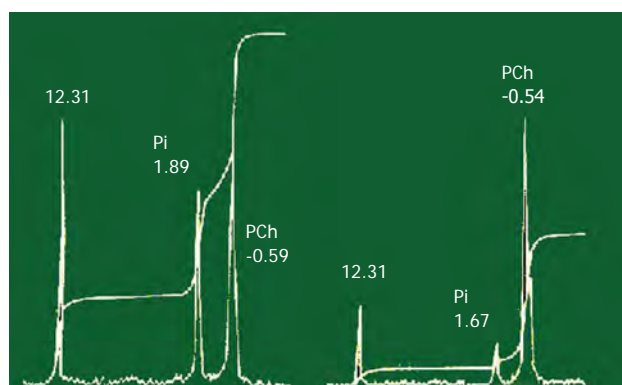
demonstrated that specific vesicle trafficking machinery is required for membrane polarity. The overall functions based on hepatocyte polarity are not attributable to the mere presence of transporters in both poles of these cells<sup>[128]</sup> but also to their intracellular trafficking and temporary anchorage to the different hepatocyte membranes (Figure 7).

**Molecular physiology of the transporting systems of the hepatocyte canalicular membrane:** After traversing the cell by Fick's diffusion, cholephilic compounds mostly bound to high-affinity cytosolic proteins are excreted into the bile mainly by ATP-dependent pumps of the superfamily of ABC transporters; in particular those belonging to the family of multidrug-resistance proteins, MDR/Mdr, or to the family of multidrug-resistance-associated proteins, MRP/Mrp<sup>[57,71]</sup>.

MDRs/Mdrs are members of the ABC superfamily that were originally described in cancer cell lines, where they confer resistance to therapeutic agents. Three gene products were identified in rodents, Mdr1a (Abcb1a), Mdr1b (Abcb1b) and Mdr2 (Abcb4), and two in humans, MDR1 (ABCB1) and MDR 3 (ABCB4). MDR1/Mdr1 functions as an efflux pump for a wide range of amphiphilic, bulky type II cationic drugs, together with other hydrophobic compounds, such as endogenous and exogenous metabolites or toxins, steroid hormones, hydrophobic peptides and even glycolipids<sup>[113]</sup>. Two closely related but functionally distinct Mdr1 isoforms, mdr1a and mdr1b, are present in the murine but not human phenotype<sup>[129]</sup>. MDR3/Mdr2 functions as a flippase, which translocates PC from the inner to the outer hemileaflet of the canalicular membrane, followed by release of PC-containing vesicles from the outer hemileaflet into bile; a process facilitated by the detergent properties of luminal bile salts<sup>[58]</sup>.

Monoanionic bile salts are excreted in the canalicular pole by the bile salt export pump (BSEP/Bsep; ABCB11/abcb11); another member of the MDR family<sup>[130]</sup>. In contrast, canalicular efflux of divalent, bipolar sulfated or glucuronidated bile salts is mediated by the multidrug-resistance-associated protein 2 (MRP2/Mrp2; ABCC2/Abcc2)<sup>[131,132]</sup>. This carrier is also engaged in the biliary excretion of many other organic anions, including glutathione-S-conjugates (*e.g.*, leukotriene C4 or sulfobromophthalein, among others), glucuronides (*e.g.*, bilirubin and estrogens), and reduced (GSH) and oxidized glutathione (GSSG) - the former with low affinity<sup>[133,134]</sup>. Both GSSG and GSH are major determinants of the so-called "canalicular bile-salt-independent bile flow"<sup>[135]</sup>.

The canalicular membrane domain also contains the electroneutral anion exchanger 2 (AE2/Ae2; SLC4A2/slca4a2), which extrudes HCO<sub>3</sub><sup>-</sup> by exchanging the anion for biliary Cl<sup>-</sup><sup>[136]</sup>. It functions to regulate intracellular pH when hepatocytes are exposed to an alkaline load<sup>[136]</sup>. In addition, AE2/Ae2 plays a role in bile flow generation, because HCO<sub>3</sub><sup>-</sup> excretion is thought to be an additional primary driving force of the canalicular bile-salt-inde-



**Figure 8**  $^{31}\text{P}$ -NMR spectroscopy hepatic (left part of figure) and gallbladder (right part of figure) bile. Signals: 12,31 (standard); inorganic phosphate (Pi); phosphatidylcholine (PCh). PCh signal of gallbladder bile is increased with respect to such signals from hepatic bile. Signal of standard and inorganic phosphate of gallbladder bile are decreased with respect to such signals of hepatic bile. The concentration of lipids is increased by water absorption by gallbladder mucosa.

pendent bile flow<sup>[136,137]</sup>. Both in humans and rats, three transcript variants of AE2/Ae2 have been described: the full-length transcript AE2a/Ae2a, expressed from the upstream promoter in most tissues, and the alternative transcripts AE2b<sub>1</sub>/Ae2b<sub>1</sub> and AE2b<sub>2</sub>/Ae2b<sub>2</sub>, expressed in a more tissue-restricted fashion (mainly in liver and kidney). AE2b<sub>1/2</sub>/Ae2b<sub>1/2</sub> transcription is driven from overlapping promoter sequences within intron 2, which results in AE2/Ae2 protein isoforms with short N-terminal differences<sup>[138,139]</sup>.

In water transporters, for a solute to drive blood-to-bile vectorial water transport primarily, resultant osmotic forces need to be associated with aquaporin (AQP)-mediated transcellular movement of water molecules from plasma to the bile canaliculus<sup>[140]</sup>. Both immunochemical and functional studies have demonstrated the constitutive expression of the water channel AQP9 at the basolateral membrane of rat hepatocytes, and the regulated expression of the water channel AQP8 at the hepatocellular canalicular membrane domain<sup>[140-142]</sup>. AQP8 is suggested to play a role in bile formation, facilitating the osmotic movement of water under choleretic stimulus<sup>[140,142]</sup>. AQP isoforms that mediate polarized water transport in human hepatocytes, if any, remain to be identified.

### Gallbladder

The bile formed outside the digestive periods enters the gallbladder that performs two important functions: concentration of bile and its storage up to the evacuation into the duodenum. Minor quantities of bile acids (about 1.3%) are absorbed in the gallbladder walls. Micelles and vesicles enter the gallbladder where the concentration of lipids is increased by water absorption, which causes some physicochemical changes in the bile (Figure 8).

If the gallbladder functions well due to its contraction, all agglomerated vesicles and micelles with bile flow reach the duodenum. The gallbladder mucosa actively absorbs amino acids (this has been established by  $^{35}\text{S}$ -

labelled methionine) and an albumin bile protein fraction<sup>[143]</sup>.

Thus, the gallbladder takes an active part in changing the composition of bile *via* reabsorption of its components into the blood and making the bile components circulate along the small circuit: liver-gallbladder-blood-liver.

The flow of bile is lowest during fasting, and a majority of that is diverted into the gallbladder for concentration. When chyme from an ingested meal enters the small intestine, acid and partially digested fats and proteins stimulate secretion of cholecystokinin and secretin. These enteric hormones have important effects on pancreatic exocrine secretion. They both are also important for secretion and flow of bile.

**Cholecystokinin:** The name of this hormone describes its effect on the biliary system-cholecysto = gallbladder and kinin = movement. The most potent stimulus for release of cholecystokinin is the presence of fat in the duodenum. Once released, it stimulates contractions of the gallbladder and common bile duct, resulting in delivery of bile into the gut.

**Secretin:** This hormone is secreted in response to acid in the duodenum. Its effect on the biliary system is similar to that seen in the pancreas: it stimulates biliary duct cells to secrete  $\text{HCO}_3^-$  and water, which expands the volume of bile and increases its flow into the intestine.

### Sphincter of Oddi

The sphincter of Oddi is located at the interface of the common bile duct and the main pancreatic duct at their confluence into the duodenum. Anatomical and immunohistochemical studies have indicated that the sphincter of Oddi is richly innervated with cholinergic, adrenergic, and peptidergic neurons.

The location of the sphincter of Oddi determines its function: to regulate the entry of bile and pancreatic juice into the duodenum and to prevent reflux of duodenal contents into the common bile duct and the main pancreatic duct. The sphincter coordinates the time and rate of secretion of about 3 L/d of bile and pancreatic juice into the duodenum<sup>[144]</sup>. Normally, the sphincter is characterized by considerable phase contractions throughout the interdigestive period.

### Transport of bile acids along the portal venous system

Bile acid salts come from the intestine into the portal venous system. Bile acids with venous blood enter the liver where they are apparently completely (99%) absorbed by hepatocytes. And only a small quantity (about 1%) of bile acids enters the peripheral blood. In this connection, in healthy individuals the blood concentration of circulating bile acid salts is small.

In the hepatocytes, the secondary bile acids, deoxycholic and lithocholic, are subject to hydroxylation and they conjugate with glycine or taurine. Bile acids come as



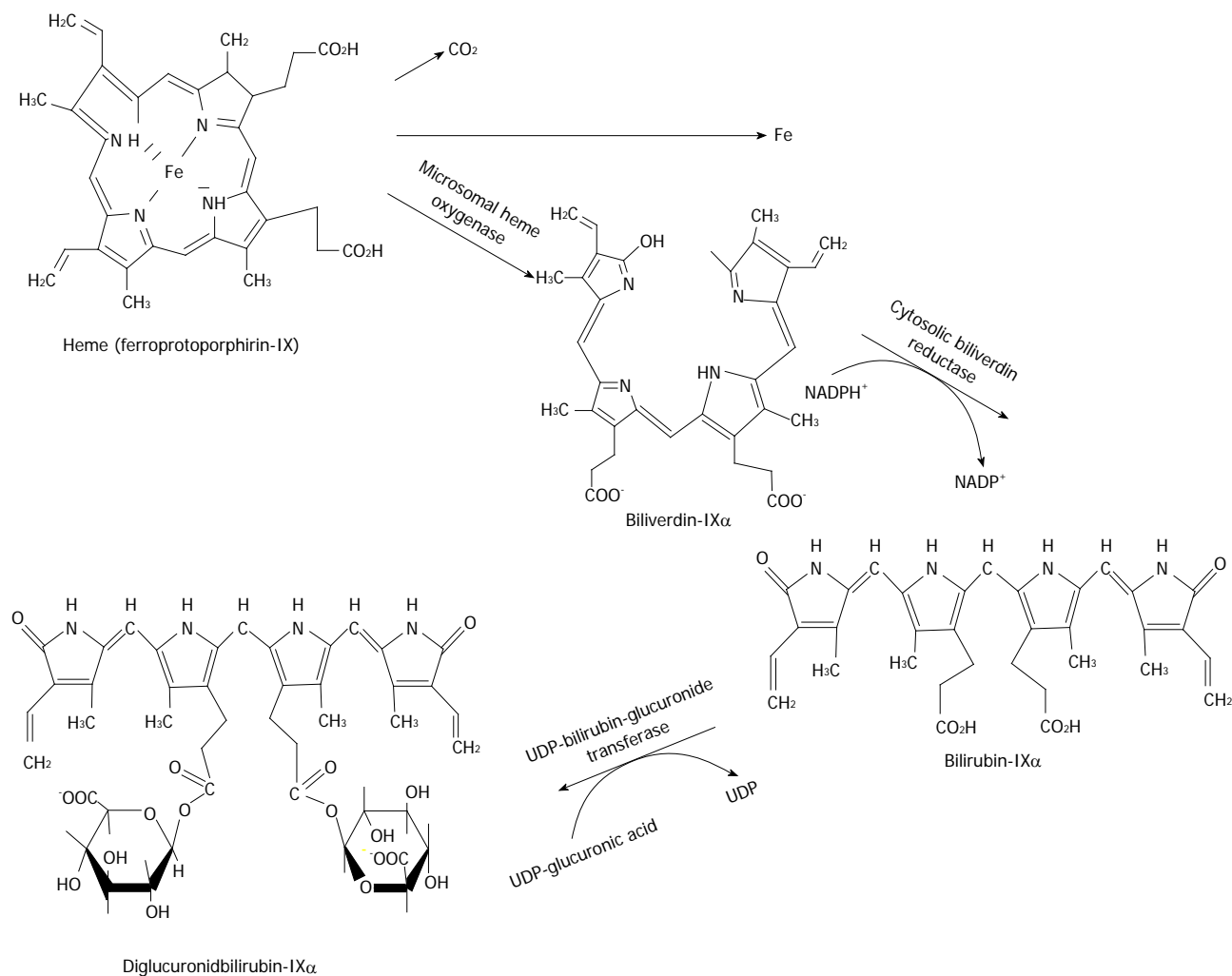


Figure 9 Formation of IX $\alpha$  bilirubin from heme.

conjugates from the liver into the bile again. The normal enterohepatic circulation of bile acids occurs 2-6 times daily depending upon the dietary regimen.

It has been shown that the normal kidneys are not involved in the excretion of bile acids from the body. Portal venous blood bile acids are bound by plasma proteins, mainly albumins<sup>[145]</sup> and, to a lesser extent, by  $\alpha$ - and  $\beta$ -globulins. The data available suggest binding of blood bile salts to lipoproteins<sup>[146,147]</sup>. The bile acids are loosely bound to lipoproteins. In healthy individuals, bile acids are detectable in all classes of lipoproteins<sup>[147]</sup>.

## METABOLISM OF BILE PIGMENTS

Bilirubin is a principal bile pigment. Bilirubin is formed in the cells of the reticuloendothelial system. Unconjugated bilirubin is a product of heme catabolism (Figure 9). Degradation of heme involves its conversion to biliverdin followed by reduction of biliverdin to bilirubin<sup>[148,149]</sup>. Two different enzyme systems are involved in the production of bilirubin from heme: (1) microsomal heme oxygenase<sup>[150-152]</sup>, and (2) cytosolic biliverdin reductase<sup>[153,154]</sup>.

The heme oxygenase activity is detectable in all body

tissues. However, the highest activity of this enzyme is found in the spleen<sup>[150]</sup>. Heme oxygenase has an evident stereospecificity for the  $\alpha$ -meso-bridge of a heme molecule, resulting in the formation of primarily IX- $\alpha$  isomers of biliverdin in man.

Cytosolic biliverdin reductase is also present in all body tissues; its activity is most pronounced in the spleen, liver, and kidney.

A gram of hemoglobin produces 36.2 mg of bilirubin<sup>[151]</sup>. In an adult, the daily bilirubin production determined by different methods is  $3.9 \pm 0.7$  mg/kg (or 250-350 mg/d) - when determined with labeled bilirubin; and  $4.4 \pm 0.7$  mg/kg established from CO<sub>2</sub> formation when heme is converted to bilirubin<sup>[149,154]</sup>. Normally, a human being generates bilirubin IX- $\alpha$  and a minor quantity of its structural isomers: bilirubins IX- $\beta$ , IX- $\gamma$  or IX- $\delta$ .

Bilirubin is liberated from the endothelial tissue cells to plasma, binds to albumin, and is delivered to the hepatocytes and taken up by liver cells in a form dissociated from albumin. Bilirubin transport across the basolateral part of a membrane within the hepatocyte is accomplished by transport systems such as organic anions

transport protein<sup>[155]</sup>, *via* the flip-flop mechanism<sup>[156]</sup>, or by simple diffusion due to chemical concentration gradient. The uptake of bilirubin is highly effective because of its rapid hepatic metabolism and excretion into the bile and of the presence of cytosolic binding proteins, such as glutathione-*S*-transferase. Unconjugated bilirubin is a nonpolar (fat-soluble) substance. In the endoplasmic reticulum of hepatocytes, bilirubin is converted by microsomal UDP-glucuronyl transferase to a polar conjugate with glucuronic acid. Mono- and diglucuronide bilirubin is formed. Water-soluble conjugate of bilirubin is easily excreted into bile. Much smaller fractions of bilirubin are conjugated to sulfates, glucose, or xylose<sup>[157]</sup>.

The physiological significance of conjugation of bilirubin to sugars or glucuronic acid is diminishing its toxic properties through increasing molecular hydrophilicity, which improves bilirubin transport across the membrane structures and ensures secretion into the bile. However, IX $\beta$ , IX $\gamma$ , and IX $\delta$  isomers of bilirubin require no conjugation for bile secretion because they have no rigid structure as compared to the molecule of bilirubin IX $\alpha$ . The latter has a rigid structure due to intramolecular hydrogen bonds. Interestingly, the destruction of the bonds can improve the biliary secretion of bilirubin IX $\alpha$ . It has been demonstrated that UV blood irradiation results in destruction of intramolecular hydrogen bonds in bilirubin. This facilitates the diffusion of bilirubin IX $\alpha$  molecules across the membrane structures without conjugation with glucuronic acid or sugars.

A greater proportion of conjugated bilirubin in the bile is present in the mixed micelles containing cholesterol, phospholipids, and bile acids. Conjugated bilirubin is hydrolyzed by bacterial glucuronidases under urobilinogens. Having a nonpolar molecule, urobilinogen is well absorbed in the small intestine, and in minimal quantities in the colon. The liver and kidneys re-excrete small quantities of absorbed urobilinogen. In hepatocyte dysfunction, hepatic urobilinogen re-excretion is impaired and renal excretion is increased<sup>[151,153,154]</sup>.

## SECRETION OF WATER AND ELECTROLYTES INTO BILE

Formation of bile and generation of bile flow are driven by the active secretion of bile salts, lipids and electrolytes into the canalicular and bile duct lumens followed by the osmotic movement of water<sup>[158]</sup>. Thus, water has to cross rapidly into and out of the cell interior driven by osmotic forces. Bile as a fluid, results from complicated interplay of hepatocyte and cholangiocyte uptake, secretion and concentration that involves various transporters of lipids, anions, cations, and water. Considering bile is composed of > 95% water, the molecular basis and regulatory mechanisms of water transport in hepatocytes during bile formation are still under evaluation<sup>[159]</sup>.

Theoretically, water can flow through the hepatocyte epithelial barrier either across tight junctions between adjacent hepatocytes (paracellular route) or across he-

patocyte plasma membranes (transcellular route). The paracellular route was traditionally proposed as the major pathway for water movement.

The tight junctions play an important role in the paracellular secretion of water and the dissolved low-molecular-weight compounds of the latter. Water and electrolytes are assumed to pass from the intercellular space through the tight junctions into the bile canalicular lumen<sup>[99]</sup>. Moreover, the selectivity of electrolyte excretion is considered to be caused by the presence of a negative charge at the site of a tight junction. This charge serves also as a barrier to regurgitation of substances from the bile canaliculus into the sinusoidal space. The molecular mechanisms of paracellular permeability are currently associated with the functioning of the specific tight junction protein ZO-1. ZO-1 is phosphorylated with protein kinases, which is of importance in the molecular mechanisms responsible for the regulation of paracellular permeability<sup>[160]</sup>. Tight junctions exhibit low water permeability but allow electrolyte permeation that enables canalicular spaces to shrink below the van't Hoff equilibrium during the osmotic maneuver<sup>[161]</sup>. Nonetheless, the experimental data supporting this view remain limited and largely indirect<sup>[162]</sup>.

The cloning and functional characterization of a family of proteins that works as membrane water channels, named AQP<sup>s</sup><sup>[163]</sup>, challenged the former concepts of water transport and contributed to the better understanding of bile physiology. The discovery of the AQP water channels has clarified the mechanisms by which water, the major component of bile, moves across the hepatobiliary epithelia<sup>[164]</sup>.

Direct osmotic water permeability assessment by stopped-flow spectrophotometry in canalicular and sinusoidal plasma membrane vesicles revealed the presence of both lipid (non-channel) and AQP-mediated pathways for sinusoidal and canalicular water movement<sup>[165]</sup>. The study demonstrated that the canalicular plasma membrane domain has lower water permeability than the sinusoidal membrane, and thus it is rate limiting for transcellular water transport in hepatocytes<sup>[159]</sup>. However, upon cAMP stimulus the intracellular AQP8 inserts to the canalicular domain and so this membrane becomes highly water permeable. Therefore, the transcellular pathway *via* water channels seems to account for most of the water entering the bile canaliculus. Hepatocytes express AQPs, a family of membrane channel proteins that facilitate the osmotically driven movement of water molecules. AQP8 is localized to canalicular membranes and modulates membrane water permeability, providing a molecular mechanism for the osmotically coupled transport of solute and water during bile formation.

There is experimental evidence suggesting that defective hepatocyte AQP8 expression leads to alterations in normal bile physiology. Thus, AQP8 protein is down-regulated (and canalicular water permeability decreased), in established rat models of cholestasis, such as sepsis-associated cholestasis, estrogen-induced cholestasis, and

extrahepatic obstructive cholestasis<sup>[166]</sup>.

The entry of inorganic cations and anions into the hepatocytes is effected with the participation of specific ion channels and carriers, which include  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Na}^+/\text{H}^+$ -exchanger,  $\text{Na}^+/\text{HCO}_3^-$  cotransporter, and  $\text{Na}^+$ -independent carrier of  $\text{SO}_4^{2-}$ <sup>[153]</sup>. It is suggested<sup>[167]</sup> that by analogy with the mechanisms of water reabsorption from the bile in the gallbladder, water is reabsorbed by the bile duct epithelial cells as a result of  $\text{Na}^+$ -coupled cotransport of  $\text{Cl}^-$ . Enhanced reabsorption after removal of the gallbladder is regarded as an adaptive mechanism that provides bile concentration.

The primary mechanism of biliary copper excretion involves ATP7B-mediated vesicular sequestration of copper rather than direct copper translocation across the canalicular membrane<sup>[168]</sup>.

The microfilament system is of no small importance in the canalicular secretion of bile. Bile is transported to the ducts *via* contraction of a canaliculus, which is associated with the activity of microfilaments. The latter play a great role in the regulation of permeability of a tight junction for water and electrolytes.

## SECRETION OF PROTEINS INTO BILE

Proteins, as bile acids, are referred to as the osmotically active components of bile, which determine the rate of its secretion<sup>[99]</sup>. Albumin and the other proteins that are close to its molecular weight make a major contribution to the creation of an osmotic gradient.

Tens of different proteins with a molecular weight of 6-220 kDa are detectable in the bile<sup>[99]</sup>. The greater part of them are blood proteins; the lesser are the proteins entering the bile directly from the hepatocytes and epithelial cells of bile ducts. The experimental data<sup>[99]</sup> suggest that many plasma proteins interact with the specific receptors available on the sinusoidal membrane of hepatocytes and form a plasma protein-receptor complex that enters the liver cells *via* endocytosis.

The intracellular transfer of proteins from the perisinusoidal area of a hepatocyte to its pericanalicular one is mainly accomplished by targeted vesicular transport. The large proportion of proteins entering the hepatocyte from blood as a receptor-plasma protein complex generate a receptosome (endosome) and are transported in such a form to the Golgi apparatus, then to the canalicular membrane<sup>[99]</sup>. The newly synthesized proteins entering the bile canaliculi are also transported just from the hepatocytes by targeted vesicular transport. It is presumed that apolipoproteins perform in the bile the same function as lipoproteins in the serum, that is, ensure lipid transport<sup>[169]</sup>.

Apoproteins are taken up from the sinusoidal space by endocytosis, transported in the hepatocytes as vesicles and released into the bile across the canalicular membrane by exocytosis<sup>[170]</sup>.

In conclusion, the cholesterol that is newly synthesized in the hepatocytes serves as a substrate for the

synthesis of bile acids. The synthesis of bile acids is a key point in the formation of bile. Acid-dependent and acid-independent bile fractions are identified. The structure of a hepatocyte provides a targeted transport of bile constituents from the basolateral to apical membrane of a liver cell. The transport of bile acids across the sinusoidal membrane is associated with the overcoming of a high concentration gradient and an electrical potential and it is accomplished by an active transport with the participation of carrier proteins. The vesicles are the basic form of the transport of lipids in and out of the cell itself. Bile lipids are secreted into the bile canaliculi as monolamellar vesicles. Bile acids pass across the apical part of a hepatocytic membrane independently, by ABC transporters, solubilize the phospholipids and cholesterol from the membrane surface in the bile canalicular lumen.

There is a constant transformation of vesicles to and from micelles in the bile canaliculi and ducts as a result of absorptive and secretory processes with the participation of bile acids. All the aforesaid suggests considerable progress made in the understanding of the mechanisms of bile formation and excretion, which has made possible due to the advances of fundamental investigations in cell biology, molecular biology and biochemistry.

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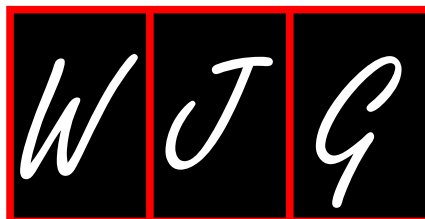


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## Role of cyclooxygenase-2 in gastric cancer development and progression

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### Abstract

Although the incidence of gastric cancer has been declining in recent decades, it remains a major public health issue as the second leading cause of cancer death worldwide. In China, gastric cancer is still the main cause of death in patients with malignant tumors. Most patients are diagnosed at an advanced stage and mortality is high. Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme in prostanoid synthesis and plays an important role in the development and progression of gastric cancer. The expression of COX-2 in gastric cancer is upregulated and its molecular mechanisms have been investigated. *Helicobacter pylori* infection, tumor suppressor gene mutation and the activation of nuclear factor-kappa B may be responsible for the elevated expression of COX-2 in gastric cancer. The mechanisms of COX-2 in the development and progression of gastric cancer are probably through promoting the proliferation of gastric cancer cells, while inhibiting apoptosis, assisting angiogenesis and lymphatic metastasis, and participating in cancer invasion and immunosuppression. This review is intended to discuss, comment and summarize recent research progress on the role of COX-2 in gastric cancer development and progression, and elucidate the

molecular mechanisms which might be involved in the carcinogenesis.

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**Key words:** Cyclooxygenase-2; Gastric cancer; Prostaglandin; Carcinogenesis; Molecular mechanism

**Core tip:** Cyclooxygenase-2 (COX-2) plays an important role in gastric cancer development and progression. The present review aims to determine the molecular mechanism of COX-2 overexpression in gastric cancer and focus on the detailed information on COX-2 involved in carcinogenesis. By reviewing research progress, this may be helpful in clarifying the internal relationship of the afore-mentioned aspects.

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### INTRODUCTION

Gastric cancer is one of the most common malignant tumors worldwide, and the morbidity and mortality associated with this disease are ranked second highest of all malignant neoplasms<sup>[1-3]</sup>. In China, the incidence of gastric cancer has been declining in recent years<sup>[4]</sup>. Since 75% of gastric cancer patients are diagnosed at an advanced stage and cannot be cured merely by surgery, chemotherapy combined with surgery is often the primary treatment. There are many factors that affect the prognosis of gastric cancer, and of these factors invasion and metastasis are leading causes of death. The role of cyclooxygenase-2 (COX-2) in gastric cancer development and progression

has been extensively studied. The expression of COX-2 is elevated in gastric cancer tissues, therefore, inhibition of COX-2 expression may prevent or reverse gastric carcinogenesis. This review focuses on the crucial role of COX-2 in gastric cancer development and progression. In addition, its mechanisms of action are illustrated.

## COX

COX, also known as prostaglandin synthase, is the rate-limiting enzyme responsible for the conversion of arachidonic acid (AA) into the various prostaglandins (PGs), a family of lipid mediators that have widespread and diverse biological functions<sup>[5]</sup>. This enzyme possesses both peroxidase activity in catalyzing prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) and COX catalytic activity in the conversion of PGG<sub>2</sub> from AA<sup>[6]</sup>. Members of the PGs family including PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGG<sub>2</sub>, and PGH<sub>2</sub> are widely distributed in organic bodies and play different roles in metabolism<sup>[7-11]</sup>. It is reported that PGE<sub>2</sub> was overexpressed in tumor tissues and was involved in carcinogenesis<sup>[12-14]</sup>.

COX, with a relative molecular mass of 71000, is a type of glycoprotein which is located on the surface of the nuclear membrane and microsomal membrane. Two COX isoforms have been found: COX-1 and COX-2. Although COX-1 and COX-2 share a high level of homology (65%), the activity and expression of these enzymes are different, and they can function independently within the same cell type<sup>[15]</sup>.

The *COX-1* gene, comprised of 11 exons and 10 introns, is a type of housekeeping gene, which is located at chromosome 9 q<sup>32-33.3</sup>. The full length of the *COX-1* gene is about 22.5 kb, and no hogness box and promoter elements are found. In most tissues, COX-1 is composed of 599-600 amino acid residues and expressed constitutively and continuously<sup>[16]</sup>. The basic functions of COX-1 are not only promoting the synthesis of PGs, but also maintaining the homeostasis of an organism such as regulating the clotting mechanism, stabilizing renal blood flow and protecting gastric mucosa<sup>[17-19]</sup>. COX-1 is expressed negatively or weakly in tumor tissues and is not involved in carcinogenesis<sup>[20]</sup>.

The *COX-2* gene, located at chromosome 1q<sup>25.2-25.3</sup>, is composed of 10 exons and 9 introns. With hogness box, CAAT/enhancer binding protein (C/EBP) and cAMP response elements in the 5'-terminal nucleotide sequence, the gene is approximately 8.3 kb in size<sup>[21]</sup>. There are also some binding sites in the gene sequence such as the activator protein-2 (AP-2) binding site and the nuclear factor-kappa B (NF-κB) binding site<sup>[22]</sup>. COX-2 is composed of 604 amino acid residues and is expressed negatively in normal tissues and organs under physiological conditions, except the constitutive expression in kidney and brain. It is inducible in response to certain stimuli such as growth factors and cytokines. COX-2 is involved in many pathological processes such as inflammation and carcinogenesis<sup>[23,24]</sup>. It was reported that more than 15% of

malignant tumors are correlated with infection<sup>[25]</sup>. Various inflammation networks have been confirmed to play crucial roles in the microenvironment of carcinogenesis<sup>[26]</sup>, and the most important network is the COX-2/PGE<sub>2</sub> pathway<sup>[27]</sup>. In addition, it has been well established that COX-2 is up-regulated in a variety of cancers and promotes their growth<sup>[28-30]</sup>.

## EXPRESSION OF COX-2 IN GASTRIC CANCER

The first report on the expression of COX-2 in gastric cancer was from Ristimaki *et al.*<sup>[23]</sup>. Their study showed that human gastric adenocarcinoma tissues contained significantly higher levels of COX-2 mRNA when compared with paired gastric mucosal specimens devoid of cancer cells. Immunohistochemical staining detected COX-2 protein expression in the cytoplasm of gastric carcinoma cells, but not in the surrounding stroma. Ue-fuji confirmed the overexpression of COX-2 protein in human gastric adenocarcinomas by immunoblotting, and reported that overexpression of COX-2 protein was independent of the histologic type of gastric cancer<sup>[31]</sup>. A study further confirmed the significant difference in COX-2 protein expression between normal tissues and gastric cancer tissues<sup>[32]</sup>. Researchers found that the overexpression of COX-2 protein was not related to the clinicopathological characteristics of gastric cancer patients<sup>[33]</sup>, but related to tumor node metastasis clinical stage, depth of invasion and metastasis<sup>[33,34]</sup>. A series of studies showed that COX-2 protein expression was associated with intestinal histological subtype, proximal location, tumor size and advanced clinical stage and lymph node involvement<sup>[35-39]</sup>. Importantly, the expression of COX-2 protein and mRNA was already detected in noninvasive gastric dysplasia<sup>[40,41]</sup>. Thus, it seems likely that COX-2 plays a role in early gastric carcinogenesis.

There are controversial results in the association between COX-2 and survival rate. Although COX-2 played a crucial role in gastric carcinogenesis and was relevant to the degree of tumor differentiation, the expression of COX-2 protein was not correlated with survival rate. In addition, it made little sense in predicting gastric cancer prognosis<sup>[42]</sup>. In contrast, other research results suggested that COX-2 was an independent prognostic factor for gastric cancer as the 5-year survival rate of COX-2 protein positively expressed patients was lower than that of negatively expressed patients<sup>[43]</sup>. In addition, early-stage gastric cancer patients with high expression of COX-2 protein were at a higher risk for cancer-related death than those with a low level of COX-2 expression<sup>[35]</sup>. Another study<sup>[44]</sup> assessed the correlation between tumor progression and epithelial mesenchymal transition using multivariate analysis, and showed that COX-2 protein overexpression was an independent prognostic factor for poor survival, due to angiogenesis, cancer invasion and metastasis. Recently, scientists also found that COX-2 protein and *p53* expression were independent prognostic

factors for poor survival, in addition to late-stage disease and non-curative surgery<sup>[45]</sup>. Most of the findings illustrated above share the similar points, while the controversial points need to be further investigated to reach a consensus.

## MECHANISM OF ELEVATED COX-2 EXPRESSION IN GASTRIC CANCER

### *Helicobacter pylori* infection

Some studies suggested that *Helicobacter pylori* (*H. pylori*) infection was significantly related to COX-2 expression<sup>[46-48]</sup>. *H. pylori* infection can lead to a local inflammatory response, phenotypic change of epithelial cells, promotion of cell proliferation and inhibition of cell apoptosis, and ultimately an increased risk of gastric cancer<sup>[49]</sup>.

*H. pylori* is classified as a class I carcinogen by the International Agency for Research on Cancer. Over-expression of COX-2 was detected in *H. pylori* positive gastritis compared with *H. pylori* negative gastritis<sup>[50]</sup>. COX-2 over-expression was found in 50%-80% of gastric cancer patients. Another study showed that 24 h after *H. pylori* infection of epithelial cells in mice, the expression of COX-2 and PGE<sub>2</sub> were significantly elevated<sup>[51]</sup>. An *in vitro* study also obtained similar results<sup>[48]</sup>. After the co-culture of MKN 28 cell lines with *H. pylori* for 24 h, the COX-2 mRNA transcription level increased five-fold and the expression of PGE<sub>2</sub> increased three-fold, suggesting that synthesis of COX-2 and PGE<sub>2</sub> was one of the factors for *H. pylori* associated gastric cancer<sup>[52]</sup>.

The mechanism of COX-2 over-expression caused by *H. pylori* infection is not entirely clear. In an *in vitro* study, *H. pylori* induced the expression of COX-2 and inducible nitric oxide synthase by activating AP-1 of AGS cells<sup>[53]</sup>. Cytokines from the *H. pylori* associated inflammatory response also promoted the upregulation of COX-2<sup>[54]</sup>. In an *in vivo* study, *H. pylori* infection influenced the expression of 385 genes, and 160 of these genes were related to COX-2, including the inflammation genes (*Icam1*), the apoptosis genes (*Cln*), the proliferation genes (*Gdf3*, *Igf2*), the gastric physiology genes (*Galr-1*) and the epithelial barrier function genes (*Tjp1*, *Aqp5*). After treatment with NS398, a COX-2 inhibitor, the expression of 140 genes changed, which indicated that COX-2 was correlated with the occurrence of gastritis<sup>[55]</sup>. Another study indicated that *H. pylori* could lead to the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and its downstream transcription factor ATF-2, and the expression of COX-2 could be inhibited by a p38 MAPK inhibitor. These results indicated that the p38/ATF-2 signal transduction pathway induced by *H. pylori* was the crucial mechanism involved in COX-2 expression<sup>[56]</sup>. Infection with *H. pylori* stimulated the secretion of gastrin which promoted the expression of COX-2 and extended the half-life of COX-2 mRNA<sup>[57]</sup>. In addition, the inhibitor of gastrin-releasing peptide decreased the expression of COX-2, which indicated that gastrin may be involved

in COX-2 expression induced by *H. pylori*<sup>[58]</sup>.

### Suppressor gene mutation

An imbalance of oncogenes and suppressor genes is responsible for carcinogenesis. The mutation of a suppressor gene can lead to the occurrence of cancer. A study revealed that COX-2 expression in patients with *P53* mutation was higher than in those without *P53* mutation. This indicated that *P53* mutation might be related to COX-2 over-expression<sup>[59]</sup>. The protein of wild-type *P53* could inhibit the formation of a complex composed of TATA box binding proteins and promoters located upstream of the gene sequences, and eventually inhibited the expression of COX-2. In contrast, the product of mutant type *P53* could elevate COX-2 expression by the Ras/Raf/MAPK signal pathway. Moreover, COX-2 could reversibly induce mutation of *P53*, and both were co-expressed in gastric cancer tissues<sup>[60]</sup>.

*P16* is a tumor suppressor gene located at chromosome 9p<sup>21</sup>. It can inhibit the function of cyclinD1/CDK4 and CDK6 complex, and cause *p53*-independent G1 arrest through the phosphorylation of pRb<sup>[61,62]</sup>. This gene is usually inactivated in human gastric cancers for different reasons. A study indicated that *p16* was found to harbor promoter methylation associated with the loss of protein expression in cancer cells, suggesting that *p16* inactivation due to promoter methylation may be important for gastric tumorigenesis<sup>[63]</sup>. Other mechanisms such as mutation and homozygous deletion, are also responsible for the inactivation of *p16*, which lead to the development and progression of tumors<sup>[64]</sup>. Researchers explored the expression of COX-2 protein and *p16* protein in gastric cancer mucosa, and found that COX-2 protein expression was negatively related to *p16* protein expression. There may be a relationship between the expression of COX-2 and *p16*. However, the mechanism involved needs to be clarified in further research<sup>[65,66]</sup>.

### NF-κB

NF-κB is a protein which has the ability to combine with the nucleotide sequence in the promoter region and enhancer region of some genes, and consequently activate or enhance the transcription of these genes. NF-κB is usually distributed in the cytoplasm in the inactive form under physiological conditions. It is then activated and enters the nucleus in response to outside stimuli. The NF-κB signal pathway is involved in many processes such as the inflammatory response, cell proliferation, apoptosis and carcinogenesis. Some inflammatory cytokines, such as tumor necrosis factor (TNF)-α, can activate NF-κB, and this activated transcription factor can induce over-expression of inflammatory factors including COX-2 and TNF-α itself, forming the inflammatory network in the tumor microenvironment<sup>[67]</sup>. The COX-2 promoter region contains several elements, with the presence of two NF-κB consensus sites<sup>[68]</sup>. COX-2 expression decreased significantly when NF-κB was blocked by chondroitin sulfate<sup>[69,70]</sup>. Expression of COX-2 and NF-κB increased



simultaneously during the process from chronic atrophic gastritis, dysplasia to gastric cancer. The co-expression of COX-2 and NF- $\kappa$ B played an important role in the angiogenesis of stomach tissues. In addition, NF- $\kappa$ B up-regulated the expression of vascular endothelial growth factor (VEGF), which is an important promoter of angiogenesis<sup>[71]</sup>.

## MECHANISM OF COX-2 IN GASTRIC CARCINOGENESIS

### Cell proliferation and apoptosis

Accumulating evidence indicates that inflammation plays an important role in the development of cancers<sup>[72,73]</sup>. TNF- $\alpha$ , which is a mediator of PGE<sub>2</sub>, plays a crucial role in mediating the inflammatory process through activation of NF- $\kappa$ B. It has been found that stromal NF- $\kappa$ B can enhance proliferation of epithelial cells by inducing cytokines, chemokines, and growth factors, such as IL-6, IL-1 $\beta$ , macrophage inflammatory protein-2 and TNF- $\alpha$ , while epithelial NF- $\kappa$ B can suppress apoptosis by inducing anti-apoptotic proteins, such as GADD45 $\beta$ , A1/Bfl1, and cIAP1<sup>[74,75]</sup>. As a product of AA catalyzed by COX-2, PGE<sub>2</sub> is involved in gene mutation and cancer cell proliferation<sup>[76]</sup>. Protein encoded by COX-2 genes is a type of oncogenic protein, which could promote the high expression of PGE<sub>2</sub>. PGE<sub>2</sub> can stimulate the growth of blood vessels, inhibit local immune function and regulate a variety of signal transduction pathways which ultimately influence the proliferation of cells and the growth of tumors<sup>[77]</sup>. It has also been confirmed that over-expression of COX-2 promoted cell proliferation by weakening the anti-proliferative effect of transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>[78]</sup>.

An *in vitro* study showed that cell proliferation was suppressed when gastric cells were treated with COX-2 small interfering RNA (siRNA)<sup>[79]</sup>. In MKN-45 cells, inhibition of COX-2 with NS-398 led to reduced proliferation and induction of apoptosis, connected with downregulation of *Bcl-2* (an anti-apoptotic gene) and upregulation of *Bax* (an apoptotic gene)<sup>[80]</sup>. COX-2 was a regulatory factor in the *Bcl-2* upstream sequences, which upregulated the expression of *Mcl-1*, a member of the *Bcl-2* family, through the phosphatidylinositol 3-kinase (PI3K) signal pathway, and eventually inhibited the apoptosis of cancer cells<sup>[81]</sup>. Some studies confirmed that COX-2 could inhibit the apoptosis of cancer cells by inducing the mutation of *P53*<sup>[82]</sup>. Other researchers indicated that COX-2 weakened the apoptotic signal mediated by *Fas* protein. After adding the COX-2 inhibitor, they detected the elevated expression of caspase, a key enzyme of death receptor signaling and apoptotic signaling pathways<sup>[83]</sup>.

### Angiogenesis and lymphatic metastasis

The growth and metastasis of tumors depend on the formation of new blood vessels. PGE<sub>2</sub> and PGF<sub>2</sub> can promote vessel formation directly or indirectly<sup>[84]</sup>. Research

has found that COX-2 over-expression was associated with increased PGE<sub>2</sub> biosynthesis and angiogenesis in gastric cancer<sup>[85]</sup>. Furthermore, COX-2 can induce cells to produce VEGF and TGF- $\beta$ , which could promote endothelial cell migration and tubular morphogenesis. In addition, VEGF was an independent prognostic factor for gastric cancer prognosis<sup>[86]</sup>. After being transfected with COX-2 siRNA, the expression of VEGF was down-regulated and the growth of gastric cancer cells was significantly inhibited<sup>[22]</sup>. Other studies indicated that COX-2 upregulated the expression of *Bcl-2* and *Akt*, which can inhibit the apoptosis of endothelial cells and promote vessel formation<sup>[87,88]</sup>.

VEGF-C has been identified as a new member of the VEGF family and is considered a specific lymphangiogenic factor. It can promote the formation and dilation of lymphatic vessels, enhance the permeability of lymphatic vessels and facilitate lymphatic metastasis. A recent study confirmed a positive correlation between the expression of COX-2 and VEGF-C in gastric cancer patients, and both were related to gastric cancer prognosis<sup>[89]</sup>.

### Invasion and metastasis

Invasion is the premise of cancer metastasis which involves a variety of cytokines. Adhesion molecule is a type of cell surface glycoprotein that mediates cell adhesion. E-cadherin adhesion which inhibits the separation of cancer cells from tissue can prevent cancer cell invasion. COX-2 can lower the activity of E-cadherin, thus the invasiveness of cancer cells is enhanced for further metastasis. The activity of E-cadherin was enhanced after inhibition of COX-2<sup>[90]</sup>. It has been confirmed that the over-expression of matrix metalloproteinase (MMP) accelerated the decomposition of collagen in local tissues, which was beneficial to the spread of cancer cells. A correlation between COX-2 and MMP upregulation was also found<sup>[91]</sup>. COX-2 inhibitors reduce the expression of MMP<sup>[92]</sup>. CD44, which acts as the membrane receptor of hyaluronic acid and is expressed in cancer stem-like cells, played an important role in cancer metastasis. A large number of CD44(+) gastric glands was found in human adenocarcinomas and adjacent metaplasias, but not in normal gastric epithelium. In addition, CD44(+) tumor cell expansion is triggered by the cooperative actions of PGE<sub>2</sub> and Wnt in gastric tumorigenesis<sup>[93]</sup>. Studies also confirmed that PGE<sub>2</sub> could upregulate the expression of CD44<sup>[94]</sup>, while COX-2 inhibitor could inhibit CD44 expression<sup>[95]</sup>. A study demonstrated that CD44v, a variant form of CD44, could protect tumor cells from oxidative stress in a mouse gastric cancer model, thus it plays an important role in tumor development<sup>[96]</sup>. Other possible mechanisms include the upregulation of urokinase-type plasminogen activator (uPA) in promoting the metastasis of cancer cells<sup>[97]</sup> and more potential pathways need to be further clarified.

### Immunosuppression

It has been found that COX-2 was involved in the im-



munosuppression in gastric cancer, where effector T cells were suppressed by regulatory T cells. In Treg cells, expression of COX-2 was correlated with that of forkhead box p3. By using a COX-2 inhibitor, the immunosuppression of effector T cells was reversed<sup>[98]</sup>. The possible mechanisms involved may be as follows: PGE<sub>2</sub> disabled the function of dendritic cells in the tumor microenvironment and the cells could not present the tumor antigen effectively, and eventually the T cells did not recognize or kill the cancer cells<sup>[99]</sup>. In addition, PGE<sub>2</sub> may also reduce the immunosurveillance of the immune system on mutant cells by inhibiting the expression of human leucocyte antigen I and II, and by reducing the production of lymphokine. The immunosurveillance effect could be enhanced by using a COX-2 inhibitor and stimulating the activity of natural killer cells<sup>[100]</sup>.

## CONCLUSION

The COX-2/PGE<sub>2</sub> pathway involved in the inflammatory response plays a critical role in the microenvironment of gastric tumorigenesis. Expression of COX-2 is elevated in gastric cancer and its over-expression is associated with *H. pylori* infection, mutation of suppressor genes and NF- $\kappa$ B. Over-expressed COX-2 participates in gastric carcinogenesis by promoting cell proliferation, inhibiting cell apoptosis, inducing vessel formation, and enhancing metastasis and immunosuppression. Although progress has been made in exploring the mechanism of gastric cancer development, some issues remain to be explored in further studies. As research continues, interventions in gastric cancer using COX-2 as a target might eventually become a specific treatment of choice.

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## Intestinal acyl-CoA synthetase 5: Activation of long chain fatty acids and behind

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### Abstract

The intestinal mucosa is characterized by a high complexity in terms of structure and functions and allows for a controlled demarcation towards the gut lumen. On the one hand it is responsible for pulping and selective absorption of alimentary substances ensuring the immunological tolerance, on the other hand it prevents the penetration of micro-organisms as well as bacterial outgrowth. The continuous regeneration of surface epithelia along the crypt-villus-axis in the small intestine is crucial to assuring these various functions. The core phenomena of intestinal epithelia regeneration comprise cell proliferation, migration, differentiation, and apoptosis. These partly contrarily oriented processes are molecularly balanced through numerous interacting signaling pathways like Wnt/ $\beta$ -catenin, Notch and Hedgehog, and regulated by various modifying factors. One of these modifiers is acyl-CoA synthetase 5 (ACSL5). It plays a key role in *de novo* lipid synthesis, fatty acid degradation and membrane modifications, and regulates several intestinal processes, primarily through different variants of protein lipidation, *e.g.*, palmitoylation. ACSL5 was shown to interact with proapoptotic molecules, and besides seems to inhibit proliferation along the crypt-villus-axis. Because of its proapoptotic and antiproliferative characteristics it could

be of significant relevance for intestinal homeostasis, cellular disorder and tumor development.

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**Key words:** Acyl-CoA synthetase; Apoptosis; Carcinogenesis; Colorectal cancer; Intestinal homeostasis

**Core tip:** Acyl-CoA synthetase 5 (ACSL5) activates long-chain fatty acids by coenzyme A linkage and plays a key role in fatty acid metabolism. On the basis of its mitochondrial localization, ACSL5 forms an exceptional member among the acyl-CoA synthetase family. Although its various functions are not yet fully understood, ACSL5 seems to represent a modifier of cellular vitality along the crypt-villus-axis.

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### INTRODUCTION

Acyl-CoA synthetase 5 (ACSL5) is part of the acyl-CoA synthetase family who plays a key role in *de novo* lipid synthesis, fatty acid degradation and subsequently membrane modifications. Their main function is the activation of long-chain fatty acids by coenzyme A linkage, these conjugates are primarily utilized for triglyceride synthesis, packed into chylomicrons and secreted through the basolateral membrane<sup>[1-3]</sup>. Along the intestinal crypt-villus-axis (CVA) they either cause cell proliferation or cell apoptosis<sup>[4]</sup>. The fatty acid activation requires a two-step reaction, catalysed through these enzymes. The first step results in an acyl-AMP intermediate of ATP. AMP

is then replaced with CoA and the activated acyl-CoA is generated. Except for the resulting acyl-CoA, free fatty acids (the substrates of these enzymes) are able to pass through membranes. The ACSL-derived esterification prevents it from leaving the cell. The length of the carbon chain (12–20 C atoms) of the fatty acids defines the substrate specificities of the different acyl-CoA synthetases. On this basis, five different subfamilies were identified<sup>[5]</sup>. Their sequences differ at N-terminus, which is presumably the reason for their diversity in sub-cellular localisation. ACSL1 and 6 are associated with the plasma membrane. They might play a role in the cellular fatty acid assimilation<sup>[6,7]</sup>. ACSL4 was mainly found in ER and peroxisomes<sup>[8]</sup>. The gene *ACSL5* lies on chromosome 10q25.1–q25.2<sup>[9,10]</sup>. The functional protein is localized on the inner mitochondrial membrane<sup>[11,12]</sup> and has a significant regulatory function in the mitochondrial energy metabolism. The pH-dependence of this molecule is especially important. It shows a high functional activity under alkaline conditions. This observation is of special interest in the case of mitochondria: In contrast to the enclosing cytoplasm their pH is more basic by one point and they show a more alkaline pH with beginning apoptosis<sup>[13]</sup>. In this context the pH conditions of tumor genesis and a differing functionality of ACSL5 should be considered as well<sup>[14]</sup>.

Analysing the role of ACSL5 in differentiation and senescence of enterocytes, a sensitization to TRAIL-dependent apoptosis was found<sup>[4]</sup>, whilst the Triacsin C-induced apoptosis induction in glioma cells was inhibited. Mashima *et al.*<sup>[9]</sup> postulated an ACSL5-dependent survival of tumor cells. There is evidence to suggest a cell regulatory involvement of ACSL5 in addition to its lipid modifying function<sup>[15]</sup>.

Triacsin C [1-hydroxy-3-(E,E,E-2',4',7'-undecatrienylidene) triazene] was identified as a potent competitive inhibitor of acyl-CoA synthetase activity<sup>[16]</sup>. Its inhibitory effects depend on the N-hydroxytriazene moiety of the molecule, resulting in a dramatic reduction in cholesterol as well as triglyceride synthesis with non-transition of macrophages to foam cells or enhanced eicosanoid release in leucocytes<sup>[17,18]</sup>. Kaemmerer *et al.*<sup>[18]</sup> showed that human ACSL5 is, unlike rat ACSL5, sensitive to the competitive inhibition by triacsin C and does not compensate for other triacsin C sensitive ACSL isoforms.

This mini review outlines the modifying role of ACSL5 in different cellular processes at the interface between proliferation and apoptosis.

## ACSL5 AS A REGULATOR OF THE CELLULAR RENEWAL ALONG THE CVA IN HUMAN SMALL INTESTINE

The CVA in adult human small intestine is important for maintaining intestinal homeostasis and this process is controlled by regulatory signaling pathways such as Wnt/ $\beta$ -catenin, Notch, Hedgehog, and apoptosis. Canonical

Wnt signaling plays a key role in regulating intestinal cell proliferation<sup>[19]</sup>. A mutation-derived activation of the cascade initiates the adenocarcinoma sequence. Blocking canonical Wnt signals leads to a proliferative arrest of epithelial cells in the crypts of Lieberkuhn. The cell sorting receptors EphB2 and B3 are also target molecules of the pathway, as they control the positioning of Paneth cells towards the crypt bottom, whereas all other differentiated epithelial cells migrate along the CVA<sup>[20]</sup>.

The highest amount of Notch activity is found in intestinal stem cells<sup>[21,22]</sup>. The transcription factors Hes-1 and Math-1 conduct the differentiation of progenitor cells into enteroendocrine, goblet and Paneth cells<sup>[23,24]</sup>. Blocking of Notch signaling with the  $\gamma$ -secretase inhibitor dibenzazepine results in a rapid conversion of proliferative crypt cells into post-mitotic goblet cells<sup>[25,26]</sup>.

The Hedgehog pathway with its main epithelial proteins Sonic (Shh) and Indian (Ihh) Hedgehog is of central relevance in villus formation and stem cell homeostasis<sup>[27,28]</sup>.

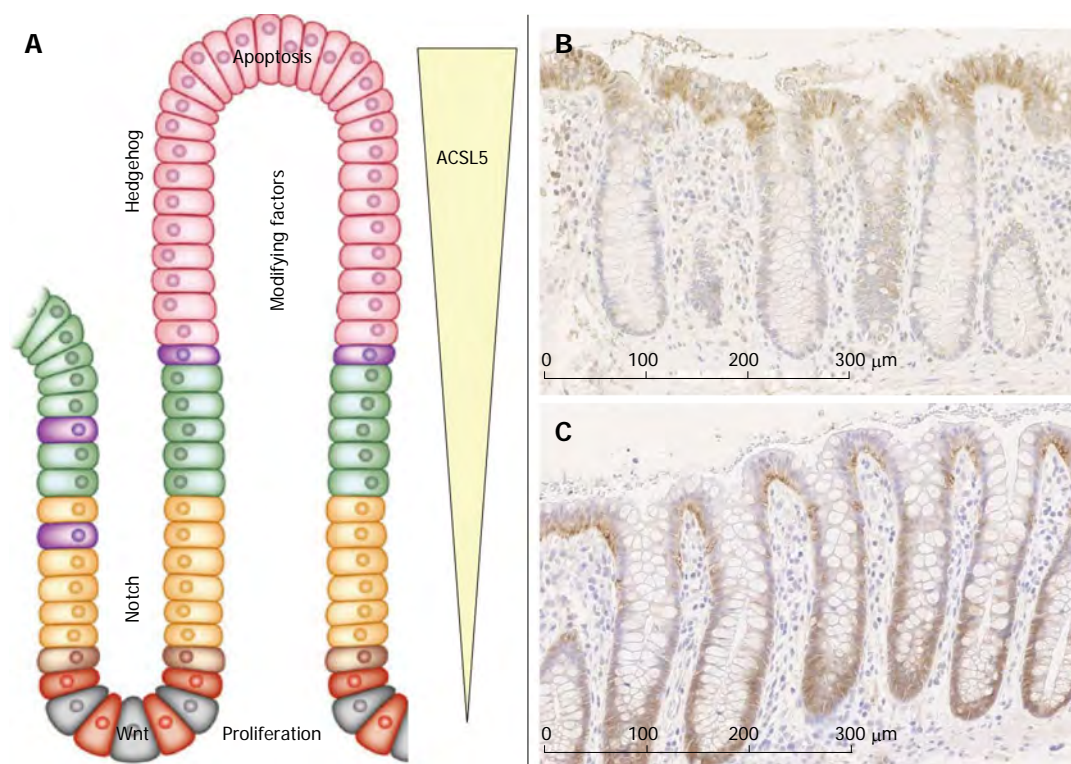
ACSL5 is expressed in an ascending gradient along the CVA with the highest expression level in enterocytes at the villus tip (Figure 1). It is assumed that ACSL5 sensitizes these enterocytes towards TRAIL-derived apoptosis susceptibility by down-regulation of the anti-apoptotic FLIP and up-regulation of TRAIL-R1 on the cell surface. TRAIL shows a corresponding gradient expression pattern<sup>[4]</sup>. Gassler *et al.*<sup>[4]</sup> suggested an ACSL5-dependent link to TRAIL-induced apoptosis by the ceramide neosynthesis, which is known to play a key role in TRAIL-induced apoptosis<sup>[29]</sup>, occurring mainly at mitochondrial membranes<sup>[30]</sup>.

Adenocarcinomas with an invasive phenotype and enhanced proliferation showed decreased levels of ACSL5<sup>[4]</sup>. A functional correlation between ACSL5-derived apoptosis susceptibility of enterocytes and increased tumor development is assumed<sup>[31]</sup>.

## LIPID MODIFICATIONS CONTROL INTESTINAL SIGNALING PATHWAYS OF CENTRAL RELEVANCE

Long-chain polyunsaturated fatty acids show significant modifying functions in the cell cycle, resulting in inflammation and cancer development<sup>[32,33]</sup>. They enter into cells by means of concentration gradients or transport proteins. The retention in the cell depends on its further metabolic function and intracellular modification. The different ACSL isoforms activate a broad range of long-chain fatty acids and channel them into specific downstream pathways that can vary in a tissue-specific manner<sup>[34]</sup>. An over-expression of ACSL5 increases the fatty acid incorporation into diacylglycerol and triacylglycerol but does not affect FA used for  $\beta$ -oxidation<sup>[35]</sup>.

A lipid modification of central relevance in several intestinal pathways is palmitoylation. Palmitate, a 16-carbon saturated fatty acid, is covalently bound to more than 100



**Figure 1** Expression of acyl-CoA synthetase 5 and  $\beta$ -catenin in normal human intestinal mucosa. Schematic overview of signaling pathways in intestinal mucosa (A) immunostaining of acyl-CoA synthetase 5 (ACSL5) (B) and  $\beta$ -catenin (C).

cellular and viral proteins. In most cases, the fatty acid is thioester (S-acyl) posttranslationally linked to cysteine. The reaction is catalysed by palmitoyl-acyltransferases, where palmitoyl-CoA serves as the donor. The palmitoylation is a dynamic process by means of its reversible linkage<sup>[36-38]</sup>. Palmitoylation has several effects on molecule functionality and is especially relevant for signaling cascades<sup>[39,40]</sup>. Protein hydrophobicity and membrane association are increased and protein/protein or protein/lipid interactions are augmented<sup>[41]</sup>. Modifications derived from fatty acids are described for the proteins associated with Wnt signaling, which are very important for secretion and activity. A fatty acid modification at serine promotes cysteine palmitoylation<sup>[42,43]</sup>. Without this fatty acid attachment at the Wnt ligand, N-glycosylation and secretion were still possible. In contrast, the absence of N-glycosylation resulted in a decreased secretion rate.

One example for an apoptosis-associated ligand that is regulated by palmitoylation is the human Fas ligand (FasL), responsible for correct signal transduction finally resulting in cell death. Palmitoylation regulates its transmembrane domain and is critical for efficient FasL-mediated killing and FasL processing<sup>[44,45]</sup>.

Another molecule regulated by S-palmitoylation is  $\gamma$ -secretase. Its diverse substrates originate from several pathways with a focus on type I integral membrane proteins, including Notch1 homologues, Notch ligands, Delta and Jagged, cell adhesion receptors N- and E-cadherins, low density lipoprotein receptor-related protein, ErbB-4, netrin receptor DCC<sup>[46,47]</sup>. Cheng *et al.*<sup>[46]</sup> identified S-pal-

mitoylation of  $\gamma$ -secretase subunits nicastrin and APH-1, and characterized its role on DRM association, protein stability, and  $\gamma$ -secretase enzyme activities.

Palmitoylation of Hedgehog proteins is important for effective signaling. Protein fatty acylation is not only found to be a regulator for membrane association of intracellular proteins, but also for the signaling activity and efficacy of secreted proteins<sup>[48,49]</sup>. Palmitoylation thus controls mechanisms leading to an altered reaction of the cells towards external signaling.

ACSL5 may play a role in channelling fatty acids towards palmitoylation and other lipid functions with high relevance for cellular behaviour<sup>[18]</sup>. An over-expression of ACSL5 in HepG2 showed an increased palmitate oxidation<sup>[50]</sup>. An ACSL5-mediated lipid modification of proliferation- and apoptosis-associated molecules might be an interface in the intestinal CVA.

## CONCLUSION

The intestinal epithelium is subject to continuous self-renewal along the CVA, and progress has been made towards understanding the task of the various signal transduction pathways in proliferation, differentiation, migration and apoptosis of epithelial enterocytes. ACSL5 is a protein localized in mitochondria that plays a key role in fatty acid metabolism and apoptosis. Although its various functions are not yet fully understood, ACSL5 seems to represent a modifier of cellular vitality along the CVA, providing fatty acids for several molecular events.



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## 1,3-Bis(2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of Resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells

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Author contributions: Das D carried out all the experiments and wrote the first draft of the manuscript; Preet R, Mohapatra P and Satapathy SR performed the statistical analysis and were involved in editing the manuscript; Kundu CN designed the study and wrote the manuscript.

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### Abstract

**AIM:** To study the mechanism of 5-fluorouracil (5-FU) resistance in colon cancer cells and to develop strategies for overcoming such resistance by combination treatment.

**METHODS:** We established and characterized a 5-FU resistance (5-FU-R) cell line derived from continuous exposure (25  $\mu\text{mol/L}$ ) to 5-FU for 20 wk in 5-FU sensitive HCT-116 cells. The proliferation and expression of different representative apoptosis and anti-apoptosis markers in 5-FU sensitive and 5-FU resistance cells were measured by the MTT assay and by Western blotting, respectively, after treatment with Resveratrol (Res) and/or 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU). Apoptosis and cell cycle arrest was measured by 4',6'-diamidino-2-phenylindole hydrochloride staining and fluorescence-activated cell sorting analysis, respectively. The extent of DNA damage was measured by

the Comet assay. We measured the visible changes in the DNA damage/repair cascade by Western blotting.

**RESULTS:** The widely used chemotherapeutic agents BCNU and Res decreased the growth of 5-FU sensitive HCT-116 cells in a dose dependent manner. Combined application of BCNU and Res caused more apoptosis in 5-FU sensitive cells in comparison to individual treatment. In addition, the combined application of BCNU and Res caused a significant decrease of major DNA base excision repair components in 5-FU sensitive cells. We established a 5-FU resistance cell line (5-FU-R) from 5-FU-sensitive HCT-116 (mismatch repair deficient) cells that was not resistant to other chemotherapeutic agents (*e.g.*, BCNU, Res) except 5-FU. The 5-FU resistance of 5-FU-R cells was assessed by exposure to increasing concentrations of 5-FU followed by the MTT assay. There was no significant cell death noted in 5-FU-R cells in comparison to 5-FU sensitive cells after 5-FU treatment. This resistant cell line overexpressed anti-apoptotic [*e.g.*, AKT, nuclear factor  $\kappa\text{B}$ , FLICE-like inhibitory protein], DNA repair (*e.g.*, DNA polymerase beta (POL- $\beta$ ), DNA polymerase eta (POLH), protein Flap endonuclease 1 (FEN1), DNA damage-binding protein 2 (DDB2)] and 5-FU-resistance proteins (thymidylate synthase) but under expressed pro-apoptotic proteins (*e.g.*, DAB2, CK1) in comparison to the parental cells. Increased genotoxicity and apoptosis were observed in resistant cells after combined application of BCNU and Res in comparison to untreated or parental cells. BCNU increased the sensitivity to Res of 5-FU resistant cells compared with parental cells. Fifty per cent cell death were noted in parental cells when 18  $\mu\text{mol/L}$  of Res was associated with fixed concentration (20  $\mu\text{mol/L}$ ) of BCNU, but a much lower concentration of Res (8  $\mu\text{mol/L}$ ) was needed to achieve the same effect in 5-FU resistant cells. Interestingly, increased levels of adenomatous polyposis coli and decreased levels POL- $\beta$ , POLH, FEN1 and DDB2 were noted after the same combined treatment in resistant cells.

**CONCLUSION:** BCNU combined with Res exerts a synergistic effect that may prove useful for the treatment of colon cancer and to overcome drug resistance.

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**Key words:** 5-fluorouracil; 1,3-Bis(2-chloroethyl)-1-nitrosourea; Resveratrol; Colon cancer; Combination therapy

**Core tip:** 5-fluorouracil (5-FU) resistance in colon cancer patients is a common phenomenon that requires immediate resolution. In this paper, we used two commonly administered clinical drugs, 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and Resveratrol (Res), and studied their effects on 5-FU sensitive and resistance colon cancer cells. The drug combination was more effective in 5-FU resistant cells than in 5-FU sensitive cells and was more effective than the individual treatments. The BCNU-Res combination mediated its action by compromising the base excision repair cascade. This combination might be useful for treatment of 5-FU resistant colon cancer patients.

Das D, Preet R, Mohapatra P, Satapathy SR, Kundu CN. 1,3-Bis(2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of Resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells. *World J Gastroenterol* 2013; 19(42): 7374-7388 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7374.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7374>

## INTRODUCTION

Colon cancer ranks as second in cancer related deaths in Western countries, and accounts for approximately 10%-15% of all cancers; half of these patients eventually metastasize<sup>[1]</sup>. The prognosis for patients who develop metastatic tumors is very poor, although several chemotherapeutic methods have been used to improve their survival and quality of life<sup>[2]</sup>. The antimetabolite 5-fluorouracil (5-FU) has been used as a first line therapy in colon cancer; however, in the clinical setting, more than 40% of cases are resistant. Combinations of 5-FU with Leucovorin (LV), Cisplatin, and Oxaloplatin, or with plant derived compounds including Curcumin, Resveratrol, and Quinacrine have increased the response rate up to 25%-30%<sup>[3-8]</sup>. 5-FU exerts anti-proliferative and cytotoxic effects on cells by inhibiting thymidylate synthase (TS) or by misincorporation into DNA and RNA<sup>[9]</sup>.

The misincorporation of 5-fluorodeoxyribosyl residues in DNA is generally repaired by mismatch repair (MMR)<sup>[10-12]</sup> and cells grow continuously. However, any disturbance of the repair system will cause incomplete removal of 5-FU, leading to cell death. Thus, resistance to 5-FU in certain cancers may be the result of highly efficient DNA repair in the 5-FU resistant cells. Several

investigators have used multiple combinations of drugs to increase the sensitivity of 5-FU resistant-cancer cells, but these studies showed very poor clinical responses<sup>[4-6,8]</sup>.

The well-known natural product Resveratrol (Res) has demonstrated high antitumoral efficiency without harmful side effects of conventional chemotherapies. Res (3,4,5-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin widely present in plants and enriched in red grapes, peanuts and other sources<sup>[13]</sup>. This compound demonstrates beneficial functions in normal cells both in *in vitro* and *in vivo* model systems<sup>[13]</sup>. On the other hand, this compound exhibits cytotoxic effects on the majority of malignant cells, blocking the three major stages of carcinogenesis (*i.e.*, initiation, promotion and progression)<sup>[13]</sup> in several cancer cell types, such as breast, colon, melanoma, uterine, lung and leukemia cells<sup>[13-21]</sup>. Recently, using HCT-116 colon cancer cells, we demonstrated that 5-FU increases the sensitivity to resveratrol by inducing DNA damage and the MAPK pathway<sup>[7]</sup>. A significant inhibition of cell proliferation, migration, and increased apoptosis were observed when moderate concentration of Res (15  $\mu\text{mol/L}$ ) were combined with very low concentration of 5-FU (0.5  $\mu\text{mol/L}$ )<sup>[7]</sup>.

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU or carmustine) belongs to the family of haloethylnitrosoureas. It is a common synthetic alkylating chemotherapeutic agent used for treating various types of cancers, including brain tumors, Hodgkins and non-Hodgkins lymphoma and multiple myeloma<sup>[22]</sup>. It is highly lipophilic, capable of readily crossing the blood-brain barrier. It reacts with DNA and forms several exocyclic DNA adducts, which includes the saturated ethanol adducts of adenine, cytosine, and guanine (1,N6-ethanol-A, 3,N4-ethanol-C, N2,3-ethanol-G, and 1,O6-ethanol-G) and produces intra strand cross-links in DNA<sup>[23-26]</sup>. It can also produce mono substituted purine bases in DNA<sup>[23]</sup>. BCNU is classified as an animal carcinogen<sup>[27]</sup>, and has been demonstrated as genotoxic both *in vivo* and *in vitro*<sup>[27]</sup>. This limits the use of this drug at higher doses, unless the drug is combined with some natural anticancer compounds that will not only decrease the toxic effect, but also synergistically increase its effectiveness. Several combinations of BCNU have been tried, such as with  $\text{As}_2\text{O}_3$ <sup>[28]</sup>,  $\text{O}^6\text{BG}$ <sup>[29]</sup> and TMZ<sup>[30]</sup>, to increase the sensitivity in cancer cells, but without success.

Recently, researchers attempted to target DNA damage/repair and cell cycle checkpoint signaling pathways as a means of cancer treatment<sup>[31-33]</sup>. This is relevant for 5-FU therapy, as TS inhibition and incorporation of the fluorinated base into DNA occurs during S-phase. The PI3K-like kinases, ataxia telangiectasia-mutated (ATM) and ATM-related (ATR), are central mediators in the response to DNA damage during S-phase<sup>[34]</sup>. Evidence suggests the involvement of S-phase checkpoint pathways in response to 5-FU treatment and TS inhibition<sup>[35]</sup>. A recent report suggested that ATR and CHK1 status influence cellular sensitivity to 5-FU in a MMR- or BER-mediated response-dependent manner, dictated by the

drug dose and exposure period<sup>[36]</sup>. It was also reported that 5-FU-mediated apoptosis also involved modulation of the major long patch base excision repair (LP-BER) protein Flap endonuclease 1 (FEN1)<sup>[10,37]</sup>. We have already reported that activation of adenomatous polyposis coli (APC) blocks the base excision repair pathway by interaction with DNA polymerase beta (POL-β) and FEN1<sup>[38-41]</sup>. Thus, one strategy for killing 5-FU resistant cells includes combining multiple drugs that will damage DNA, inhibit the DNA repair system and hamper cell cycle regulation in cancer cells.

In the present study, we developed a novel chemotherapeutic combination to increase the sensitivity of 5-FU resistant colon cancer cells using a synthetic DNA damaging agent, BCNU, and a plant derived anti-cancer agent, Res, and investigated the mechanism of anti cancer potentiality against 5-FU resistance. We first measured the anti-cancer potentiality of this combination in 5-FU sensitive human colon cancer cells. A 5-FU resistant cell line was derived from HCT-116 colon cancer cells after prolonged treatment with 5-FU, which was sensitive to other chemotherapeutic agents. These cells were treated with the BCNU and Res combination and drug efficacy was measured. The BCNU and Res combination increased apoptosis of the 5-FU resistant cell lines by inducing DNA damage and inhibiting the base excision repair pathway. We also used isobologram analysis to evaluate whether the drug combination has a synergistic, additive or antagonist effect on human colon cancer.

## MATERIALS AND METHODS

### Cell culture and treatment

The HCT-116 colon cancer cell line (obtained from American Type Culture Collection, VA, United States, Cat # CCL-247) was cultured in RPMI-1640 with 1% antibiotic (100 units of penicillin and 1 mg streptomycin per milliliter in 0.9% normal saline) and supplemented with 10% fetal bovine serum (HIMEDIA, Mumbai, India) in a humidified CO<sub>2</sub> incubator in 5% CO<sub>2</sub> at 37 °C. Drugs like Res, 5-FU, LV, BCNU were purchased from Sigma Chemical Ltd. (St. Louis, MO, United States). DNA polymerase eta (POLH) and DNA damage-binding protein 2 (DDB2) antibodies were procured from Abcam (MA, United States). Apurinic/aprimidinic (AP) endonuclease 1 (APE) and POL-β antibodies were purchased from Novus Biologicals (CO, United States). The anti-APC antibody was purchased from Calbiochem (CA, United States). All the other antibodies used in the experiments were procured from Cell Signaling Technology (Danvers, MA, United States). A 1 mmol/L stock of Res, 5-FU, BCNU and LV was prepared in absolute ethanol, DMSO, 50% ethanol and distilled water, respectively, and was stored at -20 °C. During treatment, Res, 5-FU and BCNU were diluted in RPMI and then added to the cultures to achieve the desired final concentration. LV (1 μmol/L) was added in combination with 5-FU in each treatment. When they reached 60%-70% confluency, the cells were treated with 5-FU, Res, BCNU and the combination of

BCNU and Res (in each case of the combination treatment, the cells were pre-treated with BCNU (20 μmol/L) for 24 h and then exposed to Res for another 48 h).

### Establishment of 5-FU resistant cell line

In our earlier report, we demonstrated that the minimum concentrations needed for causing fifty percent cell death in culture (LC<sub>50</sub>) in HCT-116 cells by 5-FU was 10 μmol/L<sup>[7]</sup>. To make stable 5-FU resistant cell lines, HCT-116 cells were exposed to 25 μmol/L for more than 20 wk. Every 48 h the media was replaced with fresh media and cells were treated with fresh 5-FU of same concentration. Initially, more than 80% of the cells died within a few days; the remaining 20% cells survived and developed 5-FU resistance. At the end of the exposure, 5-FU resistance was checked by a cell survival assay (MTT) in the presence of 5-FU and the transformed characteristic was confirmed by measuring several biomarkers. No significant cell death was noted after 5-FU treatment allowing the cells to be designated as 5-FU-R cells.

### MTT assay

The anchorage dependent cell viability of normal HCT-116 and 5-FU-R cells was measured after treatment with various drugs using a reliable and sensitive colorimetric assay: the MTT [3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] cell proliferation assay, as described previously<sup>[7,42]</sup>. Approximately, 10000-12000 cells were plated in a 96 well tissue culture plate in triplicate and incubated for 24 h, after which they were exposed to various concentrations of the indicated compounds for 48 h. At the end of the treatment, the cells were washed with 1 × phosphate buffered saline (PBS) and then 100 μL of 0.05% MTT reagent (Sigma) was added to each well and kept for 6 h in incubation at 37 °C to allow the formation of purple formazan crystals. 100 μL of detergent solution (10% NP-40 with 4 mmol/L HCl) was added to each well of the 96 well tissue culture plate and the reaction mixture was incubated for 1 hr at room temperature. The color intensity was measured spectrophotometrically using a microplate reader (Berthold, Germany) at 570 nm. The data were calculated as percentages of the control. All assays were performed at least three times.

### Nuclear staining with 4',6-diamidino-2-phenylindole

To determine the apoptosis of HCT-116 and 5-FU-R cells after treatment with the indicated drug, 4',6-diamidino-2-phenylindole (DAPI) nuclear staining was carried out. Cells at 70%-80% confluency were treated with the desired drug for indicated time, as mentioned in the figure legend. The cells were then washed with PBS and fixed with acetone: methanol (1:1) for 15 min at -20 °C in the dark. Fixed cells were washed once with PBS and then DAPI solution was added and incubation was continued for 1 h at 37 °C in the dark. Excess DAPI was removed by washing with PBS and the stained cells were visualized under a fluorescence microscope (Nikon, Japan) at × 40 magnification.



### Cell cycle

Regulation of cell cycle and apoptosis were measured using fluorescence-activated cell sorter (FACS) analysis. HCT-116 and 5-FU-R cells were cultured in 60 mm tissue culture discs until they reached 60%-70% confluency. They were then treated with the indicated drug. After treatment, cells were trypsinized and washed with PBS containing RNase-A and fixed with 70% ethanol. The cells were incubated at -20 °C overnight and then re-suspended in 0.1 mL of Propidium Iodide (PI, 50 µg/mL) and incubated for 1 h in the dark at room temperature. Finally, FACS was used to sort the cells (Becton and Dickinson, CA). Cell Quest Software was used to determine the DNA content of the cells at various phases of the cell cycle (Becton and Dickinson, CA).

### Single cell gel electrophoresis or comet assay

After drug treatment, DNA damage in HCT-116 and 5-FU-R cells was determined by performing a single cell gel electrophoresis assay following the method of Tice *et al.*<sup>[43]</sup>. Cells were suspended in PBS and about 5000-6000 cells were mixed with low melting agarose at 37 °C and spread on a microscopic slide. After solidifying, the slides were dipped in chilled lysis solution (10 mmol/L Tris, 100 mmol/L EDTA, 2.5 mol/L NaCl, 1% Triton × 100, 10% DMSO and pH 10.0) for 1 h. After lysis the slides were put in freshly prepared alkaline electrophoresis buffer and electrophoresis was performed at 20 V for 20 min. Slides were then dipped in neutralization buffer (0.4 mol/L Tris-HCl and pH 7.5) and washed with distilled water, followed by 70% ethanol, and allowed to dry. Later, 40 µL of SYBR green dye was added to each slide, which was incubated in the dark for 30 min at room temperature. A fluorescence microscope (Nikon, Japan) at a magnification of × 10 was used to visualize DNA migration. TriTek CometScore™ software was used to analyze the comet lengths.

### Western blotting

Cells were plated on 100 mm tissue culture discs and were treated with the indicated drug when they reached 70% confluence. After treatment, the cells were washed with PBS and were lysed using the RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5 mmol/L Deoxycholate, 1% NP-40, 0.1% SDS, 1 mmol/L NaVO<sub>4</sub>, 5 mmol/L EDTA, 1 mmol/L PMSF, 2 mmol/L DTT, 10 mmol/L β-glycerophosphate, 50 mmol/L NaF, 0.5% Triton × 100, protease inhibitor cocktail) for 45 min at 4 °C. The lysates were centrifuged at 14000 rpm for 10 min to remove the cell debris. The supernatant was collected in a separate tube and protein quantification was performed by Bradford method using BSA as a standard. Approximately 100 µg of protein were loaded in each well and separated using 10% SDS-PAGE. After separation, the proteins were transferred to a PVDF membrane. The membrane was blocked and then probed with specific antibody, according to the manufacturer's protocol.

### Analysis of combined drug effect

Synergistic, additive or antagonist drug effects were de-

termined by isobologram analysis<sup>[28]</sup>. Isobologram plots were drawn by plotting the individual LC<sub>50</sub> values of the drugs in their respective X- and Y-axis. The LC<sub>50</sub> values were obtained from the individual drugs effect of MTT assays. Then, a line was used to join both the data points and the LC<sub>50</sub> value of the combined drug was spotted on the same plot. In principle, if the spotted point (LC<sub>50</sub> value of combined drugs) falls on the line then it considered as additive, whereas if it falls below or above the line, then it considered as a synergistic or antagonist drug effect, respectively.

## RESULTS

### Cytotoxic effect of 5-FU, BCNU and Res in HCT-116 colon cancer cells

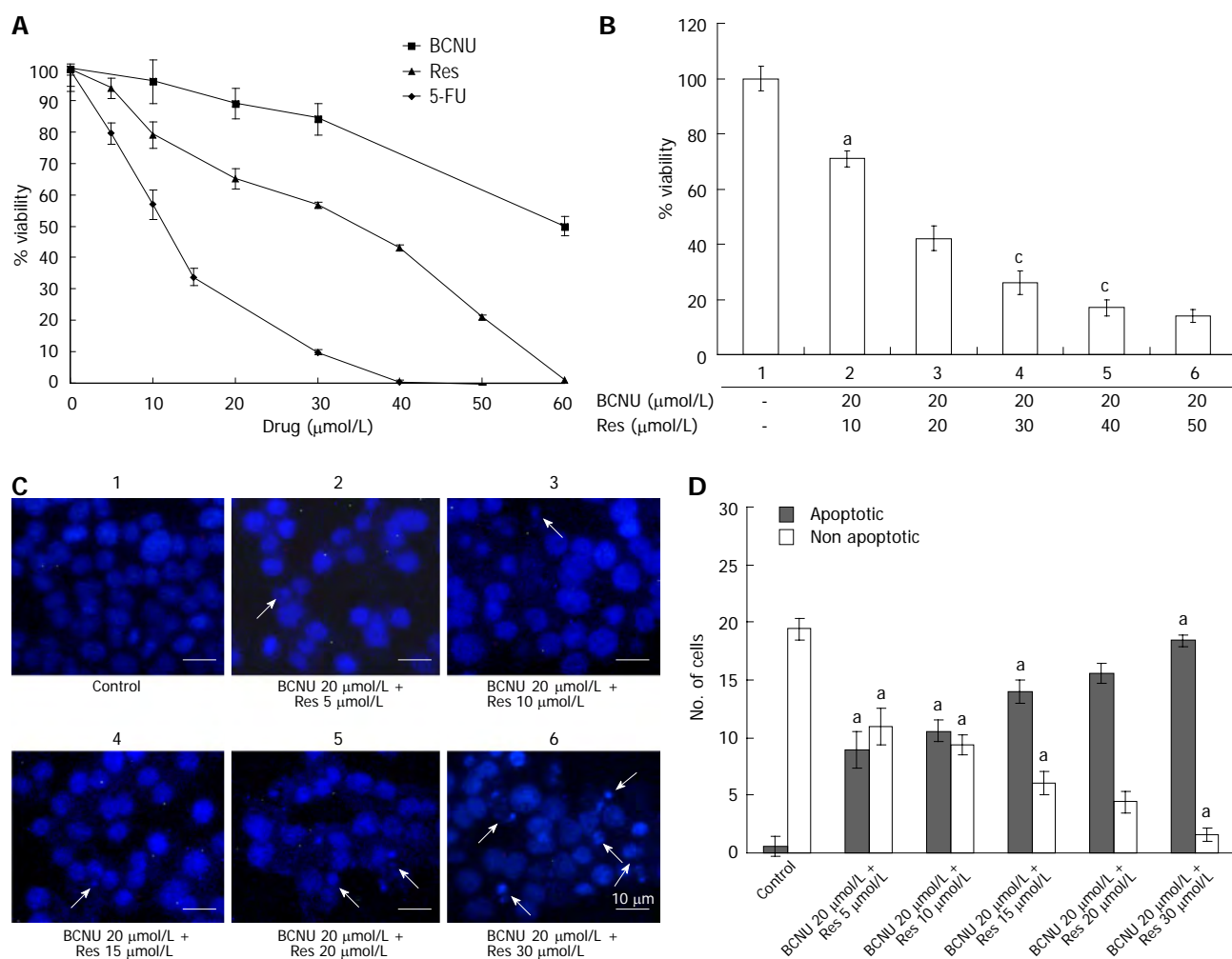
To determine the LC<sub>50</sub> (concentrations needed for fifty percent cell death in culture) and to understand the cytotoxic effect of each drug on HCT-116 cells, the MTT assay was carried out. Three known chemotherapeutic agents (5-FU, BCNU and Res) were chosen for experimentation (Figure 1A). The cells were treated for 48 h before the MTT assay. Figure 1A shows that increasing the concentrations of each drug increased cell death. 5-FU was the most effective among the three drugs and BCNU was the least effective. LC<sub>50</sub> was noted at 10 µmol/L 5-FU while 35 µmol/L Res and 60 µmol/L BCNU were needed to achieve the same amount of cell death (Figure 1A). Although 5-FU offered maximum cytotoxic effects on colon cancer cells, more than 50% of cells exhibited resistance<sup>[44]</sup>. To overcome such resistance and to increase the efficacy of Res, a combined treatment was employed comprising BCNU and Res. The BCNU concentration was kept constant at 20 µmol/L and varied concentrations of Res were added. The combination of BCNU with Res increased the sensitivity of HCT-116 cells. Fifty percent cell death was observed when 18 µmol/L Res was combined with 20 µmol/L BCNU (Figure 1B) ( $P < 0.05$ ).

### Detection of apoptosis in HCT-116 cells by DAPI staining

5-FU, BCNU and Res killed colon cancer cells, but to confirm whether the killing effect of the drugs was through apoptosis or necrosis, DAPI nuclear staining was performed. Figure 1C shows that the number of apoptotic nuclei increased with increasing concentrations of Res when combined with 20 µmol/L BCNU as compared with untreated cells. More than fifty percent apoptotic nuclei were observed when 20 µmol/L BCNU was associated with 15 µmol/L of Res. Figure 1D shows a graphical representation of the number of apoptotic and non-apoptotic nuclei ( $P < 0.05$ ).

### Combined effect of BCNU and Res caused DNA damage in HCT-116 cells

To determine whether a combination of BCNU and Res caused apoptosis through DNA damage or by another mechanism, we measured the DNA damaging efficiency of this combination in HCT-116 cells by DNA damage



**Figure 1** Anti-proliferative and apoptotic effect of 1,3-Bis(2-chloroethyl)-1-nitrosourea and/or resveratrol on HCT-116 colon cancer cells. **A:** Anchorage-dependent cell survival of HCT-116 cells after treatment with Res, 5-FU and BCNU; **B:** Bar diagram representing the % viability of HCT-116 after BCNU+ Res exposure. HCT-116 cells were cultured in 96 well plates and grown to 60%-70% confluence. The cells were then treated with different compounds according to the materials and methods. Data are the mean  $\pm$  SD of three different experiments. <sup>a</sup> $P < 0.05$  vs 20 μmol/L BCNU + 20 μmol/L Res; <sup>c</sup> $P < 0.05$  vs 20 μmol/L BCNU + 50 μmol/L Res; **C:** Apoptotic nuclei after 4',6'-diamidino-2-phenylindole hydrochloride staining. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at  $\times 40$  magnification. Arrows indicate the apoptotic nuclei. Data are the representation of one of the replicates of three different experiments; **D:** A graphical representation of apoptotic nuclei. <sup>a</sup> $P < 0.05$  vs 20 μmol/L BCNU + 20 μmol/L Res. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

assays (single cell gel electrophoresis or comet assay). The cells were pre-treated with 20 μmol/L BCNU for 24 h prior to exposure with various concentrations of Res for another 48 h. The comet formation and average comet length increased with increasing concentrations of Res (Figure 2A). Figure 2B shows the average comet length of combined treatment. Compared with untreated cells, the average comet length increased when Res was combined with BCNU (Figure 2B) ( $P < 0.05$ ). Thus, the data suggests that the DNA damaging effect of Res was magnified in presence of BCNU.

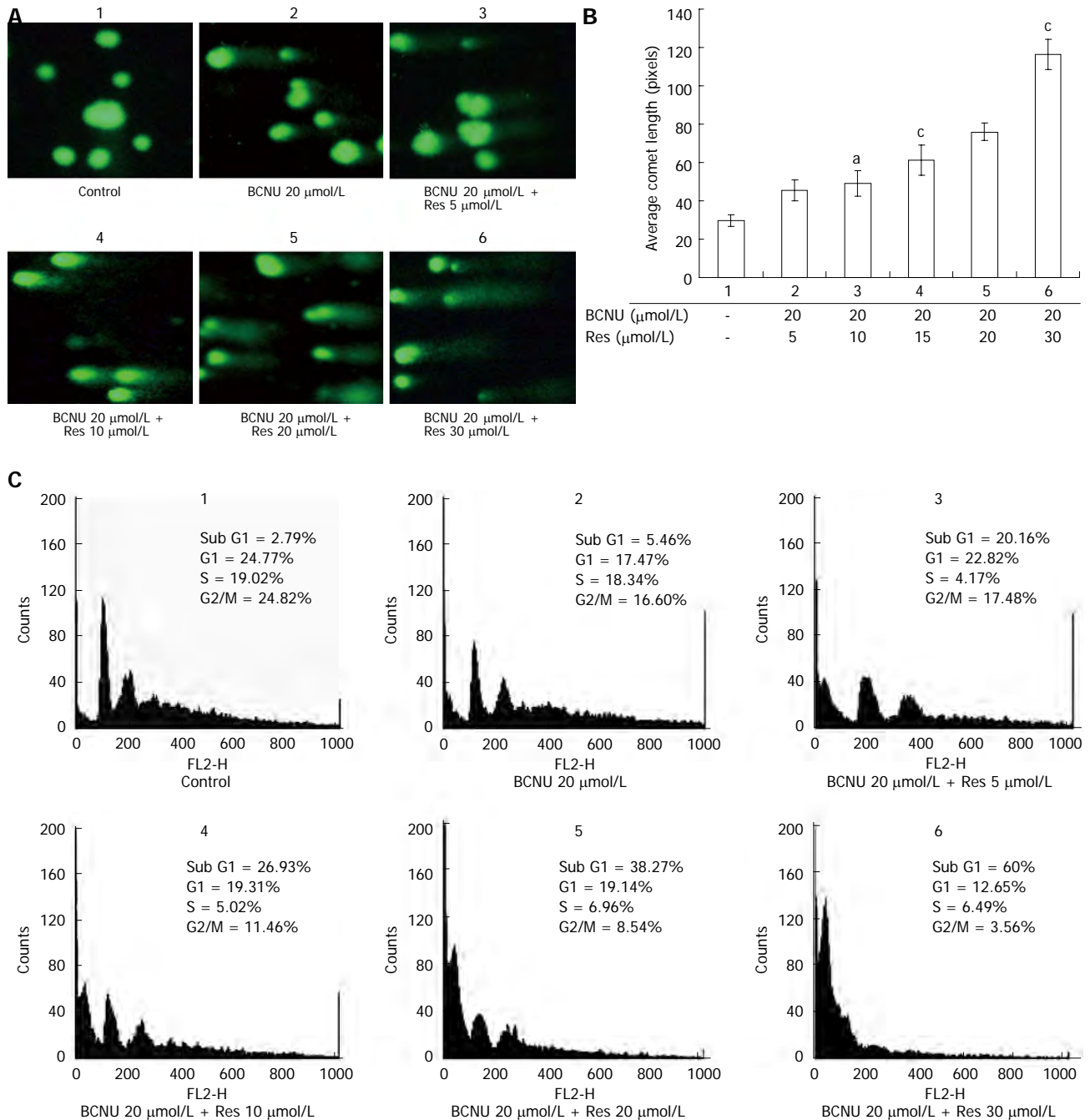
#### Effects of BCNU and Res on the cell cycle regulation and apoptosis of HCT-116

It was reported that Res halts the cell cycle at S phase in various cancer cells, such as breast, colon and pancreas<sup>[45-49]</sup>. Similarly, it was also reported that BCNU arrests the cell cycle in the G2/M phase transition<sup>[50]</sup>. To determine the regulation of cell cycle profile by BCNU

+ Res combination, we treated the cells with the above-mentioned drugs and performed FACS analysis at the end of the exposure. The percentages of G2/M population of cells decreased with increasing concentrations of Res combined with BCNU in comparison with the control (Figure 2C). The percentages of apoptotic cells (sub G1) population increased in a dose dependent manner with Res combined with a fixed concentration of BCNU. Interestingly, approximately 60% apoptosis was noted when 30 μmol/L Res was combined with 20 μmol/L of BCNU (Figure 2C).

#### Effects of BCNU and Res on the expression level of apoptotic markers in HCT-116 cells

The combined effect of BCNU and Res on apoptosis, cell cycle regulation and DNA damage repair proteins in HCT-116 cells was studied by measuring the protein expression levels of well known markers, such as Bcl-2-associated X protein (BAX), B-cell lymphoma-extra large



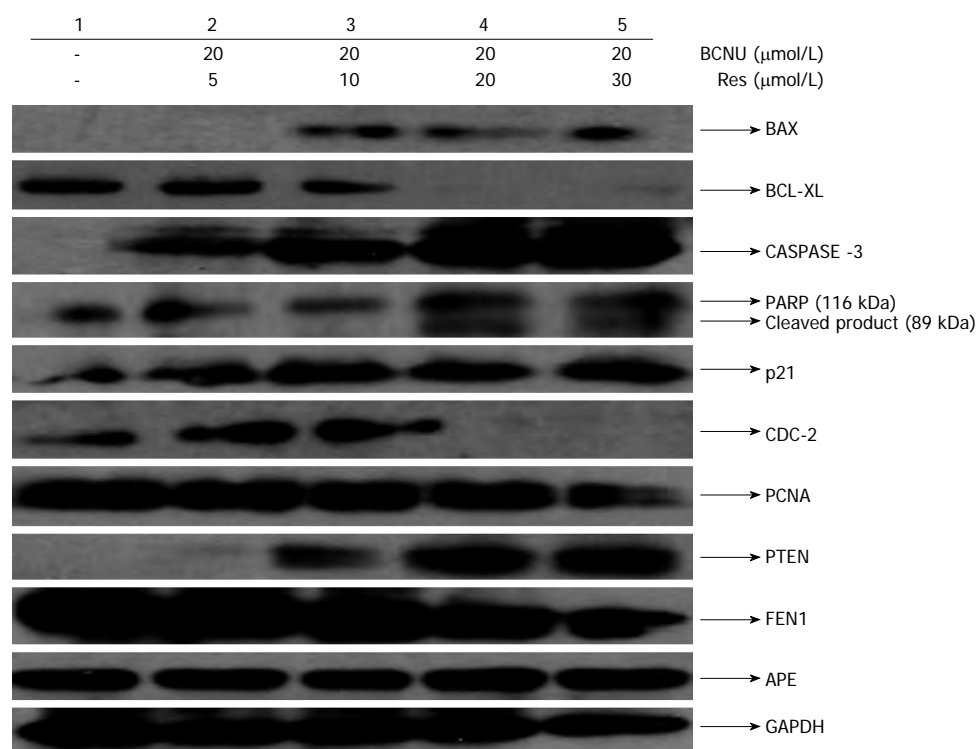
**Figure 2** Regulation of cell cycle and genotoxicity of HCT-116 cells after 1,3-Bis(2-chloroethyl)-1-nitrosourea and/or resveratrol treatments. **A:** Comet assay showing the DNA damaging effect of the drugs in HCT-116 cells. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at  $\times 20$  magnification. Data are the representation of one of the replicates of three different experiments; **B:** Bar diagram represents the average comet - length in pixels as obtained from TriTek CometScore™ software. Data are the mean  $\pm$  SD of three different experiments, <sup>a</sup> $P < 0.05$  vs 20  $\mu\text{mol/L}$  BCNU + 5  $\mu\text{mol/L}$  Res; <sup>c</sup> $P < 0.05$  vs 20  $\mu\text{mol/L}$  BCNU + 20  $\mu\text{mol/L}$  Res; **C:** Effect of the drugs on cell cycle regulation. After treatment as mentioned in the materials and method fluorescence-activated cell sorting analysis was performed and the DNA content of the cell was measured by Cell Quest Software (Becton and Dickinson, CA). Data are the representation of one of the replicates of three different experiments. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

(BCL-XL), Poly (ADP-ribose) polymerase (PARP), p21, proliferating cell nuclear antigen (PCNA), APE, phosphatase and tensin homolog (PTEN), FEN1 and human homolog of cyclin dependent kinase-2 (CDC-2) (Figure 3). The level of BAX increased compared with treatment with BCNU alone, whereas the level of BCL-XL decreased (Figure 3). The levels of CASPASE-3 and cleaved product of PARP also increased compared with BCNU alone (Figure 3). Thus, the data indicated that treatment

with the BCNU and Res combination results in apoptosis in HCT-116 cells, as reflected by the elevated BAX/BCL-XL ratio, PARP cleavage and CASPASE-3 expression (Figure 3).

#### Effects of BCNU and Res on the expression level of cell cycle regulatory and DNA repair proteins in HCT-116 cells

DNA damage repair proteins, such as FEN1, APE, and



**Figure 3** Combined effects of 1,3-Bis(2-chloroethyl)-1-nitrosourea and resveratrol on various cellular markers in HCT-116 colon cancer cells. Expression pattern of apoptotic, DNA damage/repair and cell cycle regulatory proteins after drug treatment. Data are the representation of one of the replicates of three different experiments. Glyceraldehyde phosphate dehydrogenase (GAPDH) served as a loading control. BAX: Bcl-2-associated X protein; BCL-XL: B-cell lymphoma-extra large; PARP: Poly (ADP-ribose) polymerase; PCNA: Proliferating cell nuclear antigen; PTEN: Phosphatase and tensin homolog; FEN1: Flap endonuclease 1; CDC-2: Cyclin dependent kinase-1; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea; APE: Apurinic/aprimidinic (AP) endonuclease.

PCNA, were assayed to determine the effects on DNA damage/repair in HCT-116 cells after BCNU and Res combination treatment (Figure 3). FEN1 and PCNA levels decreased when the dose of Res increased, whereas the level of APE remained almost constant during combination treatment (Figure 3). The cell cycle regulatory protein p21 and PTEN increased, while CDC-2 decreased, after combined treatment for 48 h. PTEN is considered to be a cell cycle regulatory tumor suppressor protein, and an increase in PTEN expression indicates the anti-tumor property of the combined drug. Increased p21 expression during the combination treatment indicated negative cell cycle regulation. A decrease in CDC-2 expression also indicated negative regulation of the cell cycle. Thus, these experiments demonstrated that treatment with a combination of BCNU and Res induced apoptosis in colon cancer cells by affecting cell cycle regulation and DNA damage/repair. Therefore, this novel drug combination can be used for treating 5-FU resistant cells.

#### **Establishment of a 5-FU-resistant stable cell line**

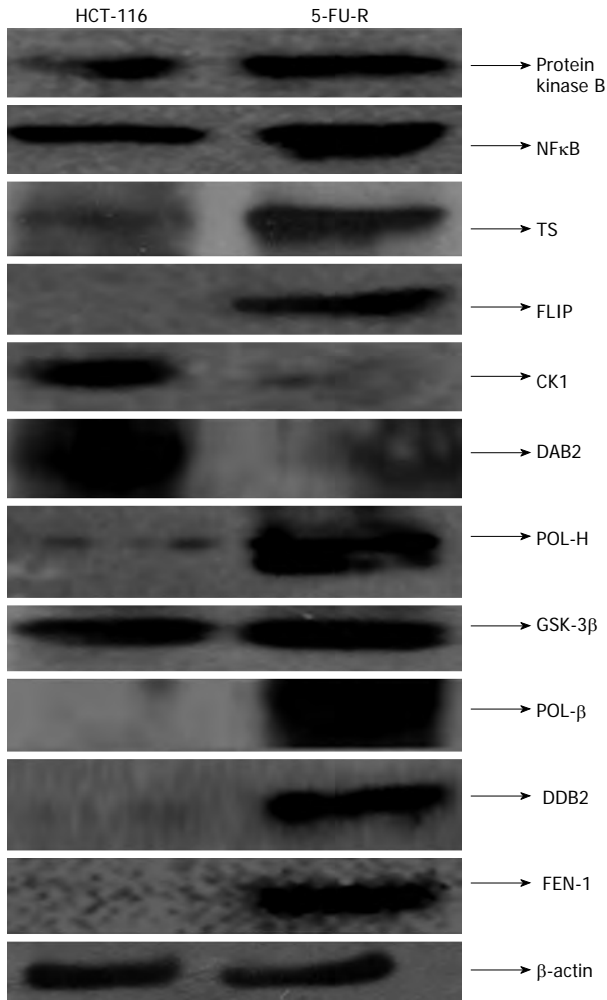
The 5-FU-R cell line was further characterized by measuring the expression pattern of anti-apoptotic, cell cycle and DNA damage repair proteins, such as nuclear factor kappa-light-chain-enhancer of nuclear factor  $\kappa\text{B}$  (NF $\kappa\text{B}$ ), FLICE-like inhibitory protein (FLIP), casein kinase 1 (CK1), disabled homolog 2 (DAB2), glycogen synthase kinase 3 beta (GSK-3 $\beta$ ), AKT, POLH, TS, POL- $\beta$ ,

DDB2, and FEN1, which were altered in stable cell line compared to normal HCT-116 cells (Figure 4). The expression levels of AKT, NF $\kappa\text{B}$ , FLIP, POLH, GSK-3 $\beta$ , POL- $\beta$ , DDB2, and FEN1 were increased in comparison with HCT-116 cells. However, the levels of CK1 and DAB2 expressions decreased compared with HCT-116 cells (Figure 4). Interestingly, widely established 5-FU resistance protein thymidylate synthase also increased in 5-FU-R cells compared with HCT-116 cells (Figure 4).

#### **Cytotoxicity of the BCNU and Res combination in 5-FU-R cells**

The experiments discussed above indicated that BCNU and Res combination significantly increased the sensitivity of HCT-116 colon cancer cells. To determine the optimum concentration of the BCNU and Res combination for cytotoxicity of 5-FU-R cells, an MTT assay was carried out. BCNU showed similar cytotoxic profile in 5-FU-R cells compared with HCT-116 cells (LC<sub>50</sub> 55  $\mu\text{mol/L}$ ) (Figure 5A); however, it was noted that 5-FU resistant cells were more sensitive to Res compared with HCT-116 parental cells. The LC<sub>50</sub> of Res in 5-FU-R cells was 18  $\mu\text{mol/L}$ , but was 35  $\mu\text{mol/L}$  in HCT-116 cells (Figure 1A *vs* Figure 5A). Interestingly, it was noted that there was little or no effect of 5-FU on 5-FU-R cells, even after treatment with 100  $\mu\text{mol/L}$  for 48 h (Figure 5A). This data suggests that 5-FU-R cells are resistant to 5-FU, but sensitive to other chemotherapeutic drug, such





**Figure 4 Characterization of 5-fluorouracil-resistant cells.** The cell lysates of HCT-116 and 5-FU-R cells were immunoblotted using specific antibody. The lower panel shows the expression of  $\beta$ -actin, which was the loading control (to ensure the same amount of protein loaded in each lane). Data are the representation of one of the replicates of three different experiments. CK1: Casein kinase 1; FLIP: FLICE-like inhibitory protein; NF $\kappa$ B: nuclear factor  $\kappa$ B; POL- $\beta$ : DNA polymerase beta; POLH: DNA polymerase eta; protein Flap FEN1: Endonuclease 1; DDB2: DNA damage-binding protein 2; 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea; TS: Thymidylate synthase.

as Res.

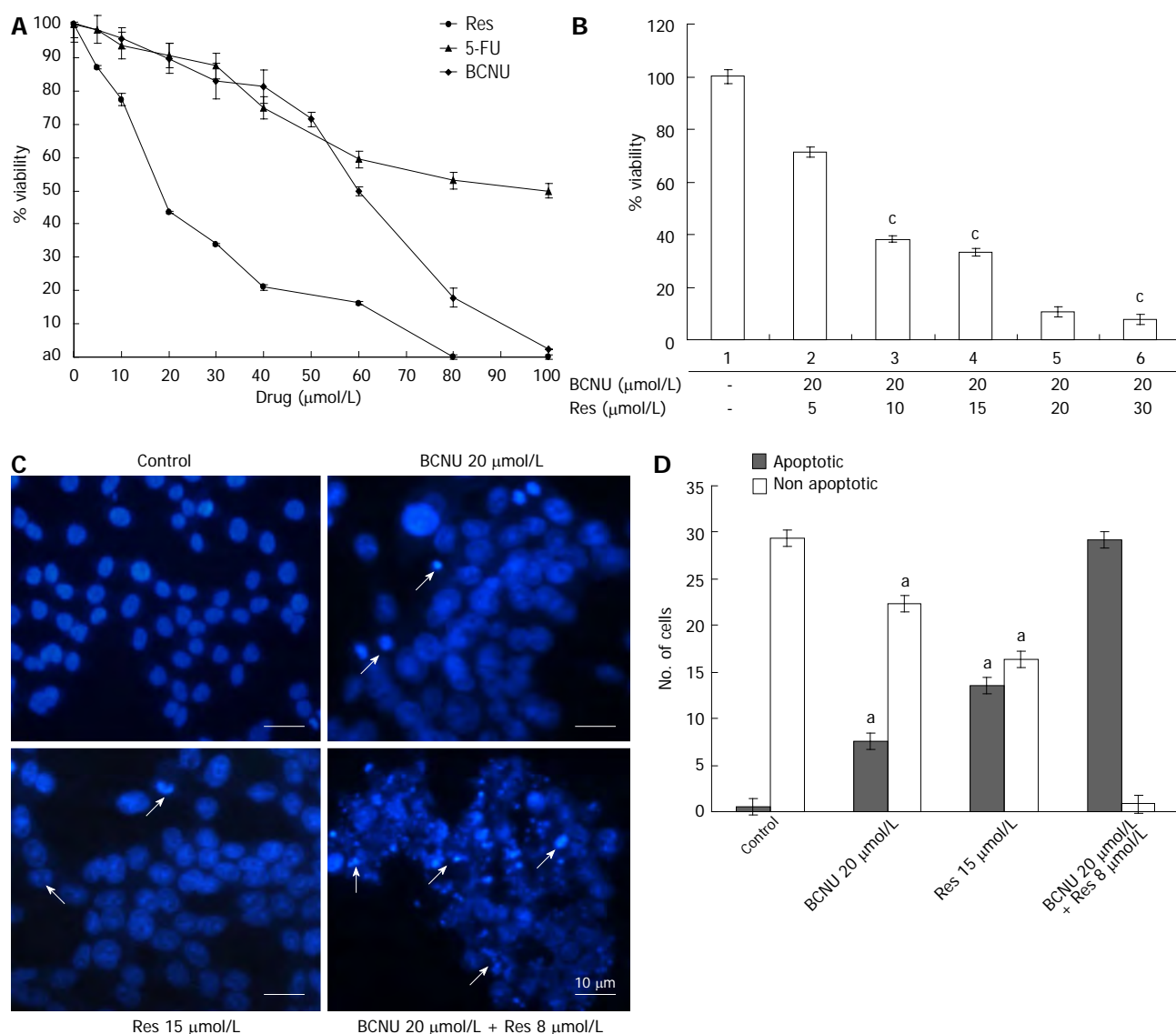
A dose dependent decrease in cell viability was observed when increasing concentrations of Res were added to the 20  $\mu$ mol/L BCNU pre-treated 5-FU-R cells (Figure 5B). Fifty percent cell death ( $LC_{50}$ ) was noticed when 8  $\mu$ mol/L of Res was combined with 20  $\mu$ mol/L of BCNU (Figure 5B) ( $P < 0.05$ ). Interestingly, it was noted that lower concentrations of Res were needed to cause fifty percent cell death in 5-FU-R cells compared with HCT-116 cells ( $LC_{50}$  for HCT-116 cells was 18  $\mu$ mol/L vs  $LC_{50}$  for 5-FU-R cells was 8  $\mu$ mol/L). Thus, it appeared that 5-FU-R resistant cells (Figure 5B) were more susceptible than HCT-116 cells (Figure 2B) to the BCNU and Res combination.

Apoptosis of 5-FU-R cells was measured by DAPI nuclear staining after exposure to the optimum formulation (BCNU 20  $\mu$ mol/L + Res 8  $\mu$ mol/L), BCNU and

Res. Although treatment with BCNU and Res alone caused cell death, increased cell death was observed by treatment with the combination compared with BCNU and Res alone (Figure 5C). However, Res alone caused more apoptosis than BCNU alone (Figure 5C). Figure 5D shows the graphical representation of the number of apoptotic and non-apoptotic nuclei ( $P < 0.05$ ).

#### **Combined effect of BCNU and Res caused increased DNA damage, and decreased cell cycle regulatory protein expression and apoptosis in 5-FU-R cells**

To elucidate the underlying mechanism of the sensitivity 5-FU-R cells to the BCNU and Res combination, a series of experiments were carried out. To determine whether the BCNU and Res combination caused DNA damage, a comet assay was performed after treatment with BCNU 20  $\mu$ mol/L + Res 8  $\mu$ mol/L for 48 h. The average comet length and number of comets increased with the combination treatment compared with BCNU and Res alone or untreated cells (Figure 6A). Figure 6B shows the average comet length, in pixels, of 5-FU-R cells treated with BCNU and Res alone and in combination [BCNU (20  $\mu$ mol/L) + Res (8  $\mu$ mol/L)] ( $P < 0.05$ ). The average comet length almost doubled when 8  $\mu$ mol/L of Res was combined with BCNU compared with BCNU alone. The percentage of apoptosis and cell cycle profile cells were also measured by FACS analysis after treatment with the combined drugs (Figure 6C). Increased accumulation of the G<sub>2</sub>/M (24%) population was noted after the BCNU treatment in comparison to Res (19.33%) and untreated cells (10%). Interestingly, the increase in G<sub>2</sub>/M population decreased when Res was combined with BCNU; however, the observed percentage of apoptosis (sub G<sub>1</sub>) was as high as 70%. To further confirm the apoptosis and to determine the status of various protein biomarkers, Western blotting was carried out. Figure 6D shows the expression patterns of apoptotic markers, such as cleaved product of PARP, BAX, CASPASE-3 and BCL-XL. The cleaved product of PARP and the BAX/BCL-XL ratio increased with increasing concentration of the combined drug. Cell cycle regulatory proteins, such as p21 and PTEN increased, while CDC-2 decreased after combined treatment for 48 h, indicating that this drug combination has adverse effect on cell cycle regulation in the 5-FU-R cells. Figure 6E represents the expression pattern of DNA repair proteins when treated with the combined drug and Res and BCNU alone. The levels of FEN1, POLH, DDB2 and POL- $\beta$  were decreased in cells that were treated with BCNU and Res alone compared with the control, but the combination treatment showed a radical decrease in the levels of these proteins compare with the control and treatment with each drug alone (Figure 6E). Interestingly, the level of tumor suppressor and DNA repair protein APC was completely abolished after individual (BCNU and Res) treatment, but increased after combined drug exposure compared with untreated cells. This result clearly indicates that the BCNU and Res works additively or synergistically and their mode of ac-



**Figure 5** Anti-proliferative and apoptotic effect of 3-Bis(2-chloroethyl)-1-nitrosourea and/or resveratrol on 5-fluorouracil-R cells. A: Anchorage-dependent cell survival of 5-FU-R cells after treatment with 5-FU, Res and BCNU; B: Bar diagram representing the % viability of 5-FU-R cells after BCNU + Res exposure. Data are the mean  $\pm$  SD of three different experiments, <sup>a</sup> $P < 0.05$  vs 20  $\mu\text{mol/L}$  BCNU + 5  $\mu\text{mol/L}$  Res; <sup>c</sup> $P < 0.05$  vs 20  $\mu\text{mol/L}$  BCNU + 20  $\mu\text{mol/L}$  Res; C: Apoptotic nuclei after DAPI staining. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at  $\times 40$  magnification. An arrow indicates the apoptotic nuclei. Data are the representation of one of the replicates of three different experiments; D: A graphical representation of apoptotic nuclei, <sup>a</sup> $P < 0.05$  vs 20  $\mu\text{mol/L}$  BCNU + 8  $\mu\text{mol/L}$  Res. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

tion was to modulate DNA damage/repair pathway.

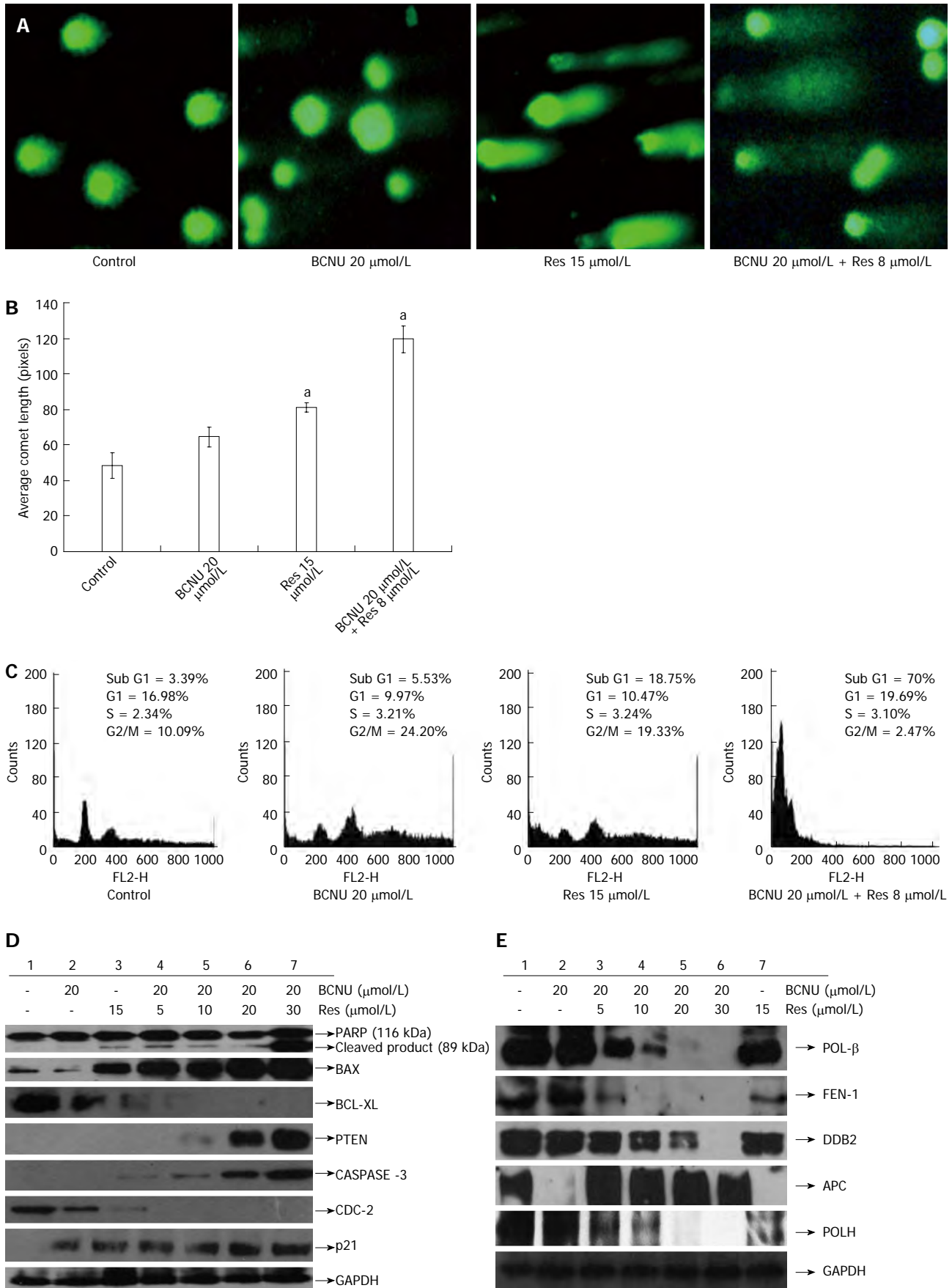
### BCNU and Res synergistically increase the sensitivity in human colon cancer cells

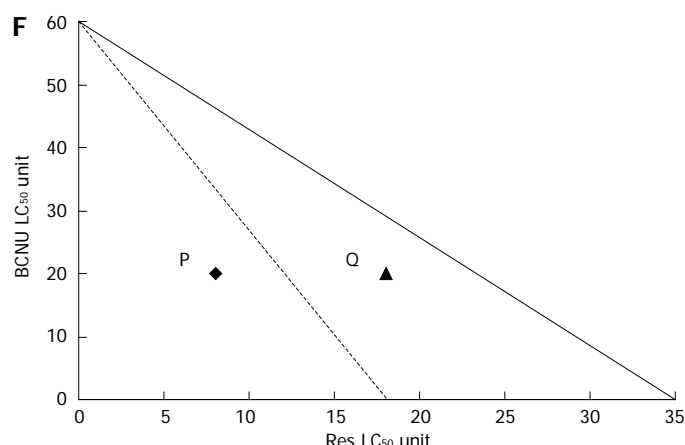
The above data showed that the BCNU and Res combination increased the sensitivity of colon cancer cells. To determine whether these two drug work additively or synergistically, we performed isobologram analysis. Figure 6F shows the occurrence of a synergistic interaction of Res and BCNU in both the HCT-116 and 5-FU-R cell lines. The  $\text{LC}_{50}$  value of the Res and BCNU in HCT-116 cells were 30 and 60  $\mu\text{mol/L}$ , respectively, but for the combination, fifty percent cell death occurred at 20  $\mu\text{mol/L}$  BCNU and 18  $\mu\text{mol/L}$  Res (Figure 2B), which were much lower than dose of each individual drug (point Q). Point Q appeared below the lines, which suggested a

synergistic interaction of these drugs. To further investigate the synergistic interaction of these drugs against the 5-FU-R cells, the same isobologram was plotted using the  $\text{LC}_{50}$  value of MTT data from Figure 5B. Interestingly, the fifty percent cell death (point P) with the combined drug appeared below the line, clearly indicating synergistic activity of BCNU and Res in 5-FU-R cells (Figure 6F).

## DISCUSSION

5-FU is widely used for the treatment of many types of cancers, including colorectal, breast, and cancers of the aerodigestive tract. Notably, 5-FU is routinely employed in the management of colorectal cancer *via* one of the two FDA-approved first line combinatorial chemothera-





**Figure 6** 1,3-Bis(2-chloroethyl)-1-nitrosourea and Res synergistically increased apoptosis in 5-fluorouracil-resistance cells. A: Comet assay. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at  $\times 20$  magnification. Data are the representation of one of the replicates of three different experiments; B: Bar diagram representing the average comet score of the treated and untreated 5-FU-R cells in pixels, as obtained from TriTek CometScore™ software. Data are the mean  $\pm$  SD of three different experiments,  $^{\ast}P < 0.05$  vs 20  $\mu\text{mol/L}$  BCNU; C: Regulation of cell cycle and apoptosis by BCNU, Res and their combination in 5-FU-R cells. Fluorescence-activated cell sorting analysis was done after the desired treatment and then the DNA content was measured by Cell Quest Software. Data are the representation of one of the replicates of three different experiments; D, E: Western blotting of 5-FU-R cell lysate after treatment with BCNU and Res individually, as well as in combination. Glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. Data are the representation of one of the replicates of three different experiments; F: Isobolograms for combinations of BCNU and Res in HCT-116 and 5-FU-R cells. The bold line represents the isobologram of BCNU and Res in HCT-116 and dotted lines represents the isobologram of BCNU and Res in 5-FU-R cells. The point P and Q represent the 50% cell death of the combined drug in case of 5-FU-R and HCT-116 cells, respectively. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

py regimes, which involve intravenous administration of the fluorinated base analog<sup>[10,11]</sup>. One problem with 5-FU treatment is that more than 50% of tumors demonstrate resistance to 5-FU in the clinical setting. Thus, novel treatments are needed to treat these resistant tumors. Novel biological agents, such as the monoclonal antibodies Cetuximab (an epidermal growth factor receptor inhibitor) and Bevacizumab (a vascular endothelial growth factor inhibitor), have recently been shown to provide additional clinical benefit for patients with metastatic colorectal cancer. However, these agents have demonstrated marginal results when treating resistant tumors<sup>[51,52]</sup>.

During the past few decades, investigators have attempted to elucidate the mechanism underlying 5-FU resistance<sup>[10-12]</sup>. Some investigators developed 5-FU resistant cell lines and studied their sensitivity to multiple chemotherapeutic agents by activation and inhibition of several signaling pathways<sup>[53,54]</sup>. Unfortunately, none of these agents showed promising results.

In the present report, we developed a new strategy for overcoming 5-FU resistance by establishing 5-FU-R cell lines and studying their sensitivity to a combination treatment with BCNU and Res. BCNU and Res display anti-cancer properties by inducing DNA damage, arresting cell cycle progression, inhibiting anti-apoptotic makers and autophagy<sup>[13,28]</sup>. We have shown recently that moderately low concentration of 5-FU (0.5  $\mu\text{mol/L}$ ) increased chemoprevention potential of Res in colon cancer cells<sup>[7]</sup>. However, in that report we could not eliminate the probability of 5-FU resistance because of presence of 5-FU. Thus, that cocktail will not be appropriate for the treatment of 5-FU resistance patients. In the present study, we treated the cells with BCNU and Res to increase the sensitivity of 5-FU resistant cell lines. Initially, we

checked whether BCNU and Res combination increase the sensitivity of 5-FU sensitive HCT-116 cells. LC<sub>50</sub> was observed at 60  $\mu\text{mol/L}$  BCNU and 35  $\mu\text{mol/L}$  Res; however, the same amount of cell death was observed when 18  $\mu\text{mol/L}$  Res was combined with 20  $\mu\text{mol/L}$  BCNU. Reduction of both BCNU and Res concentration for fifty percent cell death revealed that BCNU and Res killed HCT-116 colon cancer cells synergistically (Figure 1B). The isobologram data (Figure 6F) also support the synergistic action of BCNU and Res to kill colon cancer cells.

Cell death associated with the synergistic effects of treating cells with a combination of BCNU and Res was further confirmed by multiple parameters, such as DAPI nuclear staining, comet assay, FACS analysis and measuring the expression patterns of several DNA damage/repair, cell cycle regulatory and apoptosis related protein biomarkers. Increased numbers of apoptotic nuclei after DAPI stain, average comet formation and comet length, percentage of sub G<sub>1</sub> population were observed after combined treatment (BCNU + Res) in HCT-116 cells. An increased ratio of BAX/BCL-XL, cleaved product of PARP, tumor suppressor PTEN, cell cycle regulatory protein p21, and CASPASE-3 were also noted in BCNU and Res combination. Thus, the data suggests that the BCNU and Res combination represents a potential treatment option for 5-FU resistant colon cancer.

We have developed a 5-FU resistant cell line from 5-FU sensitive HCT-116 cells by continuous treatment with 5-FU. These cells were resistant to 5-FU, but sensitive to other chemotherapeutic agents, such as Res. The cell death effects of the BCNU and Res combination in the resistant cell lines were measured. MTT cell survival, DAPI nuclear staining and comet assays showed that the



BCNU + Res combination was highly effective to sensitize the 5-FU-R cells in comparison to individual compounds. They caused cell death synergistically. The FACS analysis showed approximately 70% cell death when 20  $\mu\text{mol/L}$  BCNU combined with 8  $\mu\text{mol/L}$  Res (Figure 6C). By contrast, approximately 60% cell death was observed when 20  $\mu\text{mol/L}$  of BCNU combined with 30  $\mu\text{mol/L}$  Res in HCT-116 sensitive cells. Increased percentages (70% *vs* 60%) of apoptosis even at lower concentration of Res (8  $\mu\text{mol/L}$  *vs* 30  $\mu\text{mol/L}$ ) indicated that Res was more effective to sensitize the 5-FU-R than HCT-116 cells when combined with BCNU. The expression of PARP cleaved product and BAX/BCL-XL ratio increased with the combined treatment (BCNU + Res) in 5-FU-R cells compared with Res and BCNU alone (Figure 6D). Isobologram analysis also indicated that BCNU and Res also killed the 5-FU-R cells synergistically.

To study the mechanism of action of the BCNU and Res combination, we have first analyzed whether the DNA damage/repair pathway was involved. Comet assays showed that the average comet length and comet formation increased in both 5-FU sensitive and resistant cell lines after treatment with the BCNU + Res combination. Interestingly, a much lower concentration of Res (8  $\mu\text{mol/L}$ ) was needed to cause the same comet length in 5-FU-R cells compared with 5-FU sensitive cells (Res 30  $\mu\text{mol/L}$ ) (Figure 2B *vs* Figure 6B). This observation suggests that Res was more effective in causing DNA damage in 5-FU-R cells than in 5-FU sensitive cells when associated with the same amount of BCNU. This data prompted us to check the level of DNA damage/repair related proteins after treatment with BCNU + Res in 5-FU-R cells. Interestingly, the major DNA repair proteins, such as FEN1, POLH, DDB2 and POL- $\beta$  decreased in case of combination treatment while the level of APC increased in comparison to the individual drug as well as untreated cells (Figure 6E).

Multiple pieces of evidence indicate that the repair of apurinic/apyrimidinic (AP)-sites in DNA occurs through two sub-pathways of BER, which differ on the basis of repair gap size and the enzymes involved in these repair pathways<sup>[55]</sup>. These sub-pathways are designated as “single-nucleotide BER” or “short patch (SP)-BER” and “multinucleotide BER” or “long patch (LP)-BER”. In both pathways, repair is started by removal of a damaged base by a DNA glycosylase leaving an abasic (AP-site) DNA. The resulting AP sites are subsequently acted upon by an APE to generate a 3' hydroxyl group and a 5'-deoxyribosephosphate (dRP) terminus. In SP-BER, Pol- $\beta$  extends the 3' terminus by a single nucleotide and removes the dRP moiety with its dRP lyase activity. Finally, the nick is sealed by DNA ligases<sup>[56]</sup>. However, the oxidized or reduced AP sites become resistant to  $\beta$ -elimination and cannot be excised by the dRP lyase activity of Pol- $\beta$ . Then the modified AP-site is repaired *via* the LP-BER pathway, in which Pol- $\beta$ ,  $\delta$  or  $\epsilon$  incorporates 2-15 nucleotides, displacing the strand containing the modified AP-site. Then, the DNA flap structure is

cleaved by FEN1, and the nick is sealed by a DNA ligase<sup>[57,58]</sup>. APC expression is induced in cancer cell lines upon exposure to DNA-damaging agents<sup>[39,40,59,60]</sup>, suggesting an interaction between APC and the DNA repair machinery. We have already shown that APC physically interacts with Pol- $\beta$ , FEN1 and blocks BER by blocking strand-displacement synthesis and then DNA repair<sup>[39-42]</sup>. Increased levels of APC and decreased levels of Pol- $\beta$  and FEN1 after combined treatment with BCNU + Res revealed that this cocktail kills cancer cells by inhibiting the BER pathway. Thus, the above result indicates that BCNU + Res in combination caused cell death through reduction of BER repair pathway. The possible cause of this effect might be activation of APC by the combination but not the individual treatments.

A new and emerging concept is to sensitize cancer cells to DNA-damaging agents by inhibiting various proteins in DNA repair pathways. Molecular docking or NMR studies identified small molecular weight inhibitors (SMIs) that target the BER pathway by inhibiting APE1 and Pol- $\beta$  activities<sup>[61]</sup>. Although a number of Pol- $\beta$  inhibitors have been reported in recent years<sup>[61,62]</sup>, more potent and selective inhibitors are still needed. Since abasic DNA damage (which may also be caused by BCNU) can also be repaired by LP-BER, there is a need for agents that can block the LP-BER pathway as well, in which 5'-flap endonuclease 1 (FEN1) plays a major role<sup>[63]</sup>. FEN1 recognizes and removes the 5'-flap structure generated by Pol- $\beta$  during the strand-displacement synthesis. The removal of this flap is essential for the joining of the newly synthesized DNA strand with the parent strand by DNA ligase to complete the repair. Interestingly, the FEN1 level decreased after BCNU and Res combination in both cell lines, indicating that the BCNU + Res combination inhibited the removal of flap from the newly synthesized step in LP-BER. BCNU + Res might cause more DNA damage that could not be repaired by the cells because of a lack of sufficient FEN1. This would result in mutations accumulating within the cells, eventually leading to apoptosis instead of survival. However, more study will be needed to understand the molecular mechanism involving the BER pathway triggered by the BCNU + Res combination.

In conclusion, this study showed that a combination of BCNU and Res could be useful to overcome 5-FU resistance in colon cancer patients. BCNU and Res caused apoptosis in both the HCT-116 cells and 5-FU-R cells by inhibiting the base excision repair, especially *via* the FEN1 protein of the LP-BER pathway. These findings represent a novel concept for overcoming 5-FU resistance in colon cancer patients.

## COMMENTS

### Background

Colon cancer is one of the most common causes of cancer-related death worldwide. 5-fluorouracil (5-FU) is a widely used chemotherapeutic drug for the treatment of colon cancer, but resistance to this drug is a barrier to successful chemotherapy.

## Research frontiers

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is a synthetic drug commonly used to treat brain tumors as it can pass through the blood-brain barrier. BCNU is highly effective against cancer, but causes toxicity at very high concentrations. Resveratrol is a plant-derived compound used to treat various forms of cancer. Res has low toxicity and efficacy. Combining BCNU with resveratrol (Res) increases the efficacy of Res and reduces the toxicity of BCNU, making the combination an good tool to inhibit HCT-116 cells and overcome 5-FU resistance. Moreover, these drugs are DNA damage specific, resembling similar mechanism of action to that of 5-FU by reducing DNA repair components. Hence, these drugs were chosen for the combination therapy.

## Innovations and breakthroughs

This report highlighted the importance of combination therapy, which surpassed the monotherapy involving BCNU and Res to inhibit the growth of HCT-116 cells and overcome 5-FU resistance. This is the first study to report a combination of BCNU and Res for the treatment of colon cancer and to overcome 5-FU resistance effectively. The authors reported the mechanism of action of the combined drug, *i.e.*, inhibition of the DNA damage/repair pathway and arresting the cell cycle, thereby leading to apoptosis. The authors also reported that the drug to be more effective in the resistant cells than the normal cancer cells.

## Applications

This study highlights the action of BCNU combined with Res, which could prove to be a major tool for the treatment of 5-FU sensitive/resistant colon cancer patients and could also be tested for its ability to overcome resistance to other drugs, as this cocktail had specificity towards drug resistant cells.

## Terminology

BCNU is a common synthetic alkylating chemotherapeutic agent belonging to the family of haloethylnitrosoureas. Resveratrol is a polyphenolic phytoalexin widely available in plants, such as red grapes.

## Peer review

The research work in this manuscript is about the tumor suppression action of BCNU in combination with resveratrol on 5-fluorouracil HCT-116 cell lines, which were made resistant to 5-FU on constant exposure of 5-FU. The study is interesting and well executed. The point is clearly made.

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## Radiofrequency ablation of hepatocellular carcinoma sized $> 3$ and $\leq 5$ cm: Is ablative margin of more than 1 cm justified?

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### Abstract

**AIM:** To investigate whether an ablative margin (AM)  $> 1.0$  cm might reduce chance of recurrence for patients with hepatocellular carcinoma (HCC) tumors 3.1 to 5.0 cm in size, compared with an AM of 0.5-1.0 cm.

**METHODS:** From October 2005 to December 2012, 936 consecutive patients with HCC who received radiofrequency ablation were screened. Of these, 281 patients, each with a single primary HCC tumor of 3.1 to 5.0 cm in size on its greatest diameter, were included in the study. Based on the AM width, we categorized patients into the 0.5-1.0 cm group and the  $> 1.0$  cm

group. Local tumor progression (LTP)-free survival, intrahepatic distant recurrence (IDR)-free survival and overall survival (OS) rates were obtained using the Kaplan-Meier method.

**RESULTS:** The 1-, 2-, 3-, 4-, and 5-year LTP-free survival rates and IDR-free survival rates were significantly higher in the  $> 1.0$  cm group compared with the 0.5-1.0 cm group (97.5%, 86.3%, 73.6%, 49.5% and 26.4% vs 91.3%, 78.4%, 49.5%, 27.8%, and 12.8%; 95.1%, 90.3%, 77.0%, 61.0% and 48.3% vs 95.2%, 85.9%, 62.6%, 47.2% and 28.5%;  $P < 0.05$ ). The 1-, 2-, 3-, 4-, and 5-year OS rates were 98.6%, 91.5%, 69.2%, 56.0% and 42.2%, respectively, in the 0.5-1.0 cm group and 100%, 98.9%, 90.1%, 68.7% and 57.4%, respectively, in the  $> 1.0$  cm group ( $P = 0.010$ ). There were no significant differences in complication rates between the two groups. Both univariate and multivariate analyses identified AM as an independent prognostic factor linked to LTP, IDR, and OS.

**CONCLUSION:** For HCC tumors  $> 3.0$  cm and  $\leq 5.0$  cm, AM  $> 1.0$  cm could reduce chances of recurrence compared with AM of 0.5-1.0 cm, emphasizing the need for a more defensive strategy using AMs  $> 1.0$  cm for ablating HCC tumors of 3.1 to 5.0 cm.

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**Key words:** Hepatocellular carcinoma; Radiofrequency ablation; Ablative margin; Recurrence; Survival

**Core tip:** Recurrence is the most important factor for prognosis of hepatocellular carcinoma (HCC) after radiofrequency ablation. Although a sufficient ablative margin (AM) is an essential way to minimize recurrence risk, the optimal AM for HCC tumors 3.1 to 5.0 cm remains controversial. This study provides evidence

that, for HCC tumors 3.1 to 5.0 cm, AMs > 1.0 cm could reduce chance of recurrence compared to AMs of 0.5-1.0 cm, which emphasizes the need for more strategic AMs that are > 1.0 cm for ablation of HCC tumors of 3.1 to 5.0 cm.

Ke S, Ding XM, Qian XJ, Zhou YM, Cao BX, Gao K, Sun WB. Radiofrequency ablation of hepatocellular carcinoma sized > 3 and  $\leq$  5 cm: Is ablative margin of more than 1 cm justified? *World J Gastroenterol* 2013; 19(42): 7389-7398 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7389.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7389>

## INTRODUCTION

Radiofrequency (RF) ablation is accepted as a potentially curative treatment modality for hepatocellular carcinoma (HCC) at an early stage when transplantation and resection are precluded<sup>[1,2]</sup>. Local tumor progression (LTP) and intrahepatic distant recurrence (IDR) have been repeatedly found to be the most important prognostic factors, with the incidence rates of 2%-53% and 43%-53%, respectively<sup>[1,3-5]</sup>. LTP that occurs after complete RF ablation is widely considered to result from residual tumor cells in the peritumoral area, which contains microvascular invasion and satellite micronodules due to an insufficient ablative margin (AM), although there is a possibility of *de novo* occurrence at the site. Pathogenesis of IDR is thought to result from intrahepatic metastasis of the primary tumor, viable residual tumor or an HCC of multicentric origin<sup>[6,7]</sup>. Therefore, a sufficient AM that encompasses both the main tumor and the area of adjacent parenchyma containing microvascular invasion or satellite micronodules, should theoretically ensure pathologically complete ablation, and would thus be an essential way to minimize risk of LTP and IDR<sup>[8,9]</sup>.

The optimal safe AM for HCC is controversial<sup>[3,10-12]</sup>. Accumulating data have demonstrated that recurrence rates, such as LTP rates, differ greatly for tumors of various sizes but similar AMs<sup>[3,13]</sup>. For HCCs  $\leq$  3.0 cm, the 3-year LTP rates for patients treated by RF ablation with AMs of 0.5-1.0 cm are reportedly 10%-20%<sup>[3,14-17]</sup>. However, for HCC tumors 3.1 to 5.0 cm treated with AMs of 0.5-1.0 cm, the 3-year LTP rates were as high as 39%<sup>[18]</sup>. This can be well explained by the data from histopathological investigations on both the scope of peritumoral microvascular invasion and satellite micronodules and the incidence rate between HCCs  $\leq$  3.0 cm and those > 3.0 cm<sup>[19,20]</sup>. Only 14.5%-19% of single small HCC ( $\leq$  3.0 cm) reportedly have satellite micronodules, located within 1.0 cm from the main tumor<sup>[21]</sup>. In contrast, for HCC tumors 3.1 to 5.0 cm, 26.3%-36.9% had peritumoral satellite micronodules, located more than 1.0 cm from the main tumor in most cases<sup>[9]</sup>. These data indicate that for HCCs  $\leq$  3.0 cm, AMs of 0.5-1.0 cm are likely to remove most peritumoral lesions. However, for HCC tumors 3.1

to 5.0 cm, AMs of  $\leq$  1.0 cm seem insufficient to ensure pathological complete tumor clearance in most cases.

Although the idea that AMs > 1.0 cm would be more likely to completely delete tumor tissues seems intuitively logical, no study to date has been conducted to determine the optimal AM for HCC tumors 3.1 to 5.0 cm. Based on the experience of the surgical requirement of a tumor-free margin  $\geq$  1.0 cm wide<sup>[9]</sup>, we supposed that for HCC tumors 3.1 to 5.0 cm, AMs > 1.0 cm might reduce chance of recurrence compared with AMs of 0.5-1.0 cm. Therefore, the purpose of this study was to elucidate the survival benefit of AMs wider than 1.0 cm for HCC tumors 3.1 to 5.0 cm.

## MATERIALS AND METHODS

### Patients

To determine whether AMs > 1.0 cm in RF ablation for HCC tumors 3.1 to 5.0 cm might greatly reduce chance of recurrence compared with AMs of 0.5-1.0 cm, a prospective cohort study was performed. From October 2005 to December 2012, 936 consecutive patients with HCC received RF ablation at the Department of Hepatobiliary Surgery, Beijing Chao-yang Hospital Affiliated to Capital Medical University, China. Among them, 327 patients suffered from a single primary HCC tumor each, 3.1 to 5.0 cm in diameter. Written informed consent was obtained from all patients. The study was approved by the investigation and ethics committee of Beijing Chao-yang Hospital, Capital Medical University according to the standards of the Declaration of Helsinki.

Among the 327 HCC patients, 291 who met the inclusion criteria were enrolled in this study. The inclusion criteria were (1) a single primary HCC tumor 3.1 to 5.0 cm at its greatest diameter on preoperative investigations; (2) no other therapy prior to RF ablation except for trans-arterial chemoembolization (TACE); (3) getting imaging-complete ablation with AM  $\geq$  0.5 cm after RF ablation; (4) no other therapy in the period of follow-up except for RF ablation or TACE for LTP or IDR; and (5) complete follow-up data. The exclusion criteria were (1) extrahepatic metastasis before LTP or IDR; and (2) follow-up period less than 6 mo. A total of 10 patients were excluded from the study for developing extrahepatic metastasis before LTP or IDR ( $n = 7$ ), or follow-up period less than 6 mo ( $n = 3$ ). The remaining 281 patients were included in this study (Table 1). There were 195 men and 86 women. Their median age was 52 years (range, 24-88 years). Of these, 231 (82.2%) were positive for serum hepatitis B virus surface antigen, 18 (6.4%) positive for serum HCV antibody, and 2 (0.7%) positive for both. Liver cirrhosis was observed in 92 patients (32.4%). In 126 of the 281 patients, preoperative diagnosis of HCC was histologically confirmed by needle biopsy under CT guidance. In the remaining 155 patients, HCC was established on the basis of compatible radiological features in contrast-enhanced multiphase helical CT scan and dynamic contrast-enhanced MRI. The median diameter of

**Table 1** Comparison of patient clinical characteristics between the 0.5-1.0 cm group and the > 1.0 cm group *n* (%)

Variable	0.5-1.0 cm group ( <i>n</i> = 158)	> 1.0 cm group ( <i>n</i> = 123)	<i>P</i> value <sup>1</sup>
Age (yr)	52 (24-88)	51 (27-84)	0.467
Gender			
Male/female	109 (69.0)/49 (31.0)	86 (69.9)/37 (30.1)	0.873
Pre-existing hepatitis			
Hepatitis B	125 (79.1)	106 (86.2)	0.133
Hepatitis C	13 (8.2)	5 (4.1)	0.161
Hepatitis B and C	1 (0.6)	1 (0.8)	0.859
Child-Pugh grade			
Class A/class B	78 (49.4)/80 (50.6)	58 (47.2)/65 (52.8)	0.754
Liver cirrhosis			
Yes/No	55 (34.8)/103 (65.2)	37 (30.1)/86 (69.9)	0.417
Serum $\alpha$ -fetoprotein level (ng/mL)			
< 20	17 (10.8)	11 (8.9)	0.622
20-200	116 (73.4)	93 (75.6)	0.681
> 200	25 (15.8)	19 (15.4)	0.938
Tumor location			
S2,S3,S4,S6,S7/S5,S8	73 (46.2)/85 (53.8)	56 (45.5)/67 (54.5)	0.923
Biochemical analysis			
AST (IU/L)	46.5 (11.3-235.2)	46.1 (12.1-202.7)	0.793
ALT (IU/L)	48.4 (15.6-240.0)	47.9 (13.8-212.4)	0.560
Alb (g/dL)	3.7 (2.9-4.2)	3.6 (2.8-4.2)	0.460
T-Bil (mg/dL)	0.9 (0.7-1.4)	0.9 (0.6-1.4)	0.594
ALP (IU/L)	83.5 (7.7-376.6)	84.2 (8.1-284.8)	0.744
PT (%)	80.5 (58-100)	83.0 (59-100)	0.262
AFP (ng/mL)	83.2 (6.3-1301.9)	82.5 (5.4-750.7)	0.849
Tumor diameter(cm)	4.1 (3.1-5.0)	4.1 (3.3-5.0)	0.652
No. of ablation sessions before getting AM $\geq$ 0.5 cm			
1 session/2 sessions	115 (72.8)/43 (27.2)	99 (80.5)/24 (19.5)	0.143
Approaches of the first ablation session			0.978
Percutaneous	121 (76.6)	94 (76.4)	
Laparoscopic	37 (23.4)	29 (23.6)	
Open	0 (0)	0 (0)	

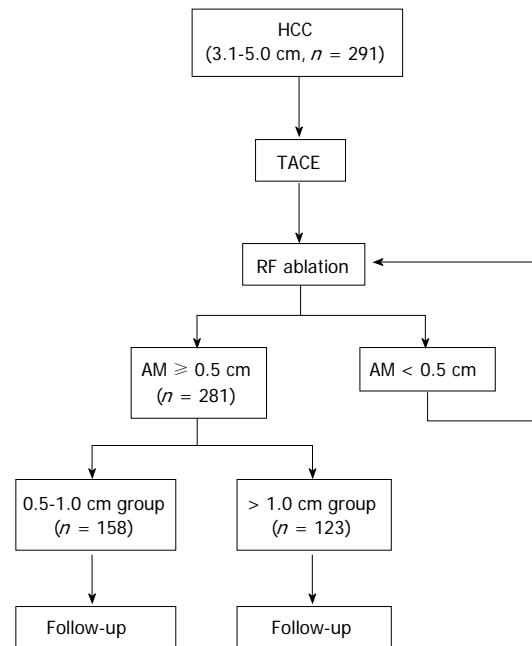
Values presented as absolute numbers (percent of cases) or median (range).

<sup>1</sup>Fisher's exact test or Mann-Whitney *U* test. AFP:  $\alpha$ -fetoprotein; Alb: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AM: Ablative margin; AST: Aspartate aminotransferase; PT: Prothrombin time; T-Bil: Total bilirubin.

the HCC nodules was 4.1 cm (range: 3.1-5.0 cm). Tumor location was described according to Couinaud segmental anatomic classification. Liver function was classified according to the Child-Pugh classification. In all, 136 (48.4%) and 145 (51.6%) showed Child-Pugh classes A and B, respectively. Randomization was not performed in this study. The aim of this study was explained to all of the approved patients in advance, and safe AMs > 1.0 cm were tried in all patients, although AMs of  $\geq$  0.5 cm are routinely considered adequate. On the basis of their AMs, we categorized patients into 2 groups: the 0.5-1.0 cm group and the > 1.0 cm group. Their demographic characteristics are shown in Table 1. The treatment algorithm of the present study is depicted in Figure 1.

### TACE

In the study, TACE was performed in all patients, both for radiological assessment of AMs, and for oncological purposes, 2-3 wk before RF ablation by two interven-



**Figure 1** Flow diagram of the enrollment and follow-up. A total of 281 patients met the inclusion criteria. HCC: Hepatocellular carcinoma; RF: Radiofrequency; TACE: transcatheter arterial chemoembolization; AM: Ablative margin.

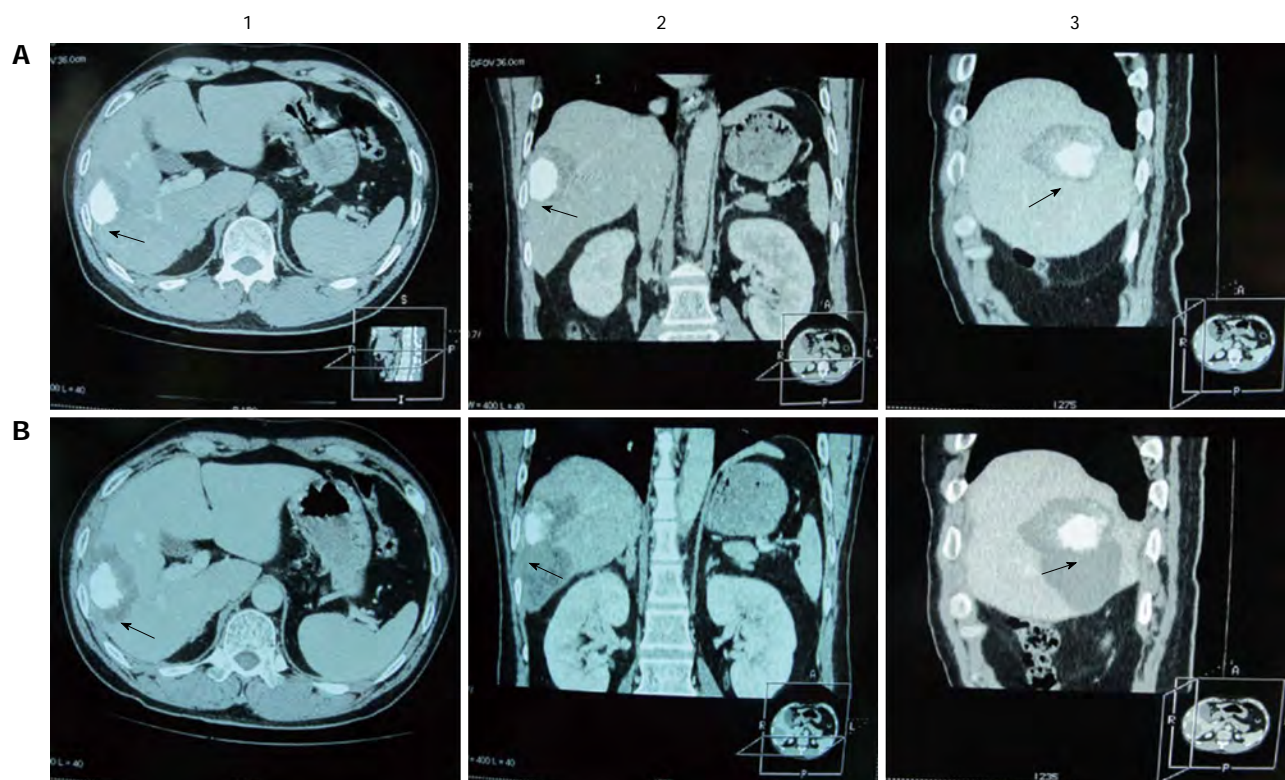
tional radiologists (YM Zhou and K Gao) with a standard regimen. TACE was performed through the femoral artery using the technique of Seldinger under local anesthesia<sup>[22]</sup> by injecting 6-10 mL of an emulsion of iodized oil (Lipiodol; Aulnay-Sous-Bois, France) and 20-40 mg of epirubicin hydrochloride (Zhejiang Haizheng, China) into the tumor feeding arteries. The selected doses of iodized oil and anticancer drug were individually based on the patient's liver function and tumor size. Injection was discontinued upon full accumulation of iodized oil into the tumor vessels. No gelatin sponge was used after TACE.

### RF ablation

A percutaneous approach was most commonly preferred in this study. Laparoscopic RF ablation was considered in the presence of the following: (1) tumors on the liver edge or surface, protruding out of the liver; (2) tumors located in the left lobe, under the bottom of the heart, for which percutaneous RF ablation might cause heart injury; or (3) tumors close to visceral organs, such as the gallbladder, small and large bowels, and stomach. If other intra-abdominal procedures were planned, then open RF ablation was used.

Percutaneous RF ablation was performed under CT guidance (GE Yokogawa Medical Systems Ltd, Tokyo, Japan). To ensure the safety and tolerance of patients, each patient had respiratory control with a tracheal tube or a laryngeal mask airway under intravenous anesthesia during RF ablation procedure. This relatively more invasive protocol also helped improve targeting accuracy by providing a transient stop of respiration at end expiratory status during the procedures of planning and





**Figure 2** Three-dimensional computed tomography for radiofrequency ablation imaging in a patient with hepatocellular carcinoma. A: The computed tomography (CT) images prior to the second radiofrequency (RF) ablation; B: The CT images after the second RF ablation. 1: Axial plane; 2: Coronal plane; 3: Sagittal plane. The ablative margin is  $< 0.5$  cm prior to the second RF ablation, and it has been ablated to  $\geq 0.5$  cm after the second RF ablation. Black arrow indicates the shortest width of the area of low density outside the iodine stained tumor.

targeting. Laparoscopic RF ablation and open RF ablation were performed with laparoscopic and open surgery techniques as usual with laparoscopic and intraoperative ultrasonographic assistance. All RF procedures in this study were performed using either a 15-gauge multitined electrode (Starburst XL; RITA Medical Systems, Manchester, GA, United States) or Cool-tip ACTC2025 or ACTC1525 electrodes, and an RF generator (RITA 1500; RITA Medical Systems Inc, Manchester, GA, United States or Covidien Healthcare, Ireland), according to their respective manufacturers' protocols. For patients treated with multitined expandable electrodes, when needle arrays are introduced into the tumor and positioned satisfactorily, the RF generator produces RF energy and maintains an average temperature of  $105^{\circ}\text{C}$ . A series of arrays, radiating from the central hollow probe, are pushed forward and unfolded gradually to 3, 4, or 5 cm until they reach the borders of the tumor. RF energy is delivered at 5-min intervals until the output power drops below 30 W in the final step of the procedure. For patients treated with Cool-tip electrodes, the RF generator (Covidien Healthcare, Ireland) was used. Unlike the RITA electrode, the Cool-tip electrode is straight, without arrays. With a 2.5 cm exposed tip, the Cool-tip electrodes can produce ablation zones of 4.5 cm with a single placement of electrodes and a maximum power of 200 W. Also, the Cool-tip RF generator continuously monitors tissue impedance throughout the procedure

and adjusts the output accordingly. For this application, the ablation protocol was preset to the automatic mode, and ablation usually was carried out for 15 to 20 min, which was a little bit longer than that suggested in the manufacturer's protocol, with the intent of attaining a satisfactory degree of tumor collapse. Cold saline continuous irrigation of the needle was provided with an external pump. In the clinical settings, we selected the type of electrode depending on the size, geometry and location of the index tumor. A multiple-overlapping ablation technique was adopted for pursuing AMs  $\geq 0.5$  cm. Electrode track ablation technique was also performed to minimize post-procedural bleeding and tumor seeding.

#### Post-treatment assessment

For post-treatment evaluation, contrast-enhanced CT scans were performed 1 mo after the procedure in all cases. The initial unenhanced CT acquisition was followed by enhanced acquisitions obtained at 30 and 70 s after the bolus administration of intravenous contrast material. The contrast agent used was 100 mL iopamidol (Iopromide Injection, Bayer Schering Pharma, Guangzhou, China). Any contrast-enhancing areas beyond the margin of the ablation zone on post-ablation CT indicated incomplete tumor ablation. Additional sessions were scheduled for ablation of residual tumors. The diagnosis and treatment procedures were repeated until imaging



**Table 2** Comparison of the recurrence pattern between the 0.5-1.0 cm group and > 1.0 cm group *n* (%)

Recurrence pattern	0.5-1.0 cm group ( <i>n</i> = 158)	> 1.0 cm group ( <i>n</i> = 123)	<i>P</i> value <sup>1</sup>
LTP only	74 (46.8)	39 (31.7)	0.01
IDR only	34 (21.5)	22 (17.9)	0.45
LTP + IDR	38 (24.1)	16 (13.0)	0.02
Total LTP	112 (70.9)	55 (44.7)	< 0.001
Total IDR	72 (45.6)	38 (30.9)	0.012

<sup>1</sup>Fisher's exact test. IDR: Intrahepatic distant recurrence; LTP: Local tumor progression.

complete ablation was achieved (Figure 1).

### Evaluation of ablative margin

Three-dimensional reconstructions of CT images were made before and after RF ablation (Figure 2). To define the AM as accurate as possible, we performed qualitative side-by-side comparison of CT scans obtained before and after RF ablation. Two radiologists (Cao BX and Qian XJ) who were blind to the results of quantitative analysis assessed in consensus whether an AM of 0.5-1.0 cm or > 1.0 cm was achieved in each case. For this analysis, the adjacent hepatic vessels or the hepatic capsule were used to facilitate comparison. The variation between the two radiologists' findings was < 5%. In the evaluation, the AM was defined as the narrowest width of the area of low density outside the iodine stain (Figure 2). When the iodine stain in the tumor was not uniform, measuring AM was more difficult. In such instances, when the radiologists did not concur (*n* = 11), we compared carefully the imaging data before and after RF ablation, outlined the contours of the tumor in this area and measured AM according to the tumor contour rather than the edge of the iodine stain.

### Follow-up

The follow-up protocol mainly included routine physical examination, laboratory tests, and measurement of AFP levels every month, as well as dynamic CT studies every 2 or 3 mo. Definitions are based on the standardization by the International Working Group on Image-Guided Tumor Ablation<sup>[23]</sup>. LTP was defined by the presence of a nodular lesion that was enhanced during the hepatic arterial phase and washed out by the delayed phase, and was found along the peripheral margin of the low-attenuated ablative zone. IDR was defined by a lesion with similar characteristics but not in contact with the original ablation zone in the liver. Overall survival (OS), defined as the interval between date of initial therapy and date of death or the last follow-up examination for living patients, was also evaluated.

In cases of LTP or IDR, other supplemental examinations like hepatic DSA, Lipiodol CT of the liver, CT of the chest and lower abdomen, and bone scintigraphy were performed for other potential tumor nodules.

When LTP or IDR was confirmed, patients were hospitalized as soon as possible. Basically, repeat RF ablation treatment cycles were administered for LTP and IDR of ≤ 4 nodules. Five or more IDR nodules, or nodules in unsuitable locations for RF ablation, were treated with TACE. TACE was performed through the femoral artery using the aforementioned technique.

### Complications

Major complications were assessed on the basis of the previously described guideline for image-guided tumor ablation<sup>[23]</sup>. Complication rates were evaluated for the total number of ablation sessions. We defined a major complication as an event that led to substantial morbidity and disability, increased the level of care required, resulted in hospital admission, or substantially lengthened the hospital stay. All other complications were considered minor.

### Statistical analysis

Continuous data are expressed as median and range. Comparisons were made with the Mann-Whitney *U* test. Categorical data were compared using the Fisher's exact test. Rates of LTP-free survival, IDR-free survival and OS rates were calculated by the Kaplan-Meier method and compared using the log-rank test. Risk factors for LTP, IDR and overall survival were evaluated by univariate analyses using Cox regression tests. If multiple risk factors were shown to be significant by this test, we performed multivariate analysis using Cox regression tests to identify independent prognostic factors for LTP, IDR and OS. All statistical analyses were performed using the SPSS 15.0 statistical software (SPSS Inc., Chicago, Illinois, United States). All reported *P* values were 2-sided. *P* < 0.05 was considered statistically significant.

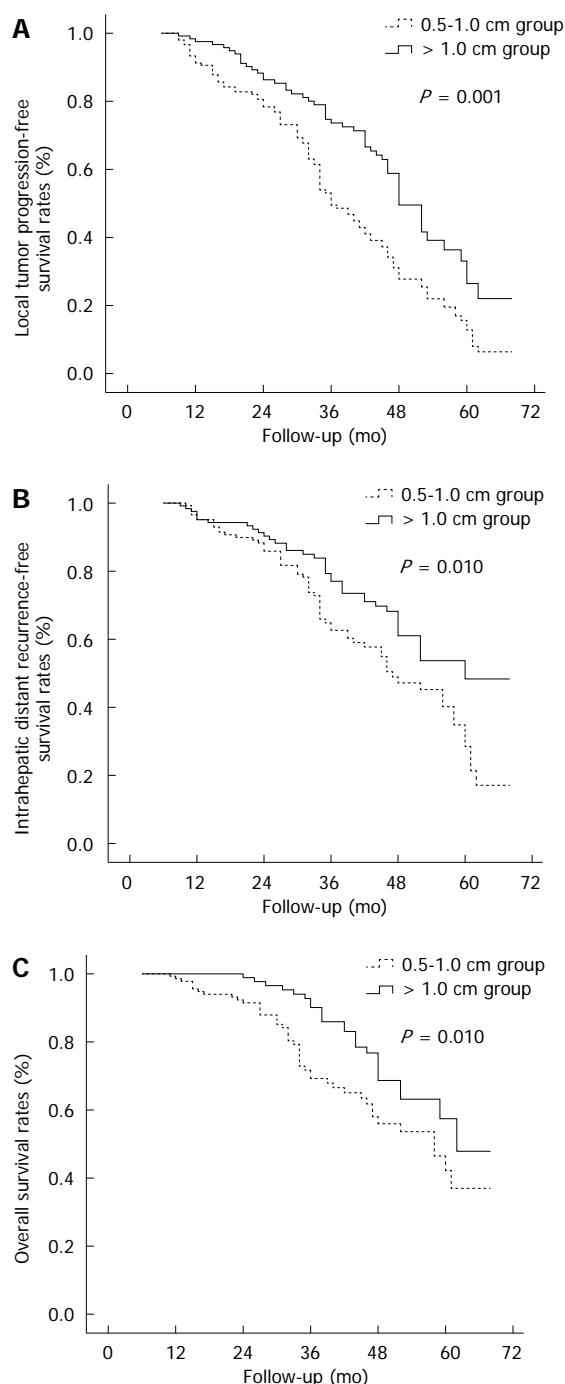
## RESULTS

### LTP-free survival

During the follow-up, LTP was found in 112 (70.9%) of 158 patients in the 0.5-1.0 cm group and in 55 (44.7%) of 123 patients in the > 1.0 cm group (*P* < 0.001) (Table 2). The rates of LTP-only and total LTP in the 0.5-1.0 cm group (46.8% and 70.9%, respectively) were significantly higher than those in the > 1.0 cm group (31.7% and 44.7%, respectively, *P* = 0.010 and < 0.001, respectively). The 1-, 2-, 3-, 4-, and 5-year LTP-free survival rates were 91.3%, 78.4%, 49.5%, 27.8% and 12.8%, respectively, in the 0.5-1.0 cm group and 97.5%, 86.3%, 73.6%, 49.5% and 26.4%, respectively, in the > 1.0 cm group (Figure 3A), and differed significantly between the two groups (*P* = 0.001).

### IDR-free survival

During the follow-up, IDR was found in 72 (45.6%) of 158 patients in the 0.5-1.0 cm group and 38 (30.9%) of 123 patients in the > 1.0 cm group (Table 2). There



**Figure 3** Kaplan-Meier analysis between the 0.5-1.0 cm group and the > 1.0 cm group. A: The local tumor progression-free survival rates; B: The intrahepatic distant recurrence-free survival rates; C: The overall survival rates.

was no significant difference in the rates of IDR-only between the 0.5-1.0 cm group and the > 1.0 cm group (21.5% *vs* 17.9%,  $P = 0.450$ ). However, the rates of total IDR in the 0.5-1.0 cm group was significantly higher than those in the > 1.0 cm group (45.6% *vs* 30.9%,  $P = 0.012$ ). The 1-, 2-, 3-, 4-, and 5-year IDR-free survival rates were 95.2%, 85.9%, 62.6%, 47.2% and 28.5%, respectively, in the 0.5-1.0 cm group and 95.1%, 90.3%, 77.0%, 61.0% and 48.3%, respectively, in the > 1.0 cm group (Figure 3B); the two groups differed statistically ( $P$

$= 0.010$ ).

### OS rates

As of December 2012 (with a median follow-up of 38.2 mo), 203 patients (72.2%) remained alive, and 78 (27.8%) had died, including 55 patients in the 0.5-1.0 cm group and 23 patients in the > 1.0 cm group. The cause of death was HCC in 58 patients (74.4%), liver failure in 7 (9.0%), upper gastrointestinal bleeding in 3 (3.8%), causes unrelated to liver disease in 4 (including 3 patients who died of cardiovascular disease and 1 of cerebral hemorrhage; 5.1%), and undetermined causes in 6 patients (who died in emergency situations at other hospitals without definite diagnoses related to death; 7.7%). The 1-, 2-, 3-, 4-, and 5-year OS rates were 98.6%, 91.5%, 69.2%, 56.0% and 42.2%, respectively, in the 0.5-1.0 cm group and 100%, 98.9%, 90.1%, 68.7% and 57.4%, respectively, in the > 1.0 cm group (Figure 3C); the two groups differed significantly ( $P = 0.010$ , log-rank test).

### Safety (complications)

No procedure-related death was observed. Major complications were observed in 5 (1.8%) of 281 patients. Of these, 2 patients in the 0.5-1.0 cm group and 1 patient in the > 1.0 cm group developed pneumothorax when laparoscopic RF ablation was performed. During the laparoscopic surgery, the presence of pneumothorax was confirmed immediately after the puncture of the RF probe. All of the pneumothorax was successfully treated by chest tube placement at the end of the operation. One patient in the 0.5-1.0 cm group suffered an intra-abdominal hemorrhage. He responded to transfusion of 2 units of packed red blood cells and required no other intervention. Two patients in the > 1.0 cm group developed hemopneumothorax and recovered from chest tightness and chest tube placement. Minor complications, including asymptomatic right pleural effusion, were noted within 3 d of the procedures in 13 patients of the 0.5-1.0 cm group and 15 patients of the > 1.0 cm group (0.5-1.0 cm group *vs* > 1.0 cm group,  $P = 0.271$ ); however, none of these patients required interventional-drainage procedures.

### Factors associated with LTP, IDR, and OS

Using univariate analysis, the tumor size (4.1-5.0 cm,  $P = 0.005$ ), AM (> 1.0 cm,  $P = 0.003$ ), AFP (> 200 ng/mL,  $P = 0.028$ ), and number of ablation sessions for imaging complete ablation (CA, 2 sessions,  $P = 0.031$ ) were found to be significant factors for predicting LTP, IDR, and OS (Table 3). In multivariate analyses of the 4 factors that were found to be significant in univariate analysis, the hazard ratios (HRs) for tumor size, AM, AFP, and number of ablation sessions for imaging CA are detailed in Table 4. Only the AM was found to be a significant independent factor linked to LTP, IDR, and OS.

**Table 3** Significant variables in the univariate analysis for local tumor progression, intrahepatic distant recurrence, and overall survival (*n* = 281)

Significant variable	<i>n</i>	<i>P</i> value <sup>1</sup>		
		LTP	IDR	OS
Age (> 65 yr), yes/no	123/158	0.376	0.282	0.103
Gender (male), yes/no	195/76	0.571	0.436	0.854
Liver cirrhosis, yes/no	92/189	0.469	0.645	0.912
Child-Pugh grade (Class B), yes/no	145/136	0.173	0.742	0.811
Tumor location (S5,S8), yes/no	152/129	0.537	0.488	0.635
Tumor size (4.1-5.0 cm), yes/no	154/127	0.007	0.011	0.005
AM (> 1.0 cm), yes/no	123/158	0.001	0.024	0.003
AST (> 40 IU/L), yes/no	166/115	0.658	0.586	0.879
ALT (> 40 IU/L), yes/no	157/124	0.672	0.460	0.734
ALP (> 110 IU/L), yes/no	120/161	0.385	0.473	0.581
Alb (> 3.5 g/dL), yes/no	202/79	0.622	0.564	0.838
T-Bil (> 1 mg/dL), yes/no	93/188	0.541	0.502	0.796
PT (> 70%), yes/no	216/65	0.336	0.573	0.636
AFP (> 200 ng/mL), yes/no	44/237	0.016	0.032	0.028
Post-RF ablation antiviral therapy, yes/no	86/195	0.275	0.547	0.201
Body mass index (> 25 kg/m <sup>2</sup> ), yes/no	83/198	0.611	0.582	0.913
No. of ablation sessions before getting AM ≥ 0.5 cm (2 sessions), yes/no	67/214	0.034	0.041	0.031
Approaches of the first ablation session (Laparoscopic), yes/no	66/215	0.459	0.383	0.728

<sup>1</sup>Cox regression analysis. AFP:  $\alpha$ -fetoprotein; Alb: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AM: Ablative margin; AST: Aspartate aminotransferase; IDR: Intrahepatic distant recurrence; LTP: Local tumor progression; OS: Overall survival; PT: Prothrombin time; T-Bil: Total bilirubin.

**Table 4** Significant variables in the multivariate analysis for local tumor progression, intrahepatic distant recurrence, and overall survival (*n* = 281)

Significant variable	LTP			IDR			OS		
	HR	95%CI	<i>P</i> value <sup>1</sup>	HR	95%CI	<i>P</i> value <sup>1</sup>	HR ratio	95%CI	<i>P</i> value <sup>1</sup>
Tumor size (4.1-5.0 cm), yes/no	1.032	0.521-1.376	0.475	0.891	0.452-1.602	0.744	0.882	0.673-1.572	0.084
AM (> 1.0 cm), yes/no	1.484	0.101-1.812	0.001	1.278	1.137-1.729	0.025	1.604	0.881-2.753	0.002
AFP (> 200 ng/mL), yes/no	0.947	0.540-1.050	0.531	0.509	0.370-1.215	0.546	1.007	0.639-1.158	0.748
No. of ablation sessions before getting AM ≥ 0.5 cm (2 sessions), yes/no	1.012	0.683-1.772	0.663	0.923	0.562-1.218	0.347	0.745	0.321-431	0.325

<sup>1</sup>Cox regression analysis. LTP: Local tumor progression; IDR: Intrahepatic distant recurrence; OS: Overall survival; AFP:  $\alpha$ -fetoprotein; AM: Ablative margin.

## DISCUSSION

Over the past years, the rapid development and refinement of RF ablation technology has led to increasing use of this treatment modality in HCC patients<sup>[24]</sup>. Extensive clinical studies support RF ablation as a preferred treatment for very early HCC<sup>[25]</sup>. However, tumor recurrence, including LTP and IDR, frequently occurs, affecting the prognosis. Furthermore, rapid tumor progression after RF ablation, which may mostly be associated with the progression of residual HCC, has been gaining increasing attention<sup>[26-29]</sup>. These experimental data indicated that any residual tumors might be a disaster for individual HCC patients who received RF ablation. Thus, we should try to remove microvascular invasions and satellite micronodules around the main tumor of HCC to decrease the likelihood of residual tumor, the incidence rates of LTP, IDR and the rapid tumor progression.

From perspective of pathological clearance of the tumor tissue, AMs should be as wide as possible. How-

ever, correctly obtaining a sufficient AM around all sides of a tumor of medium size is not easy. Specifically, to get a 1.0-cm AM for lesions sized 3.0, 4.0 and 5.0 cm in diameters, the volumes of peritumoral tissue to ablate are 3.6, 2.4 and 1.7 times, respectively, the volume of the main tumor<sup>[30]</sup>. A little increase of the dimension of the ablation zone means a tremendous increase of the amount of ablation tissue. For example, if we increase the AM from 1.0 to 1.5 cm for a lesion of 4.0 or 5.0 cm in diameter, respectively, the amounts of tissue to ablate will be theoretically increased by as much as 58.8% and 49.3% respectively, and this does not take into account the increased difficulty and time to accomplish it. So we should balance the perfect AM standard against its feasibility in clinical practice. As a compromise settlement, we take an AM of more than 1.0 cm as a minimum requirement for HCC nodules of 3.1 to 5.0 cm.

Although an AM > 1.0 cm cannot guarantee that all peritumoral tumor tissues are ablated in all cases, 2 ways can be used to compensate for the conservativeness

of this standard. First, there may be some decrease in the size of the ablation zone due to tissue healing and scar formation when measured 1 mo after ablation and the real AM might be underestimated in some extent. Second, in our performance of RF ablation treatment, we took an AM of more than 1 cm thick as the least requirement and tried to make it as wide as possible.

The precise evaluation of AM after RF ablation is rather difficult, especially for HCC lesions of medium size or larger which did not get TACE/trans-arterial embolization (TAE) pretreatment<sup>[3]</sup>. First, AM area is not clearly visualized on images because both the ablated tumor and AM appear as areas that lack contrast enhancement. Second, AMs around tumors are usually not symmetrical and the measurement of AM by subtracting the diameter of the index tumor from that of ablation zone is not as precise as expected. So in the present study, all enrolled patients received TACE pretreatment, aiming to facilitate the evaluation of AM as precise as possible.

We demonstrated that tumor size, AM, AFP, and number of ablation sessions before getting an AM > 1.0 cm were significant risk factors for LTP, IDR, and OS of HCC after RF ablation, using univariate analysis. Our results were in line with those of previous studies on risk factors related to recurrence of HCC after RF ablation<sup>[11,31]</sup>. However, only AM was found to be a significant independent factor linked to LTP, IDR, and OS of HCC after RF ablation using multivariate analysis. These findings confirmed that AMs > 1.0 cm are an important predictive factor for recurrence of HCC tumors 3.1 to 5.0 cm after RF ablation. It is easy to understand that an AM > 1.0 cm can reduce rates of LTP and IDR and eventually increase OS rate. An AM > 1.0 cm can lead to a further clearance of possible residues of microvascular invasion and satellite micronodules by ablating more viable tumor containing liver parenchyma and decrease probability of metastasis of the residual tumor cell by intrahepatic portal vein.

We are aware of the limitations of this analysis. This study was not randomized. Moreover, the AM standard of 1.0 cm referred only to the safety margin for liver resection advocated by most surgeons. Also, the biological nature of HCC as a commonly accepted important risk factor was not demonstrated in this study due to the limited number of cases with pathological examination. One further limitation is the fact that this was a single-center study; these results might not be reproducible consistently in other settings. The results may be influenced by the physicians' expertise and the institution's volume of care. Nevertheless, our data may be helpful for clinicians who treat HCC with RF ablation and may also be useful as a basis for the design of future trials. Again, more prospective, large randomized studies are needed to assess the benefit of AMs > 1.0 cm for medium-sized HCC lesions in patients who receive RF ablation.

Notwithstanding its preliminary character, this study

does provide evidence that, for HCC tumors 3.1 to 5.0 cm, AMs > 1.0 cm could reduce chance of recurrence compared with AMs of 0.5-1.0 cm, which emphasizes the need for more defensive strategies in using AMs wider than 1.0 cm for ablation of HCC tumors 3.1 to 5.0 cm. However, confirmation in a prospective multicenter randomized trial is required.

## COMMENTS

### Background

Radiofrequency (RF) ablation is becoming accepted as a promising technique for treatment of patients with hepatocellular carcinoma (HCC). Recurrence of HCC after RF ablation occurs frequently and is associated with poor prognosis. A sufficient ablative margin (AM) is an essential way to minimize the risk of recurrence. However, the optimal AM for HCC tumors 3.1 to 5.0 cm in size is unclear.

### Research frontiers

Theoretically, AMs that encompass both the main tumor and an area of adjacent parenchyma that contains microvascular invasion or satellite micronodules, ensuring a pathological complete ablation, will minimize risk of tumor recurrence. A current research hotspot is to affirm that, for HCC tumors 3.1 to 5.0 cm, AMs > 1.0 cm reduce recurrence risk compared with AMs of 0.5-1.0 cm.

### Innovations and breakthroughs

Accumulating data show that the recurrence rates after RF ablation, such as local tumor progression (LTP) or intrahepatic distant recurrence (IDR) rate, are quite different in varied-sized tumors with similar AMs. For HCC tumors ≤ 3.0 cm, an AM of 0.5-1.0 cm may remove most peritumoral lesions. However, for HCC tumors 3.1 to 5.0 cm, AMs ≤ 1.0 cm seem insufficient to ensure pathological complete tumor clearance in most cases. In this study, we confirmed that AMs > 1.0 cm are an important predictor for recurrence of HCC tumors 3.1 to 5.0 cm after RF ablation.

### Applications

The study results provide evidence that, for HCC tumors 3.1 to 5.0 cm, AMs > 1.0 cm could reduce chances of recurrence compared with AMs of 0.5-1.0 cm, emphasizing the need for a more defensive strategy that used AMs > 1.0 cm for ablation of HCC tumors 3.1 to 5.0 cm.

### Terminology

LTP was defined by the presence of a nodular lesion that was enhanced during the hepatic arterial phase and washed out by the delayed phase and was found along the peripheral margin of the low-attenuated ablative zone. IDR was defined by a lesion with similar characteristics that did not contact the original ablation zone in the liver. Overall survival was defined as the interval between date of initial therapy and date of death, or the last follow-up examination for living patients.

### Peer review

This is a good prospective cohort study in which the authors analyzed the survival benefit of radiofrequency AMs > 1.0 cm for HCC tumors 3.1 to 5.0 cm. The results are interesting and suggest that for HCC tumors 3.1 to 5.0 cm, AMs > 1.0 cm could reduce chance of recurrence compared with narrower AMs of 0.5-1.0 cm, emphasizing the need for a more defensive strategy, using wider AMs > 1.0 cm for ablation of HCC tumors 3.1 to 5.0 cm.

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## Interleukin 28B-related polymorphisms: A pathway for understanding hepatitis C virus infection?

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### Abstract

**AIM:** To analyze the role of *rs12979860* and *rs8099917* polymorphisms in hepatitis C virus (HCV) genotype 1 infection of Brazilians.

**METHODS:** A total of 145 adult patients diagnosed with genotype 1 chronic hepatitis C (CHC) who had completed a 48-wk regimen of pegylated-interferon  $\alpha$ -2a or -2b plus ribavirin combination therapy were recruited from six large urban healthcare centers and 199 healthy blood donors (controls) from a single site between January 2010 and January 2012. Data on the patients' response to treatment was collected. Polymerase chain reaction-restriction fragment length polymorphism genotyping of the interleukin (*IL*)*28B* gene fragment encompassing the single nucleotide polymorphisms (SNPs) *rs12979860* (C/T) and *rs8099917* (T/G) was carried out for 79 of the CHC patients and 199 of the controls. Bi-directional amplicon sequencing of the two SNPs was carried out for the remaining 66 CHC patients.

**RESULTS:** SNP *rs12979860* genotyping was successful in 99.5% of the controls and 97.2% of the CHC patients, whereas the SNP *rs8099917* genotyping was successful in 95.5% of the controls and 100% of the

CHC patients. The genotype and allele distributions for both rs12979860 and rs8099917 were significantly different between the control and CHC patient groups, with significantly higher genotype frequencies of CC and TT in the controls ( $P = 0.037$  and  $0.046$ , respectively) and of TT and GG in the CHC patients ( $P = 0.0009$  and  $0.0001$ , respectively). Analysis of the CHC patients who achieved sustained virological response (SVR) to treatment ( $n = 55$ ) indicated that the rs12979860 C allele and CC genotype were predictors of SVR ( $P = 0.02$ ). No significant correlation was found between rs8099917 genotypes and treatment response, but carriers of the T allele showed significantly higher rates of SVR ( $P = 0.02$ ). Linkage disequilibrium analysis of the group that achieved SVR showed a significant association between rs12979860 and rs8099917 ( $P = 0.07$ ).

**CONCLUSION:** The higher allele frequency of rs12979860 C and rs8099917 T observed in non-HCV-infected individuals may indicate a potential protective role for these *IL28B*-related polymorphisms.

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**Key words:** Hepatitis C; Interleukin 28B; Single nucleotide polymorphisms; Sustained virological response; Brazil

**Core tip:** This study investigated the differential distribution of interleukin 28B genetic variants between patients with chronic hepatitis C genotype 1 infection and non-infected healthy controls, and evaluated the association of these polymorphisms with patient response to standard antiviral therapeutic regimens. Genotype and allele frequencies of rs12979860 and rs8099917 were significantly different between the patients and controls, and the patterns suggested a potential protective role against hepatitis C virus infection. Finally, the rs12979860 CC genotype showed correlation to achievement of sustained virological response following pegylated-interferon/ribavirin-based therapy.

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## INTRODUCTION

Hepatitis C virus (HCV) infection results in a broad spectrum of clinical outcomes and symptoms, ranging from spontaneous viral elimination to chronic infection and from jaundice to end-stage liver failure. Despite the

introduction of highly efficacious antiviral drugs based on viral protease inhibition, most countries continue to rely on the established combination therapy based on an immune-modulator plus an antiviral [most frequently pegylated-interferon  $\alpha$ -2a or -2b (PegIFN- $\alpha$ -2a/2b) and ribavirin (RBV)] even though this strategy is associated with high failure rates<sup>[1]</sup>. Studies to uncover the mechanisms underlying non-/low- or unsustained response to treatment have identified a number of contributing factors, related to both the virus itself (*i.e.*, genotype and viral load) and the host (*i.e.*, age, sex, ethnicity, concomitant liver fibrosis, and metabolic abnormalities<sup>[2,3]</sup>); thus, it is theorized that interaction between the virus and host genetics may be an important determinant of the natural history of HCV infection and may be predictive of therapy outcome.

A recent genome-wide association study (GWAS) of > 600000 polymorphisms in a cohort of HCV-infected patients found a single nucleotide polymorphism (SNP) on chromosome 19 associated with therapy response<sup>[4]</sup>. Subsequent GWAS studies implicated the rs8099917 (TG) polymorphism in the genetic region of this chromosome related to the interleukin (*IL*)28B gene and showed direct associations of the SNP to spontaneous clearance of HCV and treatment outcome<sup>[5,6]</sup>. Subsequent related studies have identified an additional *IL28B*-related SNP, rs12979860, and confirmed these two polymorphisms as strong predictors of therapy response and contributors to the epidemiological and ethnical distribution of HCV infection<sup>[7-9]</sup>. In addition, comparative analysis has identified significant differential prevalence profiles of these SNPs for HCV-infected patients and healthy controls<sup>[4]</sup>, suggesting potential roles for each in disease susceptibility and/or resistance.

The prevalence rate of HCV remains high in Brazil, particularly when compared to the other Latin American countries<sup>[10]</sup>, yet little data is available on the genetic variation profile of this disease in Brazilians. This study was designed to analyze the differential genotypic and allelic distributions of the *IL28B*-related SNPs, rs12979860 and rs8099917, in a cohort of Brazilian patients infected with HCV genotype 1 and healthy controls, as well as to investigate the potential association of these polymorphisms with treatment outcome.

## MATERIALS AND METHODS

### Patients

Between January 2010 and January 2012, adult patients who had undergone combination therapy for chronic infection with HCV genotype 1 [48-wk combination of PegIFN- $\alpha$ -2a/2b and RBV (15 mg/kg)] were recruited from the following six large urban healthcare centers: Hospital Municipal São José, Joinville/SC ( $n = 25$ ); Hospital Universitário Gaffrée Guinle ( $n = 26$ ) and Hospital Universitário Clementino Fraga Filho ( $n = 66$ ), Rio de Janeiro/RJ; Hospital da Universidade Federal de Pelotas ( $n = 12$ ), Pelotas/RS; Santa Casa de Misericórdia ( $n = 2$ ),



**Table 1** Demographic characteristics of the study participants

Characteristic	HCV ( <i>n</i> = 145)	Control ( <i>n</i> = 198)
Age (yr), mean $\pm$ SD	55.5 $\pm$ 10.0	32.2 $\pm$ 10.0
Sex, M/F	52%/48%	66%/34%
Skin color		
White	75.70%	88.00%
Black	19.20%	5.00%
Brown	5.10%	7.00%

HCV: Hepatitis C virus.

Porto Alegre/RS; and Hospital da Universidade Federal do Maranhão (*n* = 14), São Luis/MA. Patients with co-infection of hepatitis B virus or human immunodeficiency virus, or with any other concomitant chronic liver disease, were denied study enrollment. Blood sample and data on treatment outcome was collected for each enrolled patient. The treatment outcome of sustained virological response (SVR) was defined by a negative result for detection of serum HCV RNA at month six post-treatment. Patients who did not achieve SVR (including those who relapsed) were collectively categorized as non-responders (NR).

### Healthy controls

Healthy individuals presenting for blood donation at HE-MOSC Joinville/SC in 2010 were recruited for the study. Study enrollment was offered to individuals with no clinical or laboratory evidence of liver disease or other major pathological conditions. All enrolled controls were > 18 years old and lacked familial relationship.

### Racial classification

To phenotypically classify each study participant by race, we employed the criteria published by the Brazilian Institute of Geography and Statistics<sup>[11]</sup>; the skin color categories (white, brown, black, yellow, and Indian) were self-reported by each participant.

### Ethics statement

The study was approved by each of the participating healthcare center's Institutional Committees, which required adherence to the Brazilian normative for ethics in research. Informed consent was obtained from each participant before blood sampling.

### Genotyping methods

The HCV patients' blood samples were collected by finger puncture and stored as blots on FTA Elute Micro Cards<sup>®</sup> (Whatman, Kent, United Kingdom) for subsequent genomic DNA extraction according to the manufacturer's protocol. The controls' blood samples were collected by venipuncture and stored in EDTA for subsequent genomic DNA extraction *via* the QIAamp DNA Miniprep Kit (Qiagen, Chatsworth, CA, United States).

The genotyping of SNPs rs12979860 and rs8099917 was performed at the Universidade da Região de Joinville and the Instituto de Biofísica Carlos Chagas Filho.

For all 199 control samples and 79 of the HCV-infected samples, genotyping was performed *via* PCR-restriction fragment length polymorphism analysis<sup>[12]</sup> on an LGC XP thermocycler (BIOER Technology Co., Tokyo, Japan) and results were documented by the Mini-Bis Pro Gel Imaging System (DNR Bio-Image Systems Ltd., Jerusalem, Israel). The remaining 66 HCV-infected samples were genotyped by bi-directional amplicon sequencing using the DYEnamic<sup>™</sup> ET Dye Terminator Cycle Sequencing Kit and the MegaBACE 1000 DNA Sequencer (GE Healthcare, Pittsburgh, PA, United States). The HCV treatment responses and *IL28B* genotyping results of this subgroup of patients were previously reported<sup>[13]</sup>.

### Statistical analysis

Data analysis was performed by the SPSS statistical software (v13.0; SPSS Inc., Chicago, IL, United States). Continuous variables were compared by Student's *t*-test for parametric variables, or by Mann-Whitney *U* test for non-parametric distributions. All *P*-values are two-tailed and values < 0.05 were considered statistically significant. The  $\chi^2$  G test for "Goodness of Fit" was used to verify whether the proportions of the polymorphisms were unequally distributed between controls and patients or in Hardy-Weinberg equilibrium (HWE). Differences in allele and genotype frequencies between different groups were assessed by Pearson's  $\chi^2$  test (with  $\chi^2$  test for linear trend when appropriate).

## RESULTS

The demographic characteristics of the HCV-infected patients and healthy controls are summarized in Table 1. SNP rs12979860 genotyping was successful in 142 (97.2%) of the patients and 198 (99.5%) of the controls, and SNP rs8099917 genotyping was successful in all 145 (100%) of the patients and 190 (95.5%) of the controls.

### Genotype and allele distributions among the HCV-infected patients and healthy controls

Analysis of rs12979860 showed that the HCV-infected group had a significantly lower prevalence of the CC genotype (30.3% *vs* control group: 47.4%, *P* = 0.037) and the C allele (0.53 *vs* 0.69, *P* = 0.0073) and T allele (0.47 *vs* 0.31, *P* = 0.0073), but a significantly higher prevalence of the TT genotype (24.8% *vs* 8.9%, *P* = 0.0009). Analysis of rs8099917 showed that the HCV-infected group had a significantly lower prevalence of the TT genotype (46.2% *vs* control group: 67.4%, *P* = 0.043) and of the T allele (0.63 *vs* 0.83, *P* = 0.0009), but a significantly higher prevalence of the GG genotype (20.0% *vs* 1.5%, *P* = 0.0001) and of the G allele (0.37 *vs* 0.17, *P* = 0.0008).

Both of the *IL28B* SNPs were in HWE among individuals belonging to the control group (rs12979860, *P* = 0.7; rs8099917, *P* = 0.1), but only the rs12979860 SNP was in HWE for the HCV group (*P* = 0.21). Linkage disequilibrium analysis showed that both SNPs were associated with the controls as well as the HCV-infected patients (*P* = 0.0001).

**Table 2** Demographic characteristics and clinical parameters of hepatitis c virus-infected patients stratified by response to treatment

Feature	SVR (n = 55)	NR (n = 89)	P value
Sex, M/F	54%/46%	51%/49%	0.072
Skin color			
White	89%	70%	0.032
Black	11%	26%	0.009
Brown	-	4%	ND
ALT in UI/mL, mean (IQR)	97 (54)	77 (64)	0.1
Baseline viral load in UI/mL <sup>1</sup>			
< 600000	47.80%	49.30%	0.760
≥ 600000	52.20%	50.70%	0.890
Fibrosis score <sup>2</sup>			
0-2	63.30%	49.40%	0.125
3-4	36.70%	50.60%	0.126

<sup>1</sup>This parameter was only available for 115 patients [46 of sustained virological response (SVR), and 69 of non-responders (NR)]; <sup>2</sup>According to histopathological analysis of liver biopsy tissues; this parameter was only available for 128 patients (49 of SVR, and 79 of NR). ND: Not done due to small numbers of cases; ALT: Alanine aminotransferase.

### Correlation between the *IL28B* polymorphisms and treatment outcomes

Fifty-five (39%) of the patients achieved SVR following the combination therapy. Stratification analysis of the SVR patients by race showed that the rate was significantly lower in the self-declared black individuals ( $P = 0.009$ ). None of the clinical parameters were significantly different between the SVR and NR groups (Table 2). However, the SVR group did have significantly higher prevalences of the rs12979860 CC genotype (45.5% *vs* NR: 21.0%,  $P = 0.02$ ) and C allele (0.64 *vs* 0.46,  $P = 0.019$ ). Conversely, the prevalences of the rs12979860 TT genotype and T allele were significantly higher in the NR group (30.0% *vs* SVR: 18.2%,  $P = 0.03$  and 0.54 *vs* 0.36,  $P = 0.019$ ).

There were no significant differences in the distributions of the SNP rs8099917 genotypes between the SVR and NR groups. However, the SVR group showed a significantly higher frequency of the T allele (0.74 *vs* NR: 0.57,  $P = 0.024$ ) but a significantly lower frequency of the G allele (0.26 *vs* 0.43,  $P = 0.024$ ). Linkage disequilibrium analysis of the SVR group only showed a significant association between rs12979860 and rs8099917 ( $P = 0.07$ ), similar analysis of the NR group only found no significant associations.

## DISCUSSION

Polymorphisms in the genomic sequence related to the *IL28B* gene have been implicated in the natural history of HCV infection and suggested as putative biomarkers for predicting an individual's therapy response<sup>[14-17]</sup>. In particular, the CC genotype of rs12979860 (*vs* CT) was associated with a two-fold difference in response to HCV treatment, both in Caucasians and African-Americans. Thomas *et al.*<sup>[18]</sup> reported the allele frequencies of rs12979860 in 2371 individuals from 51 populations

worldwide, and showed that the C allele occurred at a high frequency in East Asia, an intermediate frequency in Europe and North America, and a relatively low frequency in Africa. In a subsequent study, Fabris *et al.*<sup>[19]</sup> genotyped the rs12979860 polymorphism in 412 patients with end-stage liver disease due to hepatitis B or C, alcohol abuse, or other causes, and in 344 healthy controls. The patients with viral cirrhosis were found to have a significantly higher frequency of the T allele and of the TT/CT genotypes than the controls; ultimately, the authors theorized that the TT genotype may be associated with severe liver disease while the CC genotype may exert a protective effect. In an Australian-European cohort study of the rs12979860 polymorphism related to SVR, the TT, TG and GG genotypes were found to be associated<sup>[7]</sup>. Finally, a Swiss study yielded similar results, with a lower G allele frequency being associated to persistent infection and failure to respond to therapy<sup>[6]</sup>.

Among the few studies that have assessed the role of *IL28B* polymorphisms in a Brazilian population, the SNPs have shown various associations to disease outcome and treatment response. For example, Cavalcante *et al.*<sup>[20]</sup> analyzed rs12979860 in 222 patients infected with admixture HCV genotype 1, 2 and 3 who were treated with standard therapy and found that genotypes CC (rs12979860) and TT (rs8099917) were strongly associated with SVR, while CT/TT (rs12979860) and TG/GG (rs8099917) were associated to treatment failure. In addition, Lunge *et al.*<sup>[21]</sup> analyzed the association between CT (rs12979860) and spontaneous clearance of HCV infection in HIV-co-infected individuals and showed that the CT/TT genotypes conferred nearly 3-fold higher rates for developing CHC, compared to the CC genotype. The current study of *IL28B* polymorphisms' relationship to therapy response, presented herein, confirmed the predictive value of the C allele and CC genotype (rs12979860) that was previously suggested by Cavalcante *et al.*<sup>[20]</sup>. Even though the current study's cohort did not show a statistically significant association of rs8099917 to therapy response, the T allele distribution was related to treatment outcome. This finding may be explained by the lack of HWE in the sample.

Another intriguing finding from the current study is the significant differences in allele and genotype distributions between the healthy controls and HCV-infected patients, which was observed for both SNPs (rs12979860 and rs8099917), with higher C and T alleles as well as CC and TT genotypes in the healthy controls. These data are in accordance with a previous study that demonstrated significant associations for reduced frequencies of the C allele and the CC genotype with chronically-infected hepatitis patients compared to ethnically-matched healthy controls<sup>[4]</sup>. Together, these findings suggest a strong association between the C allele and higher rates of natural clearance of HCV and support the predicted role of this SNP as a protective factor against HCV infection or persistence.

The full array of biological pathways that are influ-

enced by the *IL28B* gene product and its polymorphisms has yet to be elucidated. Certainly, a gene variant has the ability to affect rates of protein production and it is interesting to consider that Shi *et al.*<sup>[22]</sup> demonstrated that patients with chronic HCV infection have significantly lower mRNA and serum levels of IL28B than either healthy controls or patients who have spontaneously cleared the virus, whereas individuals carrying the rs12979860 CC genotype showed a tendency towards higher levels compared to those carrying the CT or TT genotypes. The SNPs rs12979860 and rs8099917 are located respectively at 3 and 8 kb upstream from the *IL28B* locus, in a region that encodes IFN- $\lambda$ 3, a type 3 interferon with antiviral activity that is mediated through the JAK-STAT pathway upon induction of IFN-stimulated genes. IL28A (IFN- $\lambda$ 2) and IL29 (IFN- $\lambda$ 1) are genomically located adjacent to the *IL28B* gene, and it is possible that the rs12979860 and rs8099917 SNPs may also affect the function of these genes. Indeed, Langhans *et al.*<sup>[23]</sup> showed that IL29 levels were substantially lower in CHC patients and in patients that spontaneously resolved hepatitis, compared to healthy controls; moreover, the levels of *IL29* and *IL28A/B* were shown to be significantly higher in rs12979860 C allele heterozygous patients than in the TT homozygous patients.

The results from the current study of an HCV-infected Brazilian population indicate that the role of *IL28B* SNPs in patients' response to therapy is similar to that of other international populations. Furthermore, the data presented herein on the differential distribution of rs12979860 and rs8099917 polymorphisms between HCV-infected patients and a healthy population may contribute to a better understanding of the natural history of this complex disease.

## COMMENTS

### Background

Most countries continue to rely on drug therapies based on an immune-modulator plus an antiviral (most frequently pegylated-interferon  $\alpha$ -2a or -2b and ribavirin), even though this strategy is associated with high failure rates and significant side effects. Therefore, identification of predictive biomarkers of an individual's response to therapy is clinically relevant.

### Research frontiers

Since 2009, several genome-wide association studies have yielded data to support the association of two single nucleotide polymorphisms, rs12979860 and rs8099917, located near the interleukin (*IL*)28B gene with achievement of sustained virological response to treatment in patients infected with hepatitis C virus (HCV) genotype 1.

### Innovations and breakthroughs

This study represents the largest analysis of a Brazilian cohort conducted to date to investigate the potential association of the two most relevant *IL28B* single nucleotide polymorphisms (rs12979860 and rs8099917) in healthy individuals and chronic hepatitis C patients (CHC) treated with the pegylated-interferon  $\alpha$ -2a or -2b plus ribavirin combination therapy. The study's findings reveal significant differential distributions of the genotype and allele frequencies for both rs12979860 and rs8099917 between the healthy controls and CHC patients. In addition, the CC genotype of rs12979860 was shown to be associated with the CHC patients' ability to achieve sustained virological response to the standard therapeutic regimens.

### Applications

This study's findings support the predicted roles of rs12979860 C allele and

rs8099917 T allele as protective factors against HCV infection or persistence and suggest their potential for development as clinical biomarkers of HCV infection and therapeutic management. In general, the results indicate that genotyping *IL28B* polymorphisms in HCV-infected patients is likely to help improve the current standard of care, and may provide an opportunity for clinicians to improve patient outcome by individualizing treatment regimens.

### Peer review

The authors investigated the differential distribution of *IL28B* genetic variants between healthy Brazilians in the general population and HCV-infected patients undergoing standard drug therapy based on an immune-modulator plus an antiviral to demonstrate the associations of particular genotypes and alleles with infection and treatment response. This is a well-written and -discussed paper, of particular interest to the Brazil population.

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## Short and long-term outcomes of laparoscopic colectomy in obese patients

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### Abstract

**AIM:** To investigate the impact of laparoscopic colectomy on short and long-term outcomes in obese patients with colorectal diseases.

**METHODS:** A total of 98 obese (body mass index > 30 kg/m<sup>2</sup>) patients who underwent laparoscopic (LPS) right or left colectomy over a 10 year period were identified from a prospective institutionally approved database and manually matched to obese patients who underwent open colectomy. Controls were selected to match for body mass index, site of primary disease, American Society of Anesthesiologists score, and year of surgery ( $\pm$  3 year). The parameters analyzed included age, gender, comorbid conditions, American Society of Anaesthesiologists class, diagnosis, procedure, and duration of operation, operative blood loss, and amount of homologous blood transfused. Conversion rate, intra and postoperative complications as were as reoperation rate, 30 d and long-term morbidity rate were also analyzed. For continuous variables, the Student's *t* test was used for normally distributed data the Mann-Whitney *U* test for non-normally distributed data. The Pearson's  $\chi^2$  tests, or the

Fisher exact test as appropriate, were used for proportions.

**RESULTS:** Conversion to open surgery was necessary in 13 of 98 patients (13.3%). In the LPS group, operative time was 29 min longer and blood loss was 78 mL lower when compared to open colectomy ( $P = 0.03$ ,  $P = 0.0001$ , respectively). Overall morbidity, anastomotic leak and readmission rate did not significantly differ between the two groups. A trend toward a reduction of wound complications was observed in the LPS when compared to open group ( $P = 0.09$ ). In the LPS group, an earlier recovery of bowel function ( $P = 0.001$ ) and a shorter length of stay ( $P = 0.03$ ) were observed. After a median follow-up of 62 (range 12-132) mo 23 patients in the LPS group and 38 in the open group experienced long-term complications (LPS vs open,  $P = 0.03$ ). Incisional hernia resulted to be the most frequent long-term complication with a significantly higher occurrence in the open group when compared to the laparoscopic one ( $P = 0.03$ ).

**CONCLUSION:** Laparoscopic colectomy in obese patients is safe, does not jeopardize postoperative complications and resulted in lower incidence of long-term complications when compared with open cases.

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**Key words:** Obesity; Colon cancer; Laparoscopy; Right colectomy; Left colectomy; Colorectal disease

**Core tip:** The best of our knowledge, this is the first case-matched control study reporting long-term complications in obese patients' undergone laparoscopic or open colectomy.

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## INTRODUCTION

The role of laparoscopic surgery in obese patient with colorectal diseases is still object of debate, according to several papers published in the last decade, laparoscopy is feasible and safe<sup>[1-5]</sup>. However, controversial results have been reported in term of conversion rate, overall morbidity and reoperation rate, when obese patients are compared to their non-obese counterparts<sup>[1-8]</sup>.

These controversial results could be partially explained based on the relative inexperience of the surgical team possibly due to an on-going learning curve, on the small sample size of the various studies, on the specific definition of obesity and on surgical heterogeneity<sup>[3-5,9]</sup>. In order to clarify this issue while avoiding some of the aforementioned biases, we have conducted a case-matched study comparing short and long-term outcomes of standardized right or left colectomy performed by laparoscopy or by open surgery in patients with a body mass index (BMI) > 30 kg/m<sup>2</sup>. Data were extracted from a prospective collected database.

Main goal of the present study is to assess if the documented benefits of minimally invasive approach could be translated even in a high-risk patients such obese patients.

## MATERIALS AND METHODS

Adult obese patients of both sexes candidate to a standardized right or left colectomy and who were consecutively admitted to our department from January 2002 to January 2012 were considered. Patients were identified from a prospective institutionally approved database currently recording demographics, biochemistry values, nutritional status, operative variables, and co-morbidity factors on admission, postoperative outcome and histopathologic findings of patients undergoing colon resection. The case group was selected from 118 obese patients who underwent laparoscopic colectomy identified from the Institutional Colon database. Patients were excluded if they underwent an emergency procedure ( $n = 13$ ) or non-standardized colon operations ( $n = 7$ ). Overall, the case group consisted of 98 patients. The control patients were selected from the same institutional database from January 2011, backward until we had identified one control subject for each case. Each case was manually matched with a control that had undergone open resection. The same exclusion criteria used for laparoscopic (LPS) patients were applied to the control patients' selection. The identification of control patients was done by a statistician (GR) who was unaware of postoperative outcomes. Control patients were selected to match for site of primary disease, American Society of Anesthesi-

ologists (ASA) score, and year of surgery ( $\pm 3$  year). Data collected from the hospital database, medical records and telephone calls were analyzed retrospectively.

Preoperative assessment included clinical examination, serologic evaluation, total colonoscopy and body CT scan. A virtual CT colonoscopy was additionally performed in case of incomplete colonoscopy examination. Co-morbidities on admission, as well as operative risk assessed by ASA score, were analyzed in both groups. The following details of the surgical procedure were recorded in all patients: duration of operation (min), operative blood loss (mL), and amount of homologous blood transfused (mL). Three surgeons (Staudacher C, Di Palo S, Vignali A) with extensive experience in open and laparoscopic colorectal surgery performed all the interventions<sup>[10,11]</sup>. For all the surgeons, the learning curve for laparoscopic colorectal surgery was completed before starting the present trial<sup>[12]</sup>. Patients underwent laparoscopic or open resection depending on surgeon's or patient's preference.

A mechanical anastomosis was intracorporeally fashioned in case of left colectomy, while in right colectomy, the specimen was divided extra corporeally (in laparoscopic operations) and an isoperistaltic side-to-side anastomosis was manually fashioned. Conversion to open surgery was defined when an abdominal incision longer than 7 cm was performed or when an abdominal incision was made earlier or differently from what planned at the beginning of the intervention.

All patients were treated according to the same intra and postoperative protocol: epidural analgesia maintenance for 3 d, nasogastric tube removal at the end of surgery, and bladder catheter removal on postoperative 2 d. Clear liquid diet was started on postoperative 1 d as tolerated by the patient. Postoperative infusion of fluids and electrolytes was given to all patients according to clinical requirements.

Tumor classification was done according to the 7<sup>th</sup> TNM edition<sup>[13]</sup>. Microbiological analysis and positive cultures proved all infectious complications. Patients were discharged after meeting the following conditions: bowel movement and full recovery of both ambulation ability and oral feeding. Registration of complications and need for an unexpected re-admission were recorded for the first 30 d following operation. The follow-up protocol consisted in outpatient clinic visits at 3 mo intervals for the first 2 years, then at 6 mo intervals for the next 3 years, then once a year. In patients with more than five years follow-up or with a benign disease, a systematic review of chart, office records as well as patient interview were done.

### Statistical analysis

Descriptive data were reported as mean, median, standard deviation (SD), number of patients and percentage. For continuous variables, differences between groups were tested with the Student's *t* test for normally distributed data (based on Kolmogorov-Smirnov test) or the Mann-

Whitney *U* test for non-normally distributed data. The Pearson's  $\chi^2$  test, or the Fisher exact test as appropriate, was used for proportions. A two-sided significance level less than 0.05 were used to indicate statistical significance. Confidence intervals (95%CI) were reported when appropriate. The SPSS™ software package, version 18. For Windows (SPSS Inc, Chicago, IL) was used for all the statistical analyses.

## RESULTS

### Study population

Ninety-eight obese patients with colorectal diseases, identified from a prospectively maintained database, who underwent laparoscopic right or left colectomy over a 10 year period, were matched with 98 obese patients who underwent open resection.

The two groups were adequately matched for BMI, type of surgery, and operative risk as assessed by their ASA classification (Table 1). Moreover, a similar incidence of co-morbidities on admission was observed between the two groups (Table 2). The mean  $\pm$  SD (range) BMI ( $\text{kg}/\text{m}^2$ ) was  $31.9 \pm 1.6$  (range 30-35.5) in the LPS group, and  $32.3 \pm 2.1$  (range 30.1-36.4) in the open colectomy group. No significant differences were observed between the two groups with respect to demographics, indication for surgery (benign *vs* cancer), tumor stage (among cancer patients) as well as for the incidence of previous operations (Table 1).

### Technical feasibility

Conversion to open surgery was necessary in 13 of 98 patients (13.3%). Reasons for conversion were as follows: obesity-hindering vision ( $n = 5$ ), large tumour infiltrating adjacent organ/s ( $n = 2$ ), bleeding from the ileocolic pedicle ( $n = 2$ ), dense adhesions ( $n = 2$ ), urgent splenectomy ( $n = 1$ ) and ureteral damage ( $n = 1$ ). Converted patients remained in the LPS group for an intention to treat analysis. In the converted patients, mean  $\pm$  SD (range) time from beginning of the conversion procedure to open surgery was  $22.5 \pm 38.1$  (15-80) min.

Table 2 reports the surgical characteristics of the two groups. The operation time was averagely 29 min (95%CI: 24.3-49.3) longer in LPS than open group ( $P = 0.03$ ). Operative blood loss was 78 mL lower in the LPS group when compared to open surgery ( $P = 0.0001$ ; 95%CI: 47.1-108.9). No significant differences were observed with respect to perioperative blood transfusion rate between the two groups. Moreover, among cancer patients a similar mean  $\pm$  SD number of lymph nodes was retrieved in the operative specimen in the two groups ( $16.6 \pm 10.1$  in the open group *vs*  $17.7 \pm 11.5$  in the LPS group,  $P = 0.57$ ).

### Postoperative outcomes

In the LPS group, a death occurred as a sequelae of an anastomotic leakage on postoperative day 5. No significant differences between the two groups were observed with respect to overall morbidity rate as well as for tech-

**Table 1** Demographics and clinical characteristics of the two groups *n* (%)

Variable	LPS ( <i>n</i> = 98)	Open ( <i>n</i> = 98)	<i>P</i> value
Age (yr)	66.9 $\pm$ 12.2	68.7 $\pm$ 15	0.31
Male/female	52/46	47/51	0.34
ASA score	2.3 $\pm$ 0.7	2.4 $\pm$ 0.9	0.57
BMI ( $\text{kg}/\text{m}^2$ )	31.9 $\pm$ 2.1	32.3 $\pm$ 2.5	0.13
Cancer/benign/disease	85/13	79/19	0.34
Previous surgery (%) <sup>1</sup>	8 (8.1)	10 (10.2)	0.30
Type of operation			
Right colectomy	57	57	
Left colectomy	41	41	
Tumour stage	<i>n</i> = 85	<i>n</i> = 79	0.77
Stage 0	13 (15.2)	9 (11.3)	
Stage 1	14 (16.5)	13 (16.3)	
Stage 2	29 (34.2)	32 (40.6)	
Stage 3	25 (29.4)	20 (25.4)	
Stage 4	4 (4.7)	5 (6.4)	

<sup>1</sup>Only abdominal surgeries were included; Values are reported as mean  $\pm$  SD or *n* (%). BMI: Body mass index; LPS: Laparoscopic; ASA: American Society of Anesthesiologists.

nical complications such as anastomotic leakage. Moreover, a similar incidence of hospital re-admission within 30 d and of reoperation rate was observed in the two groups (Table 2). Conversely, in the open group, the occurrence of wound complications was more than double when compared to the LPS group and the difference showed a trend toward a statistical significance ( $P = 0.09$ ). In both groups, more than 30% of the postoperative infections occurred after discharge. A similar incidence of respiratory and cardiac complications was observed in the two groups (Table 3). When the converted cases were compared to the laparoscopically completed ones, a trend toward a longer duration of surgery ( $P = 0.07$ ) and a longer length of stay was observed ( $P = 0.002$ ). Converted patients experienced a higher complications rate when compared to patients who completed the operation laparoscopically; however the difference failed to reach statistical significance (22.8 *vs* 32.4,  $P = 0.62$ ). Similar results in term of length of stay and overall complication rate were observed when converted and open surgery patients were compared (Table 4). Mean  $\pm$  SD recovery of oral food intake occurred after 2.1 (1) d in the LPS and after  $3.5 \pm 1.5$  d in the open surgery group ( $P = 0.001$ ). Mean (median, SD) length of stay was 8.6 (8; 3.1) d in the LPS group and 10.4 (10; 4.9) d in the open surgery group ( $P = 0.03$ ).

### Long-term outcomes

At a median follow-up of 62 (range 12-132) mo, 23 patients in the LPS group and 38 in the open surgery group experienced long-term complications (LPS *vs* open,  $P = 0.03$ ; Table 2). Incisional hernia was the most common complication in both groups and its occurrence was more frequent in the open surgery group when compared to the laparoscopic one ( $P = 0.03$ ). Among the converted patients, a 30.8% (4/13 pts) incisional hernia rate was observed.

**Table 2 Comparison of co-morbidities, variables, complications (30 d morbidity) and Long-term complications between the two groups in study *n* (%)**

		LPS ( <i>n</i> = 98)	Open ( <i>n</i> = 98)	<i>P</i> value
Co-morbidities	Diabetes	14	11	0.66
	Coronary artery disease <sup>1</sup>	13	15	0.84
	Hypertension	43	52	0.25
	Smoker	10	14	0.51
	Chronic obstructive pulmonary disease	5	3	0.72
	Steroid use	2	3	0.91
Variable	Operative time (min)	193 (71)	164 (111)	0.03
	Blood loss (mL)	177 (76)	255 (102)	0.0001
	No of transfused patients	11 (11.2)	16 (16.3)	0.33
	Conversion rate	13 (13.3)	NA	
	Overall	27 (27.6)	33 (33.7)	0.4
Complications (30 d morbidity)	Infectious <sup>2</sup>	13 (13.7)	21 (21.7)	0.13
	Noninfectious <sup>2</sup>	9 (9.2)	10 (10.2)	0.8
	Anastomotic leak <sup>2</sup>	7 (7.1)	5 (5.1)	0.78
	Readmission	7 (7.1)	11 (11.2)	0.56
	Reoperation	8 (8.1)	9 (9.2)	0.8
	Length of stay	8 (3.1)	10.4 (4.9)	0.03
	Mortality	1 (1.02)	0 (0.0)	0.9
	Overall	23	38	0.03
Long-term complications	Incisional hernia	17	31	0.03
	Intestinal obstruction	5	6	0.48
	Anastomotic stricture	4	4	0.86
	Overall	23	38	0.03

<sup>1</sup>Including: History of angina, percutaneous cardiac intervention, cardiac operation, or myocardial infarction within 6 mo of operation; <sup>2</sup>Number of single type of complication do not add up to the number of overall complication within the two groups because of the possible occurrence of more than one type of complication in some patients; all values within parenthesis indicate percentage values. LPS: Laparoscopic.

**Table 3 Postoperative complications in details *n* (%)**

	LPS ( <i>n</i> = 98)	Open ( <i>n</i> = 98)
Infectious complications		
Wound complications <sup>1</sup>		
Wound infections	8 (8.2)	15 (15.4)
Wound disruption	-	2 (2.04)
Abdominal abscess	1 (1.06)	2 (2.04)
Pneumonia	2 (2.04)	3 (3.1)
Urinary tract	2 (2.04)	1 (1.06)
Non-infectious complications		
Cardiologic	2 (2.04)	3 (3.1)
Ileus	3 (3.1)	3 (3.1)
Intestinal obstruction	3 (3.1)	4 (4.1)
Bleeding	1 (1.01)	-

<sup>1</sup>Laparoscopic (LPS) *vs* open, *P* = 0.09.

## DISCUSSION

The global prevalence of obesity means that surgeons are increasingly faced with these high-risk patients. The choice of optimal operative approach and technique becomes extremely actual and crucial. The vast majority of the studies available in the literature addressing the issue of mini-invasive approach and obesity compares obese patients to their non-obese counterparts<sup>[2-6]</sup>. In our opinion, in order to better evaluate the effective impact of mini-invasive approach in obese patients, it is important to compare open and laparoscopic colectomy outcomes in the specific population of obese patients only. A definitive answer whether laparoscopic surgery would be preferable in the obese to the open approach can only be

obtained by a randomized controlled trial. To the best of our knowledge, there are no RCTs in the literature specifically addressing this issue. To limit the biases related to the design of the study and in an attempt to minimize surgical heterogeneity<sup>[14]</sup>, a single center case-matched study was performed including only well standardized surgical procedures.

In previous studies, obesity has been identified as one of the factors associated with a higher conversion rate<sup>[15-19]</sup>. In the present trial, a conversion rate of 13.3% has been obtained, which is within the range (0%-39%) previously reported by other studies dealing with this issue<sup>[1-6]</sup>. This rate is however higher when compared to the 2.6% conversion rate after laparoscopic right colectomy or the 5.2% conversion rate in left LPS colectomies reported in studies performed by our Institution in the non-obese population<sup>[20,21]</sup>. These findings are in accordance with results from Tekkis and co-workers who identified obesity as an independent predictor of conversion to open surgery at multivariate analysis with an odd-ratio of 2.2 for patients with a BMI > 28.5 kg/m<sup>2</sup> derived from a large series 1253 subjects<sup>[22]</sup>. Moreover an increasing BMI was associated with a proportionally higher conversion rates in data extracted from the laparoscopic colorectal surgery study group (LCSSG)<sup>23</sup> on 5853 recruited patients<sup>[23]</sup>.

A possible argument against the adoption of the mini-invasive approach in obese patients is that converted patients resulted in poor short and long-term outcomes when compared to patients who successfully completed the operation laparoscopically<sup>[24,25]</sup>. In our experience,



**Table 4 Outcomes of laparoscopically completed, converted and open cases**

Variable	LPS completed ( <i>n</i> = 85)	Converted ( <i>n</i> = 13)	Open ( <i>n</i> = 98)	<i>P</i> value
Operative time (min)	175 (61)	210 (86)	164 (111)	0.07 <sup>1</sup> 0.08 <sup>2</sup>
Morbidity rate	22.8%	32.4%	33.7%	0.55 <sup>1</sup> 0.91 <sup>2</sup>
Length of stay (d)	7.2 (2.5)	9.6 (3.2)	10.4 (4.9)	0.002 <sup>1</sup> 0.52 <sup>2</sup>

<sup>1</sup>Laparoscopic (LPS) completed *vs* converted; <sup>2</sup>Converted *vs* open. Values are expressed as mean (SD) or percentages.

in the converted patients, no significant difference was observed in term of postoperative morbidity when compared to laparoscopically completed cases. Similar results were reported by other authors<sup>[26,27]</sup>. Possible explanation may include the wide experience of the surgical team, in the appropriate patients' selection, or to the rapid decision to convert thus minimizing potentially adverse outcomes<sup>[1,4,10,22]</sup>.

In the present series, the overall morbidity rates did not differ between the two groups. Similarly, despite the incidence of infectious complications was about twice higher in patients in the open surgery group when compared to laparoscopic (23.6% *vs* 11%), the difference failed to reach statistical significance.

Only with respect to wound complications, a trend toward a lower rate was observed in the LPS group. These latter findings are consistent with the data recently reported by Wick *et al*<sup>[28]</sup> and Mustain *et al*<sup>[29]</sup>, who identified open surgery as an independent risk factors for surgical site infections in a large series of obese patients undergoing laparoscopic and open colorectal surgery<sup>[28,29]</sup>.

In the present series, the incidence of anastomotic leakage was similar in the two groups. Delaney and co-workers reported similar results in the only other, to the best of our knowledge, case-matched study comparing laparoscopic to open colectomy in obese patients. These authors reported an absence of statistical difference, both for the overall complications as well as for anastomotic leakage rate<sup>[27]</sup>. The absence of an adverse impact of laparoscopic colectomy on anastomotic leakage rate in the obese patients undergone laparoscopic colectomy has been recently supported by a review paper by Martin and Stocchi<sup>[9]</sup> reporting data from high volume institutions.

Moreover, patients in the LPS group experienced a shorter recovery of bowel function and a shorter length of stay when compared to their open surgery counterparts. These findings are consistent with the results reported by a large meta-analyses analyzing the outcomes of 2512 procedures from 12 RCT trials comparing LPS and the open approach for colorectal diseases in the non-obese population<sup>[30]</sup>. In our series, the earlier recovery of bowel function, the shorter length of stay as well as the lower intraoperative blood loss and a trend toward a reduction of wound complications observed in the LPS

group, deserve major consideration, as these findings indicate that some of the wide-reported short-term benefits of the mini-invasive approach are maintained even in high-risk patients, also suggesting that paradoxically, these are the patients who stands to benefit the most laparoscopic surgery.

With respect to long-term complications, patients in the LPS group experienced a significantly lower incidence of overall complications when compared to their open surgery counterparts. In particular a higher incisional hernia rate was observed in the open surgery group. Few data are available in the literature on this subject reporting controversial results. No difference in term of incisional hernias between laparoscopic or open approach has been reported or, conversely the mini-invasive approach was preferred when obese *vs* non obese patients are compared<sup>[31,32]</sup>. To the best of our knowledge, no study has reported data on long-term complications in obese patients treated with mini-invasive or with conventional approach. The mechanisms for incisional hernia occurrence have not been yet clarified. Potential risk factors have been identified and categorized into patient-related (advanced age, obesity, nutritional status,) disease and surgery-related such as emergency operation, post-operative wound infections, reoperations and others<sup>[33-35]</sup>. Moreover, an association was recently reported by Rullier *et al*<sup>[36]</sup> between the rate of hernia and the length of incision. These authors found an incisional hernia rate of 33.0% at median follow-up period of 51 mo in open group compared to 13.5% in the LPS group of patients who underwent rectal resection.

In conclusions, although there are some limitations to the study resulting from the non-randomized design, to the possible bias in patient selection for laparoscopic surgery, and to the fact that patients were treated in a single institution, our findings indicate that laparoscopic surgery can be safely performed in obese patients with colorectal disease. Moreover, minimally invasive approach has no adverse impact on postoperative complications, resulting in reduced length of stay and lower incidence of long-term incisional hernia when compared to conventional colectomy.

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## COMMENTS

### Background

The safety and the benefits of laparoscopic colon resection, including less post-operative pain, faster recovery of bowel function, earlier mobilization, less morbidity, reduction of hospital stay, and smaller scars, have been underlined by several studies, making it now the preferred approach in the surgical management of many colorectal diseases. Obese patients are considered to be at high perioperative risks. Therefore laparoscopic surgery may be particularly advantageous in obese patients. On the other hand a colorectal resection is more difficult in obese subjects due to obesity hindering visualization and dissection of tissue planes that lead to longer operative time and increase blood transfu-

sion requirement, thus possibly impair the benefits of laparoscopic colectomy.

### Research frontiers

The study performed a single center study in which patients were matched for body mass index, site of primary disease, American Society of Anesthesiologists score, and year of surgery. Moreover only well standardized surgical procedures (left and right colectomy) were included. However, in order to better evaluate the real impact of minimally-invasive approach in obese patients, a randomized controlled trial should be conducted.

### Innovations and breakthroughs

The vast majority of previous studies, focusing on laparoscopic colectomies in obese patients, compared obese to non-obese subjects. No randomized study has been conducted. Only one case matched study compared open and laparoscopic approach in obese patients. This is the first study that analyses long-term outcomes. The principals finding of this study is that the documented benefits of laparoscopic colon resection could be translated even in a high-risk group, such obese patients, since this approach did not impair postoperative outcomes and resulted in lower incidence of long-term complications when matched with open surgery cases.

### Applications

Providing that experienced surgeons are involved, laparoscopic colorectal resection should be offered to obese patients considering its benefits when compared to conventional open surgery.

### Peer review

The authors analyze the feasibility of laparoscopic colectomy in obese patients. There are not randomized trials comparing the outcome of colectomy in obese and non-obese patients. The results confirm previous studies on the efficacy and safety of laparoscopic left and right colectomy in obese subjects. The literature on this topic is scarce, and, although another case-match study was previously published, this is the only one that analyses long term results.

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## Is increased red cell distribution width an indicating marker of nonalcoholic steatohepatitis and fibrotic stage?

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**RESULTS:** Patients with NASH had higher RDW values compared with simple steatosis and healthy control groups [ $14.28\% \pm 0.25\%$  vs  $13.37\% \pm 0.12\%$ ,  $12.96\% \pm 0.14\%$  ( $P < 0.01$ ), respectively]. Patients with advanced fibrosis had higher RDW values than the mild fibrosis group ( $15.86\% \pm 0.4\%$  vs  $13.63\% \pm 0.67\%$ ,  $P < 0.01$ , respectively). RDW also correlated with fibrotic scores ( $r = 0.579$  and  $P < 0.01$ ). The variables that were significant in the univariate analysis were evaluated in multivariate logistic regression analysis, and RDW was an independent predicting factor of NASH (OR = 1.75, 95%CI: 1.129-2.711,  $P < 0.05$ ).

**CONCLUSION:** RDW a new non-invasive marker that can be used to demonstrate the presence of NASH and indicate advanced fibrotic scores.

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**Key words:** Non-alcoholic steatohepatitis; Liver fibrosis; Red cell distribution width; Simple steatosis; Non-invasive marker; Liver biopsy

### Abstract

**AIM:** To evaluate the red cell distribution width (RDW) as an indicator of the presence of non-alcoholic steatohepatitis (NASH) and its association with fibrotic scores.

**METHODS:** A retrospective study was carried out that included sixty-two biopsy proven NASH, 32 simple steatosis patients and 30 healthy controls. The correlation between the clinical and histopathological features of NASH patients and RDW values was evaluated. Liver fibrosis scores were measured using a 0 to 4 point scale and were divided in to two groups; fibrosis scores 0-1 were termed mild and fibrosis scores 2-4 were termed advanced fibrosis. RDW values were compared between NASH, simple steatosis and healthy controls. Univariate and multivariate analyses were performed to evaluate the independent predicting factors for the presence of liver fibrosis caused by NASH.

**Core tip:** We evaluated the role of red cell distribution width (RDW) as an indication of nonalcoholic steatohepatitis by comparing the values of biopsy proven non-alcoholic steatohepatitis (NASH) patients with simple steatosis and healthy controls. Independent predictors of the presence of NASH and advanced liver fibrosis were evaluated by using multivariate logistic regression analyses and RDW was a statistically significant independent predictor.

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## INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is part of the spectrum of non-alcoholic fatty liver disease (NAFLD), which comprises simple fatty liver, NASH, associated advanced fibrosis and cirrhosis<sup>[1]</sup>. NAFLD has become one of the most common forms of chronic liver diseases in the United States and worldwide, with a prevalence of 10%-24%<sup>[2-5]</sup>. NASH is characterized by ballooning, degeneration, and lobular inflammation with various stages of fibrosis. Lobular inflammation is a hallmark of NASH, which is characterized by infiltration of lymphocytes, mononuclear cells and polymorphonuclear neutrophils. Steatosis is present in all cases and affects the hepatic lobules<sup>[6]</sup>. Simple steatosis is a benign condition with minimum progression, despite a high rate of progression to cirrhosis in 15%-25% of patients with NASH<sup>[7]</sup>. Liver fibrosis is a result of chronic injury<sup>[8]</sup>. In the absence of approved treatment modalities for NASH, care should be taken on the detection of advanced fibrosis. A liver biopsy is the only method for distinguishing NASH from other diagnoses and permits the evaluation of the damage and fibrosis of the liver. However a biopsy is an invasive and expensive procedure with important complications, such as bleeding and perforation. Therefore, there has been a search for noninvasive methods of detecting the presence of NASH and liver fibrosis to avoid these procedure-related complications.

Chronic inflammation plays a significant role in disease progression to NASH<sup>[9]</sup>. In addition, some studies have shown that certain inflammatory cytokines were higher in patients with NASH<sup>[10,11]</sup>.

Red cell distribution width (RDW) is a parameter of the variation of circulating red cells. This parameter demonstrates the heterogeneity of red cell volume and is a component of the complete blood count (CBC). The RDW is also a widely available, inexpensive and easily repeatable marker that measures red blood cell (RBC) volume variability. Recent reports have demonstrated that elevated RDW values were related to negative outcomes in cardiovascular and metabolic disorders, colon cancer and stroke independent of hemoglobin (HGB) values<sup>[12-16]</sup>. The association between RDW values and the severity of liver diseases has been also reported in two recent studies<sup>[17,18]</sup>.

In this study, we aimed to investigate the clinical utility of RDW for indicating the presence of NASH in comparison with simple steatosis and healthy controls. We also evaluated whether there is an association between RDW values, fibrotic stages and histological features of NASH.

## MATERIALS AND METHODS

### Patients

This retrospective study was performed in Gazi University Department of Gastroenterology, a tertiary reference center (Ankara, Turkey), between January 2010 and May 2013. All the patients who had persistently elevated liver enzymes and hepatosteatosis on ultrasonography, in the absence of any causes of elevated aminotransferases,

were evaluated in the study. Among them, patients who had histopathology consistent with NASH and simple steatosis were included in the study. The control group was created among individuals who had normal aminotransferase levels and normal abdominal ultrasonography. Patients who were diagnosed with viral hepatitis, sclerosing cholangitis, primary biliary cirrhosis, autoimmune hepatitis, hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency, malignancy and drug-induced liver disease were excluded. Other exclusion criteria were alcohol consumption of > 20 g/d for men and > 10 g/d for women, presence of infectious diseases on admission, chronic renal diseases, collagen vascular diseases, malignancies, hematological diseases that might impair red cell production (such as iron deficiency, B12 or folate deficiency), increased red cell destruction, hemoglobinopathies, blood transfusions, bone marrow depression, usage of anticoagulant drugs, non-steroid anti-inflammatory drugs and hepatotoxic drugs. Patients who had diabetes mellitus were on oral antidiabetics and/or insulin therapy. Patients who had hypertension were all using angiotensin-converting enzyme inhibitors and were under control in terms of hypertension.

### Clinical and laboratory assessment

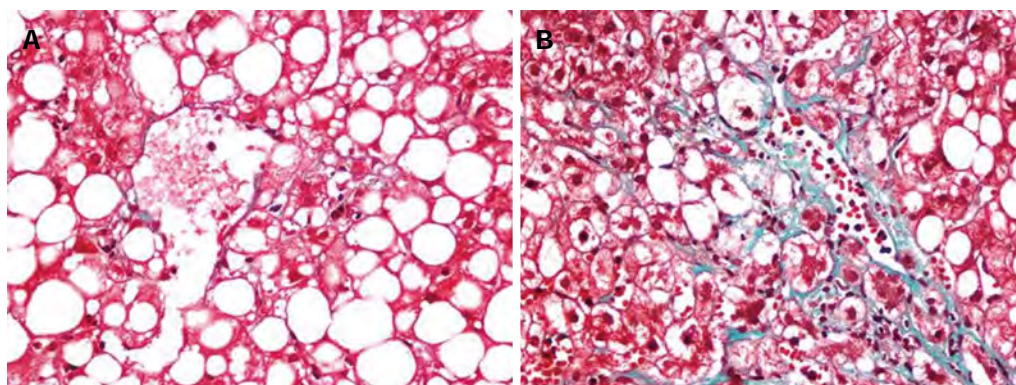
Demographical, clinical and laboratory data were collected and registered in a database by an uninformed clinician to prevent bias. Body mass index was calculated by dividing weight in kilograms (kg) by the square of height in meter (m) as (kg/m<sup>2</sup>).

Before the liver biopsy, venous blood samples were obtained from the antecubital vein from all patients between 8.30 and 10.00 am, after fasting for at least 8-12 h. A CBC containing RDW, hemoglobin (HB), white blood cell (WBC), neutrophils, lymphocytes, platelets and mean platelet volume (MPV) was performed with a Beckman Coulter Gen-S automated analyzer (High Wycombe, United Kingdom) within 2 h after obtaining the blood samples. RDW was calculated by the Beckman Coulter automated analyzer as  $RDW = SD/MCV \times 100\%$ . (The standard deviation of the volume of red blood cell (SD)/[(mean corpuscle volume) (MCV)]  $\times 100\%$ . RDW was calculated by dividing the SD by the MCV and then multiplying that result by 100. The SD represented the volume of erythrocytes or RBCs that were in the blood smear.

A Roche Modular System auto analyzer (Roche COBAS INTEGRA 800 (Indianapolis, United States) determined the Albumin, creatinine, blood urine nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$  glutamyl aminotransferase (GGT) and alkaline phosphatase (ALP) levels. All the laboratory analyses were performed in our hospital's hematology laboratory.

### Histopathological evaluation of the liver

Percutaneous liver biopsy was performed using a 16-G disposable needle by a skilled clinician. All liver biopsy specimens included 12 or more complete portal tracts



**Figure 1** Mild perivenular-perisinusoidal fibrosis (A) and advanced perivenular-perisinusoidal, and periportal fibrosis (B) in non-alcoholic steatohepatitis. A, B: Trichrome stain, x 400.

and were longer than 20-25 mm. All liver tissue samples were evaluated by the same experienced hepatopathologist who was blinded to the patients' clinical and laboratory data. Hematoxylin and eosin (HE) and Masson trichrome stains were used for histopathological diagnoses of formalin-fixed paraffin-embedded liver tissues. The diagnosis of NASH was evaluated on Brunt's Criteria<sup>[19]</sup>. Histological characteristics were graded according to the NAFLD scoring system recommended by the National Institute of Diabetes and Digestive and Kidney Diseases NASH Clinical Research Network<sup>[20]</sup>. Steatosis was graded as 0  $\leq$  5%; 1  $\geq$  33%-66%; 3  $\geq$  66% lobular inflammation was graded as 0 = no foci; 1  $\leq$  2 foci; 2 = 2-4 foci; 3  $\geq$  4 foci; and ballooning was graded as 0 = none; 1 = few ballooning cells; 2 = many ballooning cells, respectively.

Depending on the recommendations of Brunt's criteria steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2) were then combined to establish the NAFLD activity score (0-8). Fibrosis was also scored as 0 = no fibrosis; 1 = periportal or perisinusoidal fibrosis; 2 = perisinusoidal and portal/periportal fibrosis; 3 = bridging fibrosis; and 4 = cirrhosis. While mild fibrosis was defined with fibrotic score  $\leq$  1 advanced fibrosis was defined with fibrotic score  $>$  1. Mild fibrosis caused by NASH is shown in Figure 1A and advanced fibrosis in Figure 1B.

### Ethics

The study was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board.

### Statistical analysis

Statistical analyses were performed using the SPSS software version 17 and MedCalc version 12. The variables were investigated by using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's test) to determine whether or not they were normally distributed. Non-normally distributed and non-parametric variables were compared between groups using the Mann-Whitney *U* test where appropriate. The  $\chi^2$  test, where appropriate, was used to compare

the propositions in different groups. As the RDW was not normally distributed, the Kruskal-Wallis test was used for comparisons among NASH, simple steatosis and healthy control groups. The Mann-Whitney *U* test was used to test the significance of pairwise differences, using Bonferroni correction to adjust for multiple comparisons. For the multivariate analysis, the possible factors identified with univariate analyses were further entered into the logistic regression analysis to determine independent predictors of NASH. To assess model fit, we used Hosmer-Lemeshow goodness of fit statistics. Spearman rank correlation coefficients (*r*) were calculated to assess the correlation between RDW and liver histopathological features (inflammation, NAS score, the degree of steatosis and degree of fibrosis) and clinical characteristics of NASH patients. Receiver operating characteristics (ROC) analysis was used to evaluate the role of RDW in distinguishing subjects with NASH and fibrosis. ROC analysis was also used to distinguish advanced and mild fibrotic stage groups. Cutoff values that maximized both sensitivity and specificity were chosen for the NASH group. *P* < 0.05 was considered statistically significant.

## RESULTS

### Patient characteristics and liver histology

Sixty-two biopsy proven NASH patients, 32 biopsy proven simple steatosis patients and 30 healthy controls were recruited. The median ages of patients with NASH, simple steatosis and healthy controls were 49.5 years (22-77 years), 48 years (24-71 years) and 48 years (24-72 years), respectively. There was no statistically significant difference between the ages of participants. The mean BMI ( $\text{kg}/\text{m}^2$ ) of NASH, simple steatosis and healthy controls were  $27.13 \pm 0.37$ ,  $25.97 \pm 0.67$  and  $24.22 \pm 0.45 \text{ kg}/\text{m}^2$ , respectively (*P* < 0.01). The platelet counts among NASH, simple steatosis and healthy controls were  $245.5 \pm 34.73$ ,  $244.5 \pm 13.8$  and  $260 \pm 4.47 \times 10^3/\text{mL}$ , respectively (*P* > 0.05). However, there was an inversely significant correlation between platelet counts and fibrotic scores (*r* = -0.335 and *P* < 0.01). Among the NASH group 30/62 (48.3%), steatosis group 18/32 (56.2%) and

**Table 1** Demographic and laboratory features of non-alcoholic steatohepatitis, simple steatosis and healthy control groups

Factor	NASH group (n = 62)	Steatosis group (n = 32)	Healthy control group (n = 30)	P-value
Age mean $\pm$ SD	48.81 $\pm$ 12.21	47.25 $\pm$ 12.58	48.03 $\pm$ 9.54	NS
Gender (male)	30 (48.3)	18 (56.2)	18 (60)	NS
BMI (kg/m <sup>2</sup> )	27.13 $\pm$ 0.37	25.97 $\pm$ 0.67	24.22 $\pm$ 0.45	< 0.01
Hemoglobin (g/dL)	14.10 $\pm$ 0.16	14.51 $\pm$ 0.28	13.91 $\pm$ 0.11	NS
Platelet ( $\times 10^3$ /mL)	245.5 $\pm$ 34.73	244.5 $\pm$ 13.8	260 $\pm$ 4.47	NS
WBC (k/UL)	6950 $\pm$ 319	7250 $\pm$ 330	7350 $\pm$ 146	NS
Neutrophil ratio	59.39% $\pm$ 1.20%	59.87% $\pm$ 1.20%	59% $\pm$ 1.1%	NS
Lymphocyte ratio	31.29% $\pm$ 1.09%	29.88% $\pm$ 1.10%	32% $\pm$ 0.78%	NS
RDW	14.28% $\pm$ 0.25%	13.37% $\pm$ 0.12%	12.96% $\pm$ 0.14%	< 0.01
MPV (fL)	8.63 $\pm$ 1.30	8.49 $\pm$ 1.06	8.20 $\pm$ 0.59	NS
MCV (fL)	86.34 $\pm$ 0.88	87.98 $\pm$ 1.37	85.5 $\pm$ 0.82	NS
ALT (IU/L)	54 $\pm$ 8.1	45 $\pm$ 3.97	32.5 $\pm$ 1.49	< 0.01
AST (IU/L)	40.05 $\pm$ 6.04	40.05 $\pm$ 3.01	24 $\pm$ 1.39	< 0.01
ALP (IU/L)	87.5 $\pm$ 9.09	81.5 $\pm$ 3.91	61 $\pm$ 1.87	< 0.01
GGT (IU/L)	61 $\pm$ 13.4	32 $\pm$ 3.4	27 $\pm$ 2.5	< 0.01
Albumin (g/dL)	4.36 $\pm$ 0.73	4.5 $\pm$ 0.42	4.9 $\pm$ 0.75	< 0.01
Creatinine	0.78 $\pm$ 0.033	0.79 $\pm$ 0.023	0.72 $\pm$ 0.014	NS
BUN (mg /dL)	12.25 $\pm$ 0.85	14.2 $\pm$ 0.67	13.46 $\pm$ 0.71	NS
Diabetes mellitus	19 (30.6)	9 (28.1)	0 (0)	NS
Hypertension	22 (35.4)	7 (21.8)	0 (0)	NS

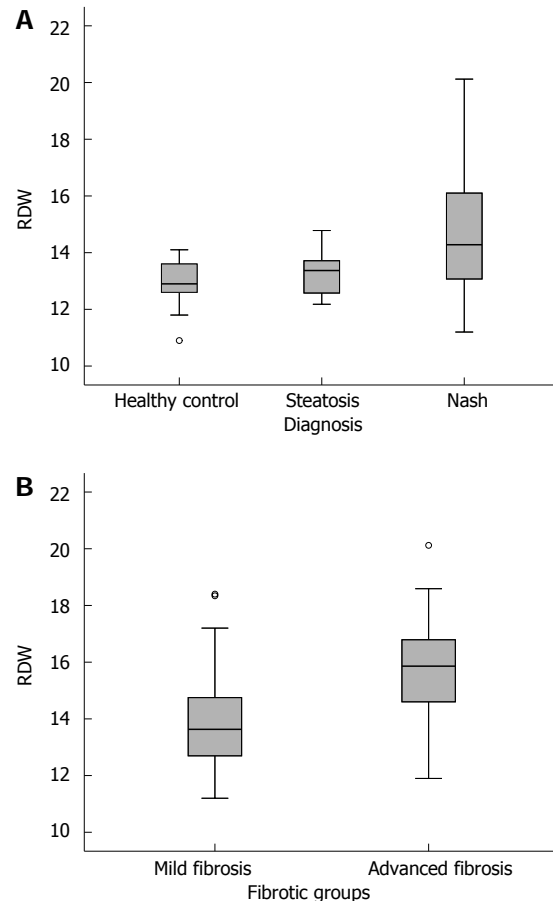
Values are presented as *n* (%) frequencies, median  $\pm$  SE for skewed distributed variables. BMI: Body-mass index; RDW: Red cell distribution width; MPV: Mean platelet volume; NS: Not significant; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT:  $\gamma$  glutamyl transferase; WBC: White blood cell; MCV: Mean corpuscle volume; BUN: Blood urine nitrogen.

healthy controls 18 (60%) were men ( $P > 0.05$ ). The median RDW values were  $14.28\% \pm 0.25\%$ ,  $13.37\% \pm 0.12\%$  and  $12.96\% \pm 0.14\%$ , respectively ( $P < 0.01$ ). The clinical and laboratory data of patients with NASH, simple steatosis and healthy control groups are summarized in Table 1. The comparison of RDW values among NASH, simple steatosis and healthy control groups is also shown in Figure 2A.

#### Relationship between RDW and the presence of NASH and fibrosis

The ROC analysis suggested that a cutoff value of 13.56 has the highest sensitivity (61.3%) and specificity (72.6%) for detecting patients with NASH with an area under the curve (AUC) of 0.70 (95%CI: 0.600-0.805), ( $P < 0.01$ ), as shown in Figure 3A.

According to the liver histopathological features, while the mild fibrosis group consisted of 40 patients, there were 22 patients in advanced fibrosis group. We compared RDW values between the mild and advanced fibrosis subgroups of NASH and found a statistically significant difference ( $13.95\% \pm 1.74\%$  and  $15.85\% \pm 1.89\%$ , respectively,  $P < 0.01$ ), as seen in Figure 2B. The ROC curve of RDW for the identification of advanced fibrosis in NASH was statistically significant (AUC = 0.78, 95%CI: 0.660-0.903,  $P < 0.01$ ), as shown in Figure 3B.



**Figure 2** Red cell distribution width values of healthy controls, simple steatosis and non-alcoholic steatohepatitis groups (A), and fibrotic subgroups of non-alcoholic steatohepatitis (B). RDW: Red cell distribution width.

#### Correlation between RDW and clinical/histopathological features of patients with NASH

In Spearman correlation analysis, there were statistically significant correlations between RDW values and fibrotic scores, age and gender, as seen in Table 2. The correlation between fibrotic scores and RDW values reached statistically significance, such that Spearman's correlation coefficients were  $r = 0.42$ , and  $P < 0.001$ , as shown in Figure 4.

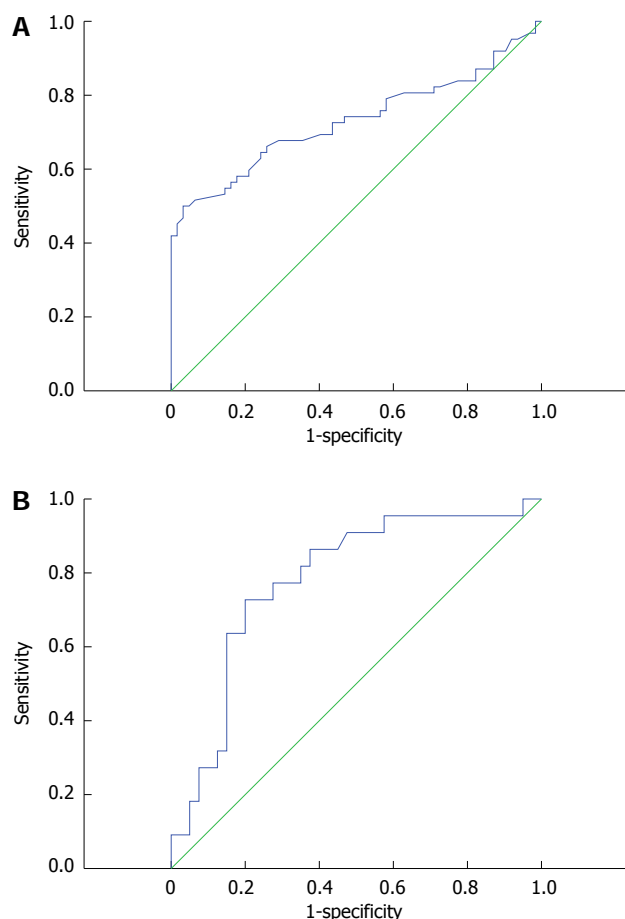
Univariate analysis for the presence of NASH indicated that age, gender, platelet counts, ALT, AST, ALP, GGT, albumin, BMI and RDW were statistically significant predictor factors of the presence of the NASH. The results of univariate analysis are shown in Table 3.

Those variables that were statistically significant in univariate logistic regression analyses were furthered subjected to multivariate logistic regression analysis, and RDW continued to be statistically significant and an independent predictor of fibrosis (OR = 1.75, 95%CI: 1.129-2.711,  $P < 0.001$ ), as seen in Table 4.

## DISCUSSION

In the present study, we concluded that patients with biopsy proven NASH have higher RDW values compared





**Figure 3** Receiver operating characteristics curve of red cell distribution width values for the identification of patients with non-alcoholic steatohepatitis (A), and the identification of fibrosis in non-alcoholic steatohepatitis (B).

with simple steatosis patients and healthy controls. The increase in RDW values correlated with disease progression because the patients with advanced fibrosis had higher RDW values than the mild fibrosis group. There was a positive correlation between RDW values and fibrotic scores. As a result of multivariate logistic regression analysis RDW was identified as an independent predictor of NASH and advanced liver fibrosis.

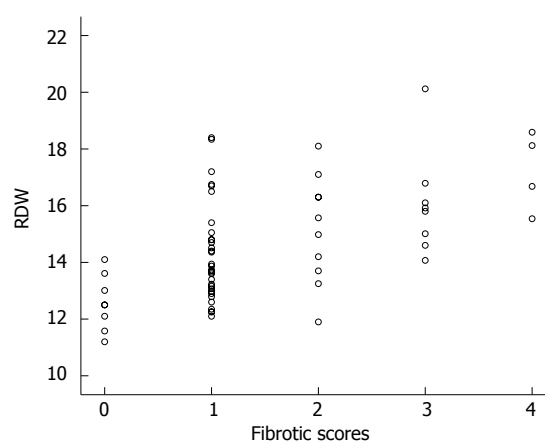
The search for a noninvasive diagnostic marker indicating the histological changes and fibrotic stages observed in NASH is very important. The absence of approved treatment methods for NASH means that care must be taken on the detection of advanced fibrosis. The risk stratification requires an adequate evaluation of fibrosis, which only currently possible by performing a liver biopsy. Biopsy is an invasive method with natural risks, costs, and is disturbing for the patients; therefore, considerable efforts have been made in the development of noninvasive alternatives of determining the degree of liver fibrosis.

Lou *et al*<sup>[21]</sup> found that RDW values were significantly increased in patients with hepatitis B and that high RDW values were associated with disease severity. Similarly Chen *et al*<sup>[22]</sup> concluded that RDW to platelet index is a

**Table 2** Red cell distribution width correlated with clinical characteristics and histological features of non-alcoholic steatohepatitis

Factor	r-value	P-value
Age (yr)	0.245	< 0.01
BMI (kg/m <sup>3</sup> )	0.131	NS
Gender	-0.251	< 0.01
Fibrosis	0.579	< 0.01
Inflammation	0.207	NS
Steatosis	0.121	NS
Ballooning	0.175	NS
NASH	0.217	NS

BMI: Body mass index; RDW: Red cell distribution width; NASH: non-alcoholic steatohepatitis; NS: Not significant.



**Figure 4** Correlation between red cell distribution width values and fibrotic scores of non-alcoholic steatohepatitis. RDW: Red cell distribution width. Score 0, *n* = 8; Score 1, *n* = 32; Score 2, *n* = 10; Score 3, *n* = 8; Score 4, *n* = 4.

routinely available and easily calculated index that could predict significant liver fibrosis caused by chronic hepatitis B with a high accuracy. They also estimated that this index may reduce the requirement of liver biopsy in patients infected with chronic hepatitis B. High RDW values are associated with poor outcomes in patients with cardiovascular diseases<sup>[23,24]</sup>. Although the main mechanism that causes elevation of RDW levels in these different conditions is unknown, it is speculated that inflammation may play an important role<sup>[25]</sup>. Some pathways may explain the increase in RDW values, such as impaired iron metabolism, suppressed erythropoietin gene expression, inhibition of proliferation of erythroid progenitor cells, downregulation of erythropoietin receptor expression and reduced erythrocyte circulatory half-life<sup>[26]</sup>.

Alkhouri *et al*<sup>[27]</sup> found that the neutrophil to lymphocyte ratio was higher in patients with NASH and advanced fibrosis. They hypothesized that this ratio could be used as a novel noninvasive marker to predict advanced liver fibrosis caused by NASH. Tonelli *et al*<sup>[28]</sup> reported that higher RDW values might reflect an underlying chronic inflammation, which could result in an increased risk of cardiovascular disease.

These results suggest that inflammation may be a



**Table 3** Univariate analyses for predicting liver fibrosis

	OR	95%CI	P-value
Age	1.05	1.004-1.109	< 0.05
Gender	1.46	0.516-4.171	NS
Platelet	0.98	0.988-0.996	< 0.01
ALT	1.08	0.976-1.004	NS
AST	0.98	0.963-1.007	NS
ALP	1.015	1.002-1.028	< 0.05
GGT	1.002	0.998-1.007	NS
RDW	1.73	1.242-2.409	< 0.01
Albumin	0.418	0.158-1.105	NS
BMI	1.012	0.847-1.209	NS

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT:  $\gamma$  glutamyl aminotransferase; RDW: Red cell distribution width; BMI: Body mass index; NS: Not significant.

potential underlying biological mechanism for increased RDW values. Chronic inflammation may play a role in disease progression of NASH and fibrosis. Indeed, multiple inflammatory cytokines have been assessed in different studies as noninvasive markers for the presence of NASH and fibrosis<sup>[29]</sup>. Thus, increased RDW values may be considered as a chronic inflammatory process in the pathogenic basis of NASH. Moreover our findings revealed that RDW could discriminate fibrotic scores of NASH, making it a potentially useful marker for predicting the progression and fibrotic stages of NASH.

We hypothesized that the relationship between RDW and NASH may be a result of an effect of an inflammatory process that suppresses mature erythrocytes and secretes young erythrocytes into the circulation, leading to anisocytosis and high RDW values. To the best of our knowledge, this is the first study to evaluate an association between high RDW values and high fibrotic scores in a well-designed, specifically organized patient group of biopsy proven NASH.

There are some limitations to our study that: RDW was assessed on a single situation instead of multiple consecutive measurements, therefore we could not evaluate any biological variabilities and measurement errors that could be a result of this. Also erythropoietin, reticulocyte count, inflammatory markers such as interleukin (IL)-1, IL-6, tumor necrosis factor- $\alpha$  were not provided; they might provide some important information on the pathophysiology of underlying high RDW physiology. A lack of a marked fall in the platelet count of NASH patients may reflect the small sample size and small number of cirrhotic patients. This must be evaluated in large population based prospective studies.

In summary, our study showed that in patients with biopsy proven NASH, RDW is associated with histological severity and could be used to identify patients with advanced liver fibrosis. Unlike many other noninvasive markers of NAFLD, RDW is inexpensive, widely available and easily repeatable. Although the accuracy of RDW for detecting NASH and significant fibrosis is sufficient, RDW in combination with other markers may help to identify patients at increased risk of having

**Table 4** Multivariate analyses for advanced liver fibrosis

	OR	95%CI	P-value
ALP	1.005	0.993-1.018	NS
RDW	1.75	1.129-2.711	< 0.05
Age	1.02	0.957-1.086	NS
Platelet	0.991	0.981-1.001	NS
Albumin	1.016	0.291-3.545	NS

ALP: Alkaline phosphatase; RDW: Red cell distribution width; NS: Not significant.

advanced disease. Perhaps independently of anemia and other factors, RDW could be used to estimate the fibrotic process of different diseases. It may become one of the cornerstones of inflammation and fibrosis if confirmed in large population based studies in the future.

## COMMENTS

### Background

Non-alcoholic steatohepatitis (NASH) is one of the most common causes of chronic liver diseases. Liver biopsy is the only method for distinguishing NASH from other diagnoses and evaluating the damage and fibrosis of liver, but it may have procedure-related clinical complications.

### Research frontiers

Therefore, there has been an attempt to find noninvasive methods for detecting the presence of NASH, liver fibrosis and avoiding procedure-related complications. In this study, the authors demonstrated that red cell distribution width (RDW) may indicate the presence of NASH and advanced liver fibrosis.

### Innovations and breakthroughs

Recent reports have demonstrated that elevated RDW values were related to negative outcomes in cardiovascular and metabolic disorders, colon cancer and stroke independent of hemoglobin values. The association between RDW values and the severity of liver diseases has also been reported in two recent studies. This is the first study to demonstrate the importance of RDW in the diagnosis of NASH and predicting advanced liver fibrosis.

### Applications

By understanding that high RDW values can be seen in the presence of NASH and advanced liver fibrosis, this study may represent a future strategy for non-invasive diagnostic method of patients with NASH.

### Terminology

RDW is a widely available, inexpensive and easily repeatable parameter of variation of circulating red cells, reflecting the heterogeneity of red cell volume, a component of the complete blood count. Chronic inflammation plays a significant role in disease progression to NASH. Liver fibrosis is a result of chronic injury and can only be diagnosed invasively by liver biopsy.

### Peer review

The results present a new laboratorial and non-invasive marker of NASH and fibrotic stage. The authors concluded that in patients with biopsy proven NASH, RDW is associated with histological severity and could be used to identify patients with advanced liver fibrosis disease. This result is interesting and RDW may be used as a non-invasive marker of indicating the presence of NASH and advanced liver fibrosis.

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## Real-time virtual sonography visualization and its clinical application in biliopancreatic disease

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3 good (pancreatic cancer and gallbladder cancer). Compared with conventional B-mode ultrasonography and CT, RVS images achieved a rate of 80% superior visualization and 20% better visualization.

**CONCLUSION:** RVS has potential usefulness in objective visualization and diagnosis in the field of biliary and pancreatic diseases.

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**Key words:** Biliary and pancreatic disease; Computed tomography-multiplanar reconstruction image; Navigation; Real-time ultrasound image; Real-time virtual sonography

### Abstract

**AIM:** To evaluate the usefulness of real-time virtual sonography (RVS) in biliary and pancreatic diseases.

**METHODS:** This study included 15 patients with biliary and pancreatic diseases. RVS can be used to observe an ultrasound image in real time by merging the ultrasound image with a multiplanar reconstruction computed tomography (CT) image, using pre-scanned CT volume data. The ultrasound used was EUB-8500 with a convex probe EUP-C514. The RVS images were evaluated based on 3 levels, namely, excellent, good and poor, by the displacement in position.

**RESULTS:** By combining the objectivity of CT with free scanning using RVS, it was possible to easily interpret the relationship between lesions and the surrounding organs as well as the position of vascular structures. The resulting evaluation levels of the RVS images were 12 excellent (pancreatic cancer, bile duct cancer, cholecystolithiasis and cholangiocellular carcinoma) and

**Core tip:** The real-time virtual sonography (RVS) system combined with ultrasonography (US) and computed tomography (CT) compensates for each of the deficiencies of US and CT. The visualization in biliary and pancreatic diseases using RVS added objectivity and detailed imaging for diagnosis lacking in conventional B-mode US and CT, and it becomes possible to make a more precise diagnosis. Moreover, it is possible to consider this system as providing images of detailed processes conveniently and in real time. RVS has potential usefulness in objective visualization and diagnosis in the field of biliary and pancreatic diseases.

Sofuni A, Itoi T, Itokawa F, Tsuchiya T, Kurihara T, Ishii K, Tsuji S, Ikeuchi N, Tanaka R, Umeda J, Tonozuka R, Honjo M, Mukai S, Moriyasu F. Real-time virtual sonography visualization and its clinical application in biliopancreatic disease. *World J Gastroenterol* 2013; 19(42): 7419-7425 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7419.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7419>

## INTRODUCTION

Recent advancements in technology have expanded the role of diagnosis by ultrasonography (US) in biliopancreatic diseases. Ultrasonography is useful because it is non-invasive and images can be obtained in real time. However, visualization is sometimes difficult in the presence of bone, gas and air, and thus has the problem of diminished objectivity. Real-time virtual sonography (RVS) (Hitachi-Aloka Medical Ltd., Tokyo, Japan) is a technological system that was developed in an attempt to resolve such problems.

This method merges ultrasound images with information from computed tomography (CT) images to produce the displayed image. In other words, the CT volume data is pre-stored into the device and the real-time ultrasound image is displayed simultaneously while the virtual view is reconstructed as a CT-multiplanar reconstruction (MPR) image from the stored volume data<sup>[1]</sup>. This is one method of compensating for the decreased objectivity in ultrasound diagnosis. This method is used during navigation in ultrasound-guided treatment and its usefulness has been reported in the field of liver disease<sup>[2-13]</sup>; however, no data has been presented in the field of biliopancreatic disease.

## MATERIALS AND METHODS

### Aim

The RVS system was applied on patients with biliopancreatic disease to investigate its usefulness and potential. The aim of the evaluation was to assess how the target lesion was correlated with the surrounding organs and blood vessels.

### Materials

RVS was performed on 15 biliopancreatic patients at our department. Among the patients, 6 had pancreatic cancer, 1 had pancreatic endocrine tumor, 2 had gall bladder cancer, 4 had bile duct cancer, 1 had cholangiocellular carcinoma, and 1 had cholecystolithiasis.

### Device

The RVS system (Figure 1) is made up of an ultrasound diagnostic device EUB-8500 (Hitachi-Aloka Medical Ltd., Tokyo, Japan), the EUP-C514 probe (Hitachi-Aloka Medical Ltd., Tokyo, Japan), which is a convex type, a personal computer (PC) and a magnetic position sensor unit to detect the position of the probe. Pre-scanned CT volume data is stored in a medium such as a compact disc read-only memory or PC using digital imaging and communication in medicine specifications *via* a local area network. The PC can display the images at a high speed of approximately 11 frames/s, with each frame being 256 × 256 pixels of virtual sonography. The PC is equipped with a magnetic position sensor unit to obtain information on the position of the probe. The magnetic position sensor unit consists of the main unit, a magnetism

generator and a magnetic sensor. When the magnetism generator is placed close to the patient, the magnetic sensor attaches to the probe. On CT, by performing imaging of very thin slices (approximately 1 mm) by multidetector CT (MDCT) of at least 16 rows, the minimum volume data required to reconstruct MPR images can be collected. In our department, 32-row MDCT is used to collect volume data by 3-planar contrast imaging.

### Obtaining information on probe position and its calibration

Information of the ultrasound probe position is obtained by the magnetic position sensor connected to the PC. The information obtained by this sensor is on its relative position to the magnetic sensor. Therefore, to prepare an MPR image that fits the ultrasound plane, it is necessary to decide on the pre-stored CT volume data and the position (reference point) of the ultrasound image. Ensiform cartilage that is palpable from the body surface is the ideal reference point. This is used to adjust the position (calibration). Apart from ensiform cartilage, calibration can also be performed using metal markers placed in the vasculature, kidneys, umbilical cord or body surface. In our department, calibration is mainly performed using ensiform cartilage. After the calibration, the ultrasound image obtained from the ultrasound scanning is displayed simultaneously. The virtual sonography-processing program in the PC detects the probe position and angle, reconstructs the corresponding planar image from the CT volume data, and displays it as an MPR image (Figure 2).

### Definitions

The respective RVS images are evaluated based on 3 levels, namely, Excellent: hardly any displacement in position, Good: midway between Excellent and Poor, and Poor: major displacement in position. The RVS images were stored in video form and were evaluated by 3 gastroenterologists (Sofuni A, Itoi T, Itokawa F) with at least 8 years of experience in abdominal sonography evaluation.

## RESULTS

The results were based on the decisions of the majority, and the result of an actual case is shown in Table 1. The resulting evaluation levels of the RVS images were 12 excellent (pancreatic cancer, bile duct cancer, cholecystolithiasis and cholangiocellular carcinoma) and 3 good (pancreatic cancer and gallbladder cancer). The evaluation of the RVS image in the patient with pancreatic endocrine tumor was excellent (Figure 3). The diagnosis by endoscopic ultrasound-guided fine needle aspiration was poorly differentiated neuroendocrine carcinoma. The relationship between the tumor and the celiac artery posed a problem in terms of choice of treatment. With the RVS image, the ultrasound showed no vascular irregularity, and the boundary between the tumor, celiac artery (CA) and splenic artery (SPA) remained intact, thus surgery was performed. Even when surgery was performed, there was



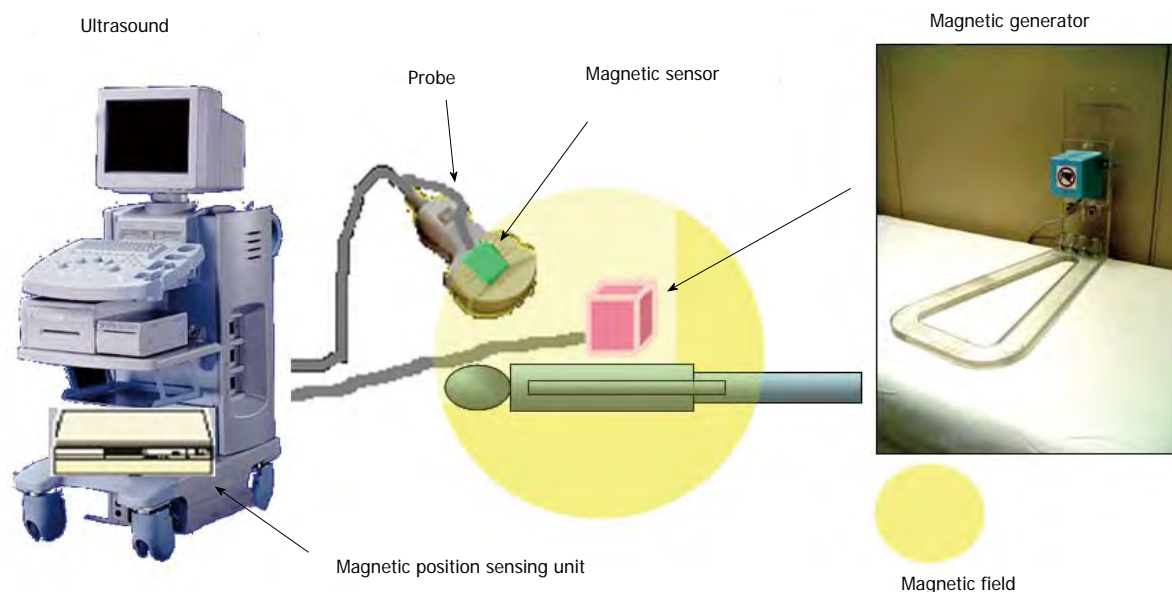


Figure 1 Real-time virtual sonography system. Pre-scanned computed tomography volume data are processed in the main body of the ultrasound machine.

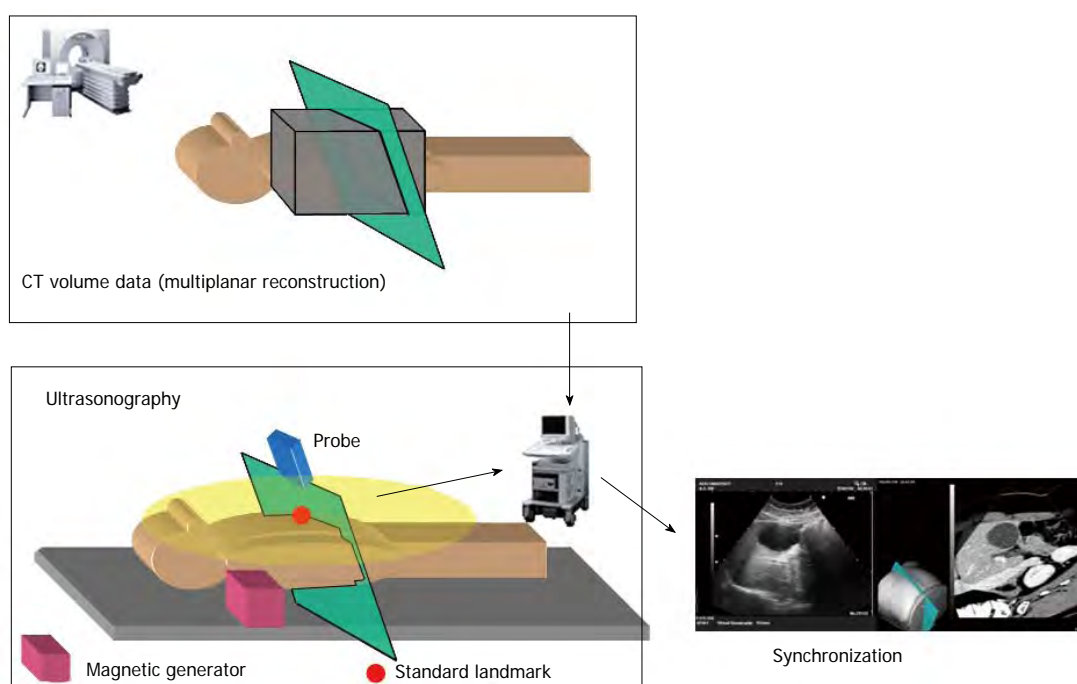


Figure 2 Calibration process. Transfer of the obtained computed tomography (CT) volume data; configuration of the settings for the standard landmark (ensiform cartilage/aorta/portal vein/other); and positional information of the probe is sensed. According to the probe sensor, the multiplanar reconstruction image is shown as an optimal angled plane.

no invasion into the arteries. With RVS, the positional relationship between the CA and the tumor could be evaluated objectively and was useful in judging whether or not surgery should be performed. Similarly, the positional relationship between the tumor mass and the vasculature could be assessed objectively, even in the cases where images of pancreatic cancer (Figure 4A, B) and bile duct cancer (Figure 4C-F) were judged as excellent. It was also useful in understanding the stage of progression and anatomical relationship. A good case (gall bladder cancer) is shown in Figure 4G.

## DISCUSSION

US is a generally subjective examination and is considered to lack objectivity, particularly in the staging of malignant tumors. By combining the objectivity of CT with the obscurity in the free scanning of US, it was possible to easily interpret the relationship between the lesions and the surrounding organs, as well as the position of vascular structures. Based on the results, incorporation of the objectivity of CT into voluntary scanning with an ultrasound probe facilitated understanding of the positional

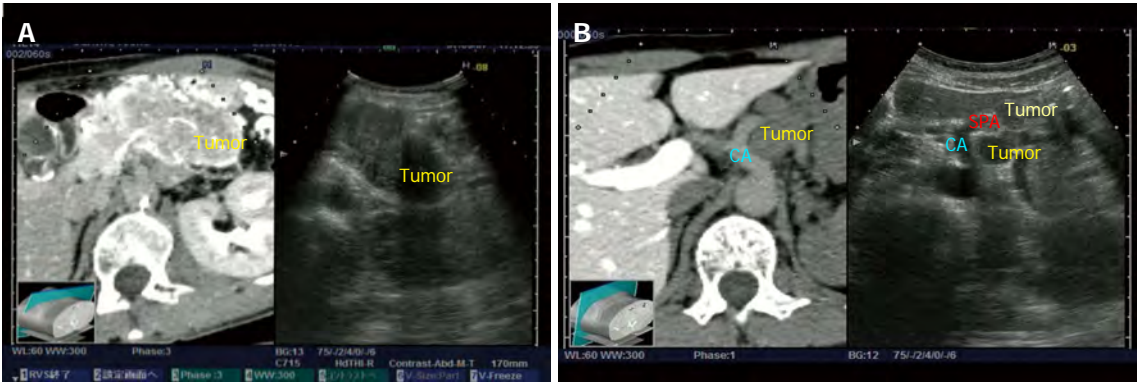


Figure 3 Pancreatic endocrine tumor (excellent case). A: Invasive pancreatic mass (60 mm) in body tail. B: Evaluation of the relationship between celiac artery (CA) and splenic artery (SPA): there was no vascular irregularity and the boundary between the tumor and CA/SPA remained intact.

Table 1 Characteristics of patients

No.	Case	Age (yr)	Sex	BMI (kg/m <sup>2</sup> )	Breath-holding	Evaluation
1	Pancreatic cancer	76	M	18.1	Possible	Excellent
2	Pancreatic endocrine tumor	65	F	23.2	Possible	Excellent
3	Pancreatic cancer	73	F	21.3	Possible	Excellent
4	Pancreatic cancer	77	M	19.2	Possible	Excellent
5	Pancreatic cancer	65	M	22.2	Possible	Good
6	Pancreatic cancer	49	M	19.7	Possible	Excellent
7	Pancreatic cancer	58	F	21.4	Possible	Excellent
8	Bile duct cancer	81	F	12.7	Impossible	Excellent
9	Bile duct cancer	79	F	22.2	Possible	Excellent
10	Bile duct cancer	76	F	21.5	Possible	Excellent
11	Bile duct cancer	68	M	23.3	Possible	Excellent
12	Gallbladder cancer	80	M	25.1	Possible	Good
13	Gallbladder cancer	63	F	21.2	Possible	Good
14	Cholelithiasis	73	M	19.6	Possible	Excellent
15	Cholangiocellular carcinoma	68	F	21.7	Possible	Excellent

M: Male; F: Female; BMI: Body mass index.

relationships among the lesion, the surrounding organs and vasculature, and the structure of the lesion itself. According to the study, this RVS system, combined with US and CT, also confirmed the comparatively precise diagnosis in US imaging. Moreover, this system compensates for each of the deficiencies of US and CT, and it becomes possible to provide more detailed information and make a more precise diagnosis.

US is usually more useful than CT in judging detailed imaging and the relationships with surrounding structures, and moreover, it is possible to consider the RVS system as providing images of detailed processes conveniently and in real time. For example, in Figure 3, it seems that the tumor invaded the blood vessel on CT imaging, but the RVS system showed that the tumor had not invaded the blood vessel. Therefore, RVS can aid in making a precise diagnosis when CT is unable to make judgments on the staging.

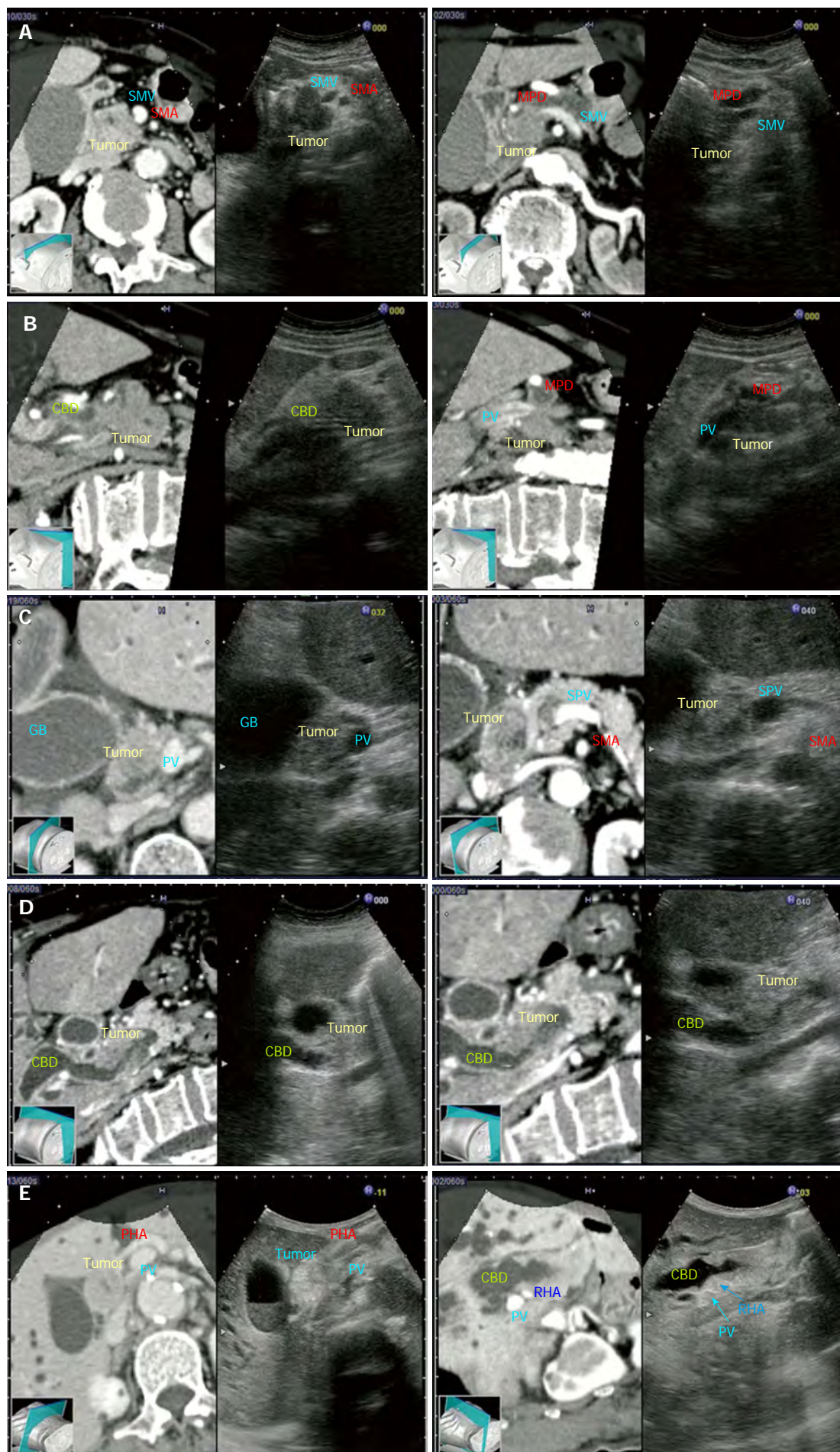
Compared with conventional B-mode US and CT, RVS images achieved a rate of 80% superior visualization and 20% better visualization, according to the statistics.

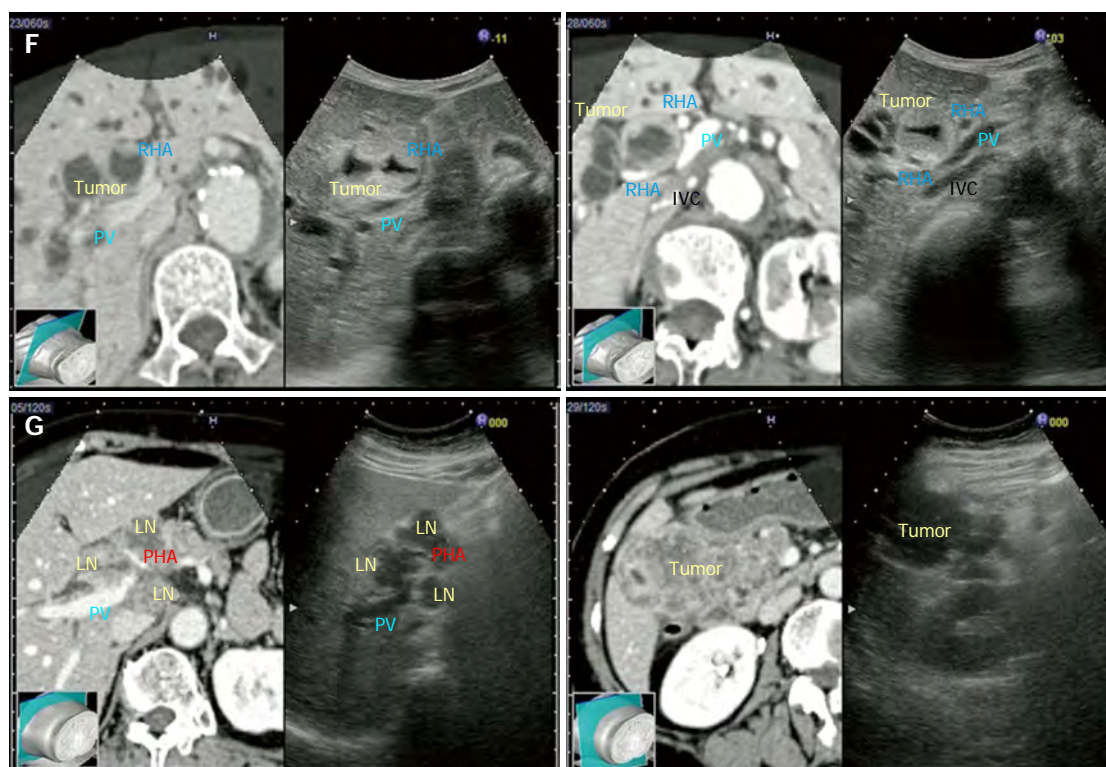
The RVS visualization of lesions and the surrounding organs of all patients provided the objectivity and detailed imaging information for diagnosis lacking in conventional B-mode US and CT. It is also suggested to be useful when applied to making a diagnosis on the existence and stage of progression of malignant tumors, and the evaluation of infiltration into major blood vessels. Further studies in more patients are required to examine the applicability of this method to the diagnosis of malignant diseases and the extent of disease progression, including assessment of infiltration into major blood vessels. This needs to be investigated further in a larger number of patients.

Considering the investigations we have performed using RVS on patients in our department to date, the advantages and disadvantages of RVS are as outlined below. Five advantages are recognized at present: (1) CT objectivity in real-time sonographic images makes it easy to understand the structure and positional relationship between the lesion and the surrounding organs and vasculature; (2) it provides supportive education for surgeons and physicians that lack experience; (3) MPR images obtained by multi-slicing using the familiar operations of the ultrasound probe can be visualized instantly; (4) the display screen can be switched between the arterial phase, portal vein phase, and equilibrium phase (applies to the hemodynamics of the lesion and evaluation of the vasculature) by a simple operation; and (5) the common bile duct and pancreas are not affected by variations in breathing as much as the liver. There are 5 disadvantages: (1) the positions of the images during CT scanning and those of the US images are easily displaced due to breathing variations (liver > biliopancreatic area); (2) with only 1 reference point, synchronization of the position tends to be inadequate; (3) progression, infiltration and vascular evaluation are time-consuming and may put a strain on patients; (4) the focus is mainly on CT, thus US evaluation tends to be poor; and (5) the calibration is time-consuming. It is preferable to perform RVS after considering these advantages and disadvantages.

The main problem in the present RVS system is that when the position is displaced, calibration becomes time-







**Figure 4** The resulting evaluation levels of the real-time virtual sonography images were 12 excellent (pancreatic cancer, bile duct cancer, cholecystolithiasis and cholangiocellular carcinoma) and 3 good (pancreatic cancer and gallbladder cancer). A: Pancreatic cancer (excellent case); the positional relationships among the tumor and vessels [superior mesenteric artery (SMA) and superior mesenteric vein, (SMV)] were well evaluated; B: Pancreatic cancer (excellent case); the positional relationships among the tumor, vessels [portal vein (PV)], common bile dilatation (CBD), and main pancreatic duct (MPD) were well evaluated; C: Bile duct cancer (excellent case); the positional relationships among the tumor and vessels (SMA, SMV and PV) were well evaluated; D: Bile duct cancer (excellent case); the positional relationship between the tumor and CBD was well evaluated; E: Bile duct cancer (excellent case); the positional relationships among the tumor and vessels [proper hepatic artery (PHA), right hepatic artery (RHA), PV] were well evaluated; F: Bile duct cancer (excellent case); the positional relationships among the tumor and vessels [RHA, PV and inferior vena cava (IVC)] were well evaluated; G: Gallbladder cancer (good case); there was a gap in the positional relationships among the tumor and vessels.

consuming. At Hitachi-Aloka Medical Ltd., an ultrasound phantom was prepared to examine the precision, and RVS was performed using simulated CT. As a result, displacement of the position of the slicing plane tended to depend on the distance between the magnetism generator and the magnetic sensor. However, the distance was reported to be approximately  $\pm 5$  mm within a range of 70 cm with the magnetism generator as the center. In addition, with only 1-point calibration, there is displacement of the position. Therefore, multiple-point calibration is important. Precision for calibration needs to be improved by taking these factors into consideration. Moreover, in actual clinical settings, the position of organs may also vary according to the posture and depth of respiration of the subject. Errors can also arise due to these factors; therefore, a corrective function for positional displacement as in the synchronization of breathing needs to be installed in the device. In addition, recent advances in computer and laser technologies have enabled the construction of MPR images of MDCT to be performed easily even with a notebook PC. Taking these points into consideration, one can say that the superiority of RVS is its potential in the areas of therapy and education.

Apart from diagnosis, RVS is now commonly used

to assist treatment under ultrasound guidance in liver disease. Even in the biliopancreatic area, particularly with pancreatic cancer, focused ultrasound treatment and high intensity focused ultrasound (HIFU) treatment are now being performed<sup>[14]</sup>. In the current HIFU, treatment progresses while looking at the 2D ultrasound image. The application of this method allows easy understanding of the range of treatment, and safe treatment can be performed objectively.

This study suggests that RVS is useful when applied to making a diagnosis on the existence and stage of progression of malignant tumors, and making evaluations of infiltration into major blood vessels, in spite of some disadvantages. Further improvement and progress of RVS is expected in the field of therapy.

## COMMENTS

### Background

Ultrasonography (US) is non-invasive, and images can be obtained in real time. However, visualization is sometimes difficult with the presence of bone, gas and air, and thus has the problem of diminished objectivity. Real-time virtual sonography (RVS) is a technological system that was developed in an attempt to resolve such problems. This is one method of compensating for the decreased objectivity in ultrasound diagnosis. This method is used during navigation in



ultrasound-guided treatment and its usefulness has been reported in the field of liver disease; however, no data has been presented in the field of biliopancreatic disease.

### Research frontiers

The frontiers of research are in the fields of diagnosis and therapy for biliopancreatic disease.

### Innovations and breakthroughs

The usefulness of the RVS system has been reported in the field of liver disease; however, no data has been presented in the field of biliopancreatic disease. RVS is useful when applied to making a precise diagnosis on the existence and stage of progression of malignant tumors, and making evaluations of infiltration into major blood vessels. Recently, therapies using ultrasound for biliopancreatic disease have been developed. Further improvement and progress of RVS is expected in the field of therapy.

### Applications

The RVS system combined with US and computed tomography (CT) compensates for each of the deficiencies of US and CT, and it becomes possible to provide more detailed information and make a more precise diagnosis. Moreover, it is possible to consider the RVS system as providing images of detailed processes conveniently and in real time. Therefore, this system is useful in the case in which the detailed information and positional relationships among the lesions are needed for a precise diagnosis.

### Terminology

RVS can be used to observe an ultrasound image in real time by merging the ultrasound image with a multiplanar reconstruction CT image, using pre-scanned CT volume data. The RVS system combined with US and CT compensates for each of the deficiencies of US and CT.

### Peer review

This is a good study of the usefulness of RVS in biliary and pancreatic diseases. Twelve patients with biliary and pancreatic diseases in whom the combination of the objectivity of CT with free scanning using RVS was observed. This study showed that the visualization in biliary and pancreatic diseases using RVS had the objectivity and the detailed imaging for diagnosis lacking in conventional B-mode US and CT. This new technique has potential usefulness in visualizing and diagnosing biliary and pancreatic diseases in the future. Even though the patient numbers were small, the paper has the potential data to be used at the clinic for appropriate diagnosis.

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L- Editor: Cant MR E- Editor: Wu HL



## Hemorrhagic gastric and duodenal ulcers after the Great East Japan Earthquake Disaster

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### Abstract

**AIM:** To elucidate the characteristics of hemorrhagic gastric/duodenal ulcers in a post-earthquake period within one medical district.

**METHODS:** Hemorrhagic gastric/duodenal ulcers in the Iwate Prefectural Kamaishi Hospital during the 6-mo period after the Great East Japan Earthquake Disaster were reviewed retrospectively. The subjects were 27 patients who visited our hospital with a chief complaint of hematemesis or hemorrhagic stool and were diagnosed as having hemorrhagic gastric/duodenal ulcers by upper gastrointestinal endoscopy during a 6-mo period starting on March 11, 2011. This period was divided into two phases: the acute stress phase, comprising the first month after the earthquake disaster, and the chronic stress phase, from the second through the sixth month.

The following items were analyzed according to these phases: age, sex, sites and number of ulcers, peptic ulcer history, status of *Helicobacter pylori* (*H. pylori*) infection, intake of non-steroidal anti-inflammatory drugs, and degree of impact of the earthquake disaster.

**RESULTS:** In the acute stress phase from 10 d to 1 mo after the disaster, the number of patients increased rapidly, with a nearly equal male-to-female ratio, and the rate of multiple ulcers was significantly higher than in the previous year (88.9% vs 25%,  $P < 0.005$ ). In the chronic stress phase starting 1 mo after the earthquake disaster, the number of patients decreased to a level similar to that of the previous year. There were more male patients during this period, and many patients tended to have a solitary ulcer. All patients with duodenal ulcers found in the acute stress phase were negative for serum *H. pylori* antibodies, and this was significantly different from the previous year's positive rate of 75% ( $P < 0.05$ ).

**CONCLUSION:** Severe stress caused by an earthquake disaster may have affected the characteristics of hemorrhagic gastric/duodenal ulcers.

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**Key words:** Great East Japan Earthquake Disaster; Hemorrhagic gastric; Duodenal ulcer; *Helicobacter pylori* infection; Stress

**Core tip:** We determined the characteristics of hemorrhagic gastric/duodenal ulcers in the post-earthquake period within one medical district. We divided hemorrhagic gastric/duodenal ulcers into two groups, the acute stress phase group, consisting of the first month after the earthquake disaster, and the chronic stress phase group, from the second through the sixth month. We concluded that severe stress caused by this

earthquake disaster exacerbated the characteristics of hemorrhagic gastric/duodenal ulcers.

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## INTRODUCTION

Iwate Prefectural Kamaishi Hospital is located in the southern coastal area of Iwate Prefecture and is a regional core hospital with 15 departments and 272 beds in the Kamaishi medical district (Figure 1). This hospital is a core disaster hospital in the medical district, which has a population of approximately 55000. In the Great East Japan Earthquake Disaster that occurred on March 11, 2011, the Pacific coastal regions of East Japan suffered devastating damage from the earthquake itself and the consequent tsunami. Our hospital remained functional enough to accept all patients transported by ambulance from within the medical district, even after the disaster. The East Japan Earthquake Disaster significantly increased the occurrences of cardiovascular disease, respiratory disease, and cerebrovascular disease<sup>[1-9]</sup>. Since the number of patients visiting our hospital with hemorrhagic gastric/duodenal ulcers increased after this disaster, we compared those patients with the analogous patient population in 2010, the pre-earthquake period. This study aimed to elucidate the characteristics of ulcers in the post-earthquake period within our medical district.

## MATERIALS AND METHODS

This study included 27 patients who visited our hospital with a chief complaint of hematemesis or hemorrhagic stool and were diagnosed as having hemorrhagic gastric/duodenal ulcers by upper gastrointestinal endoscopy during a 6-mo period starting on March 11, the day of the earthquake disaster, until September 10, 2011. This period was divided into 2 phases: the acute stress phase, comprising the first month after the earthquake disaster, and the chronic stress phase, from the second through the sixth month. The following items were analyzed according to these phases: age, sex, sites and number of ulcers, peptic ulcer history, status of *Helicobacter pylori* (*H. pylori*) infection, intake of non-steroidal anti-inflammatory drugs (NSAIDs), and degrees of impact of the earthquake disaster. Moreover, these data were compared with those obtained from 27 consecutive patients who visited our hospital with a chief complaint of hematemesis or hemorrhagic stool and were diagnosed as having hemorrhagic gastric/duodenal ulcers by upper gastrointestinal

endoscopy during the same period of the previous year (March 11 to September 10, 2010). Rates of hemorrhagic gastric/duodenal ulcers reportedly increased after the earthquake disaster<sup>[10]</sup>. We performed endoscopy only on patients who presented with a chief complaint of hematemesis or hemorrhagic stool during the first few months after the earthquake disaster, so only patients with confirmed hemorrhagic gastric/duodenal ulcers were included in this study. The status of *H. pylori* infection was determined by serum antibody titer. If the antibody titer was more than 10 U/mL, the patient was considered *H. pylori* positive. The titers of two patients were not examined because they were immediately transferred to other hospitals.

## RESULTS

### Hemorrhagic gastric ulcer

No patients with hemorrhagic gastric ulcers visited our hospital during the first 10 d after the earthquake disaster (Table 1, Figure 2A).

Acute stress phase (the first month after the earthquake disaster): There were 9 patients with ulcers. Compared to the mean number of patients in the previous year (3.3 patients/mo in 2010), this number represented a marked increase of approximately 3-fold after the earthquake disaster, but the difference was not statistically significant (Table 2). The mean patient age was 68.3 years, similar to that of the previous year (69.2 years). The male-to-female ratio was 6:3. Although it was not significantly different from that of the previous year (15:5), the percentage of women was higher in the study period. Regarding ulcer morphology, multiple ulcers were observed in 8 patients (88.9%), and the incidence of this form was significantly higher than in the previous year (25%) ( $P < 0.005$ ). The most common ulcer site was the gastric corpus, seen in 7 patients (77.8%), and the rate for this site was similar to that of the previous year (85%). Four patients had an ulcer history (44.4%), and the proportion of these patients was similar to that of the previous year (35%). Five patients were positive for serum *H. pylori* antibodies (55.6%), which was slightly lower than the previous year (64%) (not statistically significant).

Chronic stress phase (the second to the sixth month after the earthquake disaster): There were 11 patients with ulcers, with the mean number being 2.2 patients/mo, indicating a significant decrease compared to the number in the acute stress phase ( $P < 0.05$ ). Moreover, the number in the chronic stress phase was smaller than that of the previous year (3.3 patients/mo) (not statistically significant). The mean patient age was 65.8 years, again similar to that of the previous year (69.2 years). The male-to-female ratio was 8:3, which was also similar to that of the previous year (15:5). Regarding ulcer morphology, multiple ulcers were observed in 5 patients (45.5%), which was a significantly lower rate than in the acute stress phase ( $P < 0.05$ ). Moreover, the incidence

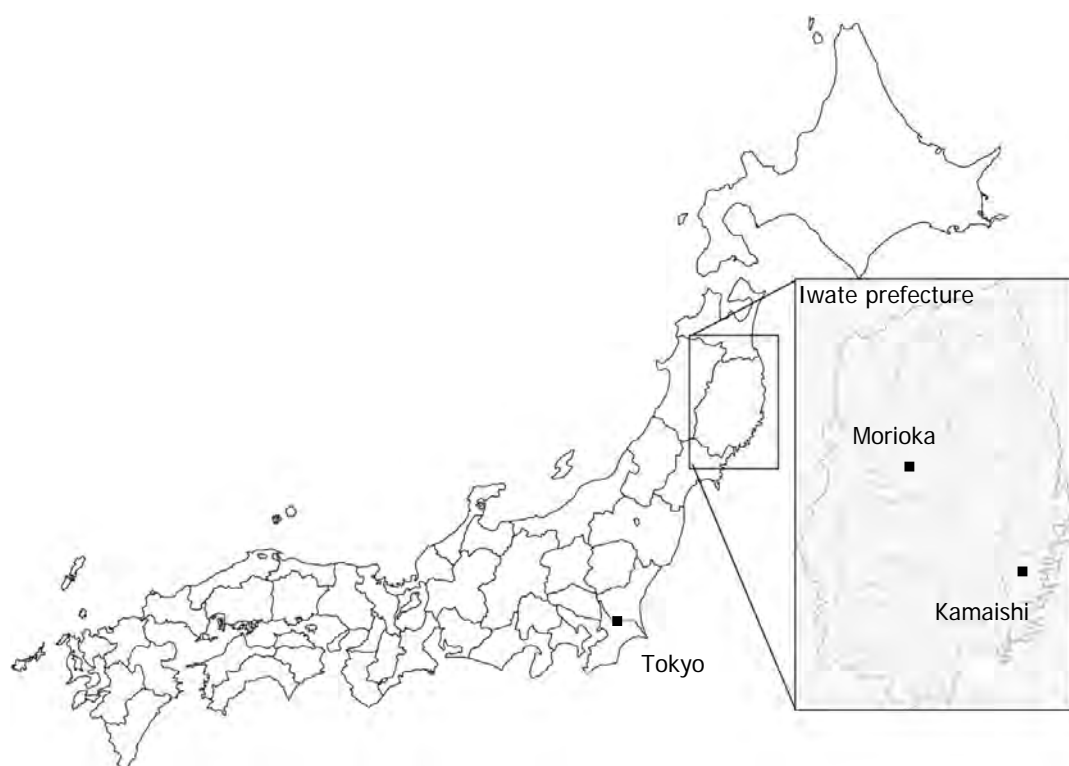


Figure 1 Location of Kamaishi city in Iwate prefecture in Japan.

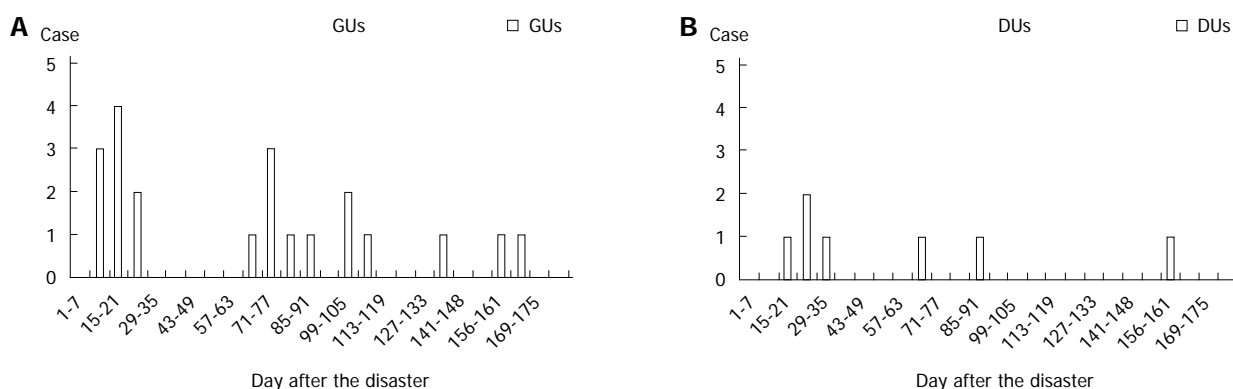


Figure 2 Incidence of hemorrhagic gastric (A) and duodenal (B) ulcers. GUs: Gastric ulcers; DUs: Duodenal ulcers.

in the chronic stress phase was nearly the same as that of the previous year (25%). Regarding ulcer site, ulcers were observed in the gastric corpus in 8 patients (72.7%), a rate that was similar to that of the previous year (85%). Three patients had an ulcer history (27.2%), and the proportion of these patients was nearly the same as that of the previous year (35%). Eight patients were positive for serum *H. pylori* antibodies (72.7%), and the proportion was similar to that of the previous year (64%).

Four of 6 patients who were negative for serum *H. pylori* antibodies used neither oral NSAIDs nor steroids at any time during the study period.

#### Hemorrhagic duodenal ulcer

No patients with hemorrhagic duodenal ulcers visited our hospital during the first 19 d after the earthquake

disaster (Table 3, Figure 2B).

Acute stress phase (the first month after the earthquake disaster): Three patients had ulcers. Compared to the mean number of patients in the previous year (1.2 patients/mo), this number was higher after the earthquake disaster, though it was not statistically significant (Table 2). The mean patient age was 68.6 years, similar to that of the previous year (57.3 years). The male-to-female ratio was 2:1, which was almost the same as that of the previous year (5:2). All patients showed multiple ulcers on the posterior wall. None of the patients had an ulcer history, which was lower than the previous year (75%), though not significantly. All patients were negative for serum *H. pylori* antibodies, and this was significantly different from the previous year (positive rate: 75%) ( $P < 0.05$ ).



**Table 1** Details of patients with gastric ulcers in 2011

Day	Age (yr)	Sex	Location	Number	p.h	<i>Helicobacter pylori</i> antibody titer	Property damage	Dead of family
Acute stage								
11	52	M	U	Multiple	-	+ 17.4	Complete	-
13	67	M	M	Multiple	+	- 6.6	Complete	-
13	64	F	L	Multiple	-	- < 3.0	-	Grandchild
17	80	F	M	Multiple	-	- < 3.0	-	Daughter
17	80	F	M	Multiple	+	+ unknown	Partial	-
17	61	F	M	Multiple	+	+ 31.0	-	-
21	67	M	M	Multiple	+	+ 18.6	-	-
25	62	M	M	Single	-	+ 16.3	Complete	-
27	82	M	M	Multiple	-	- 6.5	Partial	-
Chronic stage								
66	76	F	M	Multiple	+	- < 3.0	Complete	-
72	73	M	U	Multiple	-	+ 22.7	Complete	-
76	59	M	U	Single	-	+ 15.5	Complete	-
77	54	M	M	Single	-	+ 22.1	-	-
83	75	F	M	Single	-	+ 33.3	Complete	-
99	49	M	M	Single	-	- 5.2	-	-
101	67	M	M	Multiple	-	- 8.9	-	-
110	57	M	M	Multiple	+	+ 12.1	Complete	-
136	75	M	M	Single	-	+ unknown	-	-
158	82	F	U	Multiple	+	+ 27.4	-	-
164	54	M	M	Single	-	+ 25.3	Complete	Daughter

p.h.: Past history of peptic ulcer; U: Upper lesion; M: Middle lesion; L: Lower lesion.

**Table 2** Characteristics of patients with gastric ulcers and duodenal ulcers

	2011 acute stage	2011 chronic stage	2010	P value
GUs				
Number of patients (n)	9	11	20	NS
Average number of patient per month	9	2.2	3.3	< 0.05 <sup>a</sup>
Age (yr)	68.3	65.5	69.2	NS
Male/female ratio	1.3 (5/4)	2.7 (8/3)	3 (15/5)	NS
Simple/multiple ratio	0.13 (1/8)	1.2 (6/5)	3 (15/5)	< 0.05 <sup>a</sup> < 0.005 <sup>d</sup>
<i>Helicobacter pylori</i> (+)/(-)	6/3	8/3	7/4 (unknown 9)	NS
DUs				
Number of patients (n)	3	4	7	NS
Average number of patients per month	3	0.8	1.2	NS
Age (yr)	68.6	56.3	57.3	NS
Male/female ratio	2 (2/1)	3 (3/1)	2.5 (5/2)	NS
Simple/multiple ratio	0.5 (1/2)	3 (3/1)	1.3 (4/3)	NS
<i>Helicobacter pylori</i> (+)/(-)	0/3	1/3	4/2 (unknown 1)	< 0.05 <sup>a</sup>

GUs: Gastric ulcers; DUs: Duodenal ulcers. <sup>a</sup>*P* < 0.05 between 2011 acute stage vs 2011 chronic stage; <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 between 2011 acute stage vs 2010.

Chronic stress phase (the second to the sixth month after the earthquake disaster): Four patients had ulcers, and the mean number of 0.8 patient/mo was similar to that of the previous year (1.2 patients/mo). The mean patient age was 56.3 years, again similar to that of the previous year (57.3 years). The male-to-female ratio was 3:1, which was also quite similar to that of the previous year (5:2). Although 4 patients (100%) had an ulcer history, there was no significant difference from the previous year (43%). Three patients were positive for serum *H. pylori* antibodies (75%), and this proportion was similar to that of the previous year (67%).

## DISCUSSION

Both *H. pylori* infection and NSAIDs have major roles

in the pathogenesis of peptic ulcers<sup>[11-16]</sup>. Stress is also considered an important causal factor in peptic ulcer disease<sup>[17]</sup>, and there are studies on the involvement of *H. pylori* infection and stress in the incidence of peptic ulcers after the Great Hanshin-Awaji Earthquake Disaster in 1995<sup>[18-20]</sup>.

In the Great East Japan Earthquake Disaster that occurred on March 11, 2011, the Kamaishi medical district (population of approximately 55000), where our hospital is located, also suffered serious damage due to the tsunami, and the combined number of dead and missing persons was approximately 2300. Many disaster victims also lost their homes. They were forced to stay at shelters or to live without adequate infrastructure, such as gas, water, and electricity supplies. These disaster victims experienced severe and unprecedented stress,

**Table 3** Details of patients with duodenal ulcers in 2011

Day	Age (yr)	Sex	Location	Number	p.h.	<i>Helicobacter pylori</i> antibody titer	Property damage	Dead family
Acute stage								
19	85	M	AW/PW	Multiple	-	< 3.0	Complete	Wife
23	51	M	PW	Single	-	< 3.0	Complete	-
25	70	F	PW	Multiple	-	< 3.0	Complete	Son
Chronic stage								
35	59	M	PW	Single	+	3.4	Complete	-
65	72	F	AW/PW	Multiple	+	10.3	-	-
87	59	M	PW	Single	+	15.5	-	-
159	35	M	AW	Single	+	34.3	-	-

p.h.: Past history of peptic ulcer; AW: Anterior wall; PW: Posterior wall.

which persisted for a considerable period of time. In this study, the acute stress phase was defined as the first month, when numerous aftershocks occurred and when the disaster victims lived under extremely severe stress due to poor living conditions. The chronic stress phase was defined as the period from the second through sixth month after the earthquake disaster. We analyzed the clinical and demographic data of ulcer patients for each period.

Because the emergency transport routes were restored the day after the earthquake, many patients were transported to our hospital immediately after this disaster. Nevertheless, the day when the first patient visited our hospital with a chief complaint of hematemesis or hemorrhagic stool after the earthquake was March 22, 11 d after the disaster. Subsequently, the number of patients visiting our hospital with one of these chief complaints gradually increased, and 12 patients (mean: 3 patients/wk) with hemorrhagic gastric/duodenal ulcer visited in the first month. Because the mean number of patients with hemorrhagic gastric/duodenal ulcers for a period of 6 mo starting March 11, 2010, had been 1.13 patients/wk, there was an apparent increase after the March 11, 2011 earthquake disaster. However, the number rapidly decreased after the first month, and the subsequent mean number was 0.75 patient/wk, which was slightly lower than that of the previous year.

Matsuura *et al.*<sup>[21]</sup> studied upper gastrointestinal bleeding after the Great East Japan Earthquake Disaster and found that there were few patients immediately after the earthquake disaster but that the number increased from a few days to 1 mo after the disaster. According to a study conducted in 1998 on peptic ulcers occurring after the Great Hanshin-Awaji Earthquake Disaster<sup>[18]</sup>, Aoyama *et al.*<sup>[18]</sup> also reported that the number of patients decreased during the first 2 wk after the earthquake disaster as compared to the previous year, then increased during the third through fourth weeks, and returned to a similar level to that of the previous year from the fifth through sixth weeks. Takakura *et al.*<sup>[22]</sup> also reported that the number of patients with gastric ulcers increased by 4-fold 2 wk after the Great Hanshin-Awaji Earthquake Disaster, gradually decreased thereafter, and it had returned to near the level of the previous year 2.5 mo later. Our data show a

similar trend to these reports. The increase in the number of patients during the acute stress phase strongly suggests that severe stress due to the violent earthquake and consequent tsunami contributed to the ulcer incidence. We have no clear explanation why the incidence of hemorrhagic gastric/duodenal ulcers was low during the first 10 d after the earthquake disaster, a time when the disaster victims were under extremely severe stresses concerning matters of life and death. It was assumed that stressful conditions might need to persist for a certain period of time before the breakdown of biological defense mechanisms.

The male-to-female ratio of gastric ulcer patients was 6:3. However, the ratio in the chronic stress phase was 8:3, a higher percentage of men. The examination of the data obtained during the same period in 2010 at our hospital also shows that the male-to-female ratio was 15:5, indicating a trend for a much higher percentage of men. Moreover, a study conducted in Japan found that the male-to-female ratio among peptic ulcer patients was 71:29, indicating that this disease occurs approximately 2.4 times more frequently in men than in women<sup>[23]</sup>. Based on a comparison of these data, an approximately equal incidence between men and women might be one of the characteristics of gastric ulcer presumably caused by acute stress after an earthquake disaster. Meanwhile, the male-to-female ratios for duodenal ulcer were 2:1 in the acute stress phase and 3:1 even in the chronic stress phase. These ratios were essentially consistent with the trends observed in most studies, and we assume that there are gender differences in the incidence of gastric and duodenal ulcers caused by acute stress.

The mean age of patients with gastric ulcers was 68.3 or 65.5 years old in the acute stress phase or chronic stress phase, respectively, which were not significantly different. The mean ages of those with duodenal ulcers were 68.6 and 56.3 years, respectively, indicating that the patients tended to be older in the acute stress phase.

Regarding the number of gastric ulcer lesions, multiple ulcers were observed in 8 (88.9%) of 9 patients in the acute stress phase. On the other hand, multiple ulcers were observed in 5 (45.5%) of 11 patients in the chronic stress phase, and an increase in the number of patients with a solitary ulcer was documented. Regarding

the number of duodenal ulcer lesions, multiple ulcers were observed in 2 (66.7%) of 3 patients in the acute stress phase. In the chronic stress phase, only 1 (25%) of 4 patients had multiple ulcers. According to the data on patients at our hospital during the same period in 2010, 5 (25%) of 20 patients had multiple gastric ulcers, and 3 (42.9%) of 7 patients had multiple duodenal ulcers. Thus, both hemorrhagic gastric and duodenal ulcers in the acute stress phase were often multiple. Kanno *et al.*<sup>[24]</sup> reported that post-disaster hemorrhagic ulcers were frequently observed in the stomach as multiple ulcers at the same time.

Five (55.6%) of 9 patients in the acute stress phase and 8 (72.7%) of 11 patients in the chronic stress phase were positive for *H. pylori* antibodies. All patients of hemorrhagic gastric/duodenal ulcers had no history of eradication therapy of *H. pylori*. During the same period in 2010 (excluding unknown cases), 7 (63.6%) of 11 patients were *H. pylori* positive, a number similar to that after the earthquake disaster. In contrast, none of the 3 patients with duodenal ulcers was positive for *H. pylori* antibodies in the acute stress phase, and this incidence was significantly lower than that of the chronic stress phase, in which 4 (100%) of 4 patients were positive. During the same period in 2010, 4 (66.7%) of 6 patients with duodenal ulcers were positive for *H. pylori* antibodies. The rate of peptic ulcer not associated with the use of drugs, such as NSAIDs, in Japanese patients negative for *H. pylori* infection was reported by Aoyama *et al.*<sup>[25]</sup> to be 1.9% to 5.1% and by Nishikawa *et al.*<sup>[26]</sup> to be 1.3%. However, Kanno *et al.*<sup>[10]</sup> reported that patients with non-*H. pylori* and non-NSAID gastroduodenal ulcers accounted for as many as 24% of cases after the Great East Japan Earthquake Disaster, and their analysis showed that there were many cases with ulceration attributable to stress. In this study, 8 (29.6%) of 27 patients, including those with gastric/duodenal ulcers, were negative for *H. pylori* antibodies and had not taken oral NSAIDs. The incidence of such cases was extremely high. All 8 patients were experiencing overwhelming stress. For example, they had dead or missing family members or were forced to stay at shelters due to the complete destruction of their homes. This raises the possibility that hemorrhagic gastric/duodenal ulcers can be caused by stress alone.

We did not encounter patients with hemorrhagic acute gastric mucosal lesion that might have been induced by primary infection by *H. pylori*. There is a minor possibility that those patients got acutely infected by *H. pylori* and were not yet serologically positive. However, no incident of waterborne infection was reported in this area during the period, and the quality of most sources of drinking water was maintained in a hygienic state. We do not think that a majority of them got infected by *H. pylori* during the study period.

As to the location of hemorrhagic duodenal ulcers, all patients had ulcers in the posterior wall of the bulb in the acute stress phase. The proportion of these patients was higher than in the chronic stress phase and the previous year. Although the difference between

these proportions was not statistically significant, stress-induced duodenal ulcers without *H. pylori* infection may tend to occur in the posterior wall of the bulb.

Our study has some limitations. This was an observational analysis that was performed on a limited closed lesion with a small sample size. Under the confused and extraordinary circumstances in the aftermath of the great disaster, detailed examinations were difficult to perform. Endoscopic examinations were mainly performed for hemorrhagic gastric/duodenal ulcers. Therefore, the pathophysiology and incidence of non-hemorrhagic and asymptomatic peptic ulcers are unknown. However, we expect that this study has some useful information for the care of hemorrhagic gastro/duodenal injury at times of great disasters.

In conclusion, severe stress caused by an earthquake disaster may have affected the characteristics of hemorrhagic gastric/duodenal ulcers.

## COMMENTS

### Background

Under severe stress, the incidence of hemorrhagic gastric/duodenal ulcers increases. However, there are few reports on the incidence and characteristics of hemorrhagic gastric/duodenal ulcers after a great earthquake disaster. Since the number of patients visiting our hospital with hemorrhagic gastric/duodenal ulcers increased after the Great East Japan Earthquake Disaster, the authors compared those patients with hemorrhagic gastric/duodenal ulcer patients in 2010, the pre-earthquake period. This study aimed to elucidate the characteristics of ulcers in the post-earthquake period within one medical district.

### Research frontiers

It is not well known whether severe stress independently causes hemorrhagic gastric/duodenal ulcers without *Helicobacter pylori* (*H. pylori*) infection and non-steroidal anti-inflammatory drug (NSAID) use.

### Innovations and breakthrough

In this medical district, all patients who developed gastric/duodenal ulcers were admitted to one hospital. Therefore, interesting differences in the characteristics of hemorrhagic ulcers before and after the earthquake disaster were observed. Moreover, it is presumed that severe stress caused by this earthquake disaster may have affected the characteristics of hemorrhagic gastric/duodenal ulcers without *H. pylori* infection and NSAIDs.

### Applications

This study will provide some useful information for the care of hemorrhagic gastro/duodenal injuries at the time of a great disaster.

### Terminology

The Great East Japan Earthquake Disaster occurred on March 11, 2011. The Pacific coastal regions of East Japan suffered devastating damage by the earthquake itself and the consequent tsunami.

### Peer review

This is an important study in which dynamic changes in the characteristics of complicated peptic ulcer patients is depicted. Taking care of patients and performing such an analysis in a devastating circumstance is encouraging to all.

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## Differentiation between dysplastic nodule and early-stage hepatocellular carcinoma: The utility of conventional MR imaging

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### Abstract

**AIM:** To elucidate the variety of ways early-stage hepatocellular carcinoma (HCC) can appear on magnetic resonance (MR) imaging by analyzing T1-weighted, T2-weighted, and gadolinium-enhanced dynamic studies.

**METHODS:** Seventy-three patients with well-differentiated HCC (wHCC) or dysplastic nodules were retrospectively identified from medical records, and new histological sections were prepared and reviewed. The tumor nodules were categorized into three groups: dysplastic nodule (DN), wHCC compatible with Edmondson-Steiner grade I HCC (w1-HCC), and wHCC compatible with

Edmondson-Steiner grade II HCC (w2-HCC). The signal intensity on pre-contrast MR imaging and the enhancing pattern for each tumor were recorded and compared between the three tumor groups.

**RESULTS:** Among the 73 patients, 14 were diagnosed as having DN, 40 were diagnosed as having w1-HCC, and 19 were diagnosed as having w2-HCC. Hyperintensity measurements on T2-weighted axial images (T2WI) were statistically significant between DNs and wHCC ( $P = 0.006$ ) and between DN and w1-HCC ( $P = 0.02$ ). The other imaging features revealed no significant differences between DN and wHCC or between DN and w1-HCC. Hyperintensity on both T1W out-phase imaging ( $P = 0.007$ ) and arterial enhancement on dynamic study ( $P = 0.005$ ) showed statistically significant differences between w1-HCC and w2-HCC. The other imaging features revealed no significant differences between w1-HCC and w2-HCC.

**CONCLUSION:** In the follow-up for a cirrhotic nodule, increased signal intensity on T2WI may be a sign of malignant transformation. Furthermore, a noted loss of hyperintensity on T1WI and the detection of arterial enhancement might indicate further progression of the histological grade.

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**Key words:** Dysplastic nodule; Hepatocellular carcinoma; Histological grading; Magnetic resonance imaging; Well-differentiated hepatocellular carcinoma

**Core tip:** The aim of this article was to differentiate between early-stage hepatocellular carcinoma (HCC) and dysplastic nodules using conventional magnetic resonance (MR) imaging. We found that conventional MR imaging could provide additional information to dif-

ferentiate between early-stage HCC and dysplastic nodules in equivocal lesions. During follow-up for a cirrhotic nodules, increased signal intensity on T2-weighted axial images may be a sign of malignant transformation. Loss of hyperintensity on T1WI and the detection of arterial enhancement may indicate further progression of the histological grade.

Chou CT, Chou JM, Chang TA, Huang SF, Chen CB, Chen YL, Chen RC. Differentiation between dysplastic nodule and early-stage hepatocellular carcinoma: The utility of conventional MR imaging. *World J Gastroenterol* 2013; 19(42): 7433-7439 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7433.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7433>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed malignant tumors in the world. HCC occurs primarily in patients with chronic liver disease such as hepatitis B and C infections<sup>[1,2]</sup>. HCC develops by means of a multi-step dedifferentiation process that progresses from regenerative nodule to dysplastic nodule (DN) and then to HCC<sup>[3]</sup>. Early detection of HCC in cirrhotic livers is important to improve patient outcomes and decision-making to determine optimum therapeutic strategies<sup>[4]</sup>. A follow-up system for high-risk HCC populations has been established. Additionally, research and technology have enabled increasing numbers of small nodular lesions to be detected. According to the practice guidelines of the American Association for the Study of Liver Diseases (AASLD), HCC can be diagnosed noninvasively in at-risk patients, who typically demonstrate arterial-phase enhancement and venous- or delayed-phase washout on dynamic computed tomography (CT) or magnetic resonance imaging (MRI)<sup>[5]</sup>. However, most of these nodules with characteristic CT or MR patterns are overt HCC. The percentage of well-differentiated HCCs showing typical hypervascularity in the arterial-phase followed by washout in the delayed-phase on a dynamic study ranges from 13% to 50%<sup>[6,7]</sup>. Therefore, well-differentiated HCCs are increasingly detected in our daily practice, yet many represent diagnostic difficulties.

Gadoxetic acid (Gd-EOB-DTPA)-enhanced MRI has been demonstrated to be useful in differentiating between early HCC and dysplastic nodules in several recent studies<sup>[8,9]</sup>. However, the overlap between dysplastic nodules and HCC on gadoxetic acid-enhanced MR presentations has also been mentioned in the literature<sup>[10,11]</sup>. Due to the facts that gadoxetic acid is not available in every country and is costly, conventional MRI remains an important tool in differentiating between cirrhotic nodules and early HCC. Conventional MR imaging protocols rely on T1-weighted and T2-weighted imaging and multiphase dynamic gadolinium-enhanced imaging to depict and characterize tumors<sup>[12,13]</sup>. To the best of our knowledge,

the role of conventional MR sequences in differentiating between early-stage HCC and dysplastic nodule in cirrhotic liver has not yet been established. The purpose of our study was to elucidate the variety ways of early-stage HCC can appear on MR imaging by analyzing T1-weighted, T2-weighted, and gadolinium-enhanced dynamic studies.

## MATERIALS AND METHODS

### Patient population

Approval for this retrospective study was obtained from the institutional review board of our hospital. A flow-chart for the patient selection process is shown in Figure 1. Ultimately, 73 patients (51 men and 22 women; mean age, 61 years old; range, 26-82 years) were enrolled in this study. Among them, 70 patients underwent liver biopsy, and 3 patients underwent hepatectomy. All 73 patients had liver cirrhosis: 29 patients had hepatitis B virus; 28 patients had hepatitis C virus; 9 patients had hepatitis B and C co-infections; and 7 patients had alcoholic cirrhosis. Among the 73 patients, 49 patients had Child-Pugh class A cirrhosis, and 24 patients had Child-Pugh class B cirrhosis. The diagnoses of liver cirrhosis were all based on histopathological examination.

### Histopathological diagnosis

New histological sections from the original paraffin blocks of all 73 patients' liver tumors were prepared using hematoxylin and eosin stain. The diagnostic features for HCC included the following: (1) increased cell density >2 times the density of the surrounding tissue, with an increased nuclear:cytoplasmic ratio; (2) thickened liver cell plates (2 cells or more); (3) pseudoglandular structure formations; (4) unpaired arteries; (5) sinusoidal capillarization; and (6) stromal invasion without ductular reaction at the periphery of the nodules<sup>[14,15]</sup>. Immunohistochemical stains for CD10 (Biocare Medical, Concord, CA, United States), CD34 (DAKO, Glostrup, Denmark) and cytokeratin 7 (DAKO, Glostrup, Denmark) were also performed to facilitate interpretation. The tumor nodules were categorized into three groups: DN, well-differentiated HCC compatible with Edmondson-Steiner grade I HCC (w1-HCC)<sup>[16]</sup>, and well-differentiated HCC compatible with Edmondson-Steiner grade II HCC [which also included well-differentiated HCC in the World Health Organization system (w2-HCC)]<sup>[17]</sup>.

Two pathologists with more than 10 years of experience in hepatic pathology reviewed all the histological sections independently. Differences between the two reviewers were resolved by a third pathologist to reach a final consensus. All three of the pathologists were blinded to the original pathology diagnoses and the clinical information.

The presence of high cellularity, diffuse capillarization, abnormal biliary canaliculi and stromal invasion were considered features of w1-HCC. The additional presence of nuclear pleomorphism and thicker trabecular growth further upgraded a lesion to, w2-HCC.

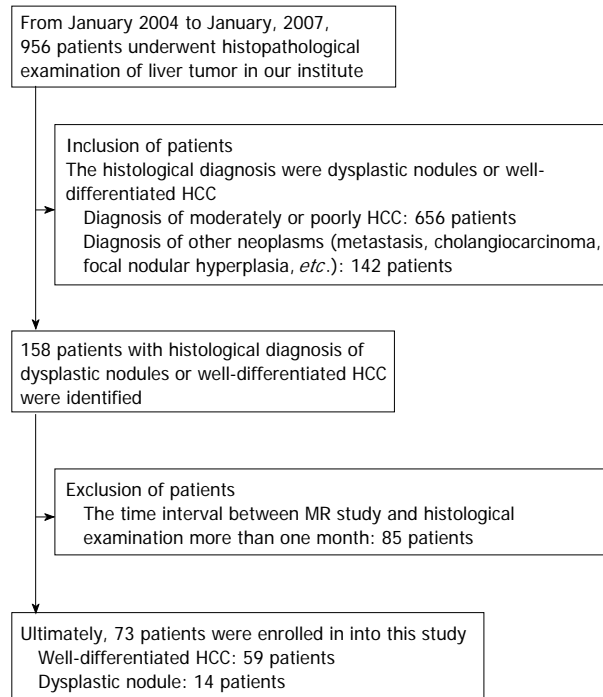


Figure 1 Flowchart of the patient selection process. HCC: Hepatocellular carcinoma; MR: Magnetic resonance.

### MR imaging

MR imaging of livers was performed using a 1.5-T MR scanner (Philips Gyroscan ACS-NT Powertrak 6000, release version 6.7.2, Best, the Netherlands) and a phased-array body coil. Turbo spin-echo (TSE) T2-weighted axial images [T2WI, TR/TE: 2500/90 ms, slice thickness/gap: 8/0.8 mm, matrix:  $192 \times 256$ , number of average (NEX): 2, TSE factor: 23, field of view (FOV): 38-40 cm, typical scanning time: 2 min 20 s] with and without fat saturation (FS, spectral fat saturation inversion recovery) and coronal T2-weighted images were obtained under respiratory trigger. Dual-echo T1-weighted imaging (TR/TE: 210/2.3 ms and 4.6 ms, slice thickness/gap: 8/0.8 mm, matrix:  $192 \times 256$ , NEX: 1, FOV: 38-40 cm, typical scanning time: 24 s) was also performed during one breath hold. Automatic shimming was applied for fat-suppression imaging to maximize magnetic field homogeneity, and flow compensation was also used.

For contrast-enhanced MR imaging, gadodiamide (Omniscan, GE Healthcare, Oslo, Norway) was administered by bolus injection (approximate rate of 2 mL/s) through a peripheral vein at a dosage of 0.1 mmol/kg. Dynamic T1-weighted fast field echo imaging (175-210/1.3-2.1, flip angle:  $80^\circ$ , matrix:  $192 \times 256$ , NEX: 1, FOV: 38-40 cm) was performed just before, 18-20 s after and 50-55 s after the contrast agent was injected. An equilibrium phase FS-T1W (TR/TE 241-344/2.7 ms, flip angle:  $70^\circ$ , slices thickness/gap: 8/0.8 mm) imaging was performed 180 s after the contrast agent injection.

### Imaging analysis

The imaging analysis was performed at a dual-screen

diagnostic workstation (GE Healthcare, Milwaukee, WI, United States). In each image assessment, liver maps were completed by drawing each individual liver lesion on a respective map according to the Couinaud system of liver anatomy. These drawings were made as accurately as possible by one investigator. All the imaging results were analyzed using visual assessment by two radiologists who each had more than 10 years of experience in abdominal MR imaging. The two observers were blinded to the clinical information and final diagnoses, and they recorded the lesion signal intensities on pre-contrast T1WI and T2WI, post-contrast T1WI and the enhancement pattern during dynamic study. The signal intensity of the focal liver nodule on dual-echo T1WI and T2WI was classified as hypointense, isointense, or hyperintense compared with adjacent liver parenchyma. The enhancement pattern of the HCC was visually classified into one of the following patterns: hypovascular, isovascular or hypervascular enhancement compared with the adjacent liver parenchyma. Any disagreements between the two reviewers were resolved by consensus with a third radiologist who was also blinded to the clinical information and final diagnoses.

### Statistical analysis

The interobserver agreement was evaluated using the kappa statistic<sup>[18]</sup>. Continuous variables such as age, tumor size, and alpha-fetoprotein were analyzed using the Kruskal-Wallis test. Categorical variables such as signal intensity and enhancement pattern were analyzed using Pearson's  $\chi^2$  test. The diagnostic performance of the HCC diagnostic criteria was evaluated along with a receiver operating characteristic analysis. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

Among the 73 patients in our study, 14 were diagnosed with a DN, 40 were diagnosed with w1-HCC, and 19 were diagnosed with w2-HCC. The clinicopathological characteristics of the growths are shown in Table 1, which shows no significant differences between the three patient groups. The interobserver agreement for the imaging analysis between the two radiologists was either good or excellent (Table 2).

Univariate analyses of MR findings for patients with DNs and wHCC are shown in Table 3. Only a hyperintense signal on T2WI was a statistically significant predictor of wHCC ( $P = 0.006$ ), whereas the other imaging features revealed no significant associations with DN and wHCC. Therefore, well-differentiated HCCs were divided into w1-HCC and w2-HCC, and the differences between DN *vs* w1-HCC and w1-HCC *vs* w2-HCC were analyzed. Only hyperintensity on T2WI was a statistically significant differentiator of DN from w1-HCC ( $P = 0.02$ ). The other imaging features revealed no significant differences between DN and w1-HCC. Hyperintensity on T1W out-phase imaging ( $P = 0.007$ ) and arterial enhancement on dynamic study ( $P = 0.005$ ) showed statistically significant

**Table 1** Clinical characteristics of the 73 cirrhotic patients with liver nodules

	Histopathological diagnosis			P value
	DN (n = 14)	w1-HCC (n = 40)	w2-HCC (n = 19)	
Age (mean ± SD, yr)	54.9 ± 12.9	62.4 ± 12.2	63.4 ± 10.8	0.129
Sex				0.629
Male	11	27	12	
Female	3	13	7	
Underlying liver disease				0.640
HBV	7	15	7	
HCV	3	15	10	
HBV + HCV	2	6	1	
Alcoholism	2	4	1	
Child-Pugh class				0.645
A	10	25	14	
B	4	15	5	
Tumor size (mean ± SD, cm)	1.8 ± 0.6	2.1 ± 0.7	2.9 ± 2.3	0.365
AFP (mean ± SD, ng/mL)	17.1 ± 27.9	55.5 ± 117.9	64.2 ± 169.7	0.202
< 20 ng/mL	12	28	13	0.790
≥ 20 ng/mL	2	12	6	
> 20, ≤ 100 ng/mL	2	7	4	
> 100, ≤ 200 ng/mL	0	0	1	
> 200 ng/mL	0	5	1	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetal protein; DN: Dysplastic nodule; w1-HCC: Well-differentiated hepatocellular carcinoma compatible with Edmondson-Steiner grade I; w2-HCC Well-differentiated hepatocellular carcinoma compatible with Edmondson-Steiner grade II.

differences between w1-HCC and w2-HCC. However, T2WI and other imaging sequences showed no significant differences between w1-HCC and w2-HCC.

Using hyperintensity on T2WI as the sole criteria in differentiating wHCC from a dysplastic nodule, 25 of 59 wHCCs could be correctly diagnosed. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 42%, 93%, 96% and 28%, respectively. Solely using the AASLD criteria (arterial enhancement followed by washout on dynamic imaging) to differentiate between wHCC and dysplastic nodules, only 9 of 59 wHCCs (w1-HCC: 2, w2-HCC: 7) were correctly diagnosed. The sensitivity, specificity, PPV and NPV were 15%, 93%, 90% and 21%, respectively. If the diagnostic criteria were changed to either a hyperintense nodule on T2WI or a lesion that demonstrated arterial enhancement followed by washout on dynamic imaging, 27 of 59 wHCCs were correctly diagnosed. The sensitivity, specificity, PPV, and NPV were 46%, 86%, 93%, and 27%, respectively. Eighteen wHCCs were further detected by the additional hyperintense-on-T2WI criterion according to the AASLD criteria. The diagnostic performance of the AASLD criteria with additional hyperintense findings on T2WI was superior to the AASLD criteria alone in the characterization of early-stage HCC ( $P = 0.013$ ).

## DISCUSSION

The development of HCC in the cirrhotic liver is de-

**Table 2** Interobserver agreement for magnetic resonance features

MR features	k value
T1-weighted in-phase imaging	0.853
T1-weighted opposed-phase imaging	0.870
Fatty metamorphosis	0.819
T2-weighted imaging	0.828
Arterial enhancement	0.806
Late-phase T1-weighted imaging	0.743

MR: Magnetic resonance.

scribed as either *de novo* hepatocarcinogenesis or as the result of a multistep progression. The stages of the multistep progression originate from a dysplastic nodule, which progresses to a dysplastic nodule with HCC foci, followed by a small HCC, and finally to an overt carcinoma<sup>[19,20]</sup>. In our study, typical arterial enhancing features were only detected in 12 (12/40) w1-HCCs, and there were no significant differences between DN and w1-HCC. In contrast, the arterial enhancing feature was demonstrated in 13 (13/19) w2-HCCs and showed a statistically significant difference between w1-HCC and w2-HCC ( $P = 0.005$ ). Our results might explain why the percentage of well-differentiated HCCs with arterial enhancement ranges from 43% to 66% in the current literature<sup>[21-23]</sup>. These figures may have resulted from the varied compositions of w1-HCC and w2-HCC within the studies.

The arterial enhancement during dynamic study showed a statistically significant difference between w1-HCC and w2-HCC in our study. This finding might be due to the insufficient development of non-triadal arteries in early-stage HCCs<sup>[14,24]</sup>. Kojiro<sup>[25]</sup> also reported that early HCCs tended to demonstrate histological hypovascularity. Kim *et al*<sup>[26]</sup> reported that the histological grade of HCC was an important factor influencing therapeutic results. They further suggested that treatment could be more effective after radiofrequency ablation in patients with histologically low-grade HCC. According to our results, wHCC demonstrated arterial enhancement during dynamic study, and the histological differentiation tended to be of a higher grade, so it should be treated aggressively.

In our results, only 9 wHCCs (15%) satisfied the AASLD HCC diagnostic criteria. In contrast, T2WI hyperintensity was the only imaging feature that allowed differentiation between wHCC and DN or w1-HCC and DN. The diagnostic performance of the AASLD criteria with additional hyperintensity on T2WI was superior to the AASLD criteria alone in the characterization of early-stage HCCs ( $P = 0.013$ ). Ouedraogo *et al*<sup>[27]</sup> also reported that adding T2W hyperintensity to the AASLD criteria increased the detection of HCC, especially in nodules smaller than 20 mm. According to our results, hyperintensity on T2WI alone could offer additional information in dynamic studies to differentiate between wHCC and DN, and a cirrhotic nodule that is hyperintense on



**Table 3** Comparison of magnetic resonance features between dysplastic nodule and well-differentiated hepatocellular carcinoma

	DN (n = 14)	wHCC		P value		
		w1 (n = 40)	w2 (n = 19)	DN vs wHCC	DN vs w1	w1 vs w2
In-phase T1WI				0.469	0.405	0.074
Hyperintense	8	29	8			
Isointense	4	5	4			
Hypointense	2	6	7			
Opposed-phase T1WI				0.661	0.565	0.007
Hyperintense	8	25	4			
Isointense	3	4	6			
Hypointense	3	11	9			
Fatty metamorphosis				0.192	0.311	0.450
Positive	0	5	4			
Negative	14	35	15			
T2WI				0.006	0.020	0.109
Hyperintense	1	14	12			
Isointense	6	5	2			
Hypointense	7	21	5			
Arterial enhancement				0.767	0.753	0.005
Hypervascular	5	12	13			
Iso-/hypo-vascular	9	28	6			
Late-phase T1WI				0.112	0.286	0.105
Hyperintense	1	5	3			
Isointense	12	26	7			
Hypointense	1	9	9			

T1WI: T1-weighted imaging; T2WI: T2-weighted imaging; DN: Dysplastic nodule; wHCC: Well-differentiated hepatocellular carcinoma; w1: wHCC compatible with Edmondson-Steiner grade I; w2: wHCC compatible with Edmondson-Steiner grade II.

T2WI should be aggressively biopsied.

In our study, hyperintensity on T1W out-phase imaging showed a significant difference between w1-HCC and w2-HCC. A hyperintense HCC on unenhanced T1W images tended to be lower grade histologically. This finding might be because borderline lesions and some early HCCs/wHCCs are occasionally hyperintense on unenhanced T1W images<sup>[28,29]</sup>. Matsui *et al.*<sup>[30]</sup> correlated MR signal intensity with tumor histology, and considered cell crowding, fatty accumulation and possibly copper deposition to be responsible for the hyperintensity on T1W imaging. However, the exact histological composition responsible for the signal intensity characteristics of w1-HCC and w2-HCC remains elusive.

According to the step-wise carcinogenesis model, HCC changes in appearance through the course of its development. These changes include a steadily increasing signal intensity on T2WI with gradually increasing neovascularity in most lesions. Van den Bos *et al.*<sup>[31]</sup> suggested that the increased signal on T2W images lags behind the developing neovascularity. Our results supported the proposition that an increased signal on T2W images occurs early in the developing arterial enhancement. These discrepancies in enhancement patterns and signal intensities might be due to the different patient populations being examined and the different underlying liver diseases. However, more studies are needed for a better understanding of developing HCC using MR imaging.

Recent progress in CT angiography, Gd-EOB-DTPA-enhanced MRI, diffusion-weighted imaging (DWI), contrast-enhanced ultrasound (CEUS) has made these modalities useful in differentiating HCC from dysplastic

nodules. Lee *et al.*<sup>[11]</sup> reported that hypointensity on Gd-EOB-DTPA-enhanced hepatobiliary-phase images and hyperintensity on high-b-value DWI in the surrounding liver parenchyma were useful in differentiating wHCC from benign nodules. Kudo<sup>[3]</sup> reported that CT during hepatic angiography and CT during arterial portography were the most sensitive tools in differentiating between premalignant/borderline lesions and early HCC. Real-time CEUS has the ability to detect slowly enhancing HCCs, which on CT could be interpreted as hypovascular lesions<sup>[32]</sup>. Giorgio *et al.*<sup>[33]</sup> reported that DN, early HCC and progressed HCC could be accurately differentiated using CEUS on the basis of the vascularization pattern during the arterial phase. Kudo<sup>[34]</sup> reported that Sonazoid-enhanced US could generate both hemodynamic-phase and Kupper-phase images and offer improved diagnostic performance for focal liver lesions. CEUS with Sonazoid may play an important role in the characterization of focal hepatic lesions in the future.

This study had two main limitations. First, the study was retrospective. If we had used the original pathological reports in the medical records as a standard of reference, inaccuracies due to differing standards of pathological interpretation at that time might have led to different results. To ensure accurate histological diagnoses, new histological slides were prepared from paraffin-embedded blocks, all of which were read by two experienced pathologists independently. A third pathologist was involved as needed to reach a final consensus when disagreements arose. The second limitation was that the histological diagnoses of 70 of the nodules (70/73) were based on needle biopsies. Potential sampling errors and

sampling variation are inherent in this type of examination, and they are recognized shortcomings in most comparative studies.

In conclusion, conventional MR imaging could provide additional information to differentiate between wHCC and DN in equivocal lesions. The variable presentation of wHCC in the current literature may be due to the differing cellular compositions of w1-HCC and w2-HCC. Consequently, during follow-up of a cirrhotic nodule, increased signal intensity on T2WI may be a sign of malignant transformation. Loss of hyperintensity in T1WI and the detection of arterial enhancement may indicate further progression of the histological grade.

## COMMENTS

### Background

Most well-differentiated hepatocellular carcinomas (HCCs) at an early stage do not demonstrate hypervascularity upon dynamic computed tomography/magnetic resonance (CT/MR) study, thereby making their diagnosis difficult. Some well-differentiated HCCs are fed by the portal vein instead of the hepatic artery and may look like benign nodules with "benign-appearing" patterns of vasculature. Gadoteric acid-enhanced magnetic resonance imaging (MRI) and contrast-enhanced ultrasound have been demonstrated to be useful in differentiating between early-stage HCC and dysplastic nodules in several recent studies. Due to the facts that gadoteric acid and contrast medium for ultrasound are not available in every country and are costly, conventional MRI remains an important tool in differentiating between cirrhotic nodules and early HCCs. The authors used a retrospective analysis to show the utility of MR imaging features to differentiate between dysplastic nodules and early stage HCC.

### Research frontiers

The authors compared conventional MR imaging results with histological results to evaluate the effectiveness of conventional MR imaging in the diagnosis of early-stage HCC.

### Innovations and breakthroughs

Increased signal intensity on T2-weighted axial images (T2WI) may be a sign of malignant transformation. Loss of hyperintensity on T1-weighted axial images (T1WI) and the detection of arterial enhancement may indicate further progression of the histological grade.

### Applications

The authors believe that conventional MR imaging could provide additional information in differentiating between early-stage HCC and dysplastic nodule in equivocal lesions. During follow-up for a cirrhotic nodule, increased signal intensity on T2WI may be a sign of malignant transformation and should be biopsied. Loss of hyperintensity on T1WI and the detection of arterial enhancement may indicate further progression of the histological grade and should be treated aggressively.

### Peer review

The authors describe a retrospective analysis of 73 patients with well-differentiated HCC or dysplastic nodules. The authors address the question whether MR imaging is helpful to differentiate between a dysplastic nodules and well-differentiated HCC in high-risk patients with cirrhosis. The question addressed by the authors is of high impact in the field of liver cirrhosis. Despite the limitations of the study (retrospective analysis, 73 patients), the authors have thoroughly analyzed the data and have made new histological classifications of all the specimens. The data show that increased signal intensity on T2WI may be a sign of malignant transformation.

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## Curcumin protects against acetaminophen-induced apoptosis in hepatic injury

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### Abstract

**AIM:** To explore the effects of curcumin (CMN) on hepatic injury induced by acetaminophen (APAP) *in vivo*.

**METHODS:** Male mice were randomly divided into three groups: group I (control) mice received the equivalent volumes of phosphate-buffered saline (PBS) intraperitoneally (*ip*); Group II [APAP + carboxymethylcellulose (CMC)] mice received 1% CMC (vehicle) 2 h before APAP injection; Group III (APAP + CMN) mice received curcumin (10 or 20 mg/kg, *ip*) 2 h before or after APAP challenge. In Groups II and III, APAP was dissolved in pyrogen-free PBS and injected at a single dose of 300 mg/kg. CMN was dissolved in 1% CMC. Mice were sacrificed 16 h after the APAP injection to determine alanine aminotransferase (ALT) levels in serum and malondialdehyde (MDA) accumulation, superoxide dismutase (SOD) activity and hepatocyte apoptosis in liver tissues.

**RESULTS:** Both pre- and post-treatment with curcumin resulted in a significant decrease in serum ALT compared with APAP treatment group (10 mg/kg:  $801.46 \pm 661.34$  U/L; 20 mg/kg:  $99.68 \pm 86.48$  U/L vs  $5406.80 \pm 1785.75$  U/L,  $P < 0.001$ , respectively). The incidence of liver necrosis was significantly lowered in CMN treated animals. MDA contents were significantly reduced in 20 mg/kg CMN pretreatment group, but increased in APAP treated group ( $10.96 \pm 0.87$  nmol/mg protein vs  $16.03 \pm 2.58$  nmol/mg protein,  $P < 0.05$ ). The decrease of SOD activity in APAP treatment group and the increase of SOD in 20 mg/kg CMN pretreatment group were also detected ( $24.54 \pm 4.95$  U/mg protein vs  $50.21 \pm 1.93$  U/mg protein,  $P < 0.05$ ). Furthermore, CMN treatment efficiently protected against APAP-induced apoptosis *via* increasing Bcl-2/Bax ratio.

**CONCLUSION:** CMN has significant therapeutic potential in both APAP-induced hepatotoxicity and other types of liver diseases.

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**Key words:** Acetaminophen; Acute hepatic injury; Apoptosis; Free radicals; Curcumin

**Core tip:** Acetaminophen (APAP) and curcumin (CMN) were administrated intraperitoneally. The aim of the study was to explore whether CMN has effect on APAP-induced hepatic toxicity *in vivo*. The findings revealed that CMN protects against APAP-induced lipid peroxidation, oxidative stress and hepatocyte apoptosis.

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## INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is safe and effective when taken at therapeutic dose<sup>[1]</sup>. However, it can cause severe liver damage or even acute liver failure that can be fatal in experimental animals and humans when administered in an acute or cumulative overdose<sup>[2,3]</sup>. APAP overdose is the leading cause for calls to Poison Control Centers (> 100000/year) and accounts for more than 56000 emergency visits, 2600 hospitalizations, and an estimated 458 deaths each year in the United States<sup>[3]</sup>.

APAP is metabolized by cytochrome P450 to *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI can react rapidly with glutathione (GSH), so large doses of APAP may result in a profound depletion of hepatocellular GSH<sup>[1,4]</sup>. Once GSH is exhausted, any remaining NAPQI will covalently bind to cellular proteins and induce mitochondrial dysfunction, lipid peroxidation, oxidative stress, and DNA fragmentation, eventually leads to massive hepatocyte necrosis, liver damage or death<sup>[5]</sup>. *N*-acetyl cysteine has been currently used in the treatment of APAP-induced liver toxicity<sup>[6]</sup>. In addition to its adverse reaction, a major concern when using *N*-acetyl cysteine is its relatively narrow therapeutic windows and drug toxicity<sup>[7,8]</sup>. Therefore, new and safe preventive measures against APAP toxicity are eagerly needed.

In recent years, natural products from plants have received considerable attention as a rich resource for drug development. Curcumin (CMN) is a yellow pigment purified from the root tubers of *Curcuma longa* Linn (commonly known as turmeric), which has long been used as a food colorant and preservative<sup>[9]</sup>. CMN also has a variety of biological and pharmacological activities, such as anti-inflammatory, anti-oxidant, antifungal, antibacterial and anticancer activities<sup>[10]</sup>. It was reported that CMN attenuates liver injury induced by ethanol<sup>[11]</sup>, iron overdose<sup>[12]</sup> and carbon tetrachloride intoxication<sup>[13]</sup>.

The aim of this study was to explore the effect of CMN on the prevention of APAP-induced hepatic toxicity *in vivo* and investigate whether CMN affects the production of lipid peroxidation, oxidative stress or hepatocyte apoptosis to attenuate liver damage.

## MATERIALS AND METHODS

### Materials

APAP and CMN were purchased from Sigma Aldrich (Saint Louis, MO, United States). Detection kits for superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineer Institute (Nanjing, China). Transferase-mediated dUTP-biotin nick end labeling (TUNEL) detection kit was purchased from Boster Biological Technology Co., Ltd (Wuhan, China).

### Animals and treatment

Male BALB/c mice (6-8 wk of age) were purchased from the Center for Animal Experiment of Wuhan

University (Wuhan, China). The mice were raised at an animal facility under special pathogen-free conditions with a 12-h light/dark cycle and free access to food and water at least 1 wk prior to treatment. All animal experiments were approved by the institutional animal care and use committee at the Yangtze University, and all efforts were made to minimize the number of animals used and their sufferings. Mice were randomly divided into three groups: group I (control) mice received the equivalent volumes of PBS intraperitoneally (*ip*); Group II (APAP + CMC) mice received 1% CMC (vehicle) 2 h before APAP injection; and Group III (APAP + CMN) mice received CMN (10 or 20 mg/kg, *ip*) 2 h before or after APAP challenge. In Group II and Group III, APAP was dissolved in pyrogen-free PBS and injected at a single dose of 300 mg/kg; the dose was selected on the basis of a previous related study<sup>[14]</sup>. CMN was dissolved in 1% CMC. Serum and livers were collected at 16 h after APAP treatment.

### Biochemical analysis

Alanine amino-transaminase (ALT) activity was determined using a diagnostic assay kit (Sichuan Maker Science and Technology Co., Ltd., Chengdu, China) by an automated chemistry analyzer (Olympus AU1000, Japan) by Central Laboratory of the Affiliated Jingzhou Hospital of Yangtze University.

Livers were quickly removed, washed with ice-cold PBS, blotted and weighed, and then a tissue homogenate (1% or 10%, w/v) was prepared in normal saline. The homogenates were then centrifuged at 4000 rpm (4 °C) for 20 min to collect supernatants for determination of SOD and MDA contents at 550 nm and 532 nm, respectively.

Lipid peroxidation was assessed by estimation of MDA in the liver tissues according to the method of Wills (1966). MDA was determined by the thiobarbituric acid assay using a MDA assay kit, according to the manufacturers' instructions. Liver tissue protein was measured using Coomassie Brilliant Blue protein reagent, and MDA content was expressed as nmol/mg protein. SOD activity was determined by measuring the inhibition of formation of NADPH-phenazine methosulphate nitroblue tetrazolium.

### Histochemistry

Liver tissues fixed in 10% formalin were embedded in paraffin, sectioned at 4 µm and stained with hematoxylin-eosin.

### TUNEL staining

Paraffin-embedded liver tissues were assayed for DNA fragmentation using a terminal deoxynucleotidyl TUNEL reaction, according to the manufacturer's instructions. Slides were developed with diaminobenzidine substrate, counterstained with HE, and then examined for evidence of apoptosis. The number of brown apoptotic cells was normalized to total cells as detected by HE. Four fields

of each image were counted.

### Reverse transcriptase polymerase chain reaction

RNA was extracted from the livers using TRIzol® Reagent (Invitrogen) according to the manufacturer's instruction. cDNA was synthesized from 2 µg of total RNA using PrimeScript™ 1<sup>st</sup> Strand cDNA Synthesis Kit (Takara Biotechnology, Co., Ltd., Da Lian, LiaoNing, China). PCR amplifications were performed by standard methods using following specific primers: for Bcl-2: Sense: 5'-GGC ATC TTC TCC TTC CAG-3', Anti-sense: 5'-CTA CCC AGC CTC CGT TAT-3'; for Bax: Sense: 5'-TTT CAT CCA GGA TCG AGC AGG-3', Antisense: 5'-GCA AAG TAG AAG AGG GCA ACC AC-3'<sup>[15]</sup>.

### Statistical analysis

A computer program (SPSS 13.0) was used for statistical analysis. Data were presented as mean ± SE. Student's *t* test (two groups) or one way ANOVA (multiple groups) were used. *P* < 0.05 indicated statistical significance.

## RESULTS

### CMN treatment attenuates APAP-induced liver injury

To explore the protective effect of CMN on APAP-induced hepatic toxicity, the animals were injected intraperitoneally with CMN (10 or 20 mg/kg body weight) 2 h before APAP (300 mg/kg body weight), and serum ALT was analyzed after 16 h of administration. Compared with PBS control (41.70 ± 2.82 U/L), APAP treatment significantly increased serum ALT levels (5406.80 ± 1785.75 U/L, 126.9-fold of the control, Figure 1A, *P* < 0.001 *vs* control). As expected, CMN pretreatment significantly suppressed the plasma ALT activity in a dose-dependent manner (10 mg/kg: 801.46 ± 661.34 U/L; 20 mg/kg: 99.68 ± 86.48 U/L, Figure 1A, *P* < 0.001 *vs* the model group). This protective effect was further confirmed by analysis of histological findings, as shown in Figure 1B, severe sinusoidal congestion and hemorrhage, inflammatory cell infiltration and gross necrosis were observed in the liver of mice treated with APAP. However, these pathological changes were dramatically suppressed by CMN treatment. To evaluate its potential therapeutic role, CMN was administrated after 2 h of APAP injection, and a marked reduction of serum ALT was also observed (395.40 ± 133.52 U/L, Figure 1A). Of note, CMN alone did not influence serum transaminase (Figure 1A) and urea, creatinine (data not shown) in normal control mice.

### CMN pretreatment inhibits production of MDA

As lipid peroxidation has been reported to be closely related to APAP-induced toxicity, the content of malondialdehyde, the end product of lipid peroxidation, in the liver tissues was detected at 16 h after APAP treatment. Only low levels of MDA were observed in the control mice (10.81 ± 1.46 nmol/mg protein), but a significant

increase was found in APAP-treated mice (16.03 ± 2.58 nmol/mg protein). As expected, MDA contents were significantly inhibited by 20 mg/kg CMN pretreatment (10.96 ± 0.87 nmol/mg protein, Figure 2).

### CMN pretreatment enhances activity of SOD

In addition to lipid peroxidation, oxidative stress is an early events related to radicals generated during the hepatic metabolism of APAP. SOD is an enzyme that neutralizes free radicals<sup>[16]</sup>. So the activity of SOD was investigated at 16 h after APAP treatment in the liver tissues. A significant decrease in SOD activity was observed in the mice treated with APAP compared with control group (24.54 ± 4.95 U/mg protein *vs* 45.64 ± 5.96 U/mg protein). However, pretreatment of mice with CMN induced a significant increase in the activity of SOD (50.21 ± 1.93 U/mg protein, Figure 3).

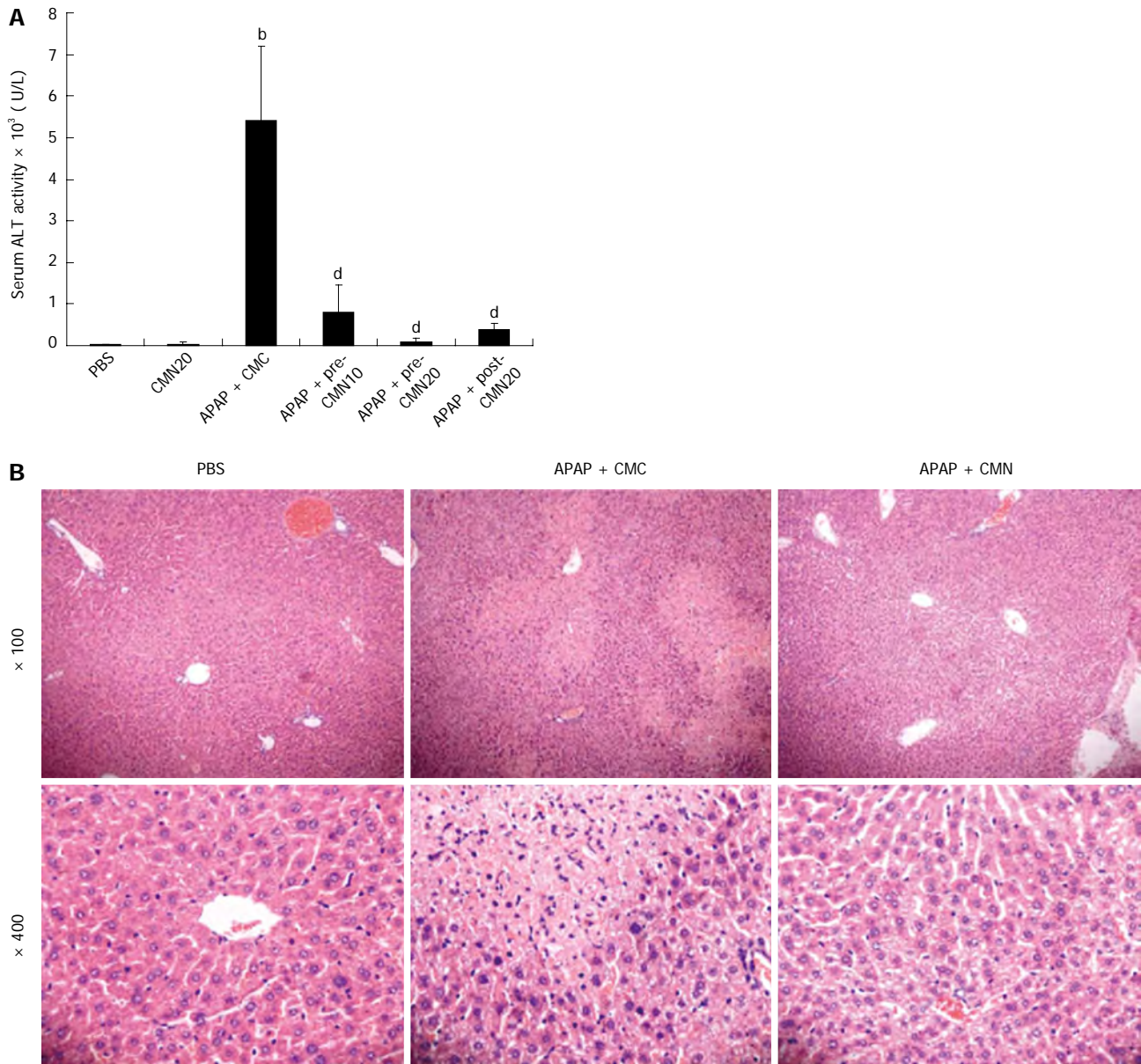
### CMN pretreatment prevents hepatocyte apoptosis

Based on the above observations, we further explored the possible mechanisms by which CMN attenuates liver injury induced by APAP. Given the importance of apoptosis in APAP-induced liver injury, the extent of hepatocyte apoptosis was determined by TUNEL assay. As shown in Figure 4A, massive hepatocyte apoptosis was detected in the livers of mice treated with APAP. CMN pretreatment markedly prevented the apoptosis induced by APAP. Furthermore, we examined the mRNA expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax in the livers. As shown in Figure 4B, CMN pretreatment down-regulated the mRNA expression of Bax and up-regulated the mRNA expression of Bcl-2 compared with APAP-treated group. These data suggest that CMN inhibits hepatocyte apoptosis *via* regulating the gene expression of Bcl-2 family and then protects the mice from APAP-induced hepatic injury.

## DISCUSSION

In the present study, for the first time, we examined the effect of CMN on liver injury induced by APAP and the possible mechanisms in mice. Our data demonstrated that CMN pretreatment dose-dependently alleviated APAP-induced acute liver injury. CMN pretreatment markedly decreased ALT levels in plasma and inhibited the necrosis of hepatocytes in Con A-treated mice. Furthermore, CMN could be also considered as a rescue therapy, for it significantly decreased APAP-induced hepatotoxicity when administered 2 h after APAP overdose.

NAPQI is the reactive metabolite product generated from APAP-induced hepatic toxicity, and it was found to be formed by cytochrome P-450 by a direct two electron oxidation of APAP. At low doses, the metabolite was efficiently detoxified by GSH. However, at high doses, NAPQI leads to GSH depletion and subsequently covalently binds to cysteine residues on proteins, which results in lipid peroxidation reaction<sup>[14,17]</sup>. As a metabolite of free radical, MDA is generally considered as an impor-



**Figure 1** Curcumin treatment protects against acetaminophen-induced hepatic injury in mice. **A:** Serum alanine aminotransferase (ALT) levels were determined 16 h after acetaminophen injection. Data are expressed as mean  $\pm$  SE;  $n = 10$  mice per group. <sup>b</sup> $P < 0.01$  vs control; <sup>d</sup> $P < 0.001$  vs acetaminophen (APAP) + carboxymethylcellulose (CMC); **B:** Hematoxylin-eosin stained liver sections from animals treated with PBS, APAP + CMC and APAP + CMN (original magnification:  $\times 100$  and  $\times 400$ ). Severe inflammatory cell infiltration and gross necrosis of the entire centrilobular areas were obvious in APAP group, and the results were significantly ameliorated in CMN-treated animals. CMN: Curcumin.

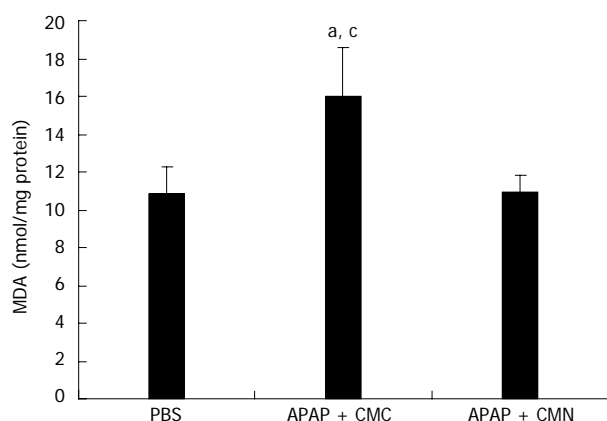
tant indicator of lipid peroxidation. In this study, in line with previous reports, we found that MDA in liver tissues was increased significantly 16 h after APAP administration. As expected, our results demonstrated that CMN treatment could inhibit the increase of MDA induced by APAP, suggesting that CMN has potent beneficial effects on lipid peroxidation.

Our organism has a function to neutralize and scavenge the free radical in order to prevent oxidative damage to cells. Such endogenous mechanisms are mainly provided by a set of antioxidant enzymes such as SOD, glutathione peroxidase, and catalase. SOD represents the first line of defense against free radicals, it converts superoxide anion into hydrogen peroxide, and then hy-

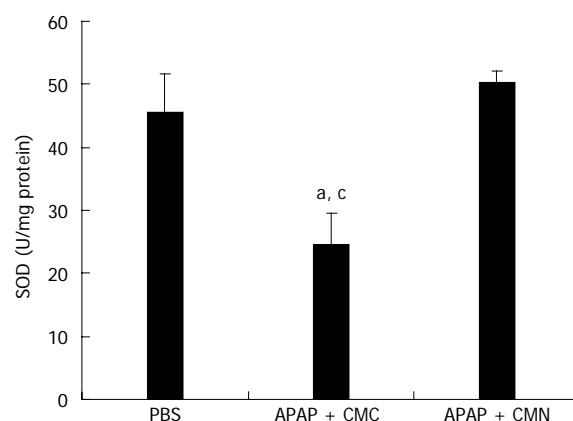
drogen peroxide is converted into oxygen and water by glutathione peroxidase, making reduced GSH as its substrate<sup>[18]</sup>. As a antioxidant, CMN has been demonstrated to effectively prevent the decrease in SOD activity in a variety of experimental models, including inflammation, cardiotoxicity and carbon tetrachloride-induced liver injury models<sup>[19]</sup>. In line with these concepts, we found that the major scavenger enzyme SOD activity was significantly decreased in the liver of APAP-treated mice. As expected, pretreatment with CMN restored SOD activity. Therefore, it is suggested that the protective effect of CMN on APAP-induced injury is associated with its inhibition of oxidative stress.

Accumulating evidence suggests that hepatocyte

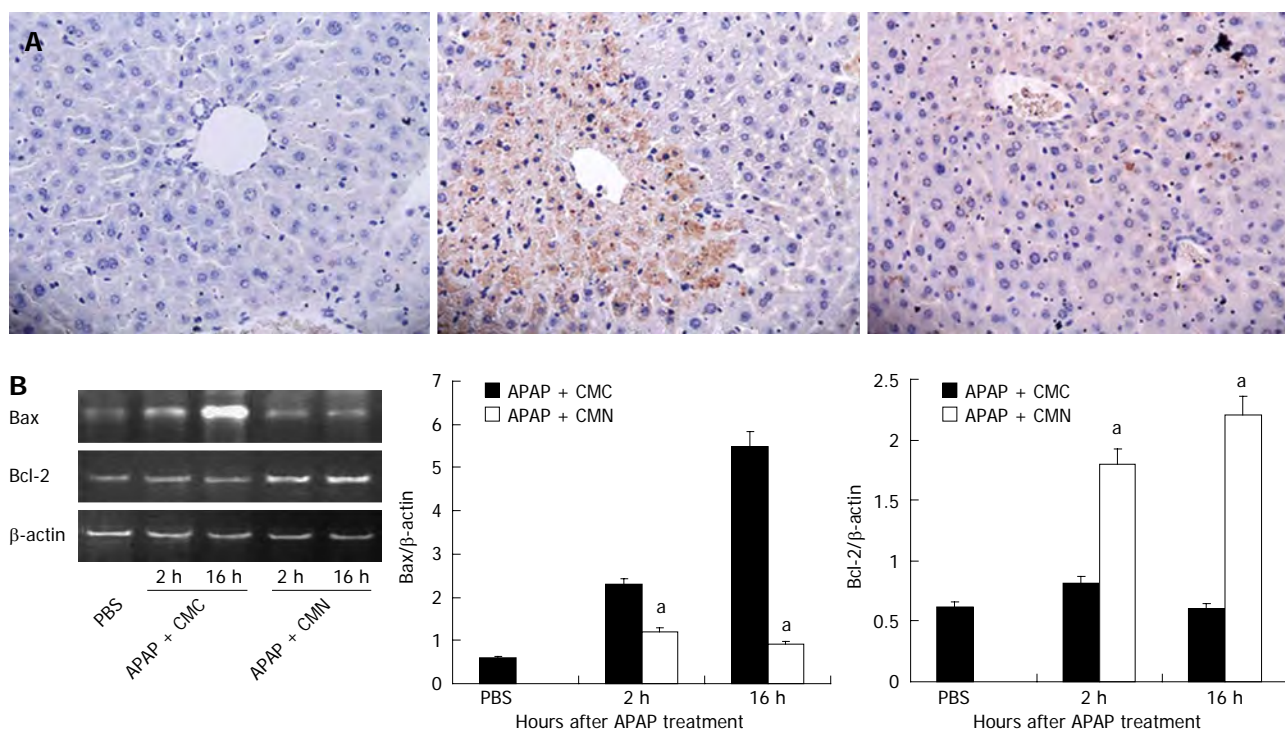




**Figure 2** Curcumin pretreatment inhibits malondialdehyde production after acetaminophen induction. Liver homogenate was prepared to analyze the content of malondialdehyde (MDA) 16 h after acetaminophen (APAP) administration. Data are expressed as mean  $\pm$  SE;  $n = 10$  mice per group. <sup>a</sup> $P < 0.05$  vs control; <sup>c</sup> $P < 0.05$  vs APAP + curcumin (CMN). CMC: Carboxymethylcellulose.



**Figure 3** Curcumin pretreatment enhances activity of superoxide dismutase after acetaminophen. Liver homogenate was prepared to analyze the activity of superoxide dismutase (SOD) 16 h after acetaminophen (APAP) administration. Data are expressed as mean  $\pm$  SE;  $n = 10$  mice per group. <sup>a</sup> $P < 0.05$  vs control; <sup>c</sup> $P < 0.05$  vs APAP + curcumin (CMN). CMC: Carboxymethylcellulose.



**Figure 4** Curcumin pretreatment prevents hepatocyte apoptosis induced by acetaminophen. A: Transferase-mediated dUTP-biotin nick end labeling stained liver sections from animals treated with PBS, acetaminophen (APAP) + carboxymethylcellulose (CMC) and APAP + curcumin (CMN) (original magnification:  $\times 400$ ); B: Liver samples were collected 2 h and 16 h after APAP injection, and the mRNA expression of Bax and Bcl2 was determined by reverse transcriptase polymerase chain reaction. Data are expressed as mean  $\pm$  SE;  $n = 6$  mice per group. <sup>a</sup> $P < 0.05$  vs APAP + CMC.

apoptosis plays a critical role in APAP-induced hepatic injury, although the mode of cell death inflicted by APAP is still controversial<sup>[20,21]</sup>. APAP-induced apoptosis is observed not only in primary hepatocytes<sup>[22]</sup>, but also in livers of mice treated with toxic doses of APAP<sup>[23]</sup>. Also, a recent report showed that hepatic caspase-3 and caspase-9 are activated in both wild type and CXCR2 knock out mice within one hour of APAP treatment<sup>[21]</sup>. Moreover, inhibiting apoptosis prevents the development of acute liver failure<sup>[23]</sup>. Based on these concepts, we found

that apoptotic hepatocytes were significantly increased in the liver of mice 16 h after APAP treatment and the hepatocyte apoptosis was significantly reduced by CMN pretreatment. Bcl-2 protein is commonly recognized as an anti-apoptotic factor, it inhibits cell apoptosis by preventing mitochondrial membrane depolarization. As a member of the Bcl-2 family, Bax inactivates Bcl-2 by interacting with it to form a heterodimer<sup>[24]</sup>. In this study, we found that CMN pretreatment down-regulated the mRNA expression of Bax and up-regulated the mRNA



expression of Bcl-2 compared with APAP-treated group, suggesting that CMN can increase Bcl-2/Bax ratio, thus reducing APAP-induced apoptosis. The mechanisms of CMN-mediated anti-apoptotic effect remained unclear. A recent study showed that CMN exerts a potent anti-apoptotic effect *via* inhibition of TGF- $\beta$  as inducer of caspase-3 mediated apoptosis in kidney and lung tissues<sup>[25]</sup>, however, the precise mechanisms by which CMN modulates cell apoptosis in APAP-induced liver injury need to be further investigated.

In summary, our study revealed that CMN has a protective effect on the acute hepatic injury induced by APAP. Both pre- and post-treatment with CMN resulted in a significant reduction in serum ALT and hepatocyte necrosis. The protection of CMN may be related to its inhibition of lipid peroxidation and oxidative stress. Moreover, we found that CMN restored Bcl-2/Bax ratio, thus reducing the APAP-induced hepatocyte apoptosis.

## COMMENTS

### Background

Acetaminophen (APAP) can cause severe liver damage or even acute liver failure when administered in an acute or cumulative overdose. *N*-acetyl-*p*-benzoquinone imine (NAPQI) is the metabolite of APAP by cytochrome P450. Accumulation of NAPQI could induce lipid peroxidation, oxidative stress, mitochondrial dysfunction and DNA fragmentation, even liver failure or death. Thus, new and safe preventive measures against APAP-induced hepatic damage are eagerly needed.

### Research frontiers

Previous studies have shown that curcumin (CMN) exerts anti-inflammatory, anti-oxidant and anticancer pharmacological activities. APAP lead to liver injury through increasing oxidative stress, lipid peroxidation and pro-apoptosis. In this study, the authors showed that CMN improved hepatic injury through inhibiting oxidative stress, lipid peroxidation and hepatic apoptosis in APAP induced liver damage model.

### Innovations and breakthroughs

This study investigated the effect of CMN on the prevention of APAP-induced hepatic toxicity *in vivo* and examined whether CMN affects the production of lipid peroxidation, oxidative stress or hepatocyte apoptosis to attenuate liver damage. The results indicated that CMN could protect mice from APAP-induced liver injury.

### Applications

CMN was abundant in the root tubers of *Curcuma longa* Linn and can be purified by modern technology. It has a broad application prospect.

### Peer review

It is an interesting study investigating the protection effect of CMN on APAP-caused hepatitis. The experimental evidences presented that APAP induced hepatic injury, with elevated alanine aminotransferase, lipid peroxidation, oxidative stress and apoptosis were improved by CMN.

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## Prognostic factors in non-malignant and non-cirrhotic patients with portal cavernoma: An 8-year retrospective single-center study

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### Abstract

**AIM:** To evaluate the outcome of non-malignant and non-cirrhotic patients with portal cavernoma and to determine the predictors for survival.

**METHODS:** Between July 2002 and June 2010, we retrospectively enrolled all consecutive patients admitted to our department with a diagnosis of portal cavernoma without abdominal malignancy or liver cirrhosis. The primary endpoint of this observational study was death and cause of death. Independent predictors of survival were identified using the Cox regression model.

**RESULTS:** A total of 64 patients were enrolled in the study. During a mean follow-up period of  $18 \pm 2.41$  mo, 7 patients died. Causes of death were pulmonary

embolism ( $n = 1$ ), acute leukemia ( $n = 1$ ), massive esophageal variceal hemorrhage ( $n = 1$ ), progressive liver failure ( $n = 2$ ), severe systemic infection secondary to multiple liver abscesses ( $n = 1$ ) and accident ( $n = 1$ ). The cumulative 6-, 12- and 36-mo survival rates were 94.9%, 86% and 86%, respectively. Multivariate Cox regression analysis demonstrated that the presence of ascites (HR = 10.729, 95%CI: 1.209-95.183,  $P = 0.033$ ) and elevated white blood cell count (HR = 1.072, 95%CI: 1.014-1.133,  $P = 0.015$ ) were independent prognostic factors of non-malignant and non-cirrhotic patients with portal cavernoma. The cumulative 6-, 12- and 36-mo survival rates were significantly different between patients with and without ascites (90%, 61.5% and 61.5% vs 97.3%, 97.3% and 97.3%, respectively,  $P = 0.0008$ ).

**CONCLUSION:** The presence of ascites and elevated white blood cell count were significantly associated with poor prognosis in non-malignant and non-cirrhotic patients with portal cavernoma.

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**Key words:** Extrahepatic portal vein obstruction; Portal cavernoma; Survival; Prognostic factors; Ascites

**Core tip:** Little is known regarding the prognostic factors of non-malignant and non-cirrhotic patients with portal cavernoma. We conducted a retrospective single-center study of 64 patients admitted to our department between July 2002 and June 2010 to evaluate this issue. Multivariate Cox regression analysis demonstrated that the presence of ascites was an independent prognostic factor of non-malignant and non-cirrhotic patients with portal cavernoma.

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## INTRODUCTION

Portal cavernoma, also known as cavernous transformation of the portal vein, is a rare entity, which is characterized by a tangle of tortuous hepatopetal collateral veins in the hilum<sup>[1]</sup>. It is traditionally considered a sequela of extrahepatic portal vein obstruction (EHPVO) to compensate for the interrupted portal blood flow<sup>[2,3]</sup>. Current treatment strategies for portal cavernoma focus on the prevention and treatment of variceal hemorrhage, prevention of recurrent thrombosis, and treatment of symptomatic portal biliopathy<sup>[4-6]</sup>. Given the rarity of portal cavernoma, controlled studies are unavailable, and therapeutic options vary in different centers. Previous studies, in which malignancy and cirrhosis were not excluded, have revealed that the increased mortality in EHPVO patients is closely associated with advanced age, presence of malignancy and cirrhosis, high bilirubin and deterioration of liver function<sup>[7-9]</sup>. However, little information is known regarding the prognostic factors in non-malignant and non-cirrhotic patients with portal cavernoma due to its low morbidity and mortality.

We conducted a retrospective study to determine the predictors for survival of non-malignant and non-cirrhotic patients with portal cavernoma managed by a uniform therapeutic strategy at our center.

## MATERIALS AND METHODS

### Study design

Between July 2002 and June 2010, all consecutive patients with a diagnosis of portal cavernoma without abdominal malignancy or liver cirrhosis who were admitted to our department were enrolled in this observational study<sup>[10-12]</sup>, regardless of age. Baseline data were collected upon admission or referral. Regular blood tests, hepatic and renal function tests, prothrombin time, internationalized normalized ratio (INR), color Doppler ultrasound (CDUS), computed tomography (CT) and endoscopy were performed in all patients. Thrombotic risk factors of EHPVO, including *JAK2 V617F* mutation, CD55 and CD59 deficiencies, anti-cardiolipin IgG antibodies, and factor V Leiden or prothrombin gene G20210A mutation, were detected at our department after September 2009<sup>[13,14]</sup>. Additionally, we recorded the date of diagnosis of portal cavernoma at our own hospital or an outside facility. Follow-up data were obtained every six months through outpatient or phone conversations with the patient or his or her family members. The primary endpoint was death. Follow-up continued either until death or July 2010. The study protocol was approved by the ethics committee of our hospital.

### Diagnosis and definitions

Portal cavernoma was characterized by a tangle of tortuous hepatopetal collateral veins in the hilum. An acute thrombotic episode was defined as fulfillment of both of the following criteria: (1) recent onset of abdominal pain; and (2) a high intraluminal density within the portal vein on non-enhanced CT scans, while contrast-enhanced CT scans displayed cavernous vessels around the obstructed portal vein<sup>[15,16]</sup>.

As previously described, the degree of portal venous obstruction was classified as partial obstruction, complete obstruction and fibrotic cord instead of original main portal vein<sup>[12,17,18]</sup>. The extent of obstruction within the portal venous system was also evaluated.

Liver cirrhosis and hepatocellular carcinoma were excluded on the basis of a history of chronic liver disease, clinical presentation, liver function, alpha-fetoprotein and positive findings on imaging (*i.e.*, ultrasound and CT scans)<sup>[18]</sup>. A liver biopsy was obtained, if a diagnosis of cirrhosis was inconclusive or if hepatocellular carcinoma was suspected. Other abdominal malignancy was excluded by imaging.

The degree of variceal size was based on the general rules established by the Japanese Research Society for Portal Hypertension (low-risk varices: F1 or F2 with negative red color sign; high-risk: F1 or F2 with positive red color sign, or F3 irrespective of red color sign)<sup>[19]</sup>. The presence of ascites was diagnosed by physical examination, ultrasound and CT scans. The grade of ascites was based on the definitions of the International Ascites Club (grade I: mild ascites only detectable by ultrasound; grade II: moderate symmetrical abdominal distension; grade III: marked abdominal distension)<sup>[20]</sup>.

### Therapeutic strategy

Our therapeutic strategy was aimed at minimal invasiveness and maximal beneficial effects through symptom resolution.

After diagnosis of an acute thrombotic episode, the patients received a continuous intravenous infusion of unfractionated heparin followed by oral warfarin. Initially, heparin was regularly administered intravenously at a starting dose of 1000-1400 U/h for 5 d. Subsequently, oral warfarin was prescribed at the dosage of 2.5-5 mg/d for at least 6 mo and was adjusted to maintain the INR at a target of 2.5 (range 2.0-3.0)<sup>[21]</sup>. A three-day overlap between intravenous and oral anticoagulation was required. Life-long oral anticoagulants were prescribed to patients with thrombophilia. If abdominal pain was progressive, we either indirectly infused thrombolytic agents into the superior mesenteric artery or performed direct thrombolysis in the portal vein by a percutaneous transhepatic approach. If ischemic intestinal infarction was diagnosed, emergency bowel resection was performed.

Once acute variceal bleeding was diagnosed, medical or endoscopic therapy was adopted as the first-line treatment option. If active bleeding was uncontrolled or if repeated hospitalizations were necessary to control recur-



rent variceal bleeding, a transjugular intrahepatic porto-systemic shunt (TIPS) insertion was performed through a transjugular approach alone or in combination with a transhepatic or transsplenic approach, as previously described<sup>[12,18,22]</sup>. If a TIPS procedure failed or was refused, splenectomy and devascularization were considered.

Other symptomatic treatments included anticoagulation for prevention of recurrent thrombosis, diuretics and/or paracentesis plus albumin for grade II and III ascites, splenectomy for hypersplenism and massive splenomegaly and prophylactic endoscopic therapy for high-risk varices. Additionally, if patients presented with a long history of repeated gastrointestinal syndromes unresponsive to conservative therapies, the TIPS procedure was considered.

### Statistical analysis

Quantitative data were reported as mean  $\pm$  SE and were compared with the independent sample *t* test or one-way analysis of variance; qualitative data were reported as frequencies and were compared with the  $\chi^2$  test or the Fisher exact test, as appropriate. Cumulative survival rates were assessed by the Kaplan-Meier curves and were compared with a log-rank test. Independent predictors of survival were identified using the Cox regression model. The covariates incorporated into the multivariate analysis were the variables that reached statistical significance ( $P < 0.05$ ) in univariate analysis. Two-tailed  $P$  values  $< 0.05$  were considered statistically significant. All statistical calculations were performed with SPSS 12.0 (Chicago, IL, United States).

## RESULTS

### Characteristics of patients

A total of 64 patients diagnosed with portal cavernoma without liver cirrhosis or abdominal malignancy were enrolled in the study. Notably, 5 patients presented with cavernous collateral veins around a patent main portal vein<sup>[10]</sup>. Baseline patient characteristics are summarized in Table 1.

### Possible risk factors

Thrombotic risk factors of EHPVO were detected in 33 patients. Among them, 11 had positive *JAK2 V617F* mutation, none had both CD55 and CD59 deficiencies, two had weakly positive anti-cardiolipin IgG antibodies, and none had positive factor V Leiden or prothrombin gene *G20210A* mutation. Previous history of infection before admission included colitis ( $n = 1$ ), pelvic infection ( $n = 1$ ), appendicitis ( $n = 1$ ), intra-abdominal infection secondary to duodenal ulcer perforation ( $n = 1$ ), umbilical cord infection ( $n = 2$ ), megacolon disease of newborn ( $n = 1$ ), bacterial dysentery ( $n = 1$ ), and pancreatitis ( $n = 4$ ). Previous history of abdominal surgery before admission included splenectomy and devascularization for variceal bleeding ( $n = 11$ ), splenectomy for hypersplenism and/or splenomegaly ( $n = 8$ ), splenectomy for traumatic spleen

**Table 1 Patient characteristics at admission**

Sex (female/male)	26/38
Age at admission (yr)	32.05 $\pm$ 2.00
Age at first diagnosis of portal cavernoma ( $\leq 18$ yr/ $> 18$ yr)	26/38
History of portal cavernoma (yr)	3.68 $\pm$ 0.78
Clinical presentations	
Abdominal distension	37
Abdominal pain	26
Variceal bleeding	29
Degree of varices (high risk/low risk/no varices)	6/12/1946
Ascites	20
Degree of ascites (grade III/grade II/grade I / no ascites)	3/1/16/44
Hydrothorax	10
Laboratory tests	
RBC ( $10^{12}$ /L)	3.95 $\pm$ 0.11
Hb (g/L)	106.25 $\pm$ 4.00
WBC ( $10^9$ /L)	8.11 $\pm$ 1.03
PLT ( $10^9$ /L)	298.73 $\pm$ 33.67
PT (s)	14.14 $\pm$ 0.24
INR	1.15 $\pm$ 0.02
ALT (U/L)	29.97 $\pm$ 3.31
AST (U/L)	30.28 $\pm$ 2.64
ALP (U/L)	107.21 $\pm$ 7.68
GGT (U/L)	41.16 $\pm$ 5.96
ALB (g/L)	38.25 $\pm$ 0.58
TBIL ( $\mu$ mol/L)	16.05 $\pm$ 1.32
Serum Cr ( $\mu$ mol/L)	70.65 $\pm$ 2.48
Serum Na (mmol/L)	139.54 $\pm$ 0.45
Child-Pugh class (A/B/C)	12/1/1951
Child-Pugh score	5.70 $\pm$ 0.13
MELD score	4.25 $\pm$ 0.53
Location and degree of obstruction	
Main portal vein (patent/partial/complete/fibrotic cord)	5/5/12/42
Right portal vein obstruction	49
Left portal vein obstruction	51
Splenic vein obstruction and splenectomy	39
Superior mesenteric vein obstruction	34

Data are expressed as quantitative data as frequency or mean  $\pm$  SE. RBC: Red blood cell; Hb: Hemoglobin; WBC: White blood cell; PLT: Platelets; PT: Prothrombin time; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT:  $\gamma$ -glutamyl transferase; ALB: Albumin; TBIL: Total bilirubin.

rupture ( $n = 1$ ), partial splenic artery embolization for hypersplenism ( $n = 1$ ), cholecystectomy ( $n = 4$ ), surgical repair of peptic ulcer perforation ( $n = 1$ ), total hysterectomy for hysteromyoma ( $n = 1$ ), and cesarean delivery ( $n = 1$ ). Notably, 7 and 13 patients underwent splenectomy before and after the diagnosis of portal cavernoma, respectively.

### Clinical profile of patients with variceal bleeding

Compared to patients without variceal bleeding, those with variceal bleeding were younger at admission (37.54  $\pm$  2.56 years in the non-bleeding group *vs* 25.41  $\pm$  2.74 years in the bleeding group,  $P = 0.002$ ) and had a longer history of portal cavernoma (1.86  $\pm$  0.66 years in the non-bleeding group *vs* 5.89  $\pm$  1.44 years in the bleeding group,  $P = 0.009$ ). A fibrotic cord replacing the main portal vein was more frequently found on CT scans in pa-

**Table 2** Clinical profile in patients with and without variceal bleeding

Variables	Variceal bleeding ( <i>n</i> = 29)	No variceal bleeding ( <i>n</i> = 35)	<i>P</i> value
Age at admission (yr)	25.41 ± 2.74	37.54 ± 2.56	0.002
Sex (female/male)	14/15	12/23	0.257
History of portal cavernoma (yr)	5.89 ± 1.44	1.86 ± 0.66	0.009
Abdominal distension (yes/no)	11/18	26/9	0.003
Abdominal pain (yes/no)	3/26	23/12	< 0.001
Ascites (yes/no)	8/21	12/23	0.565
Hydrothorax (yes/no)	4/25	6/29	0.713
Jaundice (yes/no)	2/27	2/33	0.846
RBC (10 <sup>12</sup> /L)	3.36 ± 0.12	4.44 ± 0.12	< 0.001
Hb (g/L)	87.21 ± 4.60	122.03 ± 4.86	< 0.001
WBC (10 <sup>9</sup> /L)	7.47 ± 1.82	8.65 ± 1.16	0.574
PLT (10 <sup>9</sup> /L)	317.07 ± 50.27	283.53 ± 45.84	0.624
PT (s)	14.08 ± 0.35	14.18 ± 0.34	0.838
INR	1.15 ± 0.03	1.15 ± 0.03	0.946
ALT (U/L)	26.38 ± 4.87	32.94 ± 4.51	0.327
AST (U/L)	28.85 ± 4.28	30.93 ± 3.33	0.388
AST/ALT	1.23 ± 0.08	1.21 ± 0.11	0.874
ALP (U/L)	115.98 ± 13.43	99.94 ± 8.57	0.302
GGT (U/L)	31.69 ± 8.60	49.00 ± 8.12	0.150
ALB (g/L)	37.23 ± 0.79	39.09 ± 0.82	0.111
TBIL (μmol/L)	15.54 ± 2.13	16.48 ± 1.66	0.724
DBIL (μmol/L)	7.07 ± 1.54	7.43 ± 0.98	0.838
Serum Cr (μmol/L)	70.17 ± 3.88	71.06 ± 3.26	0.860
Serum Na (mmol/L)	139.26 ± 0.55	139.77 ± 0.70	0.577
Child-Pugh class (A/B/C)	23/5/1	28/7/0	0.529
Child-Pugh score	5.69 ± 0.21	5.71 ± 0.18	0.928
MELD score	3.93 ± 0.88	4.51 ± 0.65	0.593
Main portal vein (fibrotic cord/complete obstruction/partial obstruction/patency)	27/0/1/1	15/12/3/4	< 0.001
Right portal vein (obstruction/patency)	19/10	30/5	0.058
Left portal vein (obstruction/patency)	21/8	30/5	0.188
Splenic vein (obstruction and splenectomy/patency)	14/15	25/10	0.059
Superior mesenteric vein (obstruction/patency)	11/18	23/12	0.027

Data are expressed as quantitative data as frequency or mean ± SE. RBC: Red blood cell; Hb: Hemoglobin; WBC: White blood cell; PLT: Platelets; PT: Prothrombin time; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase; ALB: Albumin; TBIL: Total bilirubin; DBIL: Direct bilirubin; MELD: Model for end-stage liver disease.

tients with variceal bleeding than those without (27/29 *vs* 15/35, *P* < 0.001). In contrast, superior mesenteric vein obstruction was less frequently observed in patients with variceal bleeding than those without (11/29 *vs* 23/35, *P* = 0.027). No significant relationship between Child-Pugh score and variceal bleeding was observed (Table 2).

### Clinical profile of patients with ascites

The white blood cell (WBC) count, prothrombin time and INR were significantly higher, and albumin and

**Table 3** Clinical profile in the patients with and without ascites

Variables	Ascites ( <i>n</i> = 20)	No ascites ( <i>n</i> = 44)	<i>P</i> value
Age at admission (yr)	36.60 ± 3.99	29.98 ± 2.25	0.127
Sex (female/male)	7/13	19/25	0.537
History of portal cavernoma (yr)	2.38 ± 0.89	4.28 ± 1.06	0.264
Variceal bleeding (yes/no)	12/8	21/23	0.565
Varices (high risk/low risk/no varices)	32/3/9	14/3/3	0.545
Abdominal distension (yes/no)	17/3	20/24	0.003
Abdominal pain (yes/no)	9/11	17/27	0.631
Hydrothorax (yes/no)	10/10	0/44	< 0.001
Jaundice (yes/no)	2/18	2/42	0.403
RBC (10 <sup>12</sup> /L)	3.94 ± 0.21	3.96 ± 0.13	0.944
Hb (g/L)	104.60 ± 6.55	107.00 ± 5.04	0.783
WBC (10 <sup>9</sup> /L)	12.28 ± 2.83	6.22 ± 0.62	0.006
PLT (10 <sup>9</sup> /L)	275.45 ± 53.28	309.30 ± 42.86	0.645
PT (s)	15.08 ± 0.41	13.71 ± 0.28	0.007
INR	1.23 ± 0.03	1.11 ± 0.02	0.006
ALT (U/L)	24.65 ± 3.36	32.39 ± 4.54	0.282
AST (U/L)	28.85 ± 4.28	30.93 ± 3.33	0.717
AST/ALT	1.33 ± 0.18	1.17 ± 0.07	0.305
ALP (U/L)	103.55 ± 10.29	108.88 ± 10.21	0.751
GGT (U/L)	46.70 ± 10.00	38.64 ± 7.43	0.535
ALB (g/L)	35.17 ± 0.91	39.65 ± 0.64	< 0.001
TBIL (μmol/L)	17.79 ± 2.92	15.27 ± 1.39	0.379
DBIL (μmol/L)	8.35 ± 1.57	6.78 ± 1.05	0.409
Serum Cr (μmol/L)	73.40 ± 5.16	69.40 ± 2.77	0.460
Serum Na (mmol/L)	138.01 ± 1.00	140.24 ± 0.45	0.021
Child-Pugh class (A/B/C)	8/11/1	43/1/0	< 0.001
Child-Pugh score	6.85 ± 0.26	5.18 ± 0.07	< 0.001
MELD score	5.60 ± 0.93	3.64 ± 0.63	0.086
Main portal vein (obstruction/patency)	18/2	40/3	0.908
Right portal vein (obstruction/patency)	15/5	34/10	0.842
Left portal vein (obstruction/patency)	16/4	35/9	0.967
Splenic vein (obstruction and splenectomy/patency)	11/9	28/16	0.512
Superior mesenteric vein (obstruction/patency)	15/5	19/25	0.018

Data are expressed as quantitative data as frequency or mean ± SE. RBC: Red blood cell; Hb: Hemoglobin; WBC: White blood cell; PLT: Platelets; PT: Prothrombin time; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase; ALB: Albumin; TBIL: Total bilirubin; DBIL: Direct bilirubin; MELD: Model for end-stage liver disease.

serum sodium were significantly lower in patients with ascites than in those without (Table 3). A higher Child-Pugh score was also present in patients with ascites than in those without (6.85 ± 0.26 *vs* 5.18 ± 0.07, *P* < 0.001). Additionally, superior mesenteric vein obstruction was more frequently observed in patients with ascites than in those without (15/20 *vs* 19/44, *P* = 0.027).

### Treatment

Ten patients were diagnosed with acute thrombotic episodes. None presented with variceal bleeding, but four had high-risk varices detected by endoscopy. Mild ascites was found by CDUS in three patients. The main portal

vein was completely ( $n = 9$ ) or partially ( $n = 1$ ) obstructed. After intravenous anticoagulation was administered for 2-5 d, abdominal pain was alleviated in 5 patients and aggravated in another 5 patients. For the 5 patients with improved abdominal pain, oral anticoagulation was continued. During follow-up, partial recanalization of the main portal vein was found in 1 patient, while the main portal vein became unidentifiable and was replaced by cavernous collateral vessels in the remaining 4 patients. One patient with high-risk varices experienced melena 2 wk after anticoagulation. Anticoagulants were discontinued in this patient and were not resumed. For the 5 patients with increased abdominal pain, thrombolytics were indirectly infused *via* the superior mesenteric artery in 3 patients and directly *via* the portal vein in two patients. Of the 3 patients who received indirect thrombolysis, one became asymptomatic, while the other 2 patients underwent intestinal resection for ischemic intestinal infarction. One of the 2 patients died of pulmonary embolism 5 d after surgery. Of the 2 patients receiving direct thrombolysis for 3-5 d, abdominal pain completely resolved. During follow-up, partial recanalization of the main portal vein was found in 2 patients, while the main portal vein was replaced by cavernous collateral vessels in the remaining 3 patients. No adverse events were recorded.

Twenty-nine patients presented with acute ( $n = 7$ ) and recurrent variceal bleeding ( $n = 22$ ). Of the patients with acute variceal bleeding, 5 received pharmacological treatment, 1 had emergency endoscopic sclerotherapy and one underwent embolization of the gastric varices *via* a percutaneous trans-splenic approach. Active bleeding was controlled in these patients. One patient died of massive variceal rebleeding 49 d after discharge. Of the patients with recurrent variceal bleeding, TIPS placement was attempted after admission and was technically successful in eight patients. Of the remaining 14 patients with TIPS failure, 13 experienced at least one episode of rebleeding within 6 mo, and 1 was lost to follow-up. Three of these 13 patients underwent splenectomy and devascularization, and 10 had repeated endoscopic treatments.

Additionally, TIPS procedures were attempted in 4 patients who presented with repeated episodes of abdominal distension over more than 4 years, and these were technically successful in three patients. After successful TIPS insertions, two patients with SMV thrombosis developed shunt occlusions, and one of them presented with variceal hemorrhage that was controlled by endoscopic sclerotherapy. The shunt was patent in another patient. Preoperatively, his spleen was palpably enlarged 10 cm below the left costal margin. The size of the spleen was reduced, but the spleen remained clinically palpable at 20 mo after surgery. After TIPS failure, one patient underwent splenectomy for splenomegaly (6 cm below the left costal margin) and hypersplenism (a low WBC count of  $1520 \text{ cells/mm}^3$  and a low platelet count of  $32000/\text{mm}^3$ ).

### Overall survival

After a mean follow-up of  $18 \pm 2.41$  mo, a total of seven

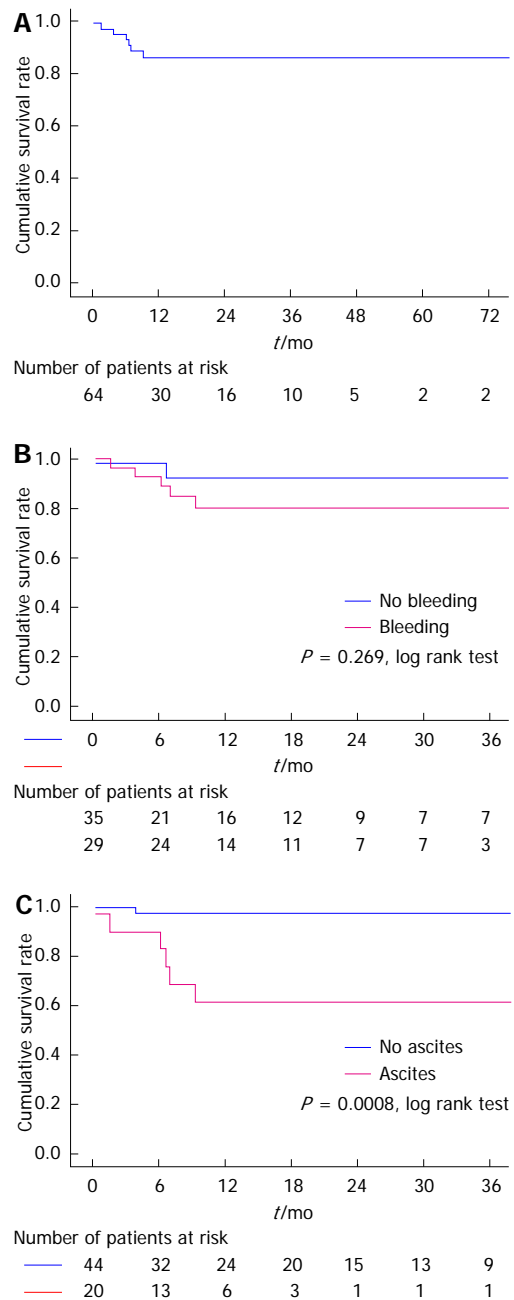


Figure 1 Overall survival in all patients (A), patients with and without variceal bleeding (B) and patients with and without ascites (C).

patients had died. Causes of death were pulmonary embolism ( $n = 1$ ), acute leukemia ( $n = 1$ ), massive esophageal variceal hemorrhage ( $n = 1$ ), progressive liver failure ( $n = 2$ ), multiple liver abscesses ( $n = 1$ ) and accident ( $n = 1$ ). Overall, 6-, 12- and 36-mo cumulative survival rates were 94.9%, 86% and 86%, respectively (Figure 1A). Cumulative 6-, 12- and 36-mo survival rates were similar between patients with and without variceal bleeding (92.8%, 80.2% and 80.2% *vs* 97.1%, 92.5% and 92.5%, respectively,  $P = 0.269$ , Log-rank test) (Figure 1B). Cumulative 6-, 12- and 36-mo survival rates were significantly different between patients with and without ascites (90%, 61.5% and 61.5% *vs* 97.3%, 97.3% and 97.3%, respectively,  $P = 0.0008$ , Log-rank test, Figure 1C).

**Table 4** Univariate analysis of baseline variables predicting overall survival

Variables	HR	95%CI	P value
Sex (female/male)	0.559	0.108-2.883	0.487
Age at admission	1.032	0.984-1.083	0.194
Age at first diagnosis (≤ 18 yr / > 18 yr)	0.840	0.187-3.772	0.820
History of portal cavernoma	0.989	0.878-1.114	0.857
Varices (yes/no)	0.428	0.083-2.213	0.311
Variceal bleeding (yes/no)	2.450	0.475-12.653	0.285
Abdominal distension (yes/no)	2.253	0.435-11.657	0.333
Abdominal pain (yes/no)	2.010	0.450-8.989	0.361
Ascites (yes/no)	15.066	1.811-125.337	0.012
Hydrothorax (yes/no)	6.638	1.452-30.346	0.015
RBC	0.681	0.288-1.614	0.383
Hb	0.987	0.964-1.011	0.302
PLT	1.000	0.997-1.003	0.900
WBC	1.099	1.040-1.162	0.001
PT	1.083	0.751-1.562	0.668
INR	5.253	0.057-480.003	0.471
ALT	0.970	0.915-1.029	0.312
AST	0.966	0.905-1.032	0.305
AST/ALT	2.328	0.891-6.083	0.085
ALP	1.003	0.993-1.013	0.558
GGT	1.003	0.989-1.017	0.684
ALB	0.932	0.795-1.094	0.392
TBIL	1.028	0.969-1.091	0.358
DBIL	1.036	0.941-1.140	0.470
Serum Cr	1.007	0.971-1.045	0.704
Serum Na	0.764	0.635-0.918	0.004
Child-Pugh score	1.573	0.976-2.535	0.063
Child-Pugh class (class A/class B and C)	0.083	0.009-0.763	0.028
MELD score	1.067	0.899-1.267	0.459
TIPS insertion (failure/success)	0.677	0.095-4.806	0.696
Main portal vein (patency/obstruction)	0.042	0.0001-1720.16	0.558
Main portal vein (no fibrotic cord/fibrotic cord)	0.798	0.155-4.113	0.787
Right portal vein (patency/obstruction)	2.269	0.507-10.151	0.284
Left portal vein (patency/obstruction)	1.639	0.318-8.455	0.555
Splenic vein (patency/ obstruction and splenectomy)	0.533	0.103-2.766	0.454
Superior mesenteric vein (patency/obstruction)	0.159	0.019-1.330	0.090

RBC: Red blood cell; Hb: Hemoglobin; WBC: White blood cell; PLT: Platelets; PT: Prothrombin time; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT:  $\gamma$ -glutamyl transferase; ALB: Albumin; TBIL: Total bilirubin; DBIL: Direct bilirubin; MELD: Model for end-stage liver disease; TIPS: Transjugular intrahepatic portosystemic shunt.

Given that the risk factors of EHPVO were detected in only half of the patients, they were not included in the prognostic analysis. In the univariate analysis, variables that were significantly associated with reduced survival included the presence of ascites and hydrothorax, a low level of serum sodium, an elevated WBC count and a high Child-Pugh class (Table 4). Multivariate backward stepwise Cox regression analysis demonstrated that the presence of ascites (HR = 10.729, 95%CI: 1.209-95.183,  $P$  = 0.033) and an elevated WBC count (HR = 1.072, 95%CI: 1.014-1.133,  $P$  = 0.015) were independent pre-

dictors of increased mortality in non-malignant and non-cirrhotic patients with portal cavernoma.

## DISCUSSION

This study was primarily designed to evaluate the outcome of non-malignant and non-cirrhotic patients with portal cavernoma and to determine prognostic factors. Our study demonstrated that most patients with portal cavernoma in the absence of cirrhosis or malignancy had a relatively benign course (overall cumulative survival rate was 86% at 60 mo), which is similar to the excellent outcomes of non-cirrhotic patients with portal vein or splanchnic vein thrombosis reported by previous studies<sup>[23-25]</sup>. However, it should be noted that all deaths occurred within the first year after admission, which is explained by the following points. First, a long history of portal cavernoma was recorded in some patients. It is possible that the underlying disease or liver dysfunction had already become severe in these patients, warranting referral to our highly specialized center. Second, as shown previously, the overall mortality of splanchnic vein thrombosis patients with intestinal infarction is high<sup>[23]</sup>. As a primary cause of death, intestinal infarction and its secondary severe complications often occur at the early stage. In our study, one of two patients who underwent bowel resection for intestinal infarction died 5 d after surgery.

Despite the high prevalence of variceal bleeding in our patients, the incidence of death was not significant (only one patient died of massive variceal bleeding), mainly due to advances in treatment modalities for controlling bleeding and well-preserved liver function in these patients. More importantly, we found that the presence of ascites might act as the most important prognostic factor for death. This finding could be explained by the higher incidence of liver dysfunction in patients with ascites. Indeed, the deterioration of liver function is caused not only by the presence of ascites itself, but also by a higher prothrombin time and INR and a lower level of serum albumin and sodium, which are closely correlated with the presence of ascites. Based on the prognostic significance of ascites in non-malignant and non-cirrhotic patients with portal cavernoma, therapeutic decision making needs to be further altered. Once ascites is detected, we should pay more attention to early diagnosis and treatment of underlying comorbidities and liver dysfunction. Accordingly, we hypothesize that the prevention and treatment of liver dysfunction should be incorporated into the treatment strategy for portal cavernoma<sup>[4]</sup>.

We also found that an elevated WBC count was an independent predictor of survival. This might be explained by the fact that comorbidities, such as acute leukemia ( $n$  = 1) and multiple liver abscesses ( $n$  = 1) could be more common in patients with an elevated WBC count. However, it should be noted that the effect size was very small (hazard ratio was very close to 1). Therefore, the significance of WBC count on patient survival might be clinically slight.



Our study has several limitations. First, only patients diagnosed with portal cavernoma were included in this study. The inclusion criteria may influence the application of prognostic factors in patients with acute EHPVO. However, we believe that it was important to differentiate between the outcomes of acute EHPVO and portal cavernoma, because of their dissimilar clinical presentations and natural history. Second, the prevalence and significance of underlying etiological factors in patients with EHPVO are discussed elsewhere<sup>[13,14,26,27]</sup>, but not in this study. Therefore, we can not demonstrate the association between survival and prothrombotic factors, including acquired and inherited factors. Further work is warranted to explore the effect of etiological factors on survival. Third, given the excellent outcome of non-malignant and non-cirrhotic patients with portal cavernoma, mean follow-up time was relatively short and a low proportion of patients met the endpoints in our study. This bias might miss other potential prognostic factors. An extended follow-up should be carried out in future studies. Fourth, the laboratory analysis of ascitic fluids was not performed. We could not exclude the possibility of spontaneous bacterial peritonitis, especially in cases with an elevated WBC. Finally, given that the number of deaths was only 7, the multivariate Cox regression analysis might be inappropriate. Indeed, the number of variables included in the multivariate analysis might introduce the risk of overfitting the data, thereby leading to a high risk of false positive results. Therefore, the conclusions of this analysis should be taken with caution and further confirmed in larger studies.

In conclusion, our study suggests that the presence of ascites and an elevated WBC count are significantly associated with increased mortality in non-malignant and non-cirrhotic patients with portal cavernoma. Further studies are needed to confirm the prognostic significance of ascites in these patients and to establish a new therapeutic strategy based on the presence of ascites.

## COMMENTS

### Background

Portal cavernoma is traditionally considered a sequela of extrahepatic portal vein obstruction (EHPVO) to compensate for the interrupted portal blood flow. Due to its low morbidity and mortality in non-malignant and non-cirrhotic patients, little information is known regarding the prognostic factors of portal cavernoma.

### Research frontiers

Previous studies, in which malignancy and cirrhosis were not excluded, have revealed that the increased mortality in EHPVO patients is closely associated with advanced age, presence of malignancy and cirrhosis, high bilirubin and deterioration of liver function.

### Innovations and breakthroughs

The authors conducted an 8-year retrospective single-center study to determine the predictors for survival of non-malignant and non-cirrhotic patients with portal cavernoma managed by a uniform therapeutic strategy at our center.

### Applications

The authors found that the presence of ascites might act as the most important prognostic factor for death in non-malignant and non-cirrhotic patients with portal cavernoma. Based on the prognostic significance of ascites in non-malignant and non-cirrhotic patients with portal cavernoma, therapeutic decision making

needs to be further altered.

### Terminology

Portal cavernoma, also known as cavernous transformation of the portal vein, is a rare entity, which is characterized by a tangle of tortuous hepatopetal collateral veins in the hilum.

### Peer review

It is an interesting review of portal cavernoma evolution. In this study, the authors investigated the prognostic factors for portal cavernoma in non-malignant and non-cirrhotic patients. They were able to show that the presence of ascites and an elevated white blood cell count were strongly associated with poor prognosis in non-cirrhotic patients with portal cavernoma.

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## Effect of low-dose amitriptyline on globus pharyngeus and its side effects

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### Abstract

**AIM:** To compare the efficacy and side effects of low-dose amitriptyline (AMT) with proton pump inhibitor treatment in patients with globus pharyngeus.

**METHODS:** Thirty-four patients who fulfilled the Rome III criteria for functional esophageal disorders were included in this study. Patients were randomly assigned to receive either 25 mg AMT before bedtime (AMT group) or 40 mg Pantoprazole once daily for 4 wk (conventional group). The main efficacy endpoint was assessed using the Glasgow Edinburgh Throat Scale (GETS). The secondary efficacy endpoints included the Medical Outcomes Study 36-item short form health survey [social functioning (SF)-36] and the Pittsburgh Sleep Quality Index. Treatment response was defined as a > 50% reduction in GETS scores. All patients entering this study recorded side effects at days 1, 8, 15, 22 and 29 using a visual analogue scale.

**RESULTS:** Thirty patients completed the study. After 4

wk of treatment, the AMT group had a greater response than the conventional group (75% vs 35.7%,  $P = 0.004$ ). At day 3, the AMT group showed significantly more improvement than the Conventional group in GETS score ( $3.69 \pm 1.14$  vs  $5.64 \pm 1.28$ ,  $P = 0.000$ ). After 4 wk of treatment, the AMT group showed significantly greater improvement in GETS score and sleep quality than the Conventional group ( $1.25 \pm 1.84$  vs  $3.79 \pm 2.33$ ,  $4.19 \pm 2.07$  vs  $8.5 \pm 4.97$ ;  $P < 0.01$  for both). Additionally, the AMT group was more likely than the Conventional group to experience improvement in the SF-36, including general health, vitality, social functioning and mental health ( $P = 0.044$ ,  $0.024$ ,  $0.049$  and  $0.005$ ). Dry mouth, sleepiness, dizziness and constipation were the most common side effects.

**CONCLUSION:** Low-dose AMT is well tolerated and can significantly improve patient symptoms, sleep and quality of life. Thus, low-dose AMT may be an effective treatment for globus pharyngeus.

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**Key words:** Amitriptyline; Globus pharyngeus; Side effect; Pantoprazole; Treatment response

**Core tip:** A literature review reveals that there is no single effective treatment for patients with globus pharyngeus. Low-dose amitriptyline (AMT) is extensively used to treat functional gastrointestinal disorders, especially in cases with prolonged severe symptoms and disorders that affect daily function. However, no data regarding the possible effects of AMT in patients with globus pharyngeus are available. In this study, we conclude that low-dose AMT is well tolerated and can significantly improve patient symptoms. Thus, we recommend the use of low-dose AMT for globus pharyngeus.

You LQ, Liu J, Jia L, Jiang SM, Wang GQ. Effect of low-dose amitriptyline on globus pharyngeus and its side-effects. *World J*

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## INTRODUCTION

Globus pharyngeus is a condition characterized by a non-painful sensation of a lump in the throat in the absence of true dysphagia or odynophagia; the sensation frequently improves with eating<sup>[1]</sup>. It is a common condition that accounts for approximately 4% of otolaryngological referrals<sup>[2]</sup>, and it is usually long-lasting, difficult to treat, recurrent and associated with a significant impairment in quality of life. Furthermore, due to the uncertain etiology of globus, it remains difficult to establish standard investigation and treatment strategies for affected patients.

Amitriptyline (AMT) is a tricyclic antidepressant with limited application due to the side effects caused by high doses (100 mg/d). In recent years, low-dose AMT has been shown to be well tolerated and significantly effective in improving the functional gastrointestinal disorders<sup>[3-5]</sup>. In 1994, Deary *et al*<sup>[6]</sup> were the first to attempt a prospective, randomized, double-blinded, placebo-controlled trial investigating the effectiveness of AMT in patients with globus pharyngeus. Most patients could not tolerate the side effects of AMT at doses of 50 mg/d to 150 mg/d, resulting in treatment failure. To the best of our knowledge, evidence supporting the possible effects of low-dose AMT in patients with globus pharyngeus has not been reported.

Therefore, the aim of this study was to investigate the response rate, onset time, side effects and clinical predictors of symptom response to low-dose AMT treatment in patients with globus pharyngeus.

## MATERIALS AND METHODS

### Patients

In this prospective study, we enrolled 34 patients who complained of globus symptoms between September 2011 and January 2013. All patients were between 12 and 65 years of age and were newly diagnosed as having functional esophageal disorders based on the following Rome III criteria<sup>[7]</sup>: (1) persistent or intermittent, nonpainful sensation of a lump or foreign body in the throat; (2) occurrence of the sensation between meals; (3) absence of dysphagia or odynophagia; (4) absence of evidence that gastroesophageal reflux is the cause of the symptom; and (5) absence of histopathology-based diagnosis of esophageal motility disorders. All included patients fulfilled the criteria for the last 3 mo, with symptom onset at least 6 mo before diagnosis. All patients underwent otolaryngological assessment with neck/thyroid palpation and gastroscopy or laryngoscopy, and none had any organic abnormality on assessment. The following exclusion criteria were adopted: hepatic or renal disease; prostatic disease; pregnancy or breast feeding; known glaucoma;

history of seizures; history of thyroid or liver dysfunction; recent use of monoamine oxidase inhibitors; use of any proton pump inhibitor (PPI) or histamine type 2 receptor antagonist during the last 2 mo; use of tranquilizers or antidepressants that may affect esophageal motor function; and moderate to severe anxiety or depression (14-item Hamilton Anxiety Rating Scale assessed from 0 to 13 points and 17-item Hamilton Depression Rating Scale assessed from 0 to 17 points).

### Study design and procedures

This study was a prospective, randomized controlled trial in globus pharyngeus patients and was approved by the hospital ethics committee (Clinical trial registration number: ChiCTR-TRC-12001968). Written informed consent was obtained from the patients according to the Declaration of Helsinki.

Thirty-four eligible patients were randomized to receive either 25 mg AMT once daily before bedtime or 40 mg Pantoprazole once daily for 4 wk. Treatment was allocated by a simple randomization method using a computer-generated randomization schedule. Ultimately, 17 patients received AMT, and 17 patients received Pantoprazole. The primary endpoint was assessed using the Glasgow Edinburgh Throat Scale (GETS) questionnaire<sup>[8]</sup>. We observed the onset time and the treatment efficiency on days 3 and 10 and week 4, and evaluated the social functioning (SF)-36 and Pittsburgh sleep quality index (PSQI) scores at baseline and at week 4 as secondary endpoints<sup>[9,10]</sup>.

All patients entering this study recorded side effects. The severity of side effects was evaluated using a visual analogue scale (VAS) administered on days 1, 8, 15, 22 and 29 after medication.

### GETS

The GETS questionnaire provided both the primary and secondary outcomes. It is a validated questionnaire used to rate globus pharyngeus symptoms. The globus symptom score component is based on 10 questions assessing various throat symptoms. Patients subjectively grade their symptoms for each question on a 7-point Likert scale, with 0 being "none" and 7 being "unbearable". These compiled questions yield a score that represents the severity of the patient's globus symptoms, with a maximum possible score of 70. The secondary outcome is the somatic distress score, which represents the psychological impact of the patient's symptoms. This component of the questionnaire is also graded on a 7-point Likert scale, with 0 being "never" and 7 being "all of the time". This yields a maximum total score of 14. Both the overall symptom score and the somatic distress score can be used over time to assess the severity of the disease.

### Medical outcomes study 36-item short form health survey

The SF-36 is the most commonly used scale for assessing patient quality of life, and it includes eight dimensions:



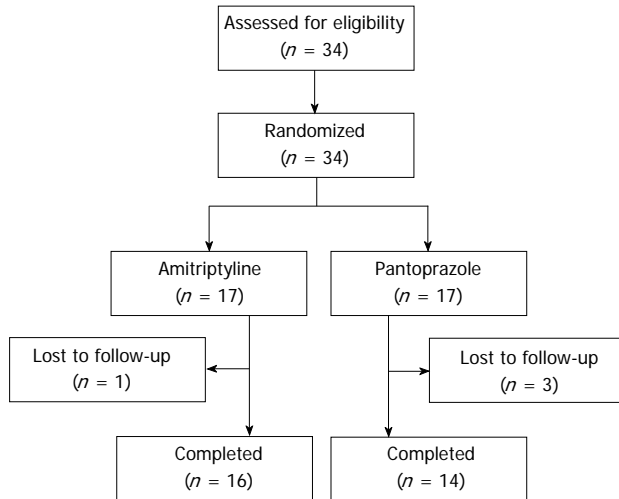


Figure 1 Consort diagram.

**Table 1 Demographic and baseline characteristics of the study patients**

Variable	AMT group n = 16	Conventional group n = 14	P value
Age (yr)	43.19 ± 10.96	42.93 ± 13.1	0.953
Gender (male/female)	6/10	5/9	1.000
BMI	21.54 ± 2.09	23.01 ± 3.49	0.166
Smoking habit	4 (18.75%)	3 (21.42%)	1.000
Alcohol consumption	6 (37.5%)	5 (35.71%)	1.000
Symptom duration (yr)	1.44 ± 0.66	1.54 ± 0.89	0.731
GETS	5.44 ± 1.63	5.71 ± 1.38	0.623
PSQI	8.75 ± 4.68	8.71 ± 5.27	0.984

Values are represented as the mean ± SD. AMT: Amitriptyline; BMI: Body mass index; GETS: Glasgow Edinburgh Throat Scale; PSQI: Pittsburgh sleep quality index.

physical functioning, role-physical (RP), bodily pain, general health (GH), vitality (VT), SF, role-emotional (RE), and mental health (MH). A higher score indicates a better quality of life.

### PSQI

Prepared by psychiatrist Buysse *et al*<sup>[9]</sup>, the PSQI assesses seven areas, including sleep quality, the time to fall asleep, sleep time, sleep efficiency, sleep disturbance, use of sleep medication and daytime dysfunction. In total, 18 items were used for the calculation of the PSQI score. Higher scores represent worse sleep quality, and a score > 7 points indicates the presence of a sleep disorder.

### Treatment response

Treatment response was defined as a > 50% reduction in the GETS score. The response was calculated as: [(score at treatment - score at baseline)/score at baseline] × 100. The treatment responses of the two groups were calculated separately.

### Statistical analysis

Data analysis was performed using SPSS 13.0 software

**Table 2 Change in glasgow edinburgh throat scale score and Pittsburgh sleep quality index in patients treated with amitriptyline vs pantoprazole and incidence of side effects n (%)**

Variable	AMT group n = 16	Conventional group n = 14	P value
GETS score			
Baseline	5.44 ± 1.63	5.71 ± 1.38	0.623
3 d	3.69 ± 1.14 <sup>1</sup>	5.64 ± 1.28	0.000
10 d	2 ± 1.71 <sup>1</sup>	5.36 ± 1.22	0.000
4 wk	1.25 ± 1.84 <sup>1</sup>	3.79 ± 2.33 <sup>1</sup>	0.002
Treatment response	12 (75)	5 (35.71)	0.004
PSQI	4.19 ± 2.07	8.5 ± 4.97	0.008
Adverse effect			
Dry mouth	12 (75)	4 (28.5)	0.026
Sleepiness	11 (68.8)	2 (14.3)	0.004
Dizziness	4 (25)	1 (7.1)	0.336
Constipation	3 (18.8)	1 (7.1)	0.602
Palpitations	1 (6.3)	0 (0)	1.000
Malaise	1 (6.3)	0 (0)	1.000

<sup>1</sup>Statistically significant difference from baseline,  $P < 0.05$  between the two groups. AMT: Amitriptyline; GETS: Glasgow Edinburgh Throat Scale; PSQI: Pittsburgh sleep quality index.

(SPSS Inc., Chicago IL, United States), and the measurement data are reported as the mean ± SD; baseline parameters and differences between the two treatments were compared using Student's *t* test. The scores at baseline and after AMT or Pantoprazole treatment were compared by paired *t* test. The VAS scores of side effects were analyzed using the  $\chi^2$  test or Fisher's exact test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Study participants

A total of 34 patients with globus symptom were enrolled in the study. All patients were randomized to receive either AMT or Pantoprazole. Of these patients, four were lost to follow-up. A total of 30 patients (AMT 16, Pantoprazole 14) completed the study (Figure 1). The baseline characteristics of the patients are shown in Table 1. No differences were observed between the two groups in age, gender, body mass index, smoking habit, alcohol consumption, symptom duration, GETS score or PSQI score.

### Primary efficacy endpoint

The rate of effectiveness was calculated in the two groups after 4 wk of treatment, the response rate of the AMT group was significantly higher than that of the Conventional group (75% *vs* 35.7%,  $P = 0.004$ , Table 2). Compared with the Conventional group, the GETS scores of the AMT group were significantly improved at days 3 and 10, and week 4 (all  $P < 0.01$ , Table 2). Compared with baseline, the GETS scores of the AMT group were significantly reduced at days 3 and 10 and week 4 (all  $P < 0.05$ ). However, the GETS scores of the Conventional group were significantly reduced only after 4 wk of treatment. The onset time in the AMT group was significantly earlier than that in the Conventional group.

**Table 3** Changes in social functioning-36 subscale scores in patients treated with amitriptyline *vs* Pantoprazole

Variable		Baseline	Week 4	P value
PF	AMT	95 ± 11.4	95.94 ± 11.14	0.945
	Conventional	93.93 ± 7.12	95.71 ± 4.75	
RP	AMT	64.06 ± 37.6	81.25 ± 26.61 <sup>1</sup>	0.929
	Conventional	66.07 ± 37.48	80.36 ± 28.04 <sup>1</sup>	
BP	AMT	89.38 ± 16.52	90.63 ± 14.55	0.498
	Conventional	85.57 ± 20.68	86.29 ± 19.96	
GH	AMT	56.75 ± 20.92	73 ± 17.57 <sup>1</sup>	0.044
	Conventional	49.86 ± 22.49	58.71 ± 19.57 <sup>1</sup>	
VT	AMT	70.63 ± 18.15	88.44 ± 7.01 <sup>1</sup>	0.024
	Conventional	71.79 ± 24.7	73.21 ± 24.39	
SF	AMT	75 ± 18.25	92.97 ± 13.67 <sup>1</sup>	0.049
	Conventional	77.68 ± 18.46	81.25 ± 17.51 <sup>1</sup>	
RE	AMT	68.76 ± 35.42	79.18 ± 23.96 <sup>1</sup>	0.384
	Conventional	61.91 ± 41.05	69.05 ± 38.04	
MH	AMT	68.75 ± 15.68	83.5 ± 10 <sup>1</sup>	0.005
	Conventional	65.43 ± 19.32	69.14 ± 15.72	

<sup>1</sup>Statistically significant difference compared with baseline,  $P < 0.05$  between the two groups. AMT: Amitriptyline; PF: Physical functioning; RP: Role-physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role-emotional; MH: Mental health.

### Secondary efficacy endpoints

Compared with the Conventional group, the AMT group exhibited significant improvements in the GH, VT, SF, MH scales of the SF-36 (all  $P < 0.05$ , Table 3). Compared with baseline, the AMT group exhibited significant improvements in RP, GH, VT, SF, RE and MH (all  $P < 0.05$ ). The Conventional group experienced significant improvements in only RP, GH and SF (all  $P < 0.05$ , Table 3).

The AMT group also showed a significant improvement in PSQI compared with the Conventional group ( $4.19 \pm 2.07$  *vs*  $8.5 \pm 4.97$ ,  $P = 0.008$ , Table 2). Compared with baseline, the AMT group exhibited significant improvement. However, there were no significant differences in PSQI at baseline and after pantoprazole treatment ( $P > 0.05$ ).

### Adverse effects

The various adverse events reported in the two treatment groups are shown in Table 2. The incidence rates of dry mouth and sleepiness in the AMT group were significantly higher than in the Conventional group ( $P = 0.026$ ,  $P = 0.004$ , Table 2). The incidence rates of dizziness and constipation were also somewhat higher in the AMT group than in the Conventional group, although the difference was not statistically significant ( $P = 0.336$ ,  $P = 0.602$ , Table 2).

As shown in Figure 2, sleepiness and dizziness almost disappeared within 1 wk. Patients who had dry mouth and constipation symptoms experienced significant relief after receiving AMT for 2 wk. No serious adverse events occurred in either group.

## DISCUSSION

There is no consistent evidence attributing globus to any

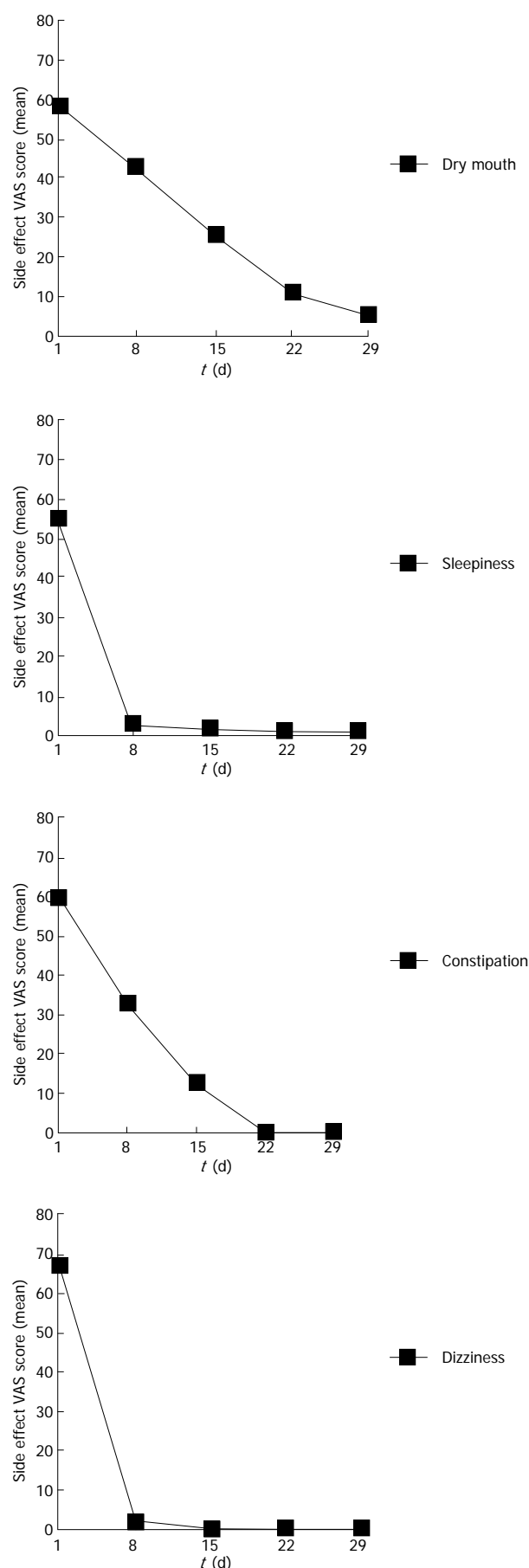


Figure 2 Side effect diagrams. VAS: Visual analogue scale.

specific anatomic abnormality, including the cricopharyngeal bar. Although it has been long since it was first described, the etiology of globus pharyngeus is poorly understood. Abnormal upper esophageal sphincter function, esophageal motility disorders, psychological factors, stress, and *Helicobacter pylori* infection of the cervical heterotopic gastric mucosa have all been suggested as potential causes of globus<sup>[11-15]</sup>. There is much controversy about the true etiology of globus pharyngeus, and it has been difficult to establish a causal relationship between globus and these other disorders<sup>[16]</sup>. In addition, esophageal balloon distention can simulate the sensation of globus at low distending thresholds, suggesting some degree of esophageal hypersensitivity<sup>[11]</sup>. Because there are few controlled studies on the treatment of globus, evidence-based treatment for this disorder is currently not available. Some studies have suggested that gastroesophageal reflux disease (GERD) is a major cause of globus<sup>[16,17-19]</sup>. Therefore, it seems practical to use anti-reflux methods as the first-line treatment for managing patients with globus<sup>[11]</sup>, although this protocol remains under considerable debate. Other established treatment options include speech and language therapy, anti-depressants, and cognitive-behavioral therapy<sup>[11,20-22]</sup>.

Low-dose AMT has been used widely in gastroenterologic practice. Morgan *et al*<sup>[23]</sup> demonstrated that the doses for the analgesic and neuromodulatory effects of AMT were below the effective doses of the antidepressant. Huang *et al*<sup>[24]</sup> observed that low-dose AMT could reduce visceral sensitivity using the noninvasive drinking-ultrasonography test in healthy volunteers. However, our understanding of the mechanism of action of this agent is limited. A commonly held hypothesis is that AMT, which indirectly stimulates norepinephrine and serotonin receptors by blocking the reuptake of norepinephrine and serotonin, works as a neuromodulator, affecting the brain-gut axis by altering neurotransmitter systems within the limbic system and other pain centers of the brain<sup>[25]</sup>. In addition, one study showed that AMT can alter sleep patterns<sup>[25]</sup>. Our study also confirmed that the AMT group showed significantly greater improvement in sleep than the Conventional group. We hypothesize that the overall effects of amitriptyline in patients with globus arise from its central nervous system activity, as altering sleep patterns modulates the regulation of the noradrenergic system of the locus coeruleus (a brain center inhibited during sleep), which alters nociception.

In our study, 5 of 14 (35.7%) patients with globus pharyngeus receiving PPI treatment as a conventional therapy were classified as responders. Dumper *et al*<sup>[26]</sup> demonstrated that there was no clinically or statistically significant difference between lansoprazole and placebo at any time point during a 3-mo treatment period. Therefore, it is important to note that although GERD is a condition that is sometimes associated with globus, this does not mean that all globus is GERD-related or that all GERD patients have globus. Thus, PPI treatment in patients with globus may not achieve satisfactory results.

To our knowledge, this is the first prospective, randomized controlled trial investigating the effectiveness of a therapeutic low dose of AMT in globus patients. The treatment efficiency of the AMT group was 75%, significantly greater than that of the Conventional group (35.7%). After 3 d of treatment, the symptoms, sleep, and quality of life of the AMT group improved more than those of the Conventional group. During treatment, average cost in the AMT group was also significantly lower than that in the Conventional group.

There are several limitations of this study. First, although many patients with globus symptoms and normal examinations were seen in our clinic, this study included only a small sample of patients with globus. A number of patients had recently started on PPI or were diagnosed with moderate to severe anxiety or depression, and excluding these patients resulted in a decreased number of cases in this study. Second, it is possible that the course of medication in our study was too short; the standard duration of AMT administration in the clinic is 4-12 wk<sup>[27]</sup>. We also evaluated only the response to short-term PPI treatment and did not investigate the response to long-term PPI treatment (3 or more months).

In conclusion, low-dose AMT is well tolerated and may be effective in reducing the symptoms of patients with globus pharyngeus while also significantly improving sleep and quality of life. However, further studies with larger sample sizes and longer follow-up periods are needed to verify our results.

## ACKNOWLEDGMENTS

We would like to thank Ming-Zhi Xu, Professor of the Guangdong Mental Disorder Research Institute, for his guidance in the use of SF-36 and the anxiety and depression scale. We also express our appreciation to Dr. Su of ENT for his assistance in diagnosing globus pharyngeus.

## COMMENTS

### Background

Low-dose amitriptyline (AMT) is extensively used for treating functional gastrointestinal disorders, although its precise mechanism of action is still not fully understood. In 1994, Deary *et al* performed the first study investigating the effectiveness of high-dose AMT (50-150 mg/d) in patients with globus pharyngeus. However, most patients could not tolerate the side effects of high doses of AMT, resulting in treatment failure. The effect of low-dose AMT in patients with globus pharyngeus has not been reported.

### Research frontiers

There is currently a lack of highly effective pharmacotherapy for globus pharyngeus. Low-dose AMT may be a good choice of treatment for patients with globus pharyngeus.

### Innovations and breakthroughs

This is the first prospective, randomized controlled trial evaluating the effectiveness of a therapeutic low dose of AMT in globus patients. The results revealed that low-dose AMT is well tolerated and can significantly improve patient symptoms, sleep and quality of life.

### Applications

Low-dose AMT can be significantly effective in improving the symptoms and quality of life of the patients, thereby supporting its clinical applicability for gastrointestinal disorders.

# Terminology

Globus pharyngeus is a physical sensation of a lump in the throat that causes difficulty or discomfort in swallowing. The sensation may also be choking or feeling as though there is a mass lodged in the esophagus. AMT is a tricyclic antidepressant that is extensively used to treat functional gastrointestinal diseases, especially in cases with prolonged severe symptoms and daily functional disorders.

# Peer review

This is an interesting paper evaluating the effect of low-dose AMT on globus pharyngeus. The study is reasonably well done, and the design and outcome assessment are standardized.

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## Combined radiochemotherapy in patients with locally advanced pancreatic cancer: A meta-analysis

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### Abstract

**AIM:** To compare the long-term clinical efficacy of chemotherapy plus radiotherapy (CRT) with that of radiotherapy alone (RT) or chemotherapy alone (CT) for locally advanced pancreatic carcinoma (LAPC).

**METHODS:** Using manual and computer-aided methods, we searched the data through the databases, including PubMed/EmBase/CNKI/CQVIP/China Journals Full Text Database and websites and proceedings of major annual meetings such as ASCO and CSCO. The methodological quality of the included studies was assessed using the Jadad scoring system. Both English and Chinese publications were searched. We collected data from controlled clinical trials on CRT vs RT or CT for LAPC, and conducted a meta-analysis of 15 included

studies. Meta-analysis was performed using RevMan4.2 Software according to the method recommended by Cochrane Collaboration.

**RESULTS:** Fifteen eligible randomized controlled trials including a total of 1128 patients were screened. Jadad score was 2 in only one article, and 3-4 in the remaining 14 studies. The meta-analysis showed that CRT was superior in the 6- and 12-mo survivals to the RT alone group or CT alone group ( $P = 0.0001$  and  $P = 0.02$ , respectively), whereas the 18-mo survival showed no significant difference ( $P = 0.23$ ). Subgroup analysis showed that the 6-, 12-, and 18-mo survivals were not significantly different between the CRT group and CT group ( $P = 0.07$ ,  $P = 0.23$ , and  $P = 0.91$ , respectively). Notably, the CRT group had significantly better 6-, 12-, and 18-mo survivals than the RT group (all  $P < 0.01$ ). CRT group had significantly more grade 3-4 treatment-related hematologic and non-hematologic toxicities than the CT group or RT group (all  $P < 0.01$ ).

**CONCLUSION:** Compared with CT or RT, CRT can benefit the long-term survival of LAPC patients, although it may also increase treatment-related toxicities.

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**Key words:** Pancreatic cancer; Chemotherapy; Radiotherapy; Meta-analysis; Survival

**Core tip:** To compare the long-term clinical efficacy of chemotherapy plus radiotherapy (CRT) with that of radiotherapy alone (RT) or chemotherapy alone (CT) for locally advanced pancreatic carcinoma (LAPC), the authors analyzed the potential impact of CRT, CT or RT on the survival of the patients using meta-analysis methodologies. Meta-analysis showed that compared with CT or RT, CRT can benefit the long-term survival of LAPC patients, although it may also increase treatment-related toxicities.

Chen Y, Sun XJ, Jiang TH, Mao AW. Combined radiochemotherapy in patients with locally advanced pancreatic cancer: A meta-analysis. *World J Gastroenterol* 2013; 19(42): 7461-7471 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7461.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7461>

## INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States<sup>[1]</sup>. Over the past decades, the standard treatment for patients with inoperable locally advanced pancreatic cancer (LAPC) was chemotherapy alone (CT) or chemo-radiation therapy (CRT)<sup>[2-4]</sup>; however, the median survival time was only 6-9 mo, and less than 10% of patients survived for 2 years<sup>[5]</sup>. Definitive results of the 2000-01 FFCD/SFRO study, in which patients randomly received CRT (60 Gy + 5-fluorouracil + cisplatin + gemcitabine) or CT, showed that the progression free survival (PFS) and overall survival (OS) were not significantly different between these two groups<sup>[6]</sup>. On the contrary, the Eastern Cooperative Oncology Group (ECOG) study, which randomly divided the LAPC patients into a gemcitabine chemotherapy group and a gemcitabine + radiotherapy group, showed that the CRT group had significantly superior OS over the CT group; meanwhile, treatment-related toxicity in the CRT group was acceptable. In the present study, we analyzed the potential impact of CRT or CT or radiotherapy alone (RT) on the survival using meta-analysis methodologies in an attempt to obtain more robust evidence.

## MATERIALS AND METHODS

### Literature collection

The search strategy was: exp pancreas/(pancreas.tw.exp); pancreatic neoplasms/(pancreas adj neoplasms, pancreas adj cancers, pancreas adj carcinomas); 30-38 exp drug therapy/exp chemotherapy adjuvant/chemotherapy; chemoradiotherapy (combin adj chemotherapy, concurrent adj chemoradiotherapy; 49-50 exp radiotherapy/exp radiotherapy adjuvant/radiotherapy.

The literature search was conducted in both English and Chinese publications. The data sources included PubMed (1964-2012), EmBase (1964-2012), CNKI (1979-2012), CQVIP(1979-2012), China Journals Full Text Database (1979-2012), websites (*e.g.*, Web of Science) and proceedings of major annual meetings such as ASCO (1995-2012) and CSCO (1995-2012). Internet searches were carried out and bibliographies of included articles were searched. Finally, the Chinese references in the selected articles were reviewed for other relevant studies. The latest updates of serial clinical studies were used. Date of last search was October 15, 2012.

### Inclusion and exclusion criteria

**Inclusion criteria:** (1) Patients were pathologically con-

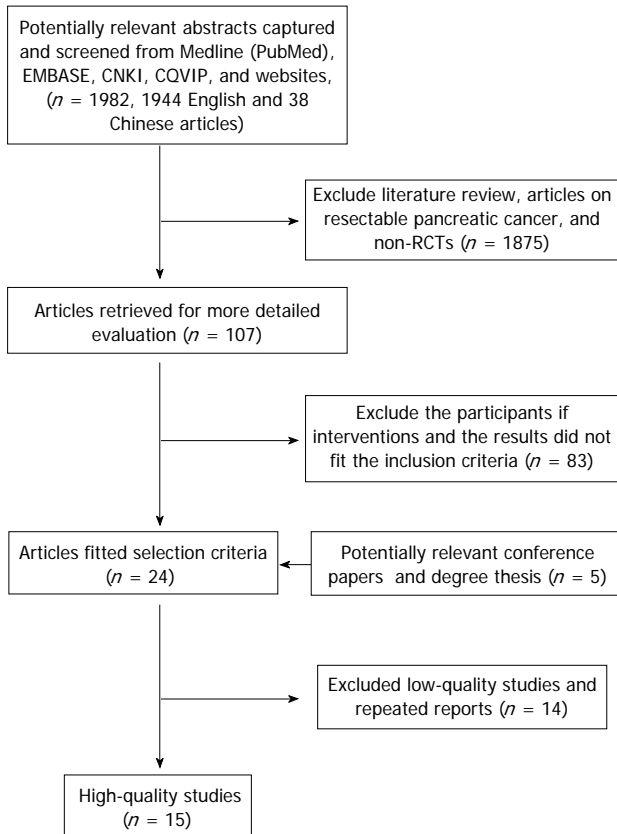
firmed to have locally advanced pancreatic malignant tumors (Tumors were judged as nonresectable due to extension to regional lymph nodes and/or vascular structures such as the superior mesenteric artery or the celiac trunk or the existence of a portal or superior mesenteric-portal venous confluent thrombosis), which were naive to surgical treatment or other anti-tumor therapies before enrollment; (2) the study was a prospective randomized controlled trial; (3) the interventions only included radiotherapy and/or chemotherapy; (4) the main endpoint was survival, and the observation lasted at least 6 mo, along with survival records; and (5) except for the treatment methods, the treatment group was parallel with the control group.

**Exclusion criteria:** (1) Patients with metastatic pancreatic cancer; individuals who suffered from relapse after anti-tumor treatment; patients who had previously received surgical treatment; patients with non-LAPC; (2) non-prospective and non-randomized/non-controlled studies; (3) other interventions were applied in addition to radiotherapy and chemotherapy; (4) only local efficacy was evaluated and no data on survival was available; and (5) low-quality studies that had a Jadad score of less than 2.

### Quality assessment

**Assessment of the included literature:** Two reviewers independently conducted methodological quality assessment and data retrieval. Cross-checking was then performed; any disagreements were resolved by discussion and, if necessary, a third reviewer. The methodological quality of the included studies was assessed using the Jadad scoring system: (1) Was the study described as randomized? Were the patients actually randomized into the treatment group or control group, and both the observers and patients did not know which group would be allocated? (2 = the appropriate randomization method was described; 1 = the author claimed the use of a randomization method); (2) Except for the targeted intervention(s), were the other procedures consistent between these two groups? (3) Was the study described as blinded (2 = both the patients and observers were blinded, and the blinding method was described; 1 = the author claimed that a double-blinding method was applied; 0 = not blinded); and (4) Was there a description of exclusion bias (*i.e.*, systematical difference of the withdrawals and drop-outs between these two groups; scored 0 or 1 for any reason of drop-outs). The final score ranged from 1 to 5. Studies scored 3 or higher were judged as "high-quality", and those scored 1 or 2 as "poor-quality". The criteria for methodological quality analysis were developed: (1) randomization; (2) blinding; (3) withdrawal or drop-outs; (4) allocation concealment; and (5) adoption of intentional analysis. The quality of the included literature was presented as weightings in a forest plot.

**Assessment of the result quality:** Using evidence-based medicine principles, we conducted a quality assessment



**Figure 1** Flow diagram depicting the process of identification and inclusion of selected studies. RCT: Randomized controlled trials.

on the design of evidence sources and finally obtained the quality of the meta-analysis results.

### Statistical analysis

Meta-analysis was performed using RevMan4.2 Software. For clinical trials reporting the summary measures or Kaplan-Meier curves, we applied the published statistical methods to analyze the risk ratio and the variance of the temporal data that cause the events<sup>[7,8]</sup>. The statistical heterogeneity of the clinical trials was analyzed using  $\chi^2$  test and  $I^2$  test;  $P > 0.05$  during  $\chi^2$  test indicates low heterogeneity. The primary analysis was completed using a fixed-effects model. If there was a significant statistical heterogeneity, a secondary confirmatory analysis was performed using a random-effects model. Meanwhile, subgroup analysis was performed to determine whether the results were affected by different interventions. By analyzing the difference between combination therapy and CT/RT, we tried to understand their different impacts on the prognosis.

## RESULTS

### Search results

The flow chart of this study is shown in Figure 1. Reviews, articles about resectable pancreatic cancer, non-randomized controlled trials (RCTs), low-quality studies and repeated reports were excluded. Finally, both review-

ers agreed to include 15 RCTs involving 1128 patients in the meta-analysis.

All of them were published in peer-reviewed international and domestic journals. The cases had been followed up for 6-36 mo. All the studies were prospective, randomized, and controlled trials. One article was claimed to be randomized, with a Jadad score of only 2, and therefore the quality was low. The remaining 14 studies scored 3-4, and belonged to high-quality literature, among which two articles used the blinding method. The meta-analysis results are listed in Table 1. There were no duplicates or mis-citations.

### Statistical analysis

**Meta-analysis on radiochemotherapy and CT/RT for LAPC:** Of these 15 RCTs ( $n = 1128$ ), 12 ( $n = 964$ ) reported the 6-mo survival; 14 ( $n = 1098$ ) reported the 12-mo survival; 9 ( $n = 805$ ) reported the 18-mo survival; the 6-, 12-, and 18-mo survival was 419, 261, and 119 cases, respectively, in the CRT group, and 238, 127, and 43 cases, respectively, in the CT group/RT group.  $P$  value was larger than 0.05 in the meta-analysis on the heterogeneity of the 6-mo survival, and a fixed-effects model was used to merge the data.  $P$  values were lower than 0.05 in the meta-analysis on the heterogeneity of the 12- and 18-mo survivals, and a random-effects model was used to merge the data. The CRT group had superior survival over the CT/RT group, while the 18-mo survival showed no such significant difference (OR = 1.78, 1.77, and 1.74; 95%CI: 1.33-2.38, 1.08-2.88, and 0.71-4.27,  $P = 0.0001$ , 0.02, and 0.23) (Table 2, Figure 2A).

**Subgroup analysis on the efficacy of radiochemotherapy and CT/RT for LAPC:** Seven RCTs ( $n = 371$ ) reported the 6-mo survival of patients after combination treatment or CT, 7 ( $n = 479$ ) reported the 12-mo survival, and 5 ( $n = 354$ ) reported the 18-mo survival. The 6-, 12-, and 18-mo survivals were reported in 129, 95, and 32 cases, respectively, in the CRT group, and 119, 91, and 37 cases, respectively, in the CT group.  $P$  value was larger than 0.05 in the meta-analysis on the heterogeneity of 6-mo survival, and a fixed-effects model was used to merge the data.  $P$  values were lower than 0.05 in the meta-analysis on the heterogeneity of the 12- and 18-mo survivals, and a random-effects model was used to merge the data. The 6-, 12-, and 18-mo survivals were not significantly different between the CRT group and the CT group (OR = 1.52, 1.49, and 1.07; 95%CI: 0.97-2.36, 0.77-2.88, and 0.33-3.45,  $P = 0.07$ , 0.23, and 0.91) (Figure 2B).

Five RCTs ( $n = 476$ ) reported the 6-mo survival after combination treatment or RT, 7 ( $n = 610$ ) reported the 12-mo survival, 4 ( $n = 451$ ) reported the 18-mo survival. The 6-, 12-, and 18-mo survivals were 246, 166, and 87 cases, respectively, in the CRT group, and 70, 36, and 37 cases, respectively, in the RT group. Meta-analysis on the heterogeneity of the 6-, 12-, and 18-mo survivals showed that the  $P$  values were larger than 0.05, and a

**Table 1** Characteristics of the eligible trials included in the meta-analysis

Ref.	Treatment arms	No. of participants	Sex (male/female)	Median age (yr)	PS 0-2/KPS $\geq 50$	Pathology (W/M/P/other)	Location of primary tumor(head of pancreas/other)	Jad score
Loehrer <i>et al</i> <sup>[9]</sup>	RT 50.4 Gy + GEM <i>vs</i> GEM	37 34	18/19 19/15	67 65.3	100% 100%	4/6/5/22 6/8/7/13	12/25 20/14	3
Klaassen <i>et al</i> <sup>[10]</sup>	RT 40 Gy + 5FU <i>vs</i> 5FU	44 47	31/13 22/25	NR NR	100% 100%	NR NR	NR NR	3
Moertel <i>et al</i> <sup>[11]</sup>	RT 35-40 Gy + saline <i>vs</i> RT 35-40 Gy + 5FU	32 32	NR NR	NR NR	NR NR	NR NR	NR NR	4
GITSG <i>et al</i> <sup>[12]</sup>	RT 60 Gy <i>vs</i> RT 60Gy + 5FU <i>vs</i> RT 40 Gy + 5FU	28 32 29	12/16 17/15 16/13	NR NR NR	100% 100% 100%	8/17/3/0 6/20/6/0 6/18/5/0	10/18 9/23 6/23	3
Cohen <i>et al</i> <sup>[13]</sup>	RT 59.4 Gy <i>vs</i> RT 59.4 Gy + 5FU + MMC	49 55	27/22 37/18	62 64	100% 100%	7/21/11/10 12/19/17/7	NR NR	3
Chauffert <i>et al</i> <sup>[6]</sup>	RT 60 Gy + 5FU + DDP + GEM <i>vs</i> GEM	59 60	31/28 34/26	60 62	100% 100%	NR NR	46/13 40/20	3
Moertel <i>et al</i> <sup>[14]</sup>	RT 60 Gy <i>vs</i> RT 60 Gy + 5FU <i>vs</i> RT 40 Gy + 5FU	25 86 83	NR NR NR	54 60 61	88% 95% 95%	5/8/2/10 20/39/9/14 13/30/9/31	8/17 68/18 64/19	3
Sun <i>et al</i> <sup>[15]</sup>	RT 45-50 Gy + GEM <i>vs</i> GEM	25 29	32/22 NR	NR NR	100% 100%	NR NR	NR NR	3
Sun <i>et al</i> <sup>[16]</sup>	RT 50-60 Gy + GEM <i>vs</i> GEM + DDP	26 30	33/23 NR	NR NR	100% 100%	NR NR	NR NR	3
Wu <i>et al</i> <sup>[17]</sup>	RT 48-56 Gy <i>vs</i> RT 48-60 Gy + GEM + DDP	31 33	50/14 NR	57 57	98% 99%	NR NR	NR NR	3
Wu <i>et al</i> <sup>[18]</sup>	RT 60 Gy <i>vs</i> RT 50 Gy + GEM	34 36	43/27 NR	NR NR	87% 90%	NR NR	NR NR	3
Ding <i>et al</i> <sup>[19]</sup>	RT 45-50 Gy + 5FU + GEM <i>vs</i> 5FU + GEM	25 29	32/22 NR	NR NR	100% 100%	NR NR	NR NR	3
Childs <i>et al</i> <sup>[20]</sup>	RT 35-40 Gy + saline <i>vs</i> RT 35-40 Gy + 5FU	12 13	11/1 8/5	58.8 56.3	NR NR	NR NR	NR NR	4
GITSG <i>et al</i> <sup>[21]</sup>	RT 54 Gy + 5FU + SMF <i>vs</i> SMF	22 21	8/14 8/13	61 60	100% 100%	NR NR	3/18 3/19	3
Hazel <i>et al</i> <sup>[22]</sup>	RT 46 Gy + 5FU <i>vs</i> 5FU + CCNU	15 15	10/5 10/5	62 62	NR NR	NR NR	NR NR	2

W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated; SMF: Streptozocin, mitomycin, and 5-fluorouracil (5FU); NR: No report; GEM: Gemcitabine; RT: radiotherapy alone; MMC: Mitomycin C.

fixed-effects model was used to merge the data. The 6-, 12-, and 18-mo survivals were significantly higher in the CRT group than in the RT group (OR = 2.49, 2.42, and 3.86; 95%CI: 1.62-3.82, 1.57-3.74, 1.66-8.99, all  $P < 0.01$ ), (Figure 2C).

**Subgroup analysis on the efficacy of CRT > 50Gy and RT for LAPC:** Due to the limited number of patients and RCTs, this subgroup analysis only covered the 12-mo survival rate. Five RCTs ( $n = 409$ ) reported the 12-mo survival of patients after CRT at a dose larger than 50Gy or RT, including 32 cases in the CRT group and 98 cases in the RT group.  $P$  value was larger than 0.05 in the meta-analysis on the heterogeneity of the 12-mo survival, and a fixed-effects model was used to pool the data. The CRT group had superior 12-mo survival over the RT group (OR = 2.32; 95%CI: 1.44-3.73,  $P = 0.0005$ ) (Figure 2D).

**Subgroup analysis on the efficacy of CRT < 50 Gy and RT for LAPC:** Due to the limited number of patients and RCTs, this subgroup analysis was only made on the 6- and 12-mo survival rates. Four RCTs ( $n = 254$ ) reported the 6-mo survival and 4 ( $n = 254$ ) reported the

12-mo survival. The 6- and 12-mo survival was reported in 108 and 68 cases in the CRT group, and 42 and 12 cases in the RT group, respectively.  $P$  value was larger than 0.05 in the meta-analysis on the heterogeneity of 6- and 12-mo survival, and a fixed-effects model was used to pool the data. The CRT group had superior 6- and 12-mo survival over the RT group (OR = 2.5 and 4.12; 95%CI: 1.45-4.30 and 2.04-8.35,  $P = 0.0009$  and  $< 0.0001$ ) (Figure 2E).

Due to the limited number of cases and RCTs included, subgroup analysis was not performed on the efficacy of different doses of CRT and CT for LAPC.

**Meta-analysis on grade 3-4 treatment-related toxicity:** In total, 12 RCTs ( $n = 874$ ) reported grade 3-4 treatment-related hematologic toxicities, and 10 ( $n = 713$ ) reported grade 3-4 treatment-related non-hematologic toxicities. Meta-analysis on the heterogeneity showed that the  $P$  value was larger than 0.05, and a fixed-effects model was used to merge the data. The CRT group had significantly more grade 3-4 treatment-related hematologic and non-hematologic toxicities than the CT group or RT group (OR = 3.74 and 1.71; 95%CI: 2.56-5.47 and 1.16-2.53, both  $P < 0.01$ ) (Figure 2F).



**Table 2 Overall survival and treatment-related toxicity of the eligible trials included in the meta-analysis**

Ref.	Treatment arms	Participants	Overall survival (n)			3-4 grade treatment-related toxicity (n)	
			6 mo	12 mo	18 mo	Hematological	Non-hematological
Loehrer <i>et al</i> <sup>[9]</sup>	RT 50.4 Gy + GEM <i>vs</i> GEM	37	28	12	4		29
Klaassen <i>et al</i> <sup>[10]</sup>	RT 40 Gy + 5FU <i>vs</i> 5FU	34	26	17	10		26
		44	25	14	11	8	NR
Moertel <i>et al</i> <sup>[11]</sup>	RT 35-40 Gy + saline <i>vs</i> RT 35-40 Gy + 5FU	47	28	12	5	1	
		32	15	3	0	12	13
GITSG <i>et al</i> <sup>[12]</sup>	RT 60 Gy <i>vs</i> RT 60 Gy + 5FU <i>vs</i> RT 40 Gy + 5FU	32	20	6	1	24	24
		28	10	2	0		
Cohen <i>et al</i> <sup>[13]</sup>	RT 59.4 Gy <i>vs</i> RT 59.4 Gy + 5FU + MMC	32	25	11	3	NR	NR
		29	19	11	4		
Chauffert <i>et al</i> <sup>[6]</sup>	RT 60 Gy + 5FU + DDP + GEM <i>vs</i> GEM	49	28	10	2	5	13
		55	36	17	5	13	17
Moertel <i>et al</i> <sup>[14]</sup>	RT 60 Gy <i>vs</i> RT 60 Gy + 5FU <i>vs</i> RT 40 Gy + 5FU	59	47	20	11	29	12
		60	49	33	21	12	11
Sun <i>et al</i> <sup>[15]</sup>	RT 45-50 Gy + GEM <i>vs</i> GEM	25	13	6	4	4	0
		86	74	52	34	53	22
Sun <i>et al</i> <sup>[16]</sup>	RT 50-60 Gy + GEM <i>vs</i> GEM + DDP	83	63	49	40	39	18
		25	18	12	NR	5	5
Wu <i>et al</i> <sup>[17]</sup>	RT 48-56 Gy <i>vs</i> RT 48-60 Gy + GEM + DDP	29	15	7		5	4
		26	21	16	NR	3	6
Wu <i>et al</i> <sup>[18]</sup>	RT 60 Gy <i>vs</i> RT 50 Gy + GEM	30	18	11		3	10
		31	NR	9	NR	NR	NR
Ding <i>et al</i> <sup>[19]</sup>	RT 45-50 Gy + 5FU + GEM <i>vs</i> 5FU + GEM	33		7			
		34	NR	5	NR	0	NR
Childs <i>et al</i> <sup>[20]</sup>	RT 35-40 Gy + saline <i>vs</i> RT 35-40 Gy + 5FU	36		11		2	
		25	18	12	NR	5	5
GITSG <i>et al</i> <sup>[21]</sup>	RT 54 Gy + 5FU + SMF <i>vs</i> SMF	29	15	7		5	4
		12	4	1	NR	2	5
Hazel <i>et al</i> <sup>[22]</sup>	RT 46 Gy + 5FU <i>vs</i> 5FU + CCNU	13	6	2		8	9
		22	19	9	4	14	3
		21	17	4	0	3	3
		15	NR	NR	2	NR	NR
		15		1			

RT: Radiotherapy alone; NR: No report.

### Sensitivity analysis

By re-analyzing the results, the sensitivity analysis was designed to re-combine the available studies to explore the effect of a certain factor on the effect, so as to understand the influences of uncertain factors and study design on the aggregate results. To avoid the effect of different chemotherapy or radiotherapy in the different randomized controlled trials on the overall results, subgroup analyses were performed to re-analyze the above studies, and the results were generally consistent with the meta-analysis findings. As shown in the sensitivity analysis, our current meta-analysis neither increased or decreased the efficacy nor exaggerated the efficacy; after the strength of the articles was changed, the results did not become negative or reversed. Therefore, our results were relatively stable and reliable.

### DISCUSSION

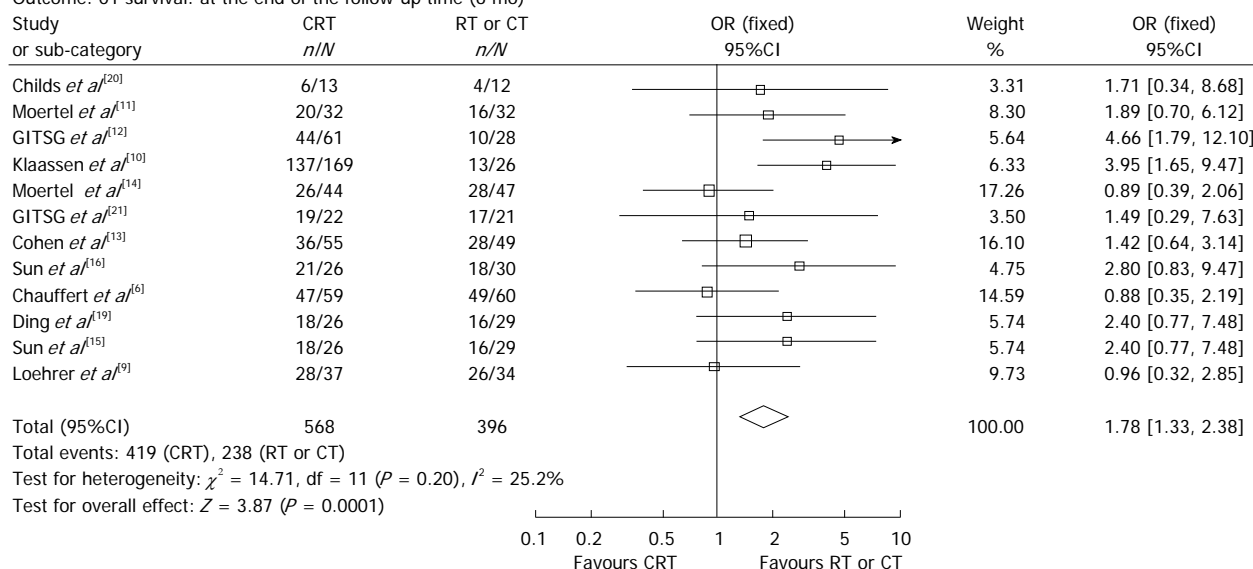
Pancreatic adenocarcinoma is among the most challenging solid malignancies to treat on account of its propensity for late presentation with inoperable disease, aggressive tumor biology and resistance to chemotherapy<sup>[23]</sup>. About a third of all pancreatic cancers is found to be locally advanced at the time of diagnosis, LAPC refers to local

tumors that have invaded the surrounding normal tissues and can not be surgically resected while no distant metastasis occurs<sup>[24]</sup>. As shown in early clinical practice, conventional radiotherapy for LAPC often can not improve the efficacy. The availability of three-dimensional conformal radiotherapy and intensity-modulated radiotherapy improves the therapeutic effectiveness, although controversies persist. Using the meta-analysis methodologies, we rigorously screened eligible randomized controlled trials for analysis. As shown in our current meta-analysis: (1) the CRT group had higher 6- and 12-mo survival rates than the RT alone and CT alone group; (2) subgroup analysis showed that the CRT group had higher 6-, 12-, and 18-mo survival rates than CT alone group; (3) subgroup analysis showed that the CRT group had higher 6-, 12-, and 18-mo survival rates than the RT alone group; and (4) CRT group had significantly more grade 3-4 treatment-related hematologic and non-hematologic toxicities than the CT group or RT group. By analyzing the results of 2000 FFCD/SFRO<sup>[6]</sup>, we found that concurrent three-dimensional conformal radiotherapy (total dosage: 60 Gy) with chemotherapy (DDP + 5FU), followed by GMZ chemotherapy achieved a median survival of 8.6 mo and a 1-year survival rate of 32%; the GMZ CT group had a survival of 13 mo and 1-year survival rate of 53%, sug-

**A** Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

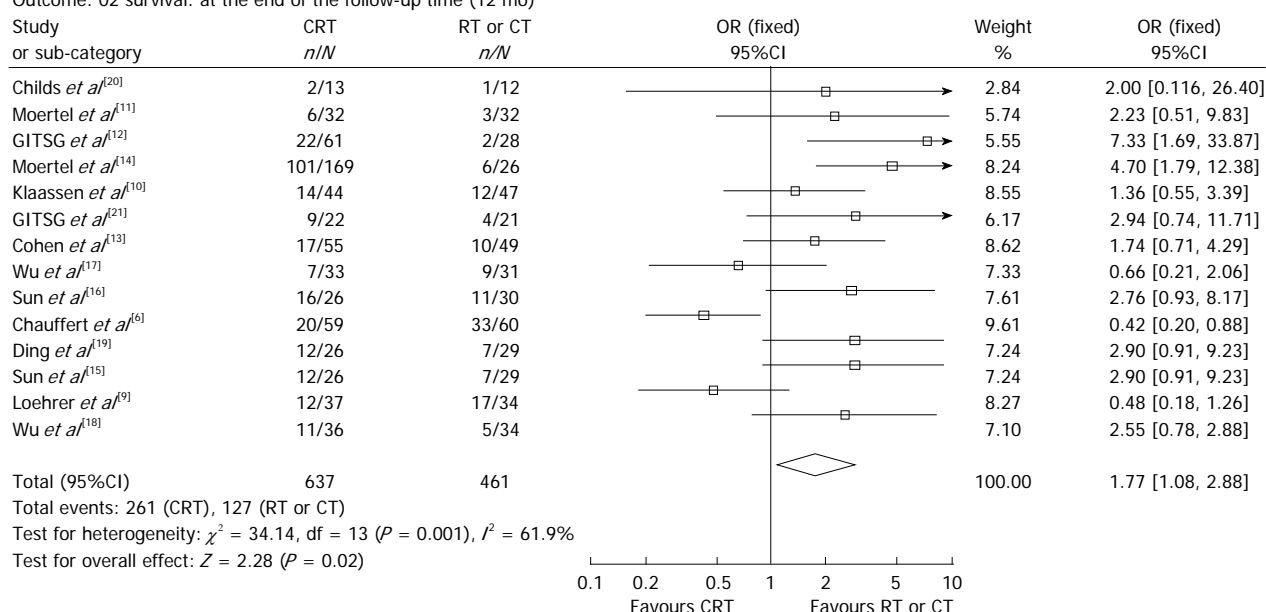
Outcome: 01 survival: at the end of the follow-up time (6 mo)



Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

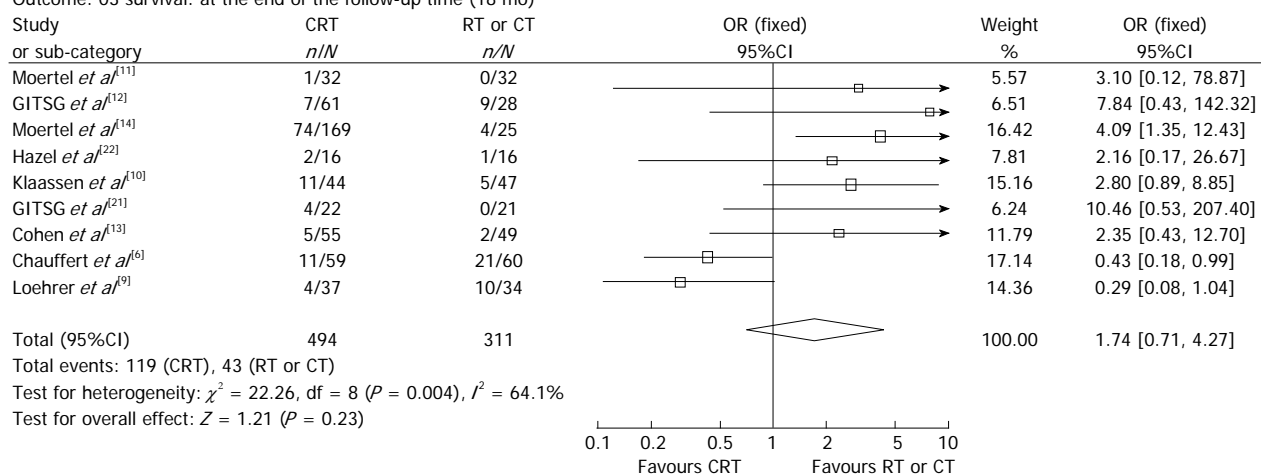
Outcome: 02 survival: at the end of the follow-up time (12 mo)



Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 03 survival: at the end of the follow-up time (18 mo)



**B** Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 04 survival: at the end of the follow-up time (6 mo) CRT vs CT

Study or sub-category	CRT n/N	RT or CT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Klaassen <i>et al</i> <sup>[10]</sup>	25/44	28/47		36.94	0.89 [0.39, 2.06]
GITSG <i>et al</i> <sup>[21]</sup>	19/22	17/21		7.50	1.49 [0.29, 7.63]
Sun <i>et al</i> <sup>[15]</sup>	21/26	18/30		10.16	2.80 [0.83, 9.47]
Chauffert <i>et al</i> <sup>[6]</sup>	0/1	0/1		0.00	3.95 [1.65, 9.47]
Ding <i>et al</i> <sup>[19]</sup>	18/26	16/29		12.29	2.40 [0.77, 7.48]
Sun <i>et al</i> <sup>[15]</sup>	18/26	16/29		12.29	2.40 [0.77, 7.48]
Loehrer <i>et al</i> <sup>[9]</sup>	28/37	26/34		20.83	0.96 [0.32, 2.85]
Total (95%CI)	180	191		100.00	1.52 [0.97, 2.36]

Total events: 129 (CRT), 119 (CT)

Test for heterogeneity:  $\chi^2 = 4.46$ ,  $df = 5$  ( $P = 0.49$ ),  $I^2 = 0\%$ Test for overall effect:  $Z = 1.83$  ( $P = 0.07$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours CT

## Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 05 survival: at the end of the follow-up time (12 mo) CRT vs CT

Study or sub-category	CRT n/N	RT or CT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Klaassen <i>et al</i> <sup>[10]</sup>	14/44	12/47		15.76	1.36 [0.55, 3.39]
GITSG <i>et al</i> <sup>[21]</sup>	9/22	4/22		11.31	2.94 [0.74, 11.71]
Sun <i>et al</i> <sup>[16]</sup>	16/26	11/30		14.00	2.76 [0.93, 8.17]
Chauffert <i>et al</i> <sup>[6]</sup>	20/59	33/60		17.56	0.42 [0.20, 0.88]
Ding <i>et al</i> <sup>[19]</sup>	12/25	7/29		13.30	2.90 [0.91, 9.23]
Sun <i>et al</i> <sup>[15]</sup>	12/25	7/29		13.30	2.90 [0.91, 9.23]
Loehrer <i>et al</i> <sup>[9]</sup>	12/28	17/34		14.78	0.75 [0.27, 2.05]
Total (95%CI)	229	250		100.00	1.49 [0.77, 2.88]

Total events: 95 (CRT), 91 (CT)

Test for heterogeneity:  $\chi^2 = 16.96$ ,  $df = 6$  ( $P = 0.009$ ),  $I^2 = 64.6\%$ Test for overall effect:  $Z = 1.20$  ( $P = 0.23$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours CT

## Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 06 survival: at the end of the follow-up time (18 mo) CRT vs CT

Study or sub-category	CRT n/N	RT or CT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Hazel <i>et al</i> <sup>[22]</sup>	2/15	1/15		13.02	2.15 [0.17, 26.67]
Klaassen <i>et al</i> <sup>[10]</sup>	11/44	5/47		24.89	2.80 [0.89, 8.86]
GITSG <i>et al</i> <sup>[21]</sup>	4/22	0/21		10.44	10.46 [0.53, 207.40]
Chauffert <i>et al</i> <sup>[6]</sup>	11/59	21/60		28.03	0.43 [0.18, 0.99]
Loehrer <i>et al</i> <sup>[9]</sup>	4/37	10/34		23.62	0.29 [0.08, 1.04]
Total (95%CI)	177	177		100.00	1.07 [0.33, 3.45]

Total events: 32 (CRT), 37 (CT)

Test for heterogeneity:  $\chi^2 = 12.65$ ,  $df = 4$  ( $P = 0.01$ ),  $I^2 = 68.4\%$ Test for overall effect:  $Z = 0.12$  ( $P = 0.91$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours CT**C** Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 07 survival: at the end of the follow-up time (6 mo) CRT vs RT

Study or sub-category	CRT n/N	RT or CT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Moertel <i>et al</i> <sup>[11]</sup>	6/13	4/12		8.68	1.71 [0.34, 8.68]
Childs <i>et al</i> <sup>[20]</sup>	20/32	16/32		21.80	1.89 [0.70, 6.12]
GITSG <i>et al</i> <sup>[12]</sup>	44/61	10/28		14.81	4.66 [1.79, 12.10]
Moertel <i>et al</i> <sup>[14]</sup>	140/169	13/25		15.06	0.89 [0.39, 2.06]
Cohen <i>et al</i> <sup>[13]</sup>	36/5	28/49		39.65	1.42 [0.64, 3.14]
Total (95%CI)	330	136		100.00	2.49 [1.62, 3.82]

Total events: 246 (CRT), 70 (RT)

Test for heterogeneity:  $\chi^2 = 5.76$ ,  $df = 4$  ( $P = 0.22$ ),  $I^2 = 30.5\%$ Test for overall effect:  $Z = 4.15$  ( $P < 0.0001$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours RT

Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 08 survival: at the end of the follow-up time (12 mo) CRT vs RT

Study or sub-category	CRT n/N	RT or CT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Moertel <i>et al</i> <sup>[11]</sup>	2/13	1/12		3.20	2.00 [0.116, 26.40]
Childs <i>et al</i> <sup>[20]</sup>	6/32	3/32		8.87	2.23 [0.51, 9.83]
GITSG <i>et al</i> <sup>[12]</sup>	22/61	2/28		6.38	7.33 [1.69, 33.87]
Moertel <i>et al</i> <sup>[14]</sup>	101/169	6/26		15.31	4.70 [1.79, 12.38]
Cohen <i>et al</i> <sup>[13]</sup>	17/55	10/49		26.60	1.74 [0.71, 4.29]
Wu <i>et al</i> <sup>[17]</sup>	7/33	9/31		26.62	0.66 [0.21, 2.06]
Wu <i>et al</i> <sup>[18]</sup>	11/36	5/34		13.00	2.55 [0.78, 2.88]
<b>Total (95%CI)</b>	<b>399</b>	<b>211</b>		<b>100.00</b>	<b>2.42 [0.78, 8.34]</b>

Total events: 166 (CRT), 36 (RT)

Test for heterogeneity:  $\chi^2 = 9.39$ ,  $df = 6$  ( $P = 0.15$ ),  $I^2 = 36.1\%$ Test for overall effect:  $Z = 3.98$  ( $P < 0.0001$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours RT

Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 09 survival: at the end of the follow-up time (18 mo) CRT vs RT

Study or sub-category	CRT n/N	RT or CT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Childs <i>et al</i> <sup>[20]</sup>	1/32	0/32		6.90	3.10 [0.12, 78.87]
GITSG <i>et al</i> <sup>[12]</sup>	7/61	0/28		8.66	7.84 [0.43, 142.32]
Moertel <i>et al</i> <sup>[14]</sup>	74/169	4/25		56.64	4.09 [1.35, 12.43]
Cohen <i>et al</i> <sup>[13]</sup>	5/55	2/49		27.80	2.35 [0.43, 12.70]
<b>Total (95%CI)</b>	<b>317</b>	<b>134</b>		<b>100.00</b>	<b>3.86 [0.43, 12.70]</b>

Total events: 87 (CRT), 6 (RT)

Test for heterogeneity:  $\chi^2 = 0.59$ ,  $df = 3$  ( $P = 0.90$ ),  $I^2 = 0\%$ Test for overall effect:  $Z = 3.13$  ( $P = 0.002$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours RT**D** Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 14 survival: at the end of the follow-up time (12 mo) RT dose &gt; 500Gy CRT vs RT

Study or sub-category	dose > 500Gy CRT n/N	CT/RT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
GITSG <i>et al</i> <sup>[21]</sup>	11/32	2/28		6.02	6.81 [1.36, 34.16]
Moertel <i>et al</i> <sup>[14]</sup>	52/86	6/25		15.80	4.84 [1.76, 13.36]
Cohen <i>et al</i> <sup>[13]</sup>	17/55	10/49		31.41	1.74 [0.71, 4.29]
Wu <i>et al</i> <sup>[17]</sup>	7/33	9/31		31.43	0.66 [0.21, 2.06]
Wu <i>et al</i> <sup>[18]</sup>	11/36	5/34		15.35	2.55 [0.78, 2.88]
<b>Total (95%CI)</b>	<b>242</b>	<b>167</b>		<b>100.00</b>	<b>2.32 [1.44, 3.73]</b>

Total events: 98 (dose &gt; 50 Gy CRT), 32 (RT)

Test for heterogeneity:  $\chi^2 = 8.84$ ,  $df = 4$  ( $P = 0.07$ ),  $I^2 = 54.8\%$ Test for overall effect:  $Z = 3.48$  ( $P = 0.0005$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours RT**E** Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 16 survival: at the end of the follow-up time (6 mo) RT dose &lt; 500Gy CRT vs RT

Study or sub-category	dose > 500Gy CRT n/N	CT/RT n/N	OR (fixed) 95%CI	Weight %	OR (fixed) 95%CI
Moertel <i>et al</i> <sup>[11]</sup>	6/13	4/12		13.84%	1.71 [0.34, 8.68]
Childs <i>et al</i> <sup>[20]</sup>	20/32	15/32		34.75%	1.89 [0.70, 6.12]
GITSG <i>et al</i> <sup>[21]</sup>	19/29	10/28		21.67%	3.42 [1.15, 10.15]
Moertel <i>et al</i> <sup>[14]</sup>	63/83	13/25		29.74%	2.91 [1.14, 7.38]
<b>Total (95%CI)</b>	<b>157</b>	<b>97</b>		<b>100.00%</b>	<b>2.50 [1.45, 4.30]</b>

Total events: 108 (dose &lt; 50 Gy CRT), 42 (RT)

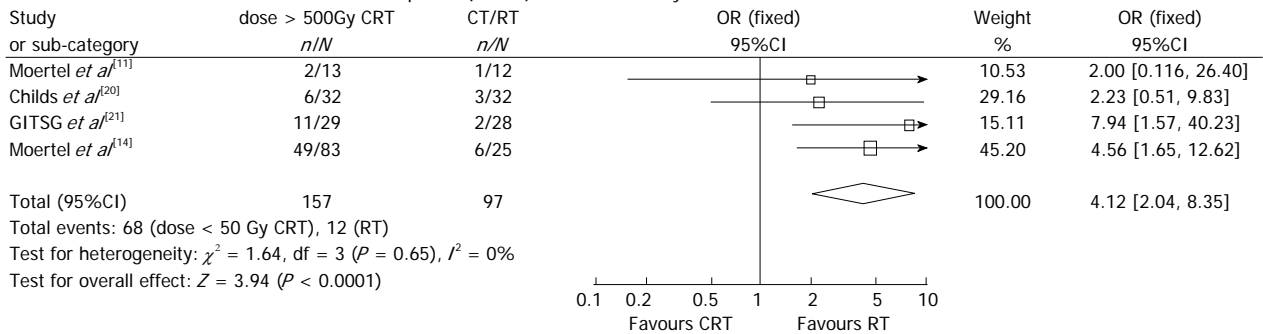
Test for heterogeneity:  $\chi^2 = 0.93$ ,  $df = 3$  ( $P = 0.82$ ),  $I^2 = 0\%$ Test for overall effect:  $Z = 3.31$  ( $P = 0.0009$ )0.1 0.2 0.5 1 2 5 10  
Favours CRT Favours RT



Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

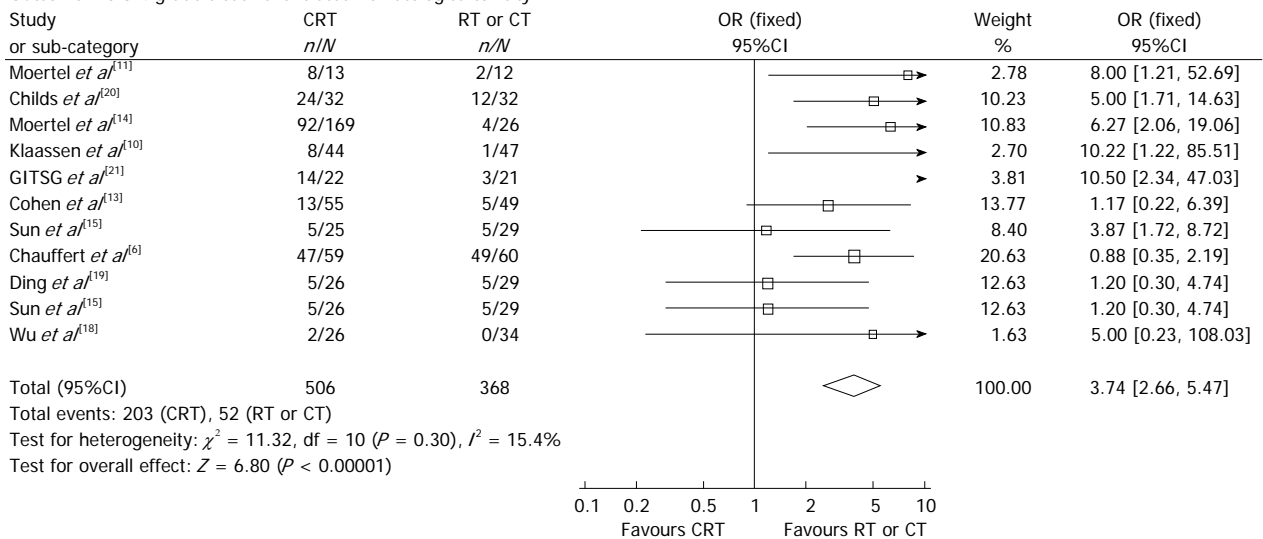
Comparison: 01 CRT vs RT or CT

Outcome: 17 survival: at the end of the follow-up time (12 mo) RT dose &lt; 500Gy CRT vs RT

**F** Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

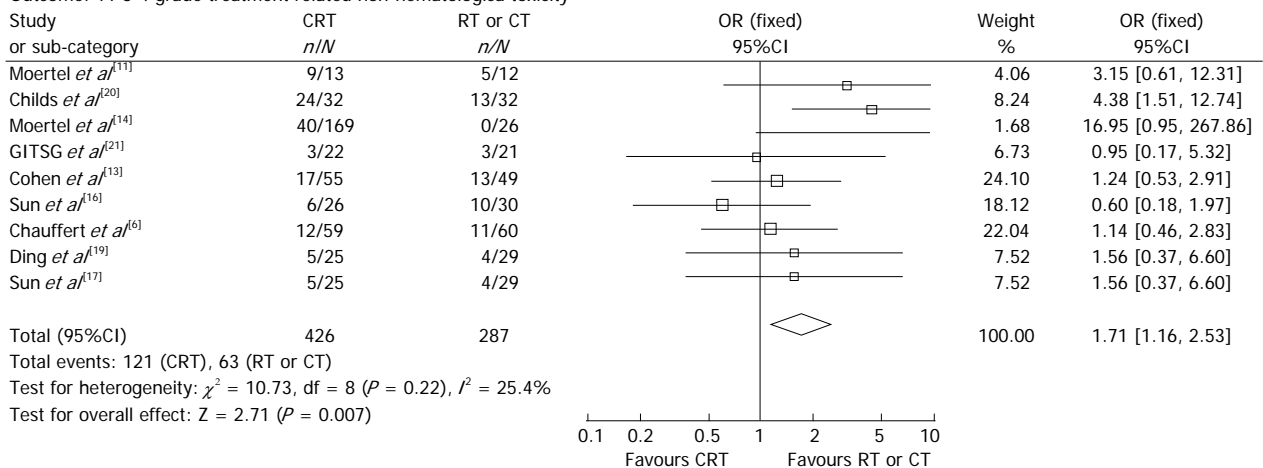
Outcome: 10 3-4 grade treatment related hematologica toxicity



Review: Radiotherapy plus chemotherapy in patients with locally advanced pancreatic cancer

Comparison: 01 CRT vs RT or CT

Outcome: 11 3-4 grade treatment related non-hematologica toxicity



**Figure 2 Meta-analysis.** A: On chemotherapy plus radiotherapy (CRT) vs chemotherapy alone (CT)/radiotherapy alone (RT) for locally advanced pancreatic carcinoma (LAPC) in the 6-, 12-, and 18-mo survivals; B: On CRT vs CT for LAPC in the 6-, 12-, and 18-mo survivals; C: On CRT vs RT for LAPC in the 6-, 12-, and 18-mo survivals; D: On CRT > 50 Gy vs RT for LAPC in the 12-mo survivals; E: On CRT < 50 Gy vs RT for LAPC in the 6- and 12-mo survivals; F: On CRT vs CT/RT for LAPC in grade 3-4 treatment-related toxicities.

gesting that the combination therapy did not change the efficacy. Meanwhile, the 60 Gy dosage induced more complications and therefore increased the mortality and

decreased the survival. The CRT protocol applied in this trial used high radiotherapy and chemotherapy dosages; furthermore, the radiation fields included not only the

cancer foci but also the peripancreatic, hilar and celiac trunk lymph nodes that had high metastatic potential (but not confirmed), which not only remarkably increased the radiotherapy-related toxic reactions but also shortened the survival. Similarly, radiochemotherapy was applied in the ECOG4201 trial, although the radiotherapy dose was lowered to 50.4 Gy. Compared with CT, concurrent radio-chemotherapy prolonged the survival (11 mo *vs* 9.2 mo) and yielded a higher 1-year survival rate (50% *vs* 32%)<sup>[25]</sup>. During concurrent radiochemotherapy, the sequencing of radiotherapy and chemotherapy can also affect the clinical efficacy. In the GERCOR study, 181 patients received 4 cycles of chemotherapy initially, among whom 53 experienced disease progression; the remaining 128 patients without disease progression were divided into a concurrent CRT group ( $n = 72$ ) and a CT group ( $n = 56$ ). The results showed that the PFS and OS were improved in the concurrent CRT group (10.8 mo and 15 mo, respectively), and these were significantly longer than those in the CT group (7.4 mo and 11.7 mo, respectively) ( $P = 0.005$ ,  $P = 0.0009$ , respectively), indicating that radiotherapy combined with chemotherapy can remarkably improve the survival<sup>[26]</sup>.

In summary, as shown in this study, the combination of chemotherapy and radiotherapy can prolong the long-term survival although it may also increase the treatment-related toxicity. More reasonably designed randomized controlled trials should be conducted to further elucidate the optimal radiotherapy dosage, the use of gemcitabine or 5-FU, and more specific chemotherapy protocols. Chemotherapy may be started for several cycles; if no disease progression occurs, concurrent radiochemotherapy may be used. By doing so, we may rule out patients with rapid disease progression to avoid the “double whammy” from chemotherapy. Along with the improvement of radiotherapy technology, the optimization of the sequencing of radiochemotherapy, and the definition of target population, the efficacy of radiochemotherapy for pancreatic cancer will be further improved, and multidisciplinary and individualized radiochemotherapy for LAPC will play a more important role.

## COMMENTS

### Background

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. Over the past decades, the standard treatment for patients with locally advanced pancreatic carcinoma (LAPC) was chemotherapy alone or chemo-radiation therapy. However, the efficacy of the combined radiotherapy and chemotherapy is currently unclear.

### Research frontiers

Over the past decades, many studies have been performed to understand the role of chemotherapy or radiation therapy alone and chemo-radiation therapy in LAPC. Some trials showed no improvement in local progression or progression-free or overall survival with the addition of radiation therapy to chemotherapy in patients with LAPC; however, some trials demonstrated improved overall survival, with acceptable toxicity. Moreover, several systematic reviews were recently published to investigate the role of chemo-radiation therapy in LAPC. However, these reviews were methodologically insufficient and thus could not achieve a comprehensive conclusion.

### Innovations and breakthroughs

Based on this meta-analysis, chemo-radiation therapy was superior in the 6- and 12-mo survivals to the radiation therapy group or chemotherapy group alone. Similar results were indicated in the subgroup analyses. The radiation therapy group had significantly more grade 3-4 treatment-related hematologic and non-hematologic toxicities than the radiation therapy group or chemotherapy group alone. These findings were not presented clearly in previous systematic reviews.

### Applications

The combination of chemotherapy and radiotherapy can prolong the long-term survival of the patients with LAPC although it may also increase the treatment-related toxicity. Chemotherapy may be started for several cycles; if no disease progression occurs, concurrent radiochemotherapy may be used.

### Terminology

Locally advanced pancreatic malignant tumors were judged as nonresectable due to extension to regional lymph nodes and/or vascular structures such as the superior mesenteric artery or the celiac trunk or the existence of a portal or superior mesenteric-portal venous confluent thrombosis. Over the past decades, the standard treatment for patients with inoperable locally advanced pancreatic cancer was chemotherapy alone or chemo-radiation therapy.

### Peer review

This is a well written manuscript analyzing therapeutic management of pancreatic cancer. In this manuscript, the authors compared the long-term clinical efficacy of chemotherapy plus radiotherapy with that of radiotherapy alone or chemotherapy alone for locally advanced pancreatic carcinoma. The data were well collected, and analyzed.

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## Massive upper gastrointestinal hemorrhage due to invasive hepatocellular carcinoma and hepato-gastric fistula

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Author contributions: Sayana H did the literature search, wrote and drafted the manuscript and reviewed; Yousef O, Clarkston WK critically reviewed and edited the manuscript; all authors reviewed and approved the final revised version.

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**Key words:** Hepatocellular carcinoma; Hepatogastric fistula; Fistula; Upper gastrointestinal bleeding

**Core tip:** Gastrointestinal bleeding is a common complication of hepatocellular carcinoma (HCC). However, HCC leading to Hepatogastric fistula presenting as massive upper gastrointestinal bleeding is uncommon. Here we report a case of HCC with direct invasion of the stomach leading to massive gastrointestinal bleeding. Patient was managed with selective arterial angiogram and coil embolization to control bleeding. HCC with local metastasis to adjacent structures such as gastrointestinal tract carries poor prognosis. With increasing incidence of HCC and recent improvements in the treatment of advanced HCC, this condition may become more common and awareness among clinicians should help consider this condition in the differential diagnosis and prompt management.

### Abstract

A 36-year-old male Asian immigrant with a history of hepatitis B and hepatitis C related unresectable hepatocellular carcinoma in the left lobe of the liver presented with hematemesis and severe anemia. He was diagnosed with a liver mass that was resected 8 years ago described as a benign tumor in his home country. He had received trans-arterial chemoembolization (TACE) four months ago after subsequent diagnosis of unresectable hepatoma, and currently was receiving chemotherapy with Sorafenib. After resuscitation, a contrast enhanced computerized tomography was performed which showed fistulization of hepatocellular carcinoma into adjacent stomach. This finding was confirmed during endoscopy with direct visualization of the fistulous opening. Hepatocellular carcinoma (HCC) invading the gastrointestinal (GI) tract is rare. We present a case and literature review of HCC with local invasion of the stomach causing massive upper GI bleeding after receiving TACE.

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive primary malignancy of the liver with 5-year survival rates as low as 5%<sup>[1]</sup>. It is the third leading causes of cancer deaths across the world and ninth leading cause of cancer deaths in United States<sup>[2]</sup>. While the incidence of other common cancers is decreasing in the United States, HCC is on the rise with an average annual percentage change of incidence rate of 3.5%<sup>[2]</sup>. The majority of cases of HCC are caused by chronic liver disease from hepatitis B and C (78%)<sup>[2]</sup>. Extra-hepatic metastasis is found at the time of diagnosis in up to 15% of patients

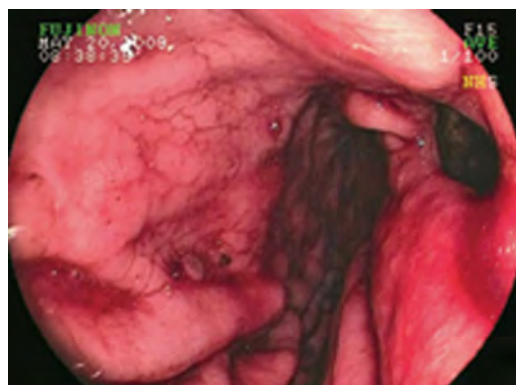


with HCC<sup>[3]</sup>. Lung is the most common site of metastasis followed by intra-abdominal lymph nodes, and bone and adrenal glands<sup>[3]</sup>. Median survival of patients with extra-hepatic manifestations is less than 6 mo<sup>[4]</sup>. Metastasis to the gastrointestinal (GI) tract is rare with rates of 0.4%-2% of cases<sup>[5]</sup> and in up to 12% of cases in autopsy series<sup>[6]</sup>. Here we report a case of HCC with direct invasion of the stomach leading to massive GI bleeding.

## CASE REPORT

A 36-year-old Asian male with hepatitis B and hepatitis C associated HCC presented with chronic melena and massive acute upper GI bleeding with large volume hematemesis. He had undergone trans-arterial chemo-embolization (TACE) four months ago for a large, diffuse, unresectable, biopsy proven HCC in the left lobe of the liver. Due to side effects from the procedure, patient declined to undergo further TACE. He had been on Sorafenib for the preceding two and one-half months prior to this presentation. Apparently, the patient had a liver mass that was resected in his home country eight years ago, described verbally to him as “a benign” tumor. Vital signs on presentation included BP 131/75 mmHg, pulse 123, RR 32, temperature 96.0 F, and oxygen saturation was 88% on 4 L *via* nasal cannula. On physical exam, he was tachycardic, chest was clear to auscultation; mucus membranes were pale, abdominal exam showed tender hepatomegaly (10 cm below right costal margin in the mid-clavicular line) and mid-epigastric tenderness. Alpha fetoprotein level was 8.3 ng/mL. Laboratory data revealed hemoglobin of 2.5 g/dL (hematocrit, 10%; mean corpuscular volume 94), leukocytosis (with white blood cell count 16.7), and platelet count of 539. Coagulation profile showed a mildly prolonged INR of 1.6 and activated partial thromboplastin time of 21. Hemoglobin from prior hospitalization 4 mo ago was 13.3 g/dL. The patient was transfused with packed red cells (7 units) and intravenous fluids were administered. Tumor marker studies including  $\alpha$ -fetoprotein (7.6 ng/mL), CEA (3.3 ng/mL), and CA 19.9 (< 0.1 U/mL), all of which were normal. Hepatitis B quantitative polymerase chain reaction was 41048 U/mL, hepatitis Be antigen was negative and hepatitis Be antibody was positive. Hepatitis C quantitative PCR was 65 IU/mL.

Abdominal computed tomography with intravenous and oral contrast noted a large left hepatic lobe mass which was increased in size (19 cm × 13 cm) compared to prior mass (16 cm × 10 cm) noted two months earlier. An EGD was performed and revealed a 1.4 cm fistula in the posterior wall of the distal gastric body with stigmata of recent bleeding *via* the fistula (Figure 1). A large amount of air was seen centrally within the mass, which appeared to communicate with the gastric lumen through a 7 mm defect along the lesser curvature near the gastric fundus (Figure 2). Subsequently, a visceral (celiac, superior mesenteric artery, and left gastric artery) arteriogram demonstrated no evidence of active

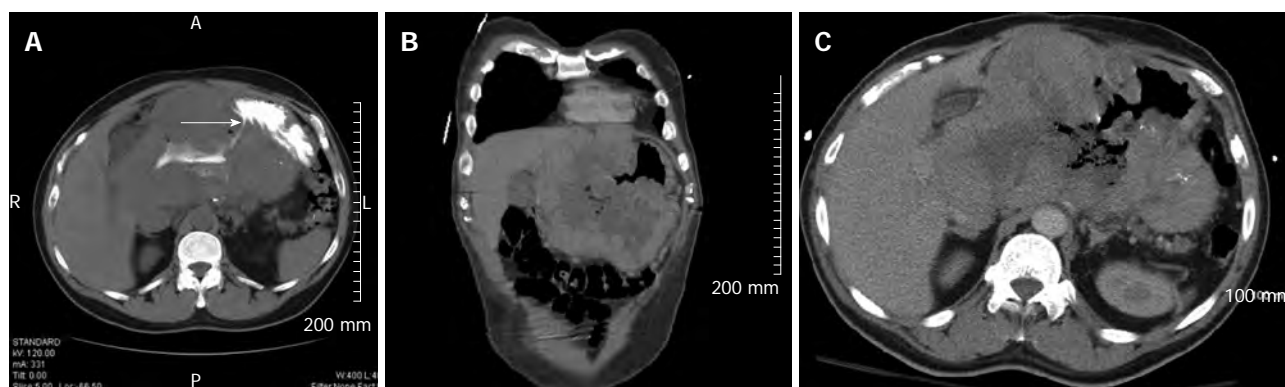


**Figure 1** Esophagogastroduodenoscopy image showing the fistulous opening of the hepatocellular carcinoma on the lesser curvature of the stomach.

bleeding. However, selective left gastric artery angiogram showed hypervascular blush within the tumor and it was selectively embolized to achieve decreased flow, utilizing a total of three, 6 mm × 10 cm long Azur detachable coils. His hemoglobin stabilized and he was discharged with plans to start second line palliative chemotherapy. He subsequently presented a month later with recurrent GI bleeding for which he had to undergo further selective embolization of celiac artery branches, which showed signs of neovascularization. The patient opted for hospice care 10 mo after diagnosis of HCC and 6 mo after treatment of acute GI bleed from hepato-gastric fistula.

## DISCUSSION

GI bleeding is a common complication in patients with HCC. Common etiologies include peptic ulcer disease, variceal bleeding due to portal hypertension from underlying cirrhosis and/or tumor invasion of portal vein causing thrombosis, and portal hypertensive gastropathy. Yeo *et al*<sup>[5]</sup> prospectively looked at 55 HCC patients who presented with GI bleeding over a period of 11 mo and found that majority had a non-variceal source of bleeding (53%) and the remainder variceal bleeding. Among patients with a non-variceal source, three patients (0.05%) bled secondary to direct invasion of tumor to gut. GI bleeding secondary to direct invasion by HCC is rare (0.05%-2%)<sup>[5,7-9]</sup>. Metastatic spread can be due to hematogenous, lymphatic or by direct invasion of the tumor into adjacent organs. The most common site of direct tumor invasion of the GI tract is stomach followed by duodenum and colon. Predisposing factors for GI tract invasion include large liver lesions (> 5 cm in size), subcapsular location and exophytic growth pattern. Suspected predisposing features include prior TACE and/or radiation therapy which can lead to ischemia and inflammatory reactions in the tumor and adjoining bowel wall. These changes can potentially lead to adhesion and local tumor invasion with subsequent fistulization<sup>[10,11]</sup>. We hypothesize a similar process in our case. Park *et al*<sup>[6]</sup> found GI tract involvement at the time of diagnosis of HCC



**Figure 2** Computerized tomography images of the abdomen. A: Showing large mass in the left lobe of the liver and oral contrast traced into liver (arrow) through fistulous opening in stomach; B: Showing large mass in the left lobe of the liver and fistulous communication between the liver mass and the stomach; C: Showing large mass in the left lobe of the liver and fistulous communication between the liver mass and the stomach. A: Anterior R: Right; L: Left; P: Posterior.

in 7/12 patients without any prior treatment. In our patient, we suspect an exophytic tumor growth pattern causing direct GI tract invasion and pressure necrosis on adjacent organs. This process was likely worsened by TACE/radiation related inflammatory reactions contributing to erosion and fistulization, with subsequent bleeding into the GI tract. Sources of gastrointestinal bleeding noted in the literature have included rupture of hepatoma into the GI tract, bleeding from the intrahepatic portion of the tumor, or bleeding from an involved vessel in the wall of the GI tract. In our case, we endoscopically documented oozing from the hypervascular edges of the gastro-hepatic fistula within the stomach.

The prognosis of patients with HCC and GI involvement is poor because of massive bleeding and/or hepatic failure. In one series, the median survival was 4 wk<sup>[7]</sup> and in a second study none survived beyond 9 mo<sup>[8]</sup>. Our patient survived 6 mo. Non-surgical techniques to achieve hemostasis include trans-arterial embolization, endoscopic injection of ethanol or adrenalin, and radiotherapy ablation. Korkolis *et al.*<sup>[12]</sup> reported a case of HCC invading the stomach and upper surface of the pancreas who underwent *en bloc* resection of left lobe of liver with liver mass (15 cm × 12 cm × 9.5 cm), total gastrectomy, distal pancreatectomy, splenectomy, cholecystectomy, radical excision of adjacent lymph node and survival of 16 mo with no local recurrence. Curative surgery is a potential option especially in good surgical candidates with reasonable functional reserve. Surgical intervention has showed prolonged survival with median survival rates of 9.7 mo as opposed to 3.0 mo in non-surgical therapy as reported in other case series<sup>[13,14]</sup>.

In conclusion, due to improvements in chemotherapy and advanced interventional radiologic chemoembolization techniques with improved short term survival in patients with unresectable HCC<sup>[15,16]</sup>, GI tract fistulization and associated upper GI bleeding are likely to become more frequent in the future. This complication may be managed *via* palliative surgical resection, or by angiographic embolization, as was performed in our patient.

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## Pseudomembranous colitis associated with a triple therapy for *Helicobacter pylori* eradication

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### Abstract

*Helicobacter pylori* (*H. pylori*) is one of the most common chronic bacterial infections in humans, affecting half of world's population. Therapy for *H. pylori* infection has proven to be both effective and safe. The one-week triple therapy including proton pump inhibitor, clarithromycin, and amoxicillin or metronidazole is still recommended as a first-line treatment to eradicate *H. pylori* infection in countries with low clarithromycin resistance. Generally, this therapy is well-tolerated, with only a few and usually minor side effects. However, rare but severe adverse effects such as pseudomembranous colitis have been reported, *Clostridium difficile* (*C. difficile*) infection being the main causative factor in all cases. We report the cases of two women who developed pseudomembranous colitis after a 1-wk triple

therapy consisting of pantoprazole 20 mg *bid*, clarithromycin 500 mg *bid*, and amoxicillin 1 g *bid* to eradicate *H. pylori* infection. A limited colonoscopy showed typical appearance of pseudomembranous colitis, and the stool test for *C. difficile* toxins was positive. Rapid resolution of symptoms and negative *C. difficile* toxins were obtained in both patients with oral vancomycin. No relapse occurred during a four and eleven-month, respectively, follow up. These cases suggest that physicians should have a high index of suspicion for pseudomembranous colitis when evaluate patients with diarrhea following *H. pylori* eradication therapy.

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**Key words:** *Helicobacter pylori* eradication; Triple therapy; *Clostridium difficile*; Pseudomembranous colitis; Vancomycin

**Core tip:** Herein are described the cases of two elderly women who developed pseudomembranous colitis after one-week triple therapy consisting of pantoprazole (20 mg *bid*), clarithromycin (500 mg *bid*), and amoxicillin (1 g *bid*) to eradicate *Helicobacter pylori* (*H. pylori*) infection. After a 10-d treatment with oral vancomycin (125 mg every 6 h) both patients had complete resolution of symptoms and negative stool test for *Clostridium difficile* toxins. Clinicians should have a high index of suspicion of pseudomembranous colitis as a rare, but severe complication of *H. pylori* therapy.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is one of the most common chronic bacterial infections in humans, affecting half of the world's population. Its prevalence is high in developing countries and low in the developed ones<sup>[1]</sup>. *H. pylori* eradication therapy is supported by numerous consensus groups around the world, and the treatment of millions of infected subjects has demonstrated that such strategy is both effective and safe. The spectrum of indications for *H. pylori* eradication therapy has steadily extended over the last decade<sup>[2]</sup> with a resultant increase in its use. The one-week triple therapy including proton pump inhibitor (PPI), clarithromycin and amoxicillin or metronidazole proposed at the first Maastricht conference<sup>[3]</sup> to eradicate *H. pylori* is still recommended as the first-line treatment by the recent Maastricht IV consensus conference<sup>[2]</sup> in countries with clarithromycin resistance rate under 15%-20% (e.g., Northern European countries)<sup>[4]</sup>. Eradication rates with standard triple therapy have fallen to 70%-80% over the past few years, mainly due to increasing resistance to clarithromycin<sup>[5]</sup>. Generally, this therapy is well-tolerated, with only a few and usually minor side effects (e.g., nausea, metallic taste). However, severe adverse effects such as pseudomembranous colitis have been reported<sup>[6-13]</sup>, *Clostridium difficile* (*C. difficile*) being the main causative agent in all cases.

We report the cases of two elderly women who developed pseudomembranous colitis after one-week triple therapy with pantoprazole, clarithromycin, and amoxicillin for *H. pylori* infection.

## CASE REPORT

The first case is a 70-year-old woman who was referred to our department with a 10-d history of watery diarrhea (6-12 stools per day) and crampy abdominal pain. Her medical history included hypertension, chronic gastritis *H. pylori* positive, and colonic diverticulosis (previously diagnosed on colonoscopy). Three weeks before admission she completed a one-week triple therapy (pantoprazole 20 mg *bid*, clarithromycin 500 mg *bid* and amoxicillin 1 g *bid*) for *H. pylori* infection. On physical examination, she looked unwell, and her abdomen was mildly tender, with no masses. Temperature and vital signs were normal. Laboratory investigations revealed leukocytosis (14800/mm<sup>3</sup>) with neutrophilia, high C-reactive protein (11.5 mg/dL), and low levels of serum albumin (2.4 mg/dL), sodium (133 mEq/L), and potassium (2.7 mEq/L). Two days before admission, stool samples examination excluded enteric bacterial pathogens (*Shigella*, *Salmonella*, *Yersinia* spp.) as well as *C. difficile* toxins. Without any prior preparation, the patient underwent a limited colonoscopy, which showed diffusely scattered off-white pseudomembranes attached to the hyperemic underlying mucosa and multiple diverticula (Figure 1). Repeated stool sample examination was positive for *C. difficile* toxins A and B. Treatment with oral metronidazole 500 mg every 8 h was initiated, replaced 72 h later with oral vancomycin 125



**Figure 1** Pre-treatment endoscopic examination. Colonoscopy revealed scattered off-white pseudomembranes, some of them around a diverticulum.

mg every 6 h due to unfavorable response. After a 10-d treatment with vancomycin, the patient had a complete resolution of the symptoms and was discharged from hospital with negative results for *C. difficile* toxins and one stool per day. During a four-month follow-up, patient remained asymptomatic.

The second case concerns a 71-year-old woman who was admitted with profuse watery diarrhea (up to 10 stools daily) and abdominal pain. Her prior medical history was unremarkable. Symptoms occurred 5 d after a one-week triple therapy (pantoprazole 20 mg *bid*, clarithromycin 500 mg *bid* and amoxicillin 1 g *bid*) for *H. pylori* eradication. Physical examination was normal, except for signs of dehydration. Microbiological examination of stools was negative for *Salmonella*, *Shigella* and *Yersinia* spp., and did not reveal any parasites. The patient had leukocytosis (12400/mm<sup>3</sup>), hypokalemia (2.8 mEq/L), and mild inflammatory syndrome. Sigmoidoscopy showed typical signs of pseudomembranous colitis, while a stool test for *C. difficile* toxins A and B proved positive. The patient received 10-d treatment with oral vancomycin 125 mg every 6 h, followed by prompt improvement in symptoms and negative test for *C. difficile* toxins. No relapse occurred during an 11-mo follow-up.

## DISCUSSION

Eradication therapy for *H. pylori* provides enormous benefits and has proved to be both effective and safe. Except from the rare and mild side-effects, eradication therapy is generally well-tolerated. Severe adverse effects such as pseudomembranous colitis following eradication therapy have very rarely been reported<sup>[6-13]</sup> which is quite surprising, taken into account the immense number of subjects treated worldwide. It is difficult to find a clear explanation why are so rare cases of pseudomembranous colitis after eradication therapy reported in the literature, but some hypotheses were put forward: (1) the use of metronidazole, an efficient drug against *C. difficile*; however, several cases of pseudomembranous colitis, as published, occurred after a regimen containing metronidazole<sup>[8-10]</sup>; (2) the short duration of the therapy; (3) almost all treat-

ments are carried out in outpatients (hospitalization is a risk factor for *C. difficile* infection); and (4) many cases with mild clinical disease were most likely not diagnosed, either because the patients did not consult a physician or the physician did not suspect the development of *C. difficile* infection<sup>[10]</sup>.

Over the last decade, *C. difficile* infection rate has increased dramatically worldwide both in incidence and severity<sup>[14,15]</sup>. In addition to broad-spectrum antibiotic therapy, there have been identified many other potential risk factors for *C. difficile* infection (advanced age, female gender, comorbidities, admission to ICU, long hospital stay, immunosuppressive therapy, and PPI use)<sup>[16-21]</sup>. Several studies and recent meta-analyses have shown that PPI therapy is associated with increased risk of *C. difficile* infection<sup>[20-27]</sup>, and United States Food and Drug Administration even issued a safety announcement to inform the public about this possible risk<sup>[28]</sup>. Newly published studies have found that *C. difficile* infection can occur outside the above mentioned well-known risk groups, in the absence of any hospitalization and even in young patients with no comorbidities<sup>[29]</sup>.

All the components of the triple eradication therapy for *H. pylori* (PPI and two antibiotics: clarithromycin and amoxicillin or metronidazole) are potential risk factors for *C. difficile* infection. The most responsible for development of pseudomembranous colitis seems to be clarithromycin, used in both our cases and in most of the published reports<sup>[6,7,10,13]</sup>.

The spectrum of *C. difficile* infection is wide, ranging from mild, self-limiting diarrhea to fulminant pseudomembranous colitis which is associated with significant morbidity and mortality. Most patients with *C. difficile* infection have a mild-to-moderate disease, but some may develop severe forms of disease such as pseudomembranous colitis, or even complicated by toxic megacolon<sup>[7]</sup>. Among variables used to define severe disease, the most important are the presence of pseudomembranes at endoscopy and age over 65 years. Both our cases met the criteria for a severe form of disease, and were treated with vancomycin according to current guidelines recommendations<sup>[30]</sup>.

Our cases, in addition to the ones published, demonstrate that pseudomembranous colitis can occur after a usually well-tolerated triple therapy for *H. pylori* eradication. Despite this very rare complication, it should be underlined that *H. pylori* eradication therapy provides huge benefits and remains effective and generally safe. However, *C. difficile* infection may occur with an eradication therapy for *H. pylori* consisting of two antibiotics and a PPI. Most likely, *C. difficile* infection cases following *H. pylori* eradication therapy are not as rare as reported by literature, considering that a significant proportion of mild form of disease does not come to physician's attention and thus may remain undiagnosed<sup>[31]</sup>. Clinicians should be aware of such complication when prescribing triple therapy for *H. pylori* eradication, and should inform the patients that they may have diarrhea during or after

treatment, and therefore should seek medical advice.

In conclusion, pseudomembranous colitis should be suspected in any patient with watery diarrhea during or after triple therapy for *H. pylori* eradication. Awareness of such complication is particularly important in the actual context when both duration and indications for *H. pylori* eradication therapy have been extended.

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## Successful treatment of multiple hepatocellular adenomas with percutaneous radiofrequency ablation

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adenoma; Radiofrequency ablation;  $\beta$ -catenin activation; Glycogen storage disease

**Core tip:** Risk stratification by pathological examination is an important step in deciding therapeutic options of multiple hepatocellular adenoma in glycogen storage disease type I a patients.

Ahn SY, Park SY, Kweon YO, Tak WY, Bae HI, Cho SH. Successful treatment of multiple hepatocellular adenomas with percutaneous radiofrequency ablation. *World J Gastroenterol* 2013; 19(42): 7480-7486 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7480.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7480>

### Abstract

Hepatocellular adenoma (HCA) is one of the important complications of glycogen storage disease type I a (GSD- I a) because it can be transformed into hepatocellular carcinoma. Although surgical resection is a standard treatment of choice for solitary HCA, multiple HCAs in GSD- I a patients present as therapeutic challenges for curative treatment. Therefore, treatment strategy according to malignant potential is important in management of HCAs in GSD- I a. The authors present a case of histologically proven multiple HCAs without  $\beta$ -catenin mutations occurred in a GSD- I a patient treated successfully with percutaneous radiofrequency ablation as a minimally invasive therapy.

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**Key words:** Glycogen storage disease; Hepatocellular

### INTRODUCTION

Glycogen storage disease type I a (GSD- I a) is an inherited disorder of carbohydrate metabolism caused by glucose-6-phosphatase deficiency in the liver, kidneys and intestinal mucosa<sup>[1]</sup>. Liver diseases associated with GSD- I a include hepatocellular adenoma (HCA) which can lead to considerable morbidity and mortality associated with malignant transformation and intratumoral hemorrhage<sup>[2]</sup>. While HCA associated with oral contraceptives or exogenous androgen is relatively large, single and encapsulated HCAs in GSD- I a tend to be small, multiple and non-capsulated<sup>[3]</sup>. Furthermore, HCAs in GSD- I a tend to transform into hepatocellular carcinoma (HCC) more frequently than sporadic HCAs<sup>[3]</sup>. Rates of transformation into HCC have been reported approximately as being up to 10%<sup>[4]</sup>. Recently, sporadic HCAs have been classified into four subgroups; hepatocytic nuclear factor 1  $\alpha$  mutated HCAs,  $\beta$ -catenin-activated HCAs, inflammatory HCA, and unclassified HCA according to molecular markers and immunohistochemistry<sup>[5]</sup>. Because  $\beta$ -catenin activation is known as a risk factor for malig-



nant transformation, subgroup classification of HCAs has been suggested as an important step in management of HCAs<sup>[6,7]</sup>. Although several strategies for management of multiple HCAs in GSD- I a are suggested in literature, the best treatment is still controversial<sup>[8]</sup>. Surgical resection has been a standard treatment for solitary HCA with high risk of malignancy. However, the role of surgical resection is limited in multiple HCAs in GSD- I a. Liver transplantation could be another treatment option for management of multiple HCAs as well as correction of most metabolic derangement in GSD- I a patients<sup>[9]</sup>. Other options such as ethanol injection and transarterial embolization have been suggested as new and less invasive treatments<sup>[10-12]</sup>. The authors present a case of multiple HCAs without  $\beta$ -catenin mutations in a patient with GSD- I a which were successfully treated by ultrasonography guided percutaneous radiofrequency ablation (RFA).

## CASE REPORT

A 24-year-old male patient was referred for multiple liver masses. He was diagnosed as GSD- I a 10 years ago. He had a family history of two brothers who died of HCC at the age of 22 and 26 years, respectively. His mother died from an undetermined cause in her twenties. After diagnosis, he had never received dietary therapy or other medical treatments. He was undersized with a height of 166 cm and weight of 51 kg. The laboratory findings revealed hyperlipidemia and hyperuricemia after a 12 h fast (Table 1). Serologic tests for viral hepatitis and autoimmune liver disease were all negative. Serum levels of  $\alpha$ -fetoprotein and prothrombin induced by vitamin K deficiency or antagonist-II were 2.4 ng/mL and 35 mAU/mL. Mutation analysis of the glucose-6-phosphatase gene revealed compound heterozygosity for p.Leu216X (c.648G > T) and p.Gly222Arg (c.664G > A) in exon 5. On subsequent gadolinium ethoxybenzyl diethylene triamine pentaacetic acid (Gd-EOB-DTPA; Primovist, Bayer Schering Pharma, Berlin, Germany) enhanced magnetic resonance imaging (MRI), four arterial-phase enhancing nodules were noted in both lobes of liver and these nodules showed clear defects on 20 min hepatobiliary phase (Figure 1). Three nodules revealed the same features with intense homogeneous arterial enhancement, nearly iso-signal intensity with capsular enhancement on 3 min delayed phase, and intermediate high signal intensity on axial T2-weighted image (T2WI). There was no evidence of fat component or internal hemorrhage, or the Atoll sign (Figure 1A-C). One nodule showed heterogeneous signal intensity on 3 min delayed phase, axial T2WI, and 20 min hepatobiliary phase (Figure 1D). These features were consistent with multiple hepatic adenomas associated with GSD- I a. We performed percutaneous liver biopsies on each nodule to identify HCA subtype. Each biopsy specimen underwent standard histopathological examination and immunohistochemistry for  $\beta$ -catenin, glutamine synthetase and amyloid A (Figure 2). Immunohistochemistry confirmed all the adenomas as

**Table 1** Laboratory findings on admission

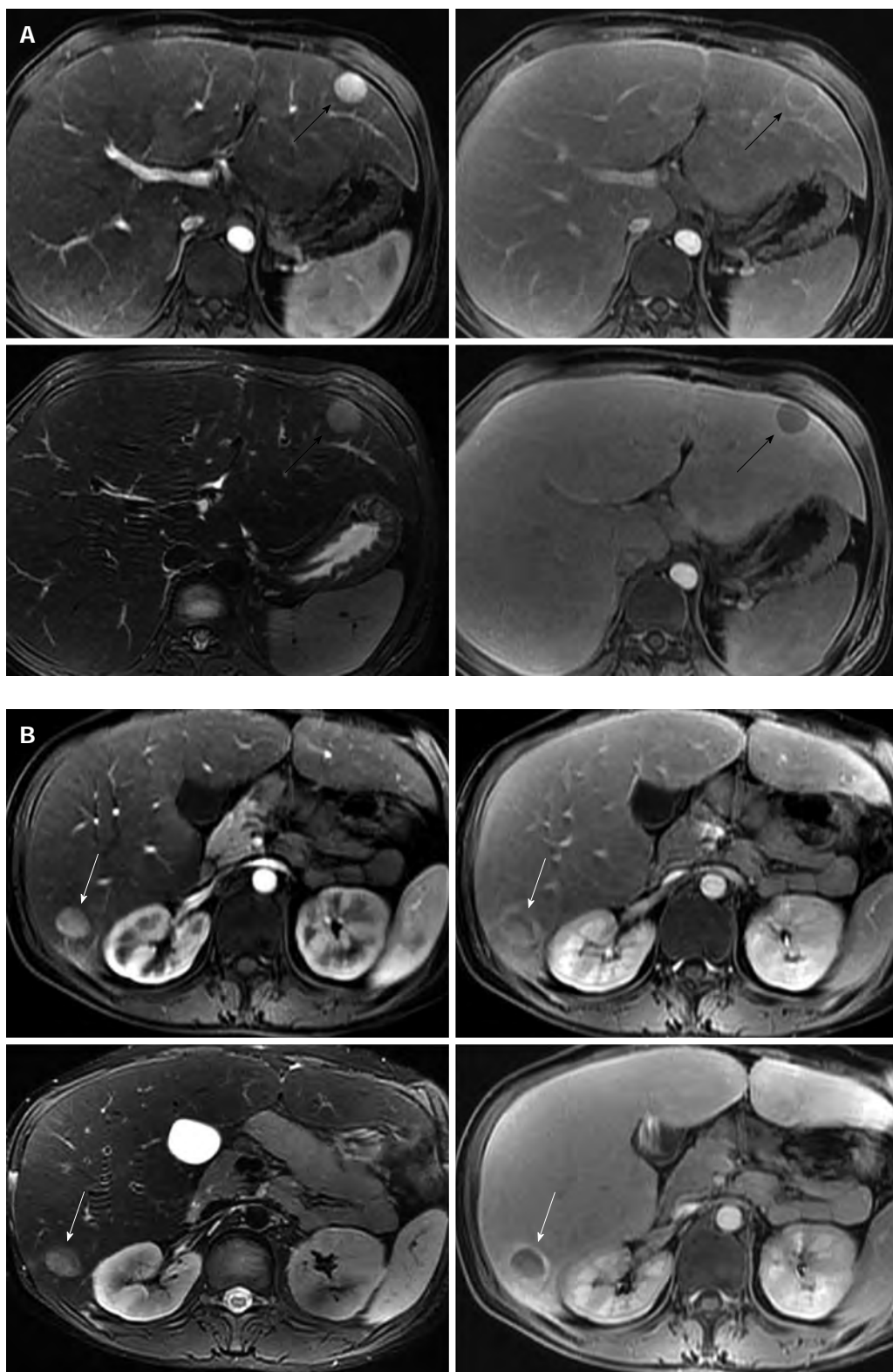
Blood chemistry	Value
Total bilirubin	0.42 mg/dL
ALP	218 U/L
AST	87 U/L
ALT	37 U/L
LDH	275 U/L
$\gamma$ -GTP	152 U/L
Total protein	8.9 g/dL
Albumin	4.8 g/dL
Total cholesterol	380 mg/dL
TG	1688 mg/dL
Uric acid	9.4 mg/dl
BUN	8.5 mg/dL
Creatinine	1.15 mg/dL
Glucose	93 mg/dL
Lactic acid	3.5 mmol/L
Hemoglobin A1c	5.0%
Hematology	
Hemoglobin	11.3 g/dL
White blood cell	$4.14 \times 10^3/\mu\text{L}$
Platelet	$269 \times 10^3/\mu\text{L}$
Serology	
AFP	2.2 ng/mL
PIVKA-II	35 mAU/mL
Hepatitis B surface antigen	Negative
Anti HBs Ab	Negative
Hepatitis B core antibody	Negative
Anti HCV Ab	Negative
Hemostasis	
Prothrombin time	11.2 s

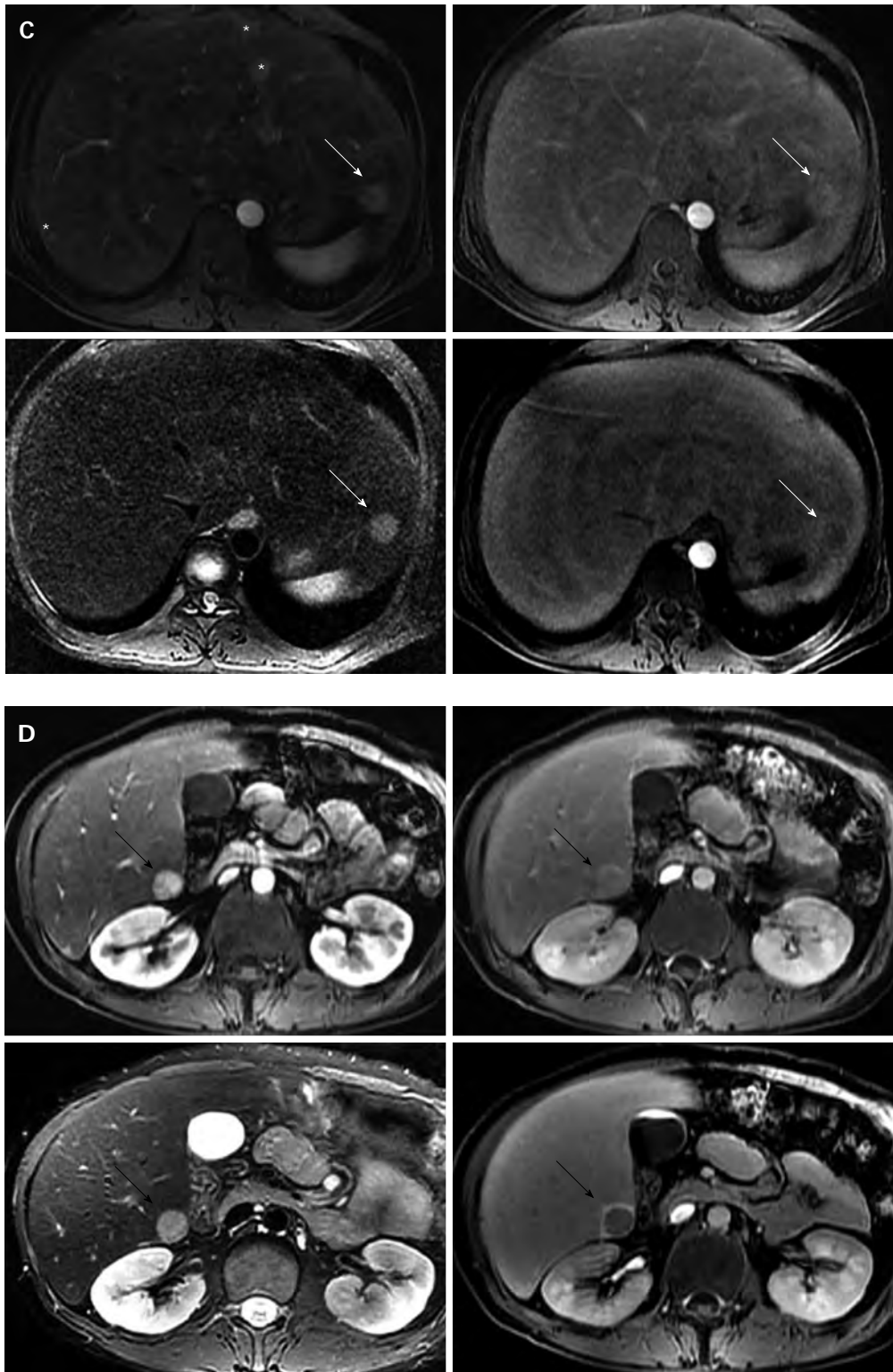
ALP: Alkaline phosphatase; AST: Aspartate transaminase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase;  $\gamma$ -GTP: Gammaglutamyl transpeptidase; TG: Triglyceride; BUN: Blood urea nitrogen; AFP:  $\alpha$ -fetoprotein; PIVKA-II: Prothrombin induced by vitamin K deficiency or antagonist-II.

inflammatory type HCA without malignant transformation. As all the nodules were small without malignant cells and  $\beta$ -catenin activation, we performed ultrasonography guided RFA as a less invasive therapeutic option. RFA was successfully performed under local anesthesia using a cool-tip electrode with a 3 cm exposed tip (ACT2030, Covidien, Mansfield, MA, United States). Multiple overlapping ablation methods were applied for each adenoma. There was no procedure-related complication after RFA. Three months follow-up computed tomogram scans revealed gradual reduction of the RFA zone without any residual tumor (Figure 3).

## DISCUSSION

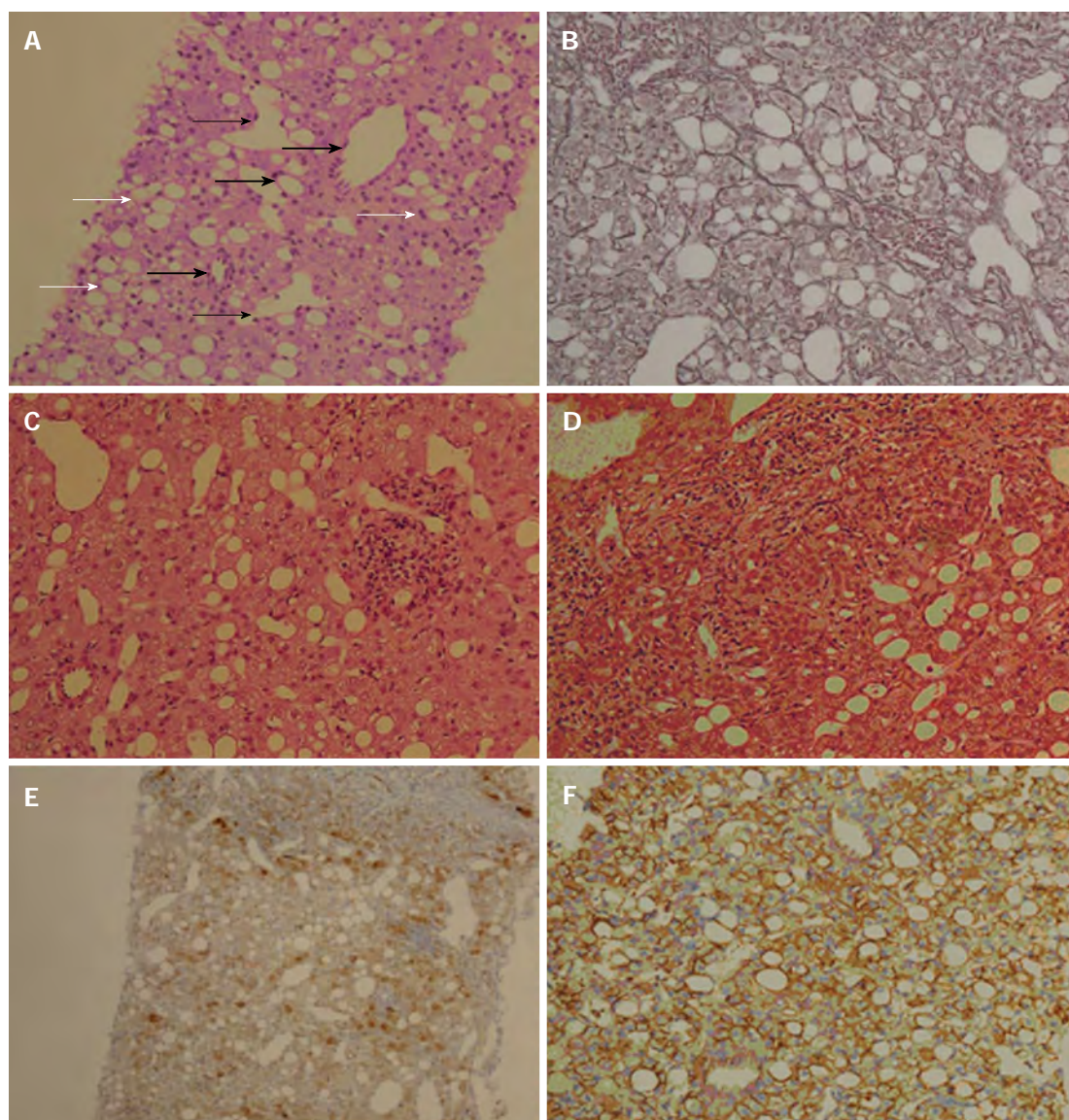
Management of HCAs in patients with GSD- I a is not well established because of the rarity of this disease. As patients with GSD- I a survive longer with intensive dietary therapy, they have more chances to develop HCAs in their expanded life span<sup>[13]</sup>. Although there is a case report that HCA in GSD- I a regressed after strict dietary therapy, maintenance of normoglycemia under intensive dietary therapy is insufficient to regress HCAs or reverse malignant transformation because patients with multiple HCAs sometimes showed sudden progression and





**Figure 1** Hepatic adenomas associated with glycogen storage disease type 1a. A: Gd-EOB-DTPA enhanced axial T1-weighted image on arterial phase (left upper) reveals approximately 2 cm intense enhancing mass in the segment 3 of liver (black arrows). The mass shows nearly iso-signal intensity with capsular enhancement on 3 min delayed phase (right upper), intermediate high signal intensity on axial T2-weighted image (left lower), and clear defect on 20 min hepatobiliary phase (right lower); B: Approximately 1.8 cm sized intense enhancing mass on arterial phase (left upper) is noted in the segment 6 of liver. This mass shows suspicious focal eccentric wash-out enhancement (white arrows) on 3 min delayed phase (right upper). This portion reveals relatively low signal intensity on axial T2-weighted image (left lower) and 20 min hepatobiliary phase (right lower), compared to other tumor area. Surrounding rim enhancement in the tumor is seen on 20 min hepatobiliary phase; C: Approximately 1.8 cm arterial enhancing mass (white arrows) is seen in the segment 2 of liver. The mass reveals slightly high signal intensity on 3 min delayed phase (right upper), intermediate high signal intensity on axial T2-weighted image (left lower), and fuzzy defect on 20 min hepatobiliary phase (right lower). Multifocal arteriportal shunts (asterisks) are noted in the segments 3 and 7 of liver; D: Approximately 1.8 cm sized intense enhancing mass (black arrows) on arterial phase (left upper) is noted in the segment 6 of liver. This mass shows slightly high signal intensity with capsular enhancement on 3min delayed phase (right upper), and intermediate high signal intensity on axial T2-weighted image (left lower). On 20 min hepatobiliary phase (right lower), the tumor reveals clear defect with surrounding rim enhancement. Gd-EOB-DTPA: Gadolinium ethoxybenzyl diethylene triamine pentaacetic acid.





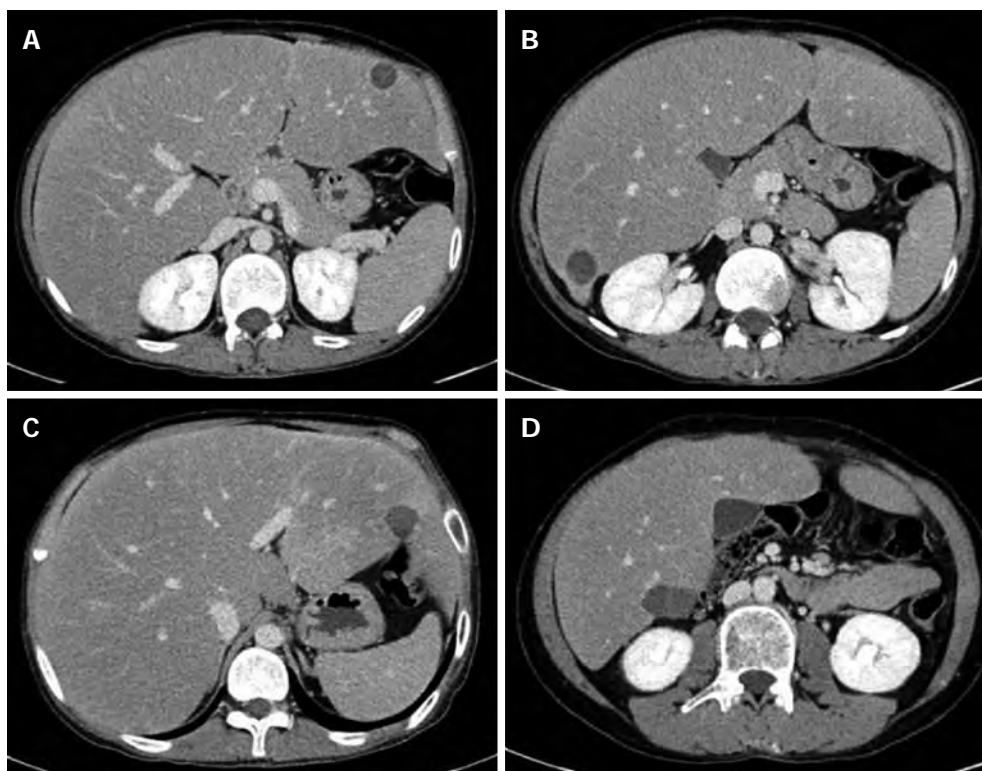
**Figure 2** The histological features are consistent with the inflammatory type of hepatocellular adenoma. A: A hepatocellular adenoma in GSD-1a has several unpaired arteries (thick black arrows), sinusoidal dilation (thin black arrows), and steatosis (white arrows), but the atypia of hepatocytes are low without a nuclear pleomorphism; B: A reticulin staining does not show thick hepatic cords; C: The lymphocytic infiltrates are found in a hepatic lobule; D: The lymphocytes infiltrate in the area surrounded a portal and a periportal tracts; E: Staining for glutamine synthetase is diffuse cytoplasmic with focal nuclear expression; F:  $\beta$ -catenin staining does not show abnormal expression. GSD-1a: Glycogen storage disease type I a.

metastatic spread<sup>[3,14-17]</sup>. As HCAs in GSD- I a tend to be multiple, involving both lobes of liver, the ultimate treatment of HCAs in GSD- I a is liver transplantation for management of HCAs and to reverse metabolic derangement<sup>[9,18]</sup>. However, liver transplantation is rarely performed due to uncertainties on timing of transplantation, limited organ availability and combined renal dysfunction with immunosuppression<sup>[13]</sup>. Surgical resection of HCAs is suggested as an effective intermediate therapy for prevention of HCC until liver transplantation<sup>[13]</sup>. However, liver resection in GSD- I a patients showed greater morbidity including intra-abdominal abscess, multi-organ failure, unstable blood glucose control and hemorrhage.

Recently, molecular characterizations of HCAs demonstrated that frequent  $\beta$ -catenin mutations are more

frequently observed in HCAs in GSD- I a and related to increased risk of malignant transformation like sporadic HCAs<sup>[19]</sup>. To assess the risk of malignant transformation, we biopsied each HCA for analysis of subtype of each tumor by immunohistochemistry. All the HCAs were small and inflammatory type with low risk of malignant transformation. There are reports of treatment of HCAs in GSD- I a by transarterial embolization and ethanol injection as a minimal invasive procedure<sup>[11,12]</sup>. As RFA can offer complete necrosis of target lesions compared to transarterial embolization and ethanol injection, we treated HCAs by percutaneous RFA successfully. The present case shows the potential role of RFA in management of multiple HCAs in GSD- I a patients along with risk stratification by pathological examination.





**Figure 3** Computed tomogram follow-up after 3 mo showed gradual reduction of radiofrequency ablation zone. A: Post-RFA follow-up CT revealed gradual contraction of ablated lesion in segment 3 to 1 cm; B: Complete ablation of enhancing lesion in segment 6 was observed in Post-RFA follow-up CT; C: Post-RFA follow-up CT showed complete ablation of 1.8 cm sized tumor in segment 2; D: Post-RFA CT revealed no residual lesion without adjacent organ damage. CT: Computed tomogram; RFA: Radiofrequency ablation.

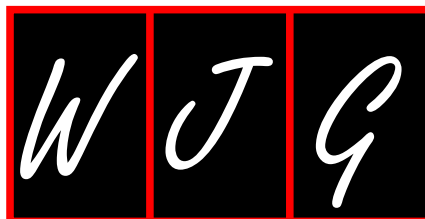
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**L- Editor:** O'Neill M **E- Editor:** Zhang DN





## Rifaximin and Crohn's disease

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Author contributions: Prantera C and Scribano ML wrote this letter.

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### Abstract

In a recent article, Longman and Swaminath analyzed our paper on the use of rifaximin in patients with moderately active Crohn's disease (CD). Here we report some considerations concerning their article. The exploratory *post-hoc* subgroup analysis showed that early-stage disease and, differently from that written by Longman and Swaminath, also colonic involvement seemed to be associated with a significant higher efficacy of rifaximin-EIR 800 mg twice daily. Early-stage disease is generally considered as the more easily treatable phase of CD, and the better response to rifaximin in Crohn's colitis is in accordance with the high concentration of bacteria in the colon. In addition, patients with C reactive protein level > 5 mg/L achieved remission more significantly than patients with normal values, thus suggesting that the symptoms were probably caused by inflammation instead of by non-inflammatory causes. We also analyze the role of rifaximin against gut bacteria and the clinical situations that could obtain the best results from antibiotics.

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**Key words:** Crohn's disease; Intestinal microbiota; Non-absorbable antibiotic; Rifaximin

**Core tip:** In this letter to the Editor we report some considerations concerning the article entitled "Microbial manipulation as primary therapy for Crohn's disease" written by Longman and Swaminath. In the article the authors analyzed our paper on the use of rifaximin as primary therapy in active Crohn's disease. The *post-hoc* analysis of our study showed that early-stage disease, colonic involvement and a C reactive protein level > 5 mg/L were associated with a significant higher efficacy of rifaximin. We also discuss the role of rifaximin against intestinal bacteria and the clinical situations to explore further in controlled studies with antibiotics.

Prantera C, Scribano ML. Rifaximin and Crohn's disease. *World J Gastroenterol* 2013; 19(42): 7487-7488 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i42/7487.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i42.7487>

### TO THE EDITOR

We would like to thank Doctor Longman and Swaminath from Columbia University for their interesting analysis of our article concerning the trial of rifamixin in Crohn's disease (CD)<sup>[1,2]</sup>.

Their expert analysis entitled "Microbial manipulation as primary therapy for Crohn's disease" merits some considerations and one slight correction: (1) The *post-hoc* explorative subgroup analysis has certainly revealed that patients with an early disease (as defined as first diagnosis ≤ 3 years before enrollment in the study) are significantly more likely to achieve remission with rifaximin-EIR 800 mg twice a day compared to placebo, but, differently from what Longman and Swaminath have written, also colonic involvement, and a baseline C-reactive protein (CRP) level > 5 mg/L showed the same statistical significant superiority. The favorable effect of these three factors is biologically plausible. Elevated CRP values suggest that the symptoms, which increase the Crohn's disease activity index values, are probably caused by inflamma-

tion instead of by non-inflammatory causes such as irritable bowel, bile salt diarrhoea or previous surgery. Many previous studies with antibiotics have shown that Crohn's colitis responds better to this therapy than does that in the small bowel location, and this is in accordance with the higher microbial content in the colon. Finally, early disease is more easily treatable because the lesions are mainly inflammatory and the structural damage has usually not yet appeared; (2) Rifaximin is a non-absorbable antibiotic and consequently it should not work on the bacteria attached to the mucosa. Rifaximin can work against bacteria present in the lumen but not against adherent *Escherichia coli* (AIEC), and this fact could be particularly important if AIEC is one of the causes of CD<sup>[3]</sup>. At most rifaximin can reduce the attachment of enteroaggregative *Escherichia coli* by biologically altering the host cell, but it should not be able to counteract the bacteria already adherent to the mucosa<sup>[4]</sup>. A study which explores the efficacy of one antibiotic together with a microbial analysis in order to ascertain which bacteria are involved in the inflammatory process, cannot be limited to investigating the stools flora, given that the bacteria found in the stools probably do not represent the adherent bacteria and could not be responsible for the inflammation; (3) From our study about 50% of the patients maintained clinical remission 12 wk after stopping rifaximin. It is probable, however, that a permanent change of the microbiota does not occur; and (4) Doctors Longman and Swaminath ask how to select patients who could ob-

tain the best benefit from antibiotics. Crohn's colitis and elevated values of acute phase reactants seem the better clinical situation to explore further in controlled studies. Given the possible role of adherent bacteria in causing CD or, at least, in being responsible for symptoms, another very promising situation should be explored: immediately after surgery, when all the diseased tracts have been removed, antimicrobial therapy could obstruct the adherence of bacteria to the mucosa reducing the recurrence of lesions.

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# World Journal of *Gastroenterology*

*World J Gastroenterol* 2013 November 21; 19(43): 7489-7824





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## Challenges in diagnosing adhesive small bowel obstruction

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### Abstract

Adhesive small bowel obstruction (ASBO) is the most frequently encountered surgical disorder of the small intestine. Up to 80% of ASBO cases resolve spontaneously and do not require invasive treatment. It is important to identify such patients that will benefit from conservative treatment in order to prevent unnecessarily exposing them to the risks associated with surgical intervention, such as morbidity and further adhesion formation. For the remaining ASBO patients, timely surgical intervention is necessary to prevent small bowel strangulation, which may cause intestinal ischemia and bowel necrosis. While early identification of these patients is key to decreasing ASBO-related morbidity and mortality, the non-specific signs and laboratory findings upon clinic presentation limit timely diagnosis and implementation of appropriate clinical management. Combining the clinical presentation findings with those from other diagnostic imaging modalities, such as abdominal X-ray, computed tomography-scan and water-soluble contrast studies, will improve diagnosis of ASBO and help clinicians to better evaluate the potential of conservative management as a safe strategy for a particular patient. Nonetheless, patients

who present with moderate findings by all these approaches continue to represent a challenge. A new diagnostic strategy is urgently needed to further improve our ability to identify early signs of strangulated bowel, and this diagnostic modality should be able to indicate when surgical management is required. A number of potential serum markers have been proposed for this purpose, including intestinal fatty acid binding protein and  $\alpha$ -glutathione S transferase. On-going research is attempting to clearly define their diagnostic utility and to optimize their potential role in determining which patients should be managed surgically.

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**Key words:** Adhesive small bowel obstruction; Diagnosis; Clinical management; Biological markers; Intestinal fatty acid binding protein;  $\alpha$ -glutathione S transferase

**Core tip:** Adhesive small bowel obstruction (ASBO) is a frequently encountered disorder of the small intestine following abdominal surgery. Accurately predicting whether ASBO patients can be treated conservatively is required to prevent exposing patients unnecessarily to surgery-related risks, including morbidity and further adhesion formation. Although recent technological developments have improved the ability to identify those patients most fit for conservative management, the remaining patients with moderate findings upon clinical presentation remain a problem. Serum markers of intestinal ischemia are promising candidates for improving early diagnosis and identification of patients with strangulated bowel, who will benefit most from surgical management.

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## INTRODUCTION

Small bowel obstruction (SBO) leading to strangulation and potential bowel necrosis is a serious condition that mandates surgical intervention<sup>[1-3]</sup>. Timely diagnosis is essential to prevent the associated morbidity and mortality that manifest as operative management is delayed<sup>[4]</sup>. This fact was highlighted by the adage among surgeons citing “never let the sun rise or set in the case of small bowel obstruction”.

Since up to 80% of SBO cases resolve without incident under conservative treatment<sup>[5,6]</sup>, identification of patients whose obstruction will spontaneously resolve is important to prevent unnecessary surgical intervention and exposure to the risks of procedure-related morbidity and further formation of adhesions<sup>[6,7]</sup>. Recent technological advances in diagnostic modalities have improved the ability to identify patients who are most likely to benefit from conservative treatment; however, accurate and early identification of those patients who will ultimately require surgical intervention remains a challenge, especially when the clinical symptoms are moderate<sup>[2]</sup>.

## BACKGROUND

SBO is the most frequently encountered surgery-related disorder of the small intestine. In up to two-third of SBO cases, adhesions from prior abdominal surgery are implicated as the direct cause, having manifested as adhesive small bowel obstruction (ASBO)<sup>[2,6,8]</sup>. Although the majority of ASBO present within one year after surgery, up to 21% can develop up to ten years later<sup>[9]</sup>. In addition, other causes of SBOs exist, including neoplasia, herniations, inflammatory disease, or congenital disorders. Regardless of the cause, however, obstructed bowel eventually becomes edematous, which leads to bowel ischemia, inflammation, and necrosis in the end-stage and requires surgical resection.

### Clinical presentation

Patients with SBO usually present with a wide range of complaints, such as nausea, vomiting, and intermittent abdominal pain. In most cases, a history of prior abdominal surgery is present<sup>[6,10]</sup>. However, the clinical symptoms only contribute partially to diagnosis of ASBO, and studies have calculated the symptom-related sensitivity and specificity of acute abdominal pain as 75% and 99%<sup>[11]</sup>. One of the more recent studies, evaluating the current diagnostic technologies and clinical routines, found a higher overall sensitivity (88%) but a lower specificity (41%) for this parameter<sup>[12]</sup>; thus, improved diagnostic modalities are still needed. Unfortunately, the clinical symptoms of SBO are also not reliable predictors of the optimal disease management strategy, and distinguishing patients with bowel strangulation who

require prompt surgical intervention remains a particular challenge to clinicians.

### Laboratory findings

Laboratory tests are often used to confirm clinical suspicions and evaluate the degree of illness. The commonly measured inflammatory markers, such as white blood cell (WBC) count and C-reactive protein (CRP)<sup>[13]</sup>, however, cannot distinguish inflammation due to obstruction from other inflammatory syndromes and are therefore of little value in the early diagnosis of ASBO<sup>[14]</sup>. Even in the case of bowel ischemia, as would be seen in bowel strangulation, studies have detected no significant differences in the WBC or CRP levels of patients who benefit from conservative management and those who require surgical intervention, making these markers useless for distinguishing these two categories of patients<sup>[14-16]</sup>.

When progression to ischemia occurs, L-lactate, lactate dehydrogenase (LDH) and creatine kinase (CK) can rise due to hypoperfusion of the intestinal tissue<sup>[15]</sup>. However, large quantities of L-lactate are cleared by the liver during splanchnic hypoperfusion, resulting in L-lactate being increased at a very late stage of the process, when extensive intestinal infarction is already well established<sup>[17]</sup>. From a clinical perspective, a rise in L-lactate level increases sensitivity for detecting bowel ischemia up to 100% and is considered a strong indicator for emergency surgical intervention<sup>[18]</sup>. In contrast, LDH and CK levels rise in any ischemic condition, and are therefore unspecific. D-dimer, however, may serve as an exclusionary indicator for the presence of ischemia, due to its role as an enzymatic degradation product of fibrin, but it also lacks specificity since it can be elevated in numerous other conditions<sup>[14]</sup>.

Since the above-mentioned markers are not specific enough for diagnosis of SBO they are also not useful for determining whether surgical intervention is needed for any particular case. Instead, these markers can be used to simply reflect severity of the disease and may contribute “circumstantial evidence” to support or deny a decision based upon a wide array of clinical findings.

### Imaging techniques

The 2010 Bologna Guidelines for Diagnosis and Management of ASBO arose from an international consensus statement. According to these guidelines, all suspected cases of ASBO should be evaluated by abdominal X-ray (level 2b)<sup>[7]</sup>. Specifically, the presence or absence of classical signs, such as distension, > 3 cm diameter dilatation of the small bowel, perturbed air-fluid levels and absence of colonic gas, is considered a sufficient means of diagnosis, and studies have calculated this approach to have overall sensitivity and specificity ranging from 60%-85%<sup>[6,7]</sup>.

In contrast, Laméris *et al*<sup>[12]</sup> showed that evaluating patients presenting with acute abdominal pain with plain radiography provided no benefit towards improving the above-mentioned sensitivity and specificity, presuming



that there is no role in the diagnostic work-up. Adding ultrasonography (US) after clinical diagnosis, however, was shown to increase the specificity from 41% to 85%. In suspected SBO cases, US can differentiate between ileus and mechanical obstruction, since peristalsis can be observed by this imaging modality<sup>[19]</sup>. Extra-luminal fluid findings are of major clinical importance as they are commonly used to make clinical decisions as to which surgical approach will be most tolerable and beneficial to a particular patient<sup>[20]</sup>. In contrast to these findings, the Bologna Guidelines state that there is limited value for US (level 2c), since entrapment of air in ASBO limits ultrasound transmission, making it a useful diagnostic tool only when applied by technical experts<sup>[2,7]</sup>.

Using computed tomography (CT)-scan as an additional imaging platform to evaluate all patients with inconclusive plain radiologic films has proven highly useful for diagnosing SBO<sup>[2,7,21,22]</sup>. CT-scan has high sensitivity and specificity for SBO (> 92% and 93% respectively); in addition, the additional information provided by CT scanning can help to detect signs of intestinal ischemia or perforation<sup>[6,23-25]</sup>. However, Maglinte *et al*<sup>[26]</sup> reported that CT-scan can be just as sensitive as a plain abdominal x-ray for differentiating between obstruction and non-obstruction (86% *vs* 82% detection levels). It is important to note that the group with possible signs of ischemia remains a clinical challenge, and making a decision for clinical management is still a problem<sup>[10,23,27,28]</sup>.

Magnetic resonance imaging (MRI) seems to have a limited role in diagnosing ASBO. MRI provides similar sensitivity and specificity as CT-scan, but no current guidelines have been established or implemented for applying MRI in standard clinical practice<sup>[2,7,29]</sup>. Interestingly, when combining abdominal films with water-soluble contrast medium, the approach can both make a diagnosis and safely rule-out the presence of a complete obstruction. In this manner, patient evaluation by water-soluble contrast studies can help to predict whether their ASBO can be treated conservatively or will require surgical intervention<sup>[7,10,22,30]</sup>. Besides being a useful diagnostic tool, water-soluble contrast may also have therapeutic potential; its ability to draw fluid into the lumen reduces edema in the gut wall, thereby relieving the obstruction and stimulating peristalsis<sup>[31]</sup>. A randomized controlled trial by Burge *et al*<sup>[3]</sup> showed an appreciable therapeutic effect when gastrografin was applied as the contrast agent to evaluate ASBO patients; specifically, a significantly accelerated resolution of the obstruction was seen in up to 75% of the patients within 24 h after the application. This result may be attributed to the hyperosmolar quality of gastrografin or other contrast mediums. While the precise benefit of contrast mediums reducing the need for surgery have yet to be systematically proven<sup>[30,32,33]</sup>, their relation to reduced length of hospital stay has been demonstrated in several trials<sup>[5,28,31,32]</sup>. Certainly, however, those ASBO patients who show no contrast being able to enter the colon will require surgical treatment.

## NEW DEVELOPMENTS

The limitations of the above-mentioned diagnostic modalities are likely to cause a delay in diagnosis. In recent years, several serum markers with potential to detect ischemic small bowel have been identified<sup>[13,14]</sup>. These markers include factors that are released by damaged enterocytes, such as intestinal fatty acid binding protein (I-FABP) and  $\alpha$ -glutathione S transferase ( $\alpha$ -GST). Enterocytes are rapidly shed in the early phases of intestinal injury and can be readily detected in both urine and plasma, providing promising possibilities for their use as early detection markers<sup>[33]</sup>.

Plasma levels of the cytosolic protein  $\alpha$ -GST rise in conjunction with ischemic intestinal damage; yet, this protein provides variable results as a diagnostic tool, with reported sensitivity ranging from 20%-100% and pooled specificity of 85%<sup>[14,15,34]</sup>. Therefore,  $\alpha$ -GST may be more useful as an exclusion criterion, rather than as an indicator for surgical intervention. The other marker I-FABP, a cytosolic protein found in tissues involved in uptake and consumption of fatty acids, is released immediately by damaged small bowel, making it a very specific marker<sup>[35]</sup>. Patients presenting with SBO but without ischemia show normal levels of serum or urine I-FABP<sup>[36]</sup>. A recent clinical trial of patients with acute abdominal pain demonstrated that serum I-FABP levels were significantly higher in those patients with small bowel ischemia than in either those with non-ischemic small bowel disease or those without small bowel disease<sup>[16]</sup>. Furthermore, a majority (57.7%) of these ischemic patients had strangulated bowel. Thus, I-FABP may have a role in selecting candidates for surgical intervention. Other putative candidate markers are D-lactate and claudin<sup>[15,37,38]</sup>; however, the low specificity of D-lactate and lack of substantial evidence for a role of claudin 3 in SBO makes it difficult to clearly define their potential.

Besides these plasma markers, the prediction model developed by Komatsu *et al*<sup>[39]</sup> has identified older age, presence of ascites, and high-volume nasogastral tube drainage on day 3 as critical factors in patients who initially received conservative treatment. Unfortunately, this study did not include findings from radiographic imaging or oral water-soluble studies in the analysis. Although the prediction model is promising, it is necessary to consider the potential impact of markers specifically released by the obstructed small bowel in an earlier stage.

## CONCLUSION

Despite the remarkable technological advances in diagnosis of ASBO, the challenge of determining how to most effectively and safely manage these cases remains. Our ability to identify patients who can be treated conservatively has improved greatly, but the same has not been achieved for patients who will require emergency surgery, especially when their presenting symptoms are moderate. Serum markers have emerged as promising

candidates for early diagnosis of strangulated bowel, but further research is necessary to clarify their clinical value in the disease management.

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## High intensity focused ultrasound, liver disease and bridging therapy

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### Abstract

High-intensity focused ultrasound (HIFU) is a non-invasive modality that uses an extracorporeal source of focused ultrasound energy. This technique was introduced by Lynn *et al* and is able to induce coagulative necrosis in selected tissues without damaging adjacent structures. Although HIFU has been studied for 50 years, recent technological developments now allow its use for tumours of the liver, prostate and other sites. In liver disease, HIFU has been used to treat unresectable, advanced stages of hepatocellular carcinoma (HCC) and liver metastases. Hepatocellular carcinoma is a serious health problem worldwide and is endemic in some areas because of its association with hepatitis B and C viruses (in 20% of cases). Liver transplantation (LT) has become one of the best treatments available because it removes both the tumour and the underlying liver disease such as cirrhosis (which is present in approximately 80% of cases). The prerequisite for long-term transplant success depends on tumour load and strict selection criteria regarding the size and number of tumour nodules. The need to obtain the optimal benefit from the limited number of organs available has prompted strict selection criteria limited to only those patients with early HCC who have a better long-term outcome after LT. The so-called "bridging therapy" has the aim of controlling disease burden for patients who

are on the organ transplant waiting list. Amongst various treatment options, transarterial chemoembolisation and radiofrequency ablation are the most popular treatment choices. Recently, Cheung *et al* demonstrated that HIFU ablation is a safe and effective method for the treatment of HCC patients with advanced cirrhosis as a bridging therapy and that it reduced the dropout rate from the liver transplant waiting list. In this commentary, we discuss the current value of HIFU in the treatment of liver disease, including its value as a bridging therapy, and examine the potential advantages of other therapeutic strategies.

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**Key words:** High-intensity focused ultrasound; Hepatocellular carcinoma; Liver transplantation; Bridging therapy; Waiting list

**Core tip:** High-intensity focused ultrasound (HIFU) is a non-invasive modality used to destroy tissue. It has been used to treat unresectable advanced stages of hepatocellular carcinoma (HCC) and liver metastases. In some HCC cases, liver transplantation has become one of the best treatments because it removes the tumour and the underlying liver disease such as cirrhosis. The so-called "bridging therapy" has the aim of controlling disease burden for patients who are on the organ transplant waiting list. Here, we discuss various treatment options including transarterial chemoembolisation and radiofrequency ablation, and we examine the utility of HIFU as a safe and effective method of bridging therapy that can reduce the dropout rate of patients who are on the liver transplant waiting list.

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## COMMENTARY ON HOT TOPICS

I have read with great interest the recent article by Cheung *et al*<sup>[1]</sup> reporting their experience in the use of high-intensity focused ultrasound (HIFU) in patients with hepatocellular carcinoma (HCC) and cirrhosis who are waiting for liver transplant. The study aim was to determine whether HIFU could reduce the patient dropout rate.

HIFU is a non-invasive modality that uses an extracorporeal source of focused ultrasound energy. The technique was introduced by Lynn *et al*<sup>[2]</sup>, and it is able to induce coagulative necrosis in targeted tissues without damaging overlying and surrounding vital structures.

Although HIFU has been studied for 50 years, recent technological developments have allowed its use in treating tumours of the liver<sup>[3]</sup>, prostate<sup>[4]</sup> and other sites<sup>[5]</sup>.

HIFU is a highly precise medical procedure that applies high-intensity focused energy to “heat” and “destroy” diseased tissues. Its precision is under investigation for a possible application as “focal” therapy in case of prostate cancer, where whole gland therapy has a negative impact in terms of incontinence and impotence<sup>[6]</sup>.

HIFU is a hyperthermia therapy, which is a class of clinical therapies [including radiofrequency ablation (RFA)] that use high temperature to treat diseases.

HIFU is also a modality of therapeutic ultrasound, involving minimally invasive or non-invasive methods to direct acoustic energy into the body. In addition to HIFU, other modalities include ultrasound-assisted drug delivery, ultrasound haemostasis, ultrasound lithotripsy and ultrasound-assisted thrombolysis.

A clinical HIFU procedure is typically performed in conjunction with an imaging procedure to enable treatment planning and targeting before applying any therapeutic or ablative levels of ultrasound energy. When diagnostic sonography is used, the technique is termed ultrasound-guided focused ultrasound (USgHIFU or USgFUS). Magnetic resonance imaging is also used for guidance; thus, the technique is sometimes called magnetic resonance-guided focused ultrasound, which is often shortened to MRgHIFU or MRgFUS.

Currently, USgHIFU is approved for use in Bulgaria, China, Hong Kong, Italy, Japan, Korea, Malaysia, Mexico, Russia, Romania, Spain and the United Kingdom. MRgHIFU is an approved therapeutic procedure to treat uterine fibroids in Asia, Australia, Canada, Europe, Israel and the United States (Food and Drug Administration, FDA approved). Research on other indications is actively underway, including clinical trials evaluating the effectiveness of HIFU for the treatment of cancers of the brain, breast, liver, bone, and prostate.

From a technical point of view, the ultrasound waves of high-intensity focused ultrasound are generated by high frequency (0.5 to 10 MHz) vibration of a piezoelectric or piezo-ceramic transducer. The ultrasound beams are then focused by spherical arrangement using an acoustic lens or parabolic reflectors into a small, discrete region that corresponds to the focal point. For clinical applications, and similar to ultrasound imaging,

an ultrasound probe is usually coupled by degassed water between the source and patient surface (skin, rectal wall). Because of the comparable acoustic properties of water and tissue, the sound waves should penetrate the surface and the pre-target tissue with only slight absorption, reflection and heating. This phenomenon occurs because the power density of the converging ultrasound increases as it approaches the focal point. The focal region is a 3-dimensional zone, whose area depends on the frequency and the geometry of the source. Generally, the focal area is approximately 10 to 50 mm in length and 1 to 5 mm in diameter.

Based on target volume, the tissue can be ablated by sequentially shifting the focal zone with incremental movements of the transducer. This approach is combined with adjustments of the focal length and is coupled with an immobile organ or with the complex real-time tracking of a moving target (such as liver). The extent of tissue ablation is approximately that of the physical focal zone, although in practice cold spots (cause by blood perfusion in the tissue), beam distortion and beam misregistration are impediments to finely controlled treatments. However, by scanning the target using multiple pulses and multiple focal points, large tissue areas can be ablated.

The effect of acoustic cavitation induced by the ultrasound beam is complex, and acoustic impedance is sometimes unpredictable. However, the result is cell necrosis induced through a combination of mechanical stress and thermal injury.

The mechanical effect is induced by cavitation, a process in which bubbles develop and increase in size to the point at which resonance is achieved. The bubble formation is a consequence of the negative pressure of the ultrasound wave. As the bubbles expand and collapse, high pressures ranging from 20000 to 30000 bars develop and damage nearby cells. The popcorn effect is the typical example of cavitation.

The thermal effect is directly induced by the ultrasound beams, and due to the significant energy deposition at the focus the temperature within the tissue can rise from 65 to 85 °C. The temperature increase destroys tissue by coagulative necrosis. Higher temperatures are typically avoided to prevent boiling of liquids inside the tissue.

Because ultrasound destroys the diseased tissue non-invasively, it is also known as a non-invasive surgery. In liver disease, HIFU has been used for the treatment of unresectable, advanced stages of hepatocellular carcinoma or for the treatment of liver metastases.

Previous studies have shown that HIFU is safe and effective for patients with hepatocellular carcinoma<sup>[7]</sup> and can improve the quality of life of patients with HCC. In a study involving 145 patients with HCC, symptoms improved or pain was relieved in 84.8% of the 145 patients. Additionally, the size of the target tumour shrank by various degrees. The 2-year survival rate was 80% in patients with stage I b HCC, 51.4% in stage II a, and 46.5% in stage III a.

Ng *et al*<sup>[8]</sup> involving 49 patients receiving HIFU for unresectable HCC showed that the technique was effective in 79.5% of cases. The study found that only tumour size ( $\geq 3.0$  cm) was a significant risk factor affecting the complete ablation rate. The 1- and 3-year overall survival rates were 87.7% and 62.4%, respectively. Moreover, HIFU is safe for the treatment of disease adjacent to or surrounding a major liver vessel. The study by Zhang *et al*<sup>[9]</sup> enrolled 39 patients with HCC. All of the treated tumours had a distance between the tumour and main blood vessel (inferior vena cava, main hepatic vein branches, portal vein) of less than 1 cm, and no major blood vessel injury was observed in any subject.

HIFU has been used in combination with transarterial chemoembolisation (TACE) in prior studies. Jin *et al*<sup>[10]</sup> reported their experience of HIFU and transarterial chemoembolisation in 73 patients with unresectable HCC. That study demonstrated that 45.2% patients achieved complete tumour ablation. By multivariate analysis, ablation response ( $P = 0.001$ ) and tumour size ( $P = 0.013$ ) were major prognostic factors in predicting response to therapy. In an interesting randomised trial comparing TACE alone *vs* TACE + HIFU, Li *et al*<sup>[11]</sup> showed that the total effective rate for tumour response was 72.8% in the TACE + HIFU group. This response was significantly higher than in the TACE group alone (44.5%,  $P < 0.05$ ). The corresponding 1-, 2-, 3- and 5-year overall survival rates for the TACE-HIFU group were 72.7%, 50.0%, 31.8% and 11.4%, respectively. These rates were higher than in the TACE alone group (47.2%, 16.7%, 2.8% and 0%, respectively,  $P < 0.01$ ).

HIFU ablation is well tolerated in HCC patients with cirrhosis. According to Cheung *et al*<sup>[12]</sup>, 13% of 100 patients developed 18 complications. Morbidity was mainly caused by skin and subcutaneous tissue injuries in nine cases. Based on the Clavien classification of surgical complications, only four complications were grade 3a, while the other 14 were below this grade. By univariate analysis, only age was found to be an independent factor for poor HIFU tolerance.

HCC is a serious health problem worldwide because of its association with hepatitis B and C viruses. Liver transplantation has become one of the best HCC treatments available because it removes both the tumour and the underlying liver disease.

A prerequisite for the long-term success of a transplantation program depends on tumour load and selection criteria regarding size and number of tumour nodules. The need to obtain the optimal benefit from the limited number of available organs has prompted the use of careful selection criteria to list only those patients with early HCC who have a prediction of superior long-term outcome after LT.

Patients who fulfil the so-called Milan criteria (single tumour  $\leq 5$  cm; two or three tumours, none  $> 3$  cm; no vascular invasion) or the expanded University of California San Francisco criteria (UCSF criteria: single tumour  $\leq 4.5$  cm; two or three tumours, none  $> 4.5$  cm; or total

tumour diameter  $\leq 8$  cm; no vascular invasion) may have a 3-year survival of up to 88%. However, the expansion of these criteria for transplantation is still a topic of discussion.

Other problems arise from the differential between the number of patients on the liver transplant waiting list and the number of available donors. Additionally, there is a time lag between patient inclusion on a waiting list and the available organ. For example, in the United States, more than 2000 candidates die each year while awaiting transplantation.

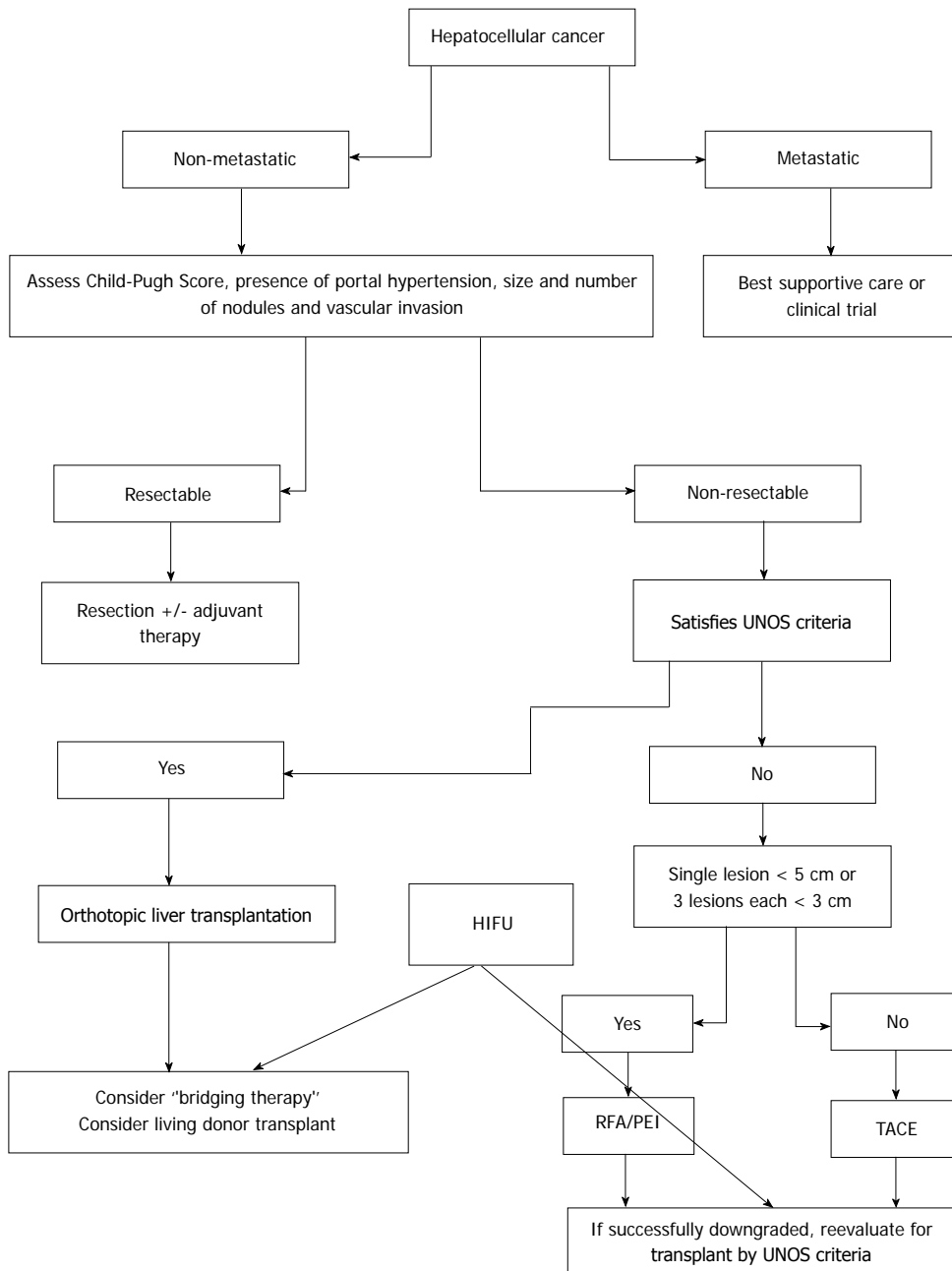
Some therapies for HCC, called “bridge therapy”, have the capacity to “fix” or suspend tumour progression and to allow HCC patients to maintain active candidacy as long as necessary to obtain a liver. Several techniques are employed as bridge therapies for HCC patients awaiting liver transplantation. Treatment options such as TACE and radio frequency ablation (RFA) are the most popular treatment choices as pre-transplant locoregional therapy.

Moreover, other goals of locoregional therapy, *e.g.*, alcohol injection, radiofrequency ablation, transarterial chemoembolisation, transarterial radioembolization, and liver resection, are intended to decrease tumour size and number in patients who initially present with tumours that do not meet locally acceptable criteria for liver transplantation.

The TACE principle is intra-arterial injection of cytotoxic drug combinations (doxorubicin and/or cisplatin and/or mitomycin into the hepatic artery), followed by lipiodol injection, gelfoam for vessel occlusion and degradable microspheres. An aggressive ablation therapy in association with a short transplant waiting time has the potential to optimise the curative intent of liver transplantation in selected cirrhotic patients. Based on the local extension of the disease and the hepatic functional reserve, TACE may be performed as a “complete”, selective or superselective procedure through a microcatheter. Contraindications for TACE include Child-Pugh C liver cirrhosis, presence of multifocal bilobar tumour spread, presence of extrahepatic metastases, portal vein thrombosis or arterio-portal fistula.

TACE has shown excellent outcomes as a bridging therapy. However, only patients with preserved liver function and asymptomatic multinodular tumours without vascular invasion or extrahepatic spread are eligible for TACE because it avoids hepatic failure and severe adverse events<sup>[13]</sup>. TACE has been used as a selective/superselective procedure and has shown excellent results that are superior to a simple lobar approach<sup>[14]</sup>. As a bridging (or down-staging) therapy, selective/superselective TACE induces a histological necrosis in 91.8% of cases and was maximal for tumours  $> 3$  cm.

RFA represents a widely applied method to treat HCC in a palliative intent, or as a “bridging” to liver transplantation. RFA may be performed under ultrasonography, Computed tomography guidance, or during laparoscopic and open surgical procedures. This procedure has more limitations than TACE, including the number of nodules



**Figure 1** Approach to the management of newly diagnosed hepatocellular cancer. Source from Parikh *et al*<sup>[19]</sup>. "Bridging therapy": Surgical resection, radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE). High-intensity focused ultrasound (HIFU) as proposal. PEI: Percutaneous ethanol injection; UNOS: United network for organ sharing.

that may be treated (up to three in most cases) or the maximal tumour diameter of the nodules (up to 5 cm). Effective treatment has been achieved<sup>[15]</sup> when 100% tumour necrosis is present. However, it is difficult to reach this goal with tumours exceeding the above-mentioned diameter or number of tumour nodules. Mazzaferro *et al*<sup>[16]</sup> showed that although the complete response rate was high (55%), tumour size (> 3 cm) and time from treatment (> 1 year) predict a high risk of tumour persistence in the targeted nodule.

As a bridging therapy, RFA showed some limitations. Schroeder *et al*<sup>[17]</sup> demonstrated that although the majority of treated patients (62%) had a solitary tumour at the be-

ginning of treatment, tumour progression was observed in a large proportion (38%) of patients. These results limit the role of RFA as a bridging treatment prior to LT.

Yttrium-90 (Y90) microsphere radio-embolisation is a recently FDA-approved, non-surgical procedure used to treat inoperable HCC. This innovative procedure delivers targeted, internal radiation therapy directly to the tumour<sup>[18]</sup>. Some promising results have been reported for this technique either as a "bridging" option before other treatment modalities (partial hepatectomy, liver transplantation) or as a main therapy for patients with diffuse intrahepatic tumour spread. Treatment with Y90 microspheres has the advantage of being able to treat all intrahepatic

HCC lesions, including otherwise undetected tumours. This treatment may also be the alternative to TACE in selected patients with contraindications for TACE.

In conclusion, with increases in waiting times for liver transplantation, it has become common practice to monitor patients to ensure that they remain within the acceptable criteria for liver transplantation. Moreover, the dropout of patients on the waiting list is common because of cancer progression or other medical reasons. Locoregional therapy as a bridging strategy for patients on the waiting list aims to decrease tumour-related dropout rates and to reduce the incidence of recurrent diseases after liver transplantation. Current available techniques show a dropout rate up to 35% for transarterial embolisation and up to 15% for radiofrequency ablation.

Cheung *et al*<sup>[12]</sup> must be congratulated for testing the utility of high-intensity focused ultrasound in this particular setting. The study examined 49 consecutive HCC patients listed for liver transplant by UCSF criteria. Twenty-nine patients received TACE as a bridging therapy, 16 patients received no treatment before liver transplantation, and five patients received HIFU as bridging therapy. The control group of five patients received HIFU but were not on the transplant list. TACE was performed using cisplatin as the chemotherapeutic agent, and it was delivered with Lipiodol, followed by gelfoam particle embolisation. Selective cannulation and embolisation of the feeding arteries of the tumours were performed whenever possible. All of the HIFU treatments were conducted by an experienced hepatobiliary surgeon and radiologist using the JC HIFU system (Chongqing Haifu Technology, Chongqing, China). The system is composed of a real-time diagnostic imaging unit, a therapeutic unit, a degassed water circulation unit, and a computer system. The real-time diagnostic imaging unit provides direct visualisation of the tumour. The therapeutic unit consists of an ultrasound energy transducer that focuses the ultrasound energy at a 12-cm focal point. The degassed water circulation unit provides a medium for ultrasound transmission outside the body. The computer system controls these three units.

Cheung demonstrated that 90% of patients receiving HIFU had complete tumour response, while 10% had partial response. There was no complete or partial tumour response in the TACE group. Fourteen (46%) patients had progressive disease, and 14 (46%) patients had stable disease. The overall dropout rate was 24.1%.

HIFU was shown to be a safe treatment, and none of the patients receiving HIFU as a bridging therapy developed complications due to intolerance after the procedure. The complication rate was 8.2%, and the complications involved mild skin oedema and injury due to energy accumulation at the ultrasound beam pathway.

HIFU ablation is an entirely extracorporeal non-invasive ablative treatment method using focused ultrasound energy. It is capable of causing coagulative necrosis of the targeted HCC *via* intact skin, without the need for surgical incision.

HIFU has been well demonstrated to be an effective ablation modality that is non-invasive. It can effectively reduce the dropout rate from the liver transplant waiting list by providing effective tumour control. The histological proof from the liver explants provides evidence that the necrosis is effective in an *in vivo* model.

Despite the low number of enrolled subjects, the preliminary study by Cheung *et al*<sup>[1]</sup> is interesting and suggests the need for more extensive clinical trials that focus on the use of HIFU as a bridging therapy (Figure 1).

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Role of innate immunity in the development of hepatocellular carcinoma

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**Key words:** Hepatocellular carcinoma; Innate immunity; Toll-like receptor; Liver cancer; Inflammation

**Core tip:** Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Growing incidence of HCC has generated immense interest to understand the mechanisms of disease at the physiological, cellular and molecular levels with the hope of developing novel therapeutics for the treatment of HCC. In the past few years, it has become clear that innate immunity plays a critical role in the development and progression of HCC. In this review, these new developments and possibilities of developing novel therapeutic options based on this newly gained knowledge are discussed.

## Abstract

Hepatocellular carcinoma (HCC) is the most common form of liver cancer worldwide. It is caused by a variety of risk factors, most common ones being infection with hepatitis viruses, alcohol, and obesity. HCC often develops in the background of underlying cirrhosis, and even though a number of interventional treatment methods are currently in use, recurrence is fairly common among patients who have had a resection. Therefore, whole liver transplantation remains the most practical treatment option for HCC. Due to the growing incidence of HCC, intense research efforts are being made to understand cellular and molecular mechanisms of the disease so that novel therapeutic strategies can be developed to combat liver cancer. In recent years, it has become clear that innate immunity plays a critical role in the development of a number of liver diseases, including HCC. In particular, the activation of Toll-like receptor signaling results in the generation of immune responses that often results in the production of pro-inflammatory cytokines and chemokines, and could cause acute inflammation in the liver. In this review, the current knowledge on the role of innate immune responses in the development and progression of HCC is examined, and emerging therapeutic strategies based on molecular mechanisms of HCC are discussed.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer, and is the third leading cause of cancer-related deaths worldwide. It accounts for approximately 70%-80% of all primary liver cancer cases<sup>[1]</sup>. A variety of risk factors such as hepatitis viruses, vinyl chloride, tobacco, foodstuffs contaminated with aflatoxin B1 toxin, heavy alcohol intake, nonalcoholic fatty liver disease (NAFLD), diabetes, obesity, oral contraceptives, and hemochromatosis cause HCC<sup>[2]</sup>. Recurrence is quite common in patients who have had a resection, and survival rate is 30%-40% at

five years post-surgery<sup>[2]</sup>. As a recent surveillance, epidemiology, and end results study using the Medicare dataset of elderly patients in the United States has shown, in addition to the human loss, there is a substantial burden of health care expense of illness associated with HCC<sup>[3]</sup>. To underscore this point, the Centers for Disease Control has recently recommended one-time health screening for the entire generation born between 1945 and 1965. The dramatic rise in the incidence of HCC in Western countries in recent years has generated intense efforts to understand the mechanisms of disease at the physiological, cellular and molecular levels with the hope of developing novel therapeutics for the treatment of HCC.

## **PATHOPHYSIOLOGY OF HCC**

The normal liver lobule is formed by hepatocytes, cholangiocytes and various non-parenchymal cells [Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs)]. Intrahepatic lymphocytes and liver-specific natural killer (NK) cells are also present in the sinusoidal lumen and perisinusoidal space of Disse<sup>[4]</sup>. Exposure to toxic substances and induction of immune responses in the liver can result in inflammation through the activation of KCs and HSCs, and can cause necrosis. In this process, liver fibrosis and cirrhosis may also occur. Even though the molecular basis for cancer-promoting effect of cirrhosis is unknown, the process of recurrent liver cell necrosis and regeneration with increased cell turn-over renders liver cells more sensitive to the adverse effects of other mutagenic agents<sup>[4]</sup>. Cirrhosis is responsible for significant morbidity and mortality, and is one of the most important risk factors for the development of HCC.

Carcinogenesis is a process that involves the transition of a normal cell into a preneoplastic lesion that develops into malignant tumor<sup>[4]</sup>. Growing evidence suggests that gradual accumulation of mutations and genetic changes in preneoplastic hepatocytes causes malignant transformation that leads to the development of HCC<sup>[5,6]</sup>. Tissue environment also plays a critical role in tumor formation<sup>[7]</sup>. Interaction of different cell types in the tumor stroma with components of the extracellular matrix (ECM), either directly or indirectly result in the acquisition of an abnormal phenotype that causes this transformation. Tumor stroma consists of fibroblasts [also referred to as “cancer-associated fibroblasts (CAFs)”], macrophages (liver resident KCs and other tumor-infiltrating cells), leukocytes, HSCs, endothelial cells, pericytes, neutrophils, and dendritic cells (DCs)<sup>[8]</sup>. Each of these cells produces growth factors, cytokines, chemokines, free radicals, and other tumorigenic substrates that contribute to tumor initiation and progression<sup>[9]</sup>.

In HCC, CAFs are involved in tumor initiation and progression. They produce epidermal growth factor (EGF), hepatocyte growth factor, fibroblast growth factor, interleukin 6 (IL-6), chemokine (C-X-C motif) ligand 12 (CXCL12), and matrix metalloproteases (MMP-3 and

MMP-9)<sup>[10]</sup>. They also produce IL-8, cyclooxygenase 2, and secrete protein acidic rich in cysteine to recruit and stimulate macrophage production, which can further increase the activation of CAFs through the secretion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and platelet-derived growth factor (PDGF)<sup>[11,12]</sup>. Tumor-associated macrophages (TAMs) are “polarized” into M2 mononuclear phagocyte-like cells by various cytokines [IL-4, IL-10, and transforming growth factor  $\beta$  (TGF- $\beta$ )] present in the tumor microenvironment<sup>[13]</sup>. These M2-like TAMs, in turn, express cytokines (IL-10 and TGF- $\beta$ ), chemokines (CCL17, CCL22 and CCL24), vascular endothelial growth factor (VEGF), and EGF to recruit regulatory T cells (Tregs) and to promote angiogenesis<sup>[14,15]</sup>. KCs are able to impair cluster of differentiation 8<sup>+</sup> (CD8<sup>+</sup>) cytotoxic T lymphocyte (CTL)-mediated immune responses through programmed death ligand 1<sup>[16]</sup>. Moreover, when stimulated with pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$  and PDGF), KCs and HSCs produce osteopontin that plays a pivotal role in various cell signaling pathways, which promote inflammation, tumor progression and metastasis<sup>[17,18]</sup>. DCs process antigens and present them to infiltrating CTLs by expressing them on their cell surface. These antigen presenting cells (APCs) possess high endocytic activity, and therefore, are critical for the induction of immune surveillance in tumors, and for immune evasion<sup>[19]</sup>. Such tumor-antigen specific CD8<sup>+</sup> T cell responses were recently shown to suppress the recurrence of HCC<sup>[20]</sup>.

In response to liver injury, HSCs transdifferentiate into myofibroblast-like cells and produce cytokines, chemokines, growth factors, and ECM<sup>[17]</sup>. This phenotypic transformation of HSCs is a key event in the development of hepatic fibrosis<sup>[21]</sup>. Hepatitis B virus (HBV)-encoded X protein, hepatitis C virus (HCV)-encoded non-structural proteins, MMP-9, PDGF, TGF- $\beta$ , Janus kinase (JNK), insulin-like growth factor binding protein 5, and cathepsins (B and D) are potent inducers of HSC activation and proliferation that enhance liver fibrosis and carcinogenesis<sup>[17]</sup>. Endothelial cells express a variety of angiogenic receptors including VEGFR, EGFR, EGF homology domains-2 (Tie-2), PDGFR, and C-X-C chemokine receptors. Tumor-associated endothelial cells express high levels of TGF- $\beta$  in HCC, which act as a chemoattractant for cluster of differentiation 105 (CD105, also known as endoglin), a type I cellular glycoprotein that is a part of the TGF- $\beta$  receptor complex, to promote tumor angiogenesis<sup>[22]</sup>. It has been shown that CD105<sup>+</sup> endothelial cells express increased angiogenesis activity with greater resistance to chemo-therapeutic agents and inhibitors of angiogenesis<sup>[23]</sup>.

Infiltration of T cells into the tumor microenvironment is an important regulator of cancer progression. In HCC tissues, CD4<sup>+</sup>/CD25<sup>+</sup> Tregs impair proliferation and activation of CTLs, degranulation, and production of granzymes (A and B), and perforin<sup>[24]</sup>. In a recent study, the infiltration of these Tregs into the tumors of HCC patients was found to correlate with an increase in

tumor size<sup>[25]</sup>. Other studies have shown that low CD8<sup>+</sup> T cell counts and high Treg numbers correlate with poor prognosis in HCC patients, especially after resection<sup>[26-28]</sup>. More recently, a 14-immune gene signature that drives the infiltration of lymphocytes into tumor has been identified<sup>[29]</sup>. This signature, which includes pro-inflammatory cytokines [TNF- $\alpha$  and interferon (IFN)- $\gamma$ ] and chemokines (CXCL10, CCL5 and CCL2), is a good predictor of patient survival at early tumor stages. In addition, dysfunctional regulation of immune response by excessive neutrophil activity was also reported as a poor prognostic indicator after resection of HCC<sup>[30]</sup>.

## INNATE IMMUNITY

To successfully detect and eliminate invading pathogens by discriminating self from non-self, the mammalian immune system has developed mechanisms that can be divided into two distinct components: the innate immunity and the adaptive immunity. In most multicellular organisms, the highly conserved innate immune system provides the first line of defense to limit infection by detecting pathogens using germline-encoded proteins<sup>[31]</sup>. The adaptive immunity, present only in vertebrates, detects non-self through the recognition of peptide antigens by receptors expressed on the surface of B and T cells<sup>[32]</sup>. The adaptive responses are much more diverse than the innate responses as each B and T lymphocyte clone expresses a distinct antigen receptor that arose by somatic gene rearrangement through a process of evolution<sup>[33]</sup>. Most often innate immune responses emanate from the host cell surface receptor with the recognition of conserved structural motifs termed pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms. Toll-like receptors (TLRs), Nucleotide-binding and oligomerization domain-like receptors (NLRs) and RIG-I-like receptors (RLRs) are the key receptors that recognize a variety of PAMPs<sup>[34]</sup>. While TLRs can recognize bacteria, viruses, fungi and protozoa, NLRs and RLRs detect bacteria and viruses, respectively. All these pattern recognition receptors (PRRs) generate innate immune responses, either by acting alone or in combination with other receptors. The focus of the review is on the role of TLRs as most of the current knowledge on the role of innate immunity in liver diseases has been obtained from studies on the TLRs.

## TLR SIGNALING IN MAMMALIAN CELLS

*Toll* was first identified as a gene important in the establishment of dorsal-ventral orientation during embryonic development in the fruit fly *Drosophila melanogaster*<sup>[35]</sup>. Later, it was found that the Toll protein plays a critical role in the fly's immunity to fungal infections<sup>[35,36]</sup>. The first mammalian homolog of Toll, Toll-like receptor 4 (TLR4), was identified as a PRR required for adaptive immunity<sup>[37]</sup>. Subsequently, TLR4 and other TLRs were shown to play critical roles in generating innate immune

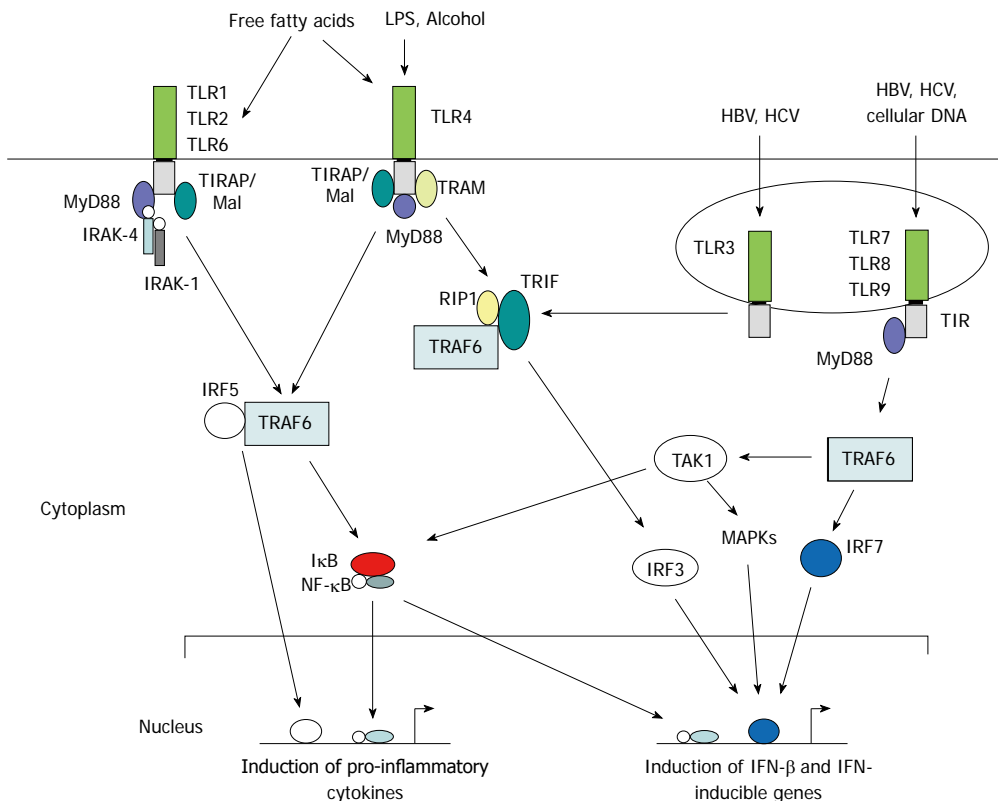
responses against microbial pathogens in mammalian systems. To date, 11 human TLRs and 13 mouse TLRs have been identified, in addition to a number of TLRs in other vertebrates, that recognize a variety of PAMPs and trigger both innate and adaptive immune responses<sup>[32,38]</sup>. TLRs are membrane-bound proteins that contain varying numbers of extracellular leucine-rich repeats and a Toll-IL receptor (TIR) domain in the cytoplasmic region that are highly conserved (Figure 1). They recognize ligands through LRRs and transmit signals through their TIR domain *via* protein-protein interactions with cellular adaptor proteins triggering a cascade of signaling events such as phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK-1) and activation of Nuclear factor kappa B (NF- $\kappa$ B) or interferon regulatory factor 3 (IRF3), resulting in the production of immune mediators and IFN-inducible genes<sup>[39]</sup>. Thus, a direct or indirect association of a ligand with its cognate TLR serves as a signal to trigger an innate immune response. Each step along the TLR signaling pathways is tightly regulated by a complex mix of phosphorylation and targeted degradation, and sequestering of various signaling molecules is dependent upon the nature of the invading pathogen<sup>[40]</sup>.

In general, vertebrate TLRs were classified into six distinct families based upon amino acid sequence homologies of LRRs<sup>[41]</sup>. Most mammalian cells express low levels of TLRs constitutively in a cell-type specific manner, and interestingly, they can be present in both membrane-bound and soluble forms. For example, the rainbow trout TLR5 is expressed constitutively as a membrane protein but upon induction with the bacterial flagellin, a soluble TLR5 is rapidly induced<sup>[42]</sup>. Normally, TLRs function as homodimers. However, some TLRs form heterodimers with other TLRs to recognize PAMPs. For instance, TLR2 associates either with TLR1 or TLR6 as a heterodimer to recognize triacylated lipoproteins and diacylated lipoproteins, respectively<sup>[32,38,40]</sup>. In addition, cellular membrane protein CD14 enhances the ligand recognition ability of TLR2<sup>[43]</sup>.

## TLR EXPRESSION IN THE LIVER

In the healthy liver, TLR expression is detectable only at very low levels<sup>[44]</sup>. Eight TLRs are expressed in the mammalian liver with varying levels of expression on hepatocytes, KCs, HSCs and LSECs<sup>[45]</sup>. These TLRs not only recognize microbial PAMPs but also the damage-associated molecular patterns (DAMPs) of dying host cells<sup>[46]</sup>. Even though hepatocytes express all TLRs, they are capable of responding to TLR2 and TLR4 ligands only, and these responses are very weak *in vivo*<sup>[47]</sup>. Under inflammatory conditions, however, hepatocyte response to TLR2 ligands was significantly enhanced but the response to TLR4 ligands was still not detectable in these cells<sup>[48]</sup>. LSECs express mRNAs of TLRs 1-9, and respond to various TLR ligands by expressing TNF- $\alpha$ , IL-6, and IFN- $\beta$ <sup>[49,50]</sup>. KCs express all TLRs and respond to a variety of ligands by producing TNF- $\alpha$





**Figure 1** Toll-like receptor signaling in liver cells. Activation of a given TLR pathway is dependent on the nature of the stimulus. LPS: Lipopolysaccharide; TLR: Toll-like receptor; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MyD88: Myeloid differentiation factor 88; IRAK: Interleukin-1 receptor-associated kinase; TIRAP: Toll-interleukin-1 receptor domain containing adaptor protein; TIR: Toll-interleukin-1 receptor; TRAM: TIR-domain-containing adaptor molecule; IRF: Interferon regulatory factor; TRAF: Tumor necrosis factor receptor-associated factor; TAM: Tumor-associated macrophage; TAK: Transforming growth factor  $\beta$ -activated protein kinase; NF- $\kappa$ B: Nuclear factor kappa B; MAPK: Mitogen-activated protein kinase; TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$ ; RIP: Receptor-interacting protein; I $\kappa$ B: Inhibitor of NF- $\kappa$ B; IFN: Interferon.

and IL-6<sup>[51,52]</sup>. When stimulated with ligands for TLRs 1, 2, 4 and 6, they produce IFN- $\gamma$  and promote the proliferation of T cells<sup>[49]</sup>. In response to TLR3 and TLR4 ligands, they produce IFN- $\beta$ , and for ligands against TLR1 and TLR8, they display a high level of major histocompatibility II expression. HSCs express low levels of TLR4 and TLR9, but activation of TLR4 has been shown to induce the expression of TLR2 as well<sup>[49]</sup>. In human HSCs, TLR4 activation results in the production of CCL2, CCL3 and CCL4<sup>[53]</sup>, and their expression of TGF- $\beta$  was implicated in the promotion of hepatic fibrosis<sup>[54]</sup>. Activation of TLR9 by DAMPs induces the differentiation of HSCs and increases the production of collagen<sup>[55]</sup>.

Among the non-parenchymal cells, hepatic DCs can be classified into different subsets as plasmacytoid DCs, myeloid DCs, lymphoid DCs, natural killer DCs, and a mixture of lymphoid and myeloid DCs<sup>[56]</sup>. Differential expression of TLRs in these hepatic DCs varies according to the subset type and the species type. For example, in humans, pDCs express TLRs 1, 3 and 7 only, whereas other DC subsets express all TLRs except TLR9<sup>[57]</sup>. On the other hand, murine pDCs express TLRs 2, 4, 7 and 9 but not TLR3<sup>[58]</sup>.

Hepatic lymphocyte population, which accounts for about 25% of non-parenchymal cells consists of B

cells, NK cells, NKT cells,  $\alpha\beta$ T cells and  $\gamma\delta$ T cells<sup>[59]</sup>. In general, T cells are activated indirectly by TLRs through APCs<sup>[60]</sup>, and NK cells that express TLRs 1 to 9, but not TLR5. They respond to various TLR ligands by producing IL-12<sup>[61]</sup>. Interestingly, expression of TLRs in B cells has no effect on antibody production, and on B cell memory responses<sup>[62]</sup>. However, TLR3 and TLR9 activation in CD4<sup>+</sup> T cells enhances their proliferation<sup>[63]</sup>, and TLR2 serves as a co-stimulatory receptor for antigen-specific T cell development and participates in T cell memory<sup>[64]</sup>.

## INVOLVEMENT OF TLRs IN LIVER DISEASES

Significant amount of evidence in recent years has demonstrated the involvement of TLRs in the pathogenesis of various liver diseases. Most of this evidence comes from the overexpression of TLRs, activation of TLRs causing enhanced disease in animal models, single nucleotide polymorphisms (SNPs) in TLR-coding genes and their adaptors linked to disease susceptibility, and TLR knockout mice being protected from disease<sup>[40]</sup>. Three most common risk factors for the development of HCC for which TLR involvement has demonstrated to play a critical role

in the disease pathogenesis are discussed in this section.

### **Viral hepatitis**

Chronic hepatitis virus infection that affects almost half a billion people worldwide is a major risk factor for HCC. The infectivity of a given virus type varies according to the geographical location<sup>[2]</sup>. Co-infection with one or more viruses may also occur contributing to a higher risk of HCC, albeit it is rare<sup>[35]</sup>. HCV is the most common blood-borne infection in the United States, with nearly 20% of chronically infected individuals developing cirrhosis and HCC<sup>[65]</sup>. It is generally acknowledged that the humoral antibody response contributes to the clearance of circulating HBV particles and the prevention of viral spread within the host while the cellular immune response eliminates infected cells<sup>[66]</sup>. The T cell response to the HBV is vigorous, polyclonal and multispecific in acutely infected patients who successfully clear the virus, and relatively weak and narrowly focused in chronically infected patients, suggesting that clearance of HBV is T cell dependent<sup>[66]</sup>. Persistent HBV infection is characterized by chronic liver cell injury, regeneration, inflammation, widespread DNA damage and insertional deregulation of cellular growth control genes, which, collectively, lead to cirrhosis of the liver and HCC<sup>[66]</sup>. Other factors that could contribute to viral persistence are immunological tolerance, mutational epitope inactivation, T-cell receptor antagonism, incomplete down-regulation of viral replication, and infection of immunologically privileged tissues<sup>[66]</sup>. However, these pathways become apparent only in the setting of an ineffective immune response, which therefore, is the fundamental underlying cause<sup>[66]</sup>. In infected cells, the HBV capsid induces cytokine production *via* TLR2 activation. It was hypothesized that TLR2 activation is involved in viral clearance based on the observation that the administration of adefovir and entricitabine in HBV patients resulted in the up-regulation of TLR2 and reduction in the viral load<sup>[46]</sup>. In HepG2 cells, HBV triggers the production of cholesterol-metabolism genes *via* the TLR2 pathway<sup>[67]</sup>, and inflammatory stress exacerbated hepatic cholesterol accumulation these cells and in mice by disrupting the PPAR-LXR-CYP7A1/ABCA1-mediated bile acid synthesis and cholesterol efflux<sup>[68]</sup>. Interestingly in HBV transgenic mice, activation of TLRs 3-5, 7, and 9, but not TLR2, inhibited HBV replication *via* IFN- $\alpha/\beta$  induction<sup>[69]</sup>.

When immune responses were compared in macrophages of patients who spontaneously cleared HCV with those who were chronically infected, it was found that the TLR3 expression was significantly up-regulated in the former group<sup>[65]</sup>. Individuals who cleared the virus had an elevated expression of IFN- $\beta$  and higher rate of STAT-1 phosphorylation. A significant association of intronic TLR3 SNP (rs13126816) in the clearance of HCV and the expression of TLR3 was found in this study, suggesting that an elevated innate immune response enhances HCV clearance and may offer a potential thera-

peutic option to increase viral clearance<sup>[65]</sup>. TLR2, in combination with TLR1 and TLR8, recognizes core and nonstructural 3 proteins of HCV in immune cells such as macrophages and monocytes, and triggers the production of pro-inflammatory immune mediators TNF- $\alpha$ , IL-6, IL-8, IL-10 and IL-1 $\beta$ <sup>[70]</sup>. On the other hand, HCV NS5 protein is recognized by TLR4 in both hepatocytes and B cells<sup>[71]</sup>. Prolonged activation of KCs in the HCV-infected liver by HCV-encoded proteins causes persistent inflammation resulting in severe liver damage and cancer<sup>[72]</sup>. It was reported recently that the expression of TLR2 and TLR4 was highly elevated in peripheral blood monocytes of HCV patients, and that the number of Tregs was significantly higher in these chronically infected individuals<sup>[73]</sup>. Similar correlation of TLR2 and TLR4 expression, and Treg numbers was also reported in HBV patients<sup>[74]</sup>. In a separate study, a strong correlation was also observed between TLR2/4 expression and TNF- $\alpha$  production causing hepatic inflammation in HCV patients<sup>[75]</sup>. Chronic infection with HBV and/or HCV, and imbalanced immunity contributes to severe inflammation, while TLR activation during this process might be a critical factor of this infection-induced prolonged inflammation. Activation of innate immune responses by viral proteins, and the interaction of HCV with cellular proteins to evade host's immune responses as well as the role of SNPs in TLRs, their adaptors and cytokine genes in altering these immune responses have been extensively reviewed in the literature<sup>[76-78]</sup>.

### **Alcoholic liver disease**

Excessive alcohol consumption that causes alcoholic liver disease (ALD) is a major risk factor for HCC worldwide<sup>[79]</sup>. ALD is an umbrella term used to describe a broad spectrum of liver abnormalities caused by alcohol that include simple steatosis, alcoholic hepatitis, fibrosis and cirrhosis, which can progress to HCC. The pathogenesis of ALD is characterized by processes such as ethanol metabolism-associated oxidative damage, glutathione depletion, abnormal methionine metabolism, ethanol-mediated induction of leakage of gut endotoxins, and inflammation<sup>[79]</sup>. Liver inflammation is known to occur with exposure to a variety of agents, including metabolites of alcohol<sup>[80]</sup>. If hepatic metabolism is impaired to any degree and fails to convert drugs and chemicals to non-reactive or non-immunogenic substances, the metabolic intermediates formed in hepatic tissues may cause liver damage<sup>[81]</sup>. In such cases, KCs and other cell types release cytokines and chemokines that result in the inflammation of the liver. This reaction coupled with deregulated hepatocyte proliferation can contribute to the pathogenesis of HCC<sup>[81]</sup>.

Simple steatosis is a benign condition that progresses to alcoholic steatohepatitis (ASH) in about 10%-20% of cases and is associated with inflammation and liver injury caused by the innate immune responses<sup>[82]</sup>. Both MyD88-dependent and MyD88-independent pathways are activated in ASH, and studies with animal models have demonstrated the up-regulation of inflammatory cytokines

in the serum, and activation of IFN and IFN-responsive genes in ASH<sup>[83,84]</sup>. Blocking of IL-1 receptor, which acts through MyD88, in advanced ASH was shown to confer protective effect in mice suggesting that IL-1 inhibitors may be used in the treatment of ALD<sup>[85]</sup>.

Excessive alcohol consumption increases gut permeability and the translocation of bacteria-derived lipopolysaccharide (LPS, also known as endotoxin) from the gut to the liver<sup>[86]</sup>. In KCs, LPS interacts with TLR4 causing oxidative stress, and the production of pro-inflammatory cytokines and reactive oxygen species (ROS) that induce hepatocellular damage<sup>[83,87]</sup>. However, this effect was not abrogated in MyD88-deficient mice suggesting that MyD88-independent pathways are involved in NF- $\kappa$ B activation by alcohol<sup>[83]</sup>. Even though IRF-3 activation was not affected by chronic alcohol treatment, IRF-7 and IRF-3-inducible genes expression was significantly induced in KCs of alcohol-fed wild type mice<sup>[83]</sup>. The activation of TLR4 has also been demonstrated to occur during the alcohol and HCV synergism<sup>[88]</sup>. Furthermore, alcohol activates complements C3 and C5, following the production of TNF- $\alpha$ , and induces hepatocyte injury<sup>[89,90]</sup>. In addition to Gram-negative bacteria that produce LPS, gut is home to a large number of other microorganisms belonging to eukaryotes, prokaryotes, archaea, and viruses<sup>[91]</sup>. It is therefore likely that Gram-positive bacteria which produce lipoproteins and activate TLR2 signaling, and viruses that activate TLRs 3, 7, 8 and 9 may also leak from the gut to the liver. However, little is known about the activation of these TLRs during alcoholic liver injury.

### Nonalcoholic steatohepatitis

NAFLD is the most common chronic liver disease that affects both adults and children worldwide<sup>[92]</sup>. Like ALD, NAFLD includes a broad spectrum of liver abnormalities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and HCC. Of these, NASH is characterized by hepatocyte injury, inflammation, and fibrosis<sup>[92-94]</sup>. Most of these disease conditions are diagnosed at a late stage, and some patients also present for the first time with cirrhosis<sup>[92]</sup>. The development of cirrhosis is significantly higher in individuals with NASH when compared to patients with simple steatosis<sup>[92-94]</sup>. Consequently, liver-related mortality is also significantly higher in NASH patients.

NASH shares immunological characteristics with ASH such as the activation of innate immune responses, crosstalk between steatosis and inflammation, activation of TLR4 by LPS and fatty acids, complement activation, production of proinflammatory immune mediators, and alteration in NK and NKT cell number and activity<sup>[95,96]</sup>. In addition, activation of KCs in response to gut microbiota, activation of TLR signaling *via* both MyD88-dependent and -independent pathways, up-regulation of type I IFN and IFN-responsive genes occur in ASH and NASH<sup>[97-100]</sup>. However, recent studies have shown that NASH differs from ASH in certain aspects: insulin resistance, crosstalk between adipose tissue and the liver,

dependence on MyD88 signaling, differential effects in mice due to MyD88 and IL-1 deficiency, and activation of inflammasome<sup>[96]</sup>.

Progression of NASH is associated with the recruitment of T cells and T1 response leading to inflammation<sup>[101]</sup>, and hepatic expression levels of inflammatory mediators are modified in morbidly obese patients even without pathohistological manifestations<sup>[102]</sup>. Accumulating evidence strongly suggests that inflammation causes the progression of NAFLD to NASH, and that innate immunity is involved in the inflammatory response. While TLR2 and TLR9 pathways are critical for the development of NAFLD<sup>[103]</sup>, deletion of either TLR4 or its co-receptor MD-2 dampened (but not abolished) necro-inflammatory activity of steatosis and fibrosis in a mouse model of NASH<sup>[104]</sup>. Furthermore, in NASH, gut microorganisms enter the liver because of the leakage in the intestinal mucosal barrier resulting in inflammation, due to the activation of TLR signaling by intestinal bacteria and their products such as LPS<sup>[45,93]</sup>. It is apparent from these reports that the activation of TLR signaling is an important factor in causing inflammation in NASH, and could be a crucial factor in the progression of NASH to cirrhosis. It was reported that the activation of TLR4 signaling and up-regulation of CD14 resulted in higher responsiveness to LPS and saturated fatty acids in KCs, and resulted in hepatic inflammation and liver complications in NASH patients<sup>[98,105]</sup>.

### Hepatocarcinogenesis

Chronic liver damage caused by excessive inflammation due the exposure to various risk factors often results in the development of fibrosis-associated HCC<sup>[106]</sup>. Since the stimulation of TLR signaling pathways result in the production of proinflammatory immune mediators, it is likely that TLRs are involved in development and progression of hepatocarcinogenesis as well. The activation of TLR signaling in liver diseases leads to the activation of NF- $\kappa$ B and JNK pathways that are critical mediators of tumor-associated cytokine production. NF- $\kappa$ B activation induces the proliferation of tumor cells through the production of TNF- $\alpha$ <sup>[107]</sup>. Tumorigenic effect of JNK in HCC is mediated through the regulation of molecules involved in cell proliferation such as MMPs and cyclins<sup>[108]</sup>. Studies in animal models of HCC have shown that ROS-mediated JNK activation is critical for tumor development<sup>[109]</sup>, and that elevated ROS levels induce hepatocyte cell death<sup>[110]</sup>. Moreover, hepatocyte-specific knockdown of the NF- $\kappa$ B inhibitor I $\kappa$ B kinase subunit NF- $\kappa$ B essential modulator causes spontaneous development of HCC in mice<sup>[111]</sup>. However, interestingly, hepatocyte-specific TGF- $\beta$ -activated protein kinase 1-deficient mice had spontaneous hepatocyte cell death, compensatory proliferation, inflammatory cell infiltration, and fibrosis in the liver<sup>[102,112]</sup>. Collectively, these results demonstrate the involvement of NF- $\kappa$ B-mediated downstream molecules in cellular homeostasis and cancer development in the liver.



## TLR POLYMORPHISMS AND THE RISK OF DEVELOPING HCC

Dysregulation of TLR expression may shift the balance between the production of pro- and anti-inflammatory cytokines, and will have a profound effect on the risk of infection, chronic inflammation and cancer. Recently, Nishalke *et al*<sup>[113]</sup> showed that the frequency of TLR2 -196 to -714del allele was significantly higher in HCV-associated HCC patients than in HCV-infected individuals demonstrating that this deletion plays a role in HCC development. In addition, a number of SNPs have also been identified in every TLR gene. Some of these have been shown to enhance the susceptibility of various cancers in humans<sup>[114]</sup>. However, only a few reports have been published on the role of TLR SNPs in the pathogenesis of HCC. In one such study, Zhang *et al*<sup>[115]</sup>, conducted a genome-wide genotyping of 440794 SNPs in chronic HBV carriers: 355 with HCC and 360 without HCC. They found that one intronic SNP rs17401966 present in the *KIF1B* gene on chromosome 1p36.22 was highly associated with HBV-associated HCC<sup>[115]</sup>. In a recent study, Junjie *et al*<sup>[116]</sup> investigated the association between SNPs of *TLR2* and *TLR9* genes, and the susceptibility of HCC in a cohort of 211 patients that included 172 HBV carriers. They found that two TLR2 SNPs rs3804099 C/T and rs3804100 C/T present in the same exon of *TLR2* were associated with HCC, whereas TLR9 SNPs have no role in tumor development<sup>[116]</sup>. Additional studies are needed to fully understand the involvement of various TLR SNPs in hepatocarcinogenesis.

## MODULATION OF TLR SIGNALING TO ALLEVIATE LIVER INFLAMMATION AND CANCER

Overwhelming evidence on the involvement of TLR signaling pathways in many human diseases has led to the efforts to develop TLRs as vaccine adjuvants<sup>[117,118]</sup> and as therapeutic targets<sup>[119-121]</sup>. As discussed earlier, chronic inflammation, which plays a critical role in the progression of liver diseases and in the development of human cancers, is mediated by TLR activation. Hence, modulation of TLR pathways using various drugs, antibodies, microRNAs (miRNAs), and small molecules that function as TLR agonists and antagonists to reduce liver inflammation and to prevent the progression of liver diseases towards cancer is a promising strategy to combat HCC.

### TLR agonists and antagonists

Initial efforts on the modulation TLR signaling with small TLR mimetics that act as agonists have produced mixed results. Using the TLR2 agonist Pam2Cys as a stimulant *in vitro*, it was shown that hepatoma cell lines HuH7 and HepG2 respond to HBV infection by producing IL-8 and by inhibiting viral replication<sup>[122]</sup>. However, in *in vivo* studies with HBV transgenic mice, it was

found that ligands specific for TLRs 3, 4, 5, 7 and 9, but not TLR2, inhibited HBV replication in the liver<sup>[69]</sup>. On the other hand, intravenous administration of the TLR7 agonist isatoribine significantly reduced viral RNA in HCV patients independent of viral genotype<sup>[123]</sup>. The decrease in viral load in this study coincided with markers indicating a heightened antiviral immune state. In similar experiments, Weeratna *et al*<sup>[118]</sup> and Ma *et al*<sup>[124]</sup> tested TLR7 agonist R-848 and CpG oligodeoxynucleotides containing CpG motifs (CpG ODN) that act as the TLR9 agonists as adjuvants for HBV vaccination in mice using the HBV surface antigen (HBsAg) as a model antigen. This study was based on the notion that the immunostimulatory sequences (ISS) containing CpG motifs would elicit strong Th1 immune responses<sup>[125]</sup>. In their studies, both these groups found that the TLR agonists trigger immune responses that are beneficial to the host<sup>[118,124]</sup>. Along similar lines, Dynavax Corporation has developed a commercial vaccine (Heplisav) against HBV by combining a synthetic ISS sequence called ISS-1018 that acts as TLR9 agonist with the recombinant HBsAg. This vaccine induced seroprotective responses when administered in HBV patients<sup>[126]</sup>. Another CpG ODN containing TLR9, CPG7909, was also successfully tested as an adjuvant to HBV vaccine and is currently undergoing a phase 2 clinical trial<sup>[125,127]</sup>.

In contrast to these TLR agonists, only a few studies have reported successful inhibition of TLR signaling using antagonists. For example, lipid A mimetics that bind directly to TLR4-MD2 were shown to inhibit LPS-mediated activation of TLR4 signaling both *in vitro* and *in vivo*<sup>[128]</sup>. TAK-242, another mimetic that targets the intracellular domain of TLR4, inhibited TLR4-mediated production of nitric oxide and TNF- $\alpha$  in murine and human cells by targeting TLR4-CD4 chimeric receptors<sup>[129]</sup>. Interestingly, however, TAK-242 had no effect on NF- $\kappa$ B activation mediated by MyD88, TRAP, TRIF or TRAM. Recently, Cowden *et al*<sup>[130]</sup> tested two inhibitors of histamine H4 receptor that interacts with TLR4 and found that they reduced TNF- $\alpha$  production and LPS-induced inflammation in mouse livers.

In addition to the strategy of inhibiting TLRs, efforts are also being made to selectively inhibit their adaptors and downstream signaling molecules<sup>[131]</sup>. In such studies, Compound 4a inhibited the interaction of MyD88 protein with the TIR domain of TLRs and type I IL-1 receptor<sup>[132]</sup>, whereas MyD88 inhibitors ST2825 and RO0884 blocked the recruitment of IRAK-1 and IRAK-4 in human cells<sup>[133,134]</sup>. Additionally, ST2825 also suppressed B cell proliferation and differentiation<sup>[133]</sup>. However, the efficacy of these molecules in reducing inflammation in the liver is yet to be determined.

### miRNAs

MiRNAs are a class of small, noncoding RNAs that act as key regulators of many cellular processes. During the past decade, it has been well established that the aberrant expression of a large number of miRNAs correlate with



disease severity and poor prognosis of HCC<sup>[135-144]</sup>. The expression of these “miRNA signatures” are unique for a given disease stage, and were useful in identifying patients with HCC who are likely to develop metastases and recurrence. In recent years, efforts to identify miRNAs involved in the regulation of innate immune genes has resulted in the identification of a few promising miRNA candidates that can be exploited for therapeutic purposes. For example, overexpression of miR-155 which is directly involved in the regulation of more than 30 innate immune genes<sup>[145]</sup> results in the significant reduction of MyD88 expression and IL-8 production induced by *Helicobacter pylori*<sup>[146]</sup>. Interestingly, in bone marrow-derived macrophages and RAW264.7 cells, IL-10 inhibits miR-155 expression after LPS stimulation and dampens inflammatory immune responses in a STAT-3 dependent manner<sup>[147]</sup>.

When THP-1 monocytes were treated with ligands for TLRs 2, 4 and 5, production of TNF- $\alpha$  correlated inversely with the up-regulation of miR-146a<sup>[148]</sup>, and when the targets of miR-146a IRAK-1 or TRAF6 were knocked down, inflammatory responses to TLR2, TLR4 and TLR5 ligands were significantly reduced in these cells suggesting a role for miR-146a in LPS-induced cross-tolerance. Similarly, miR-132 and miR-212 were shown to be involved in TLR2-mediated cross-tolerance through IRAK4 modulation<sup>[149]</sup>. In addition, miR-146a disrupts the translation of TLR4-induced TNF- $\alpha$  production in these cells<sup>[150]</sup>. miR-146b exerts this effect in monocytes by negatively regulating TLR4 *via* a IL-10-mediated STAT3 dependent loop<sup>[151]</sup>. Another study on the expression profiling of endotoxin responsive genes in human monocytes has revealed that miR-146 regulates TLR and cytokine signaling in a NF- $\kappa$ B-dependent manner through a negative feedback regulation loop involving the down-regulation of IRAK-1 and TRAF6 protein levels<sup>[152]</sup>. In THP-1 cells, miR-146a also regulates TLR2 signaling by reducing IRAK-1 and phosphorylated I $\kappa$ B $\alpha$  expression<sup>[153]</sup>.

In another study, miR-181a expression was found to be significantly elevated in mice stimulated with LPS and streptozotocin, which correlated strongly with the expression of inflammatory factors TNF- $\alpha$ , IL-6, IL1 $\beta$  in macrophages suggesting that the inhibition of miR-181a could reduce TLR4-induced inflammation<sup>[154]</sup>. After stimulation with LPS, miR-92a significantly inhibited the activation of JNK/c-Jun pathway and the production of inflammatory cytokines in macrophages by directly targeting mitogen-activated protein kinase kinase 4<sup>[155]</sup>, demonstrating that miR-92a is a negative regulator of TLR-triggered immune responses.

In other studies, Benakanakere *et al*<sup>[156]</sup> demonstrated that miR-105 binds directly to TLR2 and regulates its expression. When keratinocytes were challenged by a TLR2 agonist or when miR-105 was overexpressed, a strong inverse correlation between miR-105 expression and TLR2 protein levels was observed. Similar to miR-105, let-7i was recently shown to bind directly to

TLR4 in cholangiocytes and to control its expression by translational repression<sup>[157]</sup>. In this study, let-7i mimics inhibited *Cryptosporidium parvum* induced TLR4 production. In dendritic cells of mice with colitis, the expression of miR-10a was found to be negatively regulated by the intestinal microbiota, which correlated inversely with the production of IL-12/IL-23p40<sup>[158]</sup>. This finding suggests that miR-10a targets IL-12/IL-23p40 to maintain immune homeostasis.

When stimulated with ligands for TLRs 2-4 miR-147 is induced in murine macrophages<sup>[159]</sup>. Its expression was found to be greater after the activation of TLR4 than that of TLR2 or TLR4, and was dependent on both MyD88 and TRIF. *In vivo*, TLR stimulation induced the expression of miR-147 as a negative-feedback loop mechanism to prevent excessive inflammatory responses<sup>[159]</sup>. Likewise, miR-145 targets the adaptor protein MAL, which facilitates the interaction between TLR and TLR4 with MyD88, and inhibits its expression<sup>[152]</sup>. Recently, Wendlandt *et al*<sup>[160]</sup> demonstrated that miR-200b and miR-200c reduced the expression of MyD88 but had no effect TLR4, IRAK-1 and TRAF6 in HEK293 cells. When miR-200b and miR-200c mimics were overexpressed, LPS-induced expression of IL-6, TNF- $\alpha$ , and CXCL9 was diminished, suggesting that these miRNAs regulate TLR4 signaling *via* MyD88-dependent manner<sup>[160]</sup>. Up-regulated miR-19a and miR-19b were able to inhibit the expression of suppressor of cytokine signaling 1, a gene important in the negative regulation of TLR signaling<sup>[161]</sup>.

Programmed cell death 4 (PDCD4) is a pro-inflammatory protein that suppresses IL-10 production and activates NF- $\kappa$ B. Sheedy *et al*<sup>[162]</sup> found that PDCD4-deficient mice are protected from LPS-induced death and demonstrated that miR-21 targets PDCD4 after LPS stimulation to negatively regulate TLR4 pathway. Recently, Fabbri *et al*<sup>[163]</sup> reported that miR-21 and miR-29b secreted by lung cancer cells in exosomes function as TLR ligands. These miRNAs bind to and activate TLR7 and TLR8 signaling pathways in peritoneal macrophages cells, causing NF- $\kappa$ B activation and secretion of pro-metastatic inflammatory cytokines<sup>[163]</sup>. Secreted miR-21 was also found in the serum of HCC patients and in individuals with chronic hepatitis indicating that miR-21 is a key factor in hepatocarcinogenesis<sup>[164]</sup>. Several other circulating miRNAs have also been identified in the serum of patients with HCC<sup>[165-167]</sup>. Further studies are needed to determine whether any of these miRNAs can be used to modulate the TLR signaling pathways, and to alleviate inflammation in liver tissues.

## CONCLUSION

Hepatocarcinogenesis is a multifactorial disease in which the expression of a large number of genes, proteins, and other molecules from diverse cellular processes is altered<sup>[2]</sup>. Growing evidence suggests that inflammation plays a critical role in the progression of liver diseases to HCC. Therefore, the use of combination therapy that

targets multiple different steps and pathways, rather than a single test or a set of tests, to reduce inflammation is an appropriate therapeutic strategy to combat the development of HCC. Positive therapeutic outcomes achieved with sorafenib that can inhibit receptor tyrosine kinases of multiple signaling cascades<sup>[168]</sup>, as well as evidence that a single molecule miR-26a can significantly reduce HCC without any toxicity<sup>[169]</sup> demonstrate the success of this multi-pronged approach.

Despite recent advances in the use of miRNAs either as antagomirs or in replacement therapy, the field of miRNA therapeutics for HCC is still in its infancy. Only a handful of successful outcomes have been reported so far<sup>[135]</sup>. One such success story was that of MiraVasen, which inhibited the activity of miR-122 and suppressed HCV viraemia with no evidence for viral resistance or side effects in chimpanzees<sup>[170]</sup>. This was the first miRNA-based therapeutic drug developed to treat a liver disease, and is currently being tested by Santaris Pharma (Hørsholm, Denmark) in phase 2 clinical trials<sup>[171]</sup>. The first successful demonstration of miRNA replacement to restore the expression levels of a down-regulated miRNA by delivery to the tumor was reported using a miR-26a-encoding AAV vector in a mouse model of HCC<sup>[169]</sup>. More recently, another miRNA down-regulated in HCC, miR-34a, has been successfully tested using this approach in an orthotopic model of HCC<sup>[172]</sup>. In latter two cases, significant tumor reduction, dramatic protection from disease progression without toxicity, and prolonged survival of animals has been reported. While these reports were aimed to restore a loss of function, similar success has not yet been achieved for HCC treatment using the approaches to inhibit miRNA expression levels. In future investigations, it is important to exercise caution while designing clinical trials with miRNA mimics and TLR inhibitors for targeting multiple genes relevant to the liver diseases because of the concerns about potential toxicity in normal tissues, and as they can cause severe detrimental effects due to imbalances in TLR expression, and cellular functions that could cause immune paralysis leading to development of HCC.

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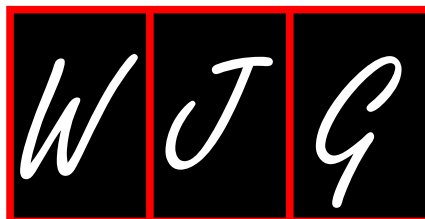
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## Bridging and downstaging treatments for hepatocellular carcinoma in patients on the waiting list for liver transplantation

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tients listed for LT within the Milan criteria, prolonging the waiting time over 6-12 mo is a risk factor for tumor spread. Bridging treatments are useful in containing tumor progression and decreasing dropout. Furthermore, the response to pre-LT treatments may represent a surrogate marker of tumor biological aggressiveness and could therefore be evaluated to prioritize HCC candidates for LT. Lastly, although a definitive conclusion can not be reached, the experiences reported to date suggest a positive impact of these treatments on both tumor recurrence and post-transplant patient survival. Advanced HCC may be downstaged to achieve and maintain the current conventional criteria for inclusion in the waiting list for LT. Recent studies have demonstrated that successfully downstaged patients can achieve a 5-year survival rate comparable to that of patients meeting the conventional criteria without requiring downstaging.

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### Abstract

Several therapeutic procedures have been proposed as bridging treatments for patients with hepatocellular carcinoma (HCC) awaiting liver transplantation (LT). The most used treatments include transarterial chemoembolization and radiofrequency ablation. Surgical resection has also been successfully used as a bridging procedure, and LT should be considered a rescue treatment in patients with previous HCC resection who experience tumor recurrence or post-treatment severe decompensation of liver function. The aims of bridging treatments include decreasing the waiting list dropout rate before transplantation, reducing HCC recurrence after transplantation, and improving post-transplant overall survival. To date, no data from prospective randomized studies are available; however, for HCC pa-

**Key words:** Hepatocellular carcinoma; Bridging treatment; Downstaging; Liver cirrhosis; Liver transplantation; Liver resection; Waiting list; Waiting time; Dropout rate

**Core tip:** The bridging treatments for patients with hepatocellular carcinoma within Milan criteria listed for liver transplantation are useful in decreasing dropout rate from the waiting list and the experiences reported to date suggest a positive impact on post-transplant tumor recurrence and patient survival. The response to treatments may represent a surrogate marker of tumor biological aggressiveness and could be evaluated to prioritize hepatocellular carcinoma candidates in the waiting list. Advanced hepatocellular carcinoma may be downstaged to achieve the current conventional criteria for inclusion in the waiting list and successfully

downstaged patients can achieve an excellent 5-year survival rate.

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## INTRODUCTION

Liver transplantation (LT) is the treatment of choice for patients with unresectable hepatocellular carcinoma (HCC) complicating liver cirrhosis because it allows the cure of both the tumor and the underlying chronic liver disease. HCC classified within the so-called Milan criteria (MC) (1 nodule smaller than 5 cm or no more than 3 nodules smaller than 3 cm)<sup>[1]</sup> is recognized everywhere as the standard indication for LT. However, after admission to the waiting list for LT, HCC patients can experience tumor growth beyond the conventional transplant criteria. Indeed, there is a high cumulative probability of drop-out from the waiting list for HCC patients due to intrahepatic or extrahepatic tumor progression. This probability has been reported to range between 7% and 11% at 6 mo and to be approximately 38% at 12 mo following enrollment by two papers published at the end of the 1990s by Llovet *et al*<sup>[2]</sup> and Yao *et al*<sup>[3]</sup>. The probability has been correlated with tumor characteristics, geographic origin, and length of time waiting for LT<sup>[4-6]</sup>.

Allocation policies for HCC patients awaiting LT remain controversial in the era of the model for end-stage liver disease (MELD) for the management of the LT waiting list. Different models have been developed to quantify the risk of death in neoplastic and non-neoplastic patients<sup>[7-11]</sup>. As the neoplastic risk assessment is not considered in MELD, patients with unresectable HCC with a neoplasm fulfilling the MC have been considered exceptions in the American allocation system. According to this rule, patients with T2-HCC fulfilling the MC (a single tumor of 2-5 cm or 2-3 tumors each < 3 cm) enter the waiting list with a MELD score equal to 22 and are therefore given priority over patients with less decompensated disease who enter the waiting list according to their laboratory MELD score (6 to 21). In addition, T2-HCC patients also receive incremental points for every 3 mo spent on the waiting list<sup>[12,13]</sup>. A similar approach has been implemented in other allocation systems<sup>[14,15]</sup>. The 22 threshold has been set to offer to HCC patients the same drop-out probability of patients without malignancy<sup>[16]</sup>. More detailed studies based on a dynamic prognosis parallelism have been published, and more complex allocation models aimed at balancing the risk of death in HCC and non-HCC patients have been proposed; however, they have not been applied in clinical practice<sup>[7,17-21]</sup>. According to these studies, LT can-

didates with HCC have dropout rates lower than non-HCC candidates, although the rate is similar to that of standard MELD candidates with a score of less than 21. Therefore, HCC patients appear to have an advantage in the current system, raising the question of whether a calculated continuous HCC priority score should be developed that also considers some biological features of the tumor such as the  $\alpha$ -fetoprotein (AFP) value, size, and rate of growth<sup>[17,18]</sup>. Indeed, HCC patients with a high AFP level achieve acceptable LT outcomes if their AFP levels can be reduced with locoregional therapy during the waiting period<sup>[22,23]</sup>. Furthermore, an inadequate response to HCC bridging therapy was shown to be a strong predictor of dropout probability in three single-center Italian studies<sup>[10,14,24]</sup>, whereas both the serum AFP level and the response to locoregional therapy were related to tumor recurrence and death in a retrospective international multicenter cohort study<sup>[25]</sup>.

Lastly, the development of the survival-benefit approach, which proposes ranking priority according to the benefit in survival between standard care and LT rather than crude survival figures, changed the perspective of the outcome evaluation system<sup>[26-28]</sup>. The practice most widely used since 2005, which is in accordance with the United Network for Organ Sharing rules, gives HCC patients with unresectable T1 neoplasms (a single nodule smaller than 2 cm) the same priority as patients listed for non-neoplastic diseases.

In this scenario, several therapeutic procedures have been proposed and largely used in the past as bridging neo-adjuvant treatments for patients listed for LT with HCC within the MC<sup>[29]</sup>. The rationale for their use is the possible decrease of the waiting list drop-out rate before transplantation and of HCC recurrence after transplantation, which is less than 15% in patients with HCC within the MC undergoing LT without any prior tumor treatment<sup>[30]</sup>. These beneficial effects could also improve the overall survival of transplanted patients. Both surgical resection and locoregional therapies can be used not only as bridging procedures to LT in T2-HCC patients but also to downstage HCC patients who do not initially meet the conventional transplant criteria<sup>[31]</sup>. According to this approach, patients can be safely listed for LT if they can reach and maintain for an adequate follow-up period the MC or slightly expanded criteria such as the University of California San Francisco criteria (UCSF) (a single HCC  $\leq$  6.5 cm or  $\leq$  3 tumors with the largest being  $\leq$  4.5 cm and a total tumor burden  $\leq$  8 cm)<sup>[32]</sup> or the up-to-7 criteria [HCCs with 7 as the sum of the size of the largest tumor (in cm) and the number of tumors]<sup>[33]</sup>. The aim of downstaging is to select HCC patients with reasonably low rates of tumor recurrence after LT among those who are initially excluded according to the current number-size criteria<sup>[34]</sup>.

In this paper, we analyzed the indications and results of the various neo-adjuvant treatment modalities currently administered to HCC patients awaiting LT to avoid exceeding the MC while on the waiting list as well as those used to downstage patients who do not meet

the conventional transplant criteria.

## NEO-ADJUVANT BRIDGING PROCEDURES FOR PATIENTS WITH HEPATOCELLULAR CARCINOMA AWAITING LIVER TRANSPLANTATION

### *Surgical resection*

Liver resection (LR) can be theoretically used as a first-line bridging procedure to LT. However, in most transplant centers, transarterial chemoembolization (TACE) and percutaneous ablation therapies are the preferred bridging therapies. The theoretical advantages of surgery in this setting are twofold. The first advantage is the best possible control of tumor growth, as TACE and percutaneous treatments do not always achieve complete tumor necrosis. The second advantage is the possibility of selecting patients in whom pathological analysis of the resected specimen shows features suggesting poor prognosis in terms of tumor recurrence, such as undifferentiated histotype, satellitosis, microvascular invasion, or capsular effraction, who should immediately undergo evaluation for LT<sup>[35]</sup>. However, compared to non-surgical therapies, the surgical bridging approach to patients listed for LT implies higher costs, entails more peri-procedural risks, can only be proposed in well-compensated patients without severe portal hypertension, and can make the ensuing LT technically more difficult, with a higher risk of post-operative complications<sup>[36]</sup>.

Moreover, important issues regarding tumor resectability should also be considered. Single exophytic or at least subcapsular neoplasms are easier to resect than multiple neoplasms or those located adjacent to the hilum or vena cava<sup>[37,38]</sup>. Furthermore, a location in the left lobe represents a more favorable condition, and the progress achieved in the laparoscopic resection of the liver has reduced the number of HCC patients with an absolute indication for LT<sup>[39]</sup>. However, the combination of LR and LT over time appears to be a reasonable strategy; HCC patients within the MC who have preserved liver function can undergo LR, limiting LT as a rescue treatment in cases of tumor recurrence or liver function failure (salvage LT)<sup>[40]</sup>. This approach allows a consequent saving of grafts, which can then be more efficaciously transplanted in other patients, and is supported by the discrepancy between the limited donor pool and the enormous number of LT candidates. However, there are important differences in access to LT according to cadaveric organ availability, blood group of the recipient, implementation of a living donor program, and degree of donor-recipient matching<sup>[41-44]</sup>.

Although during the initial experience with LR, the overall survival and disease survival rates of patients undergoing secondary LT after HCC resection were significantly lower (due to higher perioperative mortality unrelated to HCC) than those observed in cirrhotic patients with HCC undergoing primary LT<sup>[45]</sup>, favorable results

have more recently been reported by Belghiti *et al*<sup>[46]</sup>. They showed that postoperative course, complications, and the 3- and 5-year survival rates did not differ significantly between cirrhotic HCC patients undergoing primary LT or secondary LT after resection. Similarly favorable results for salvage LT have been subsequently reported by other groups in patients initially submitted to LR within the MC<sup>[15,47]</sup> or the UCSF criteria<sup>[48]</sup>.

Salvage LT has been shown to be effective not only in the setting of deceased donor LT but also in the setting of living donor LT, particularly in Asian countries. Compared to deceased donor LT, the main advantage of living donor LT is the reduction of the waiting list time, whereas the main drawback is represented by the occurrence of severe life-threatening complications among donors in approximately 1% of cases<sup>[49]</sup>. Indeed, Hwang *et al*<sup>[50]</sup> have shown that the combination of prior recipient hepatectomy and a living donor liver graft is feasible and provides excellent long-term survival in treated patients, and their results have recently been confirmed by other groups<sup>[51,52]</sup>.

Notably, the option of salvage LT cannot be offered to all patients initially treated by LR, primarily due to HCC recurrence overcoming the conventional LT criteria, age over 65 years at the time of recurrence, and the presence of comorbidities preventing the feasibility of LT. In a series reported by Poon *et al*<sup>[53]</sup>, approximately 80% of patients were still eligible for salvage LT at the time of tumor recurrence. In a recent paper by Liu *et al*<sup>[48]</sup>, among 71 patients with HCC recurrence within the UCSF criteria, salvage LT could be performed in 39 patients (54.9%). Compared to 180 HCC patients who underwent primary LT, patients treated with salvage LT for HCC recurrence showed greater intraoperative blood loss and required more blood transfusions; however, perioperative mortality, post-transplant complications, HCC recurrence rates, and overall survival did not differ significantly between the two groups.

### *Transarterial chemoembolization*

TACE is considered the standard treatment for patients with intermediate-stage HCC according to the Barcelona-Clinic Liver Cancer classification<sup>[54]</sup>, and it achieves a partial response in 15%-55% of patients and an improvement of median survival from 16 to 20 mo<sup>[55]</sup>. The most widely used conventional TACE procedure consists of an arterial infusion of a lipiodol emulsion with a chemotherapeutic agent (*e.g.*, doxorubicin or cisplatin) followed by embolization with gelfoam. However, conventional TACE is not a standardized procedure, and the optimal chemotherapeutic/embolizing agent and retreatment strategy have yet to be determined<sup>[56]</sup>. In particular, it is well known that TACE requires treatment repetition either at regular intervals or “a la demande” and that repeating conventional TACE may damage non-cancerous hepatocyte functions and affect the clinical course. Indeed, liver toxicity is a major limitation of conventional TACE regimens, and superselective TACE



is recommended in the setting of patients waiting for LT to minimize ischemic injury to non-tumoral liver tissue. Promising new data have been obtained using drug-eluting beads (DEBs), which are particles of variable size that are able to bind and elute doxorubicin in a predictable manner<sup>[57]</sup>. Compared to conventional techniques, DEBs appear to be a more standardized approach to TACE with less liver-related toxicity and fewer systemic adverse events<sup>[58]</sup>.

TACE has been extensively used in the past as a bridging treatment to LT, and a number of studies have shown that it is an effective therapy in terms of adequate tumor necrosis achievement at explant analysis. Analyzing the largest available series indicates that the rate of patients treated by TACE reaching complete tumor necrosis is quite uniform, ranging between 27% and 57% in patients within the MC<sup>[59-67]</sup>.

Interestingly, the rate of tumor necrosis appears to be higher in patients with single nodules when compared with patients with multiple nodules, in patients submitted to superselective TACE when compared with lobar TACE (complete necrosis achieved in 53.8% *vs* 29.8% of cases, respectively), and in patients with nodules 3-5 cm in size compared with patients with nodules smaller than 3 cm<sup>[65]</sup>. This last finding confirms the result obtained by Alba *et al*<sup>[64]</sup> and may be explained considering that larger nodules are typically fed by larger arteries, whereas in some instances, smaller nodules lack fully developed arterial neoangiogenesis; as a result, chemoembolization may be more effective in the former<sup>[65]</sup>. Accordingly, Kwan *et al*<sup>[66]</sup> have recently shown that the development of > 90% lesion necrosis upon pathological analysis of explanted liver was associated with avid lesion enhancement and the presence of a feeding vessel larger than 0.9 mm in diameter on the pre-TACE visceral angiogram. On post-TACE computed tomography images, a lack of residual contrast enhancement, a decrease in lesion size, a high lesion density due to an accumulation, and a diffuse distribution of ethiodized oil throughout the lesion were also correlated with near-complete lesion necrosis.

A recent small retrospective study compared tumor response in explanted liver after treatment with DEBs or standard TACE. TACE with DEBs achieved complete necrosis in 77% of the lesions, which was significantly higher than that reached after standard TACE (27.2%). More data are needed to address the better performance of DEBs compared to standard TACE in the transplant setting<sup>[68]</sup>.

Another important point to clarify is the evaluation of TACE safety in patients awaiting LT. Because arteritis of the celiac and hepatic arteries may complicate TACE as a result of endovascular trauma caused by guides and catheters, recipients could be exposed after the transplant to an increased occurrence of complications such as arterial thrombosis. However, the prevalence of such serious complications has not been found to be increased in some studies comparing patients with or without TACE performed before LT<sup>[69-71]</sup>.

## Radiofrequency ablation

Radiofrequency thermal ablation (RFA) has gained widespread use over recent years as an effective procedure for small HCCs not amenable to surgical resection. Thermal ablation may be performed using cool-tip or hook needles with comparable results<sup>[72]</sup>. Some studies have described the use of RFA as a bridge to transplantation in HCC patients in recent years. These studies have reported complete tumor necrosis at pathological evaluation of the explanted liver in 47%-75% of cases, with a mean value of 58%<sup>[73-77]</sup>. A clear difference in effectiveness can be observed when analyzing tumors according to size. Indeed, the rate of complete necrosis ranges between 50% and 78% in HCCs up to 3 cm and between 13% and 43% in larger neoplasms<sup>[73-75,77]</sup>. Furthermore, in two studies, a tumor size larger than 3 cm was the only risk factor identified for HCC persistence after treatment<sup>[73,75]</sup>.

Regarding RFA-related complications in the setting of HCC patients awaiting LT, an analysis of the largest available series demonstrated that the procedure is quite safe. In fact, considering 5 large series, the mean rate of post-ablation major complications was only as high as 4.6%, including one case of death due to peritoneal bleeding, two cases of acute peritonitis/cholecystitis, and one case each of severe liver failure treated by urgent transplantation, severe persistent liver failure, biliary stenosis, arterial hemorrhage, and small bowel perforation<sup>[73-76,78]</sup>. Additionally, the risk of tumor seeding at the level of the abdomen wall appears to be low; however, occasional cases of tumor seeding along the needle track diagnosed after LT in patients submitted to RFA as a bridging procedure have been reported in the literature<sup>[79,80]</sup>.

## Other treatments

TACE and RFA are the most used bridging treatments to LT in HCC patients, although other therapeutic options have been proposed (Table 1). Percutaneous ethanol injection (PEI) is the oldest and most used technique for the local treatment of HCC, but it has been rarely used as a bridging treatment to transplantation. In our multicenter survey, the rate of complete necrosis in tumors smaller than 3 cm was 30%<sup>[75]</sup>. Castroagudín *et al*<sup>[81]</sup>, in a series of 20 nodules in 19 patients, showed that in patients with small tumors (*i.e.*, less than 3 cm), ethanol injection induced complete necrosis in 58% of the cases. In a more recent paper, Branco *et al*<sup>[82]</sup> reported a complete necrosis rate of 64% in 59 patients within the MC and a mean tumor size of 2.4 cm (range: 0.5-5.5 cm). In these studies, PEI was not affected by procedure-related major complications and did not provide total necrosis in most tumors larger than 3 cm.

Percutaneous laser ablation (PLA) performed using multiple tiny laser fibers has recently been shown to be an effective technique for the thermal ablation of HCC in patients in whom surgical resection is not possible or appropriate<sup>[83,84]</sup>. We recently showed that in HCC patients awaiting LT, PLA provided results comparable



**Table 1 Advantages and disadvantages of the bridging and downstaging procedures for hepatocellular carcinoma in cirrhotic patients who are candidates for liver transplantation**

	Advantages	Disadvantages
Resection	Higher complete effectiveness than non-surgical procedures More simple in cases with peripheral subglissonian nodules	Unfeasible in patients with decompensated liver disease or severe portal hypertension
TACE	More effective using the selective/superselective technique in well-vascularized nodules with large feeding arteries	Unfeasible in patients with severely reduced portal vein flow, intratumoral arteriovenous fistula, or renal failure (creatinine clearance < 30 mL/min)
TARE	Possible better effectiveness than TACE in cases with multiple nodules	Less experience with TARE than TACE High cost
RFA	More effective in nodules $\leq 3$ cm	Potentially dangerous in patients with impaired clotting parameters or lesions located superficially or near the gallbladder, major bile ducts, or bowel loops
PEI	More effective in nodules $\leq 3$ cm Suitable in patients with impaired clotting parameters or lesions located in dangerous sites for thermal ablation	Less effective than RFA for nodules > 2 cm
PLA	More effective in nodules $\leq 3$ cm Suitable in patients with impaired clotting parameters	Less experience with PLA than RFA Technically complex
MWA	Possible better effectiveness than RFA in nodules $\geq 3$ cm or located near large vessels	Potentially dangerous in cases of lesions located superficially or near the gallbladder, major bile ducts, or bowel loops Less experience with MWA than RFA Potentially dangerous in patients with impaired clotting parameters or with lesions located superficially or near the gallbladder, major bile ducts, or bowel loops

TACE: Transarterial chemoembolization; TARE: Transarterial radio embolization; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; PLA: Percutaneous laser ablation; MWA: Microwave ablation.

to those of RFA; the rate of complete necrosis found at explant analysis in a series of 13 nodules up to 3 cm was 62%<sup>[85]</sup>. Due to the use of fine needles, the possible advantages of PLA in respect to RFA include the treatment of patients with either nodules in high-risk sites (*i.e.*, near vital structures)<sup>[86]</sup> or severe clotting impairment, in whom RFA may be contraindicated, and the lower overall cost of the procedure.

Microwave ablation (MWA) has been shown to be an effective thermal ablation procedure for the percutaneous treatment of HCC. Compared to RFA, this technique could theoretically provide a larger volume of necrosis and be more effective when treating nodules adjacent to large vessels; however, a clear advantage of MWA with respect to RFA has not been demonstrated<sup>[87,88]</sup>. The use of MWA as a bridging procedure to LT or a downstaging procedure in HCC patients appears to be promising. In a recent preliminary study, 6 patients with 6 HCC nodules ranging between 2.5 and 5.0 cm (mean 3.5 cm) in diameter underwent MWA before LT. At explant analysis, all of the nodules showed complete necrosis without intraoperative evidence of tumor spread in all cases or evidence of tumor recurrence at a one-year follow up in the 5 patients who could be evaluated<sup>[89]</sup>.

The effectiveness of transarterial radioembolization (TARE) with 90Yttrium microspheres has recently been evaluated by Riaz *et al.*<sup>[90]</sup>, who studied 38 nodules in 35 patients. Of 15 patients with T2-HCC, none progressed to T3-HCC (one nodule > 5 cm or up to three nodules with one > 3 cm) before LT, whereas 8 of 10 patients were downstaged from stage T3 to stage T2. At explant analysis, 23 of the 38 target lesions (61%) showed complete tumor necrosis, and its achievement was af-

fected by the size of the target lesion; indeed, complete necrosis was detected in 89%, 65%, and 33% of lesions smaller than 3 cm, between 3 and 5 cm, and larger than 5 cm, respectively.

Data regarding the use of external conformal radiotherapy (CRT) as a bridging treatment to LT in HCC patients are scarce. In a recent paper, CRT was delivered in five or six fractions to 10 patients with HCC awaiting LT with tumor diameters ranging from 2.5 to 10.8 cm. Nine patients completed the treatment, and it was well tolerated in all cases. Two tumors remained stable; the rest had 10%-50% regression, which was sustained on follow-up imaging. Five patients underwent LT, and at explant pathology, tumor necrosis ranging between 40% and 90% was demonstrated. No patients showed tumor recurrence after LT (median follow-up period of 6 mo). The main conclusions of the paper were that CRT is a safe and efficacious local bridging therapy for patients with HCC who are on the waiting list for LT and that further studies are warranted to compare the effectiveness of CRT to other local treatment regimens<sup>[91]</sup>.

### Combined treatments

Experiences with combined therapies such as TACE followed by RFA<sup>[92-94]</sup> or RFA shortly after TACE<sup>[95]</sup> have been published in recent years, typically in the setting of unresectable HCC larger than 3 cm. The rationale for the use of combined treatment rather than a single treatment is to reach a higher local tumor control rate due to higher rates of complete tumor necrosis. In this context, the question arises of how TACE and RFA should be sequenced. The advantage of performing TACE prior to RFA is the reduced heat-sink effect with the ability to create larger ablation zones more easily. The advan-

tage of using TACE after RFA is that RFA generates a hyperemic rim surrounding the ablation area, which can consequently be targeted by transarterial means more effectively. The approach of combined treatment may be applied even as a bridge to LT. A recent experience with combined TACE followed by RFA in a series of 44 HCC patients within the MC reported the absence of major complications and a 76.9% rate of complete necrosis in the 16 patients with 26 nodules who underwent LT<sup>[96]</sup>.

## IMPACT OF BRIDGING TREATMENTS ON DROPOUT FROM THE LIVER TRANSPLANTATION WAITING LIST

The impact of bridging treatments on waiting list dropout is uncertain due to the absence of prospective comparative studies, but the dropout data of treated patients should be compared with the features of HCC patients awaiting LT without any bridging treatment. In the latter case, the dropout rates were greater than 30% 12 mo after being added to the list<sup>[2,3]</sup>. In 2006, Lesurtel *et al*<sup>[97]</sup> published an interesting paper dealing with the usefulness of TACE in HCC patients undergoing LT according to the criteria of evidence-based medicine. The question was whether TACE impacted the waiting list dropout rate. They found insufficient evidence to answer this question. Hayashi *et al*<sup>[61]</sup> reported a discouraging 35% dropout rate in patients with TNM stage 1 or 2 HCC and a mean waiting time of 340 d after treatment with TACE. Similarly, among 54 listed HCC patients who underwent TACE prior to LT, Maddala *et al*<sup>[98]</sup> revealed drop-out rates of 15% and 25% at 6 and 12 mo, respectively. However, the most recent series including patients treated with TACE before LT have indicated that the dropout rate due to tumor progression is lower and ranges between 3.0% and 9.3%, with a mean waiting time on the transplantation list exceeding 6 mo in the largest available studies<sup>[63,64,69]</sup> (Table 2).

Less data are available in this setting for patients submitted to RFA. In a preliminary study published in 2002, Fontana *et al*<sup>[99]</sup> reported a dropout rate of 21% over a mean waiting period of 7.9 mo among 33 patients treated with RFA prior to LT. In more recent papers including larger numbers of patients, the dropout rate due to HCC progression was found to be 0% after a mean waiting time of 9.5 mo in one study<sup>[73]</sup> and 5.8% at 12 mo in another study<sup>[74]</sup> (Table 2). In a large study including only HCC patients within the MC awaiting LT, 77 patients who underwent RFA were compared to 93 patients without any bridging treatment; a non-specific trend toward a higher dropout rate for tumor-specific events was detected among RFA patients (21% *vs* 11%), but the mean waiting time was significantly higher in the RFA group. Using survival analysis modeling, there was no significant difference in the time to dropout between the RFA and no-treatment groups for all causes<sup>[78]</sup>.

Encouraging data have been reported following the application of multimodal schedules of treatment; in a series of 44 listed HCC patients within the MC who systematically underwent TACE followed by RFA, the intention-to-treat cumulative dropout rates were 5.5% and 11.0% at 12 and 24 mo, respectively<sup>[96]</sup>.

Lastly, the short-term response to bridging treatment has recently been reported to be crucial in the prediction of dropout. In a recent report by De Giorgio *et al*<sup>[24]</sup>, 170 HCC patients awaiting LT within the MC who underwent percutaneous ablation, TACE, or surgery as a bridging treatment were analyzed. Total tumor diameter and recurrence or persistence of tumor activity at the 6-wk follow-up after therapy were significantly correlated with progression beyond the MC and dropout from the waiting list. The finding of a significantly decreased dropout probability among T2 patients achieving a complete or partial response to bridging treatment compared with patients with an inadequate or no response to treatment has also been confirmed in two other large studies<sup>[10,14]</sup>.

In summary, there is sufficient evidence to conclude that bridging treatments yielding complete or subtotal HCC necrosis on imaging effectively reduce the rate of dropout from the waiting list.

## IMPACT OF BRIDGING TREATMENTS ON RECURRENCE OF HEPATOCELLULAR CARCINOMA AFTER LIVER TRANSPLANTATION

A less than 15% recurrence rate has been reported for HCC in patients within the MC undergoing LT without any treatment<sup>[30]</sup>. Whether the application of bridging therapies while on the waiting list decreases this rate is controversial. Again, prospective multicenter comparative studies are lacking in this field, and the only available data were obtained in single-center retrospective case series.

Regarding TACE, in a cohort of 111 HCC patients undergoing LT (54 treated preoperatively with TACE), Majno *et al*<sup>[59]</sup> showed that downstaging of tumors > 3 cm and total necrosis of the nodule at explant analysis were associated with better 5-year disease-free survival than either an inadequate response to TACE or no TACE before LT. Thereafter, low recurrence rates of 7.6% and 10.7% were reported in two large series of HCC patients within the MC who were treated with TACE before LT<sup>[63,64]</sup>. A clear trend toward longer recurrence-free survival has also been observed by Millonig *et al*<sup>[63]</sup> in patients with complete tumor necrosis when compared with patients with viable tumor at explant analysis. More recently, Tsochatzis *et al*<sup>[67]</sup> evaluated 150 consecutive patients with HCC within the MC who underwent LT. Sixty-seven patients (45%) underwent transarterial embolization (TAE) with polyvinyl alcohol particles or TACE before LT, and the remaining 83 patients were not treated before

**Table 2** Selected studies on non-surgical bridging therapy for hepatocellular carcinoma before liver transplantation *n* (%)

Ref.	Treatment	Patients	HCC stage	Dropout rate -Total -HCC progression	HCC recurrence after LT	Intention-to-treat survival	Survival after LT
Fontana <i>et al</i> <sup>[99]</sup>	RFA	33 (15 LT)	MC (30 pts)	NA	2 (13)	NA	85% at 3 yr
Graziadei <i>et al</i> <sup>[60]</sup>	TACE	48 (41 LT)	MC	0	1 (2.4)	94% at 5 yr	94% at 5 yr
Hayashi <i>et al</i> <sup>[61]</sup>	TACE	20 (12 LT)	MC	6 (35)	NA	61% at 3 yr	100% at 4 yr
Maddala <i>et al</i> <sup>[98]</sup>	TACE	54 (46 LT)	MC (47 pts)	8 (14.8)	5 (13.3)	61% at 5 yr	74% at 5 yr
				6 (11.1)			
Mazzaferro <i>et al</i> <sup>[73]</sup>	RFA	50 (50 LT)	MC (40 pts)	0 (0)	2 (4)	NA	83% at 3 yr
Lu <i>et al</i> <sup>[74]</sup>	RFA	52 (41 LT)	MC (42 pts)	6 (12)	0 (0)	74% at 3 yr	76% at 3 yr
				3 (5.8)			
Castrogaudin <i>et al</i> <sup>[81]</sup>	PEI	34 (23 LT)	UNOS T1-T2 (30 pts)	5 (14.7)	1 (4.3)	NA	19/23 (82.6%) alive (median FU 21 mo)
				2 (5.9)			
Pompili <i>et al</i> <sup>[75]</sup>	RFA, PEI	40 (40 LT)	MC (37 pts)	NA	3 (7.5)	NA	85.4% at 3 yr
Porrett <i>et al</i> <sup>[79]</sup>	TACE, RFA, TARE	31 (31 LT)	UNOS T1-T2	NA	7 (22.6)	NA	84% at 3 yr
Brillet <i>et al</i> <sup>[76]</sup>	RFA	21 (16 LT)	MC	5 (23.8)	1 (6.3)	NA	11/16 (69%) alive (median FU 25 mo)
				3 (14.3)			
Millonig <i>et al</i> <sup>[63]</sup>	TACE	68 (66 LT)	MC	2 (3)	5 (7.6)	70% at 5 yr	NA
Majno <i>et al</i> <sup>[69]</sup>	TACE	43 (43 LT)	MC	12 (27.9)	4 (9.3)	NA	NA
				4 (9.3)			
Rodríguez-Sanjuán <i>et al</i> <sup>[77]</sup>	RFA	28 (28 LT)	MC (25 pts)	NA	2 (7.1)	NA	NA
Alba <i>et al</i> <sup>[64]</sup>	TACE	63 (56 LT)	MC	7 (11)	6 (10.7)	NA	60.4% at 5 yr
				3 (4.8)			
Branco <i>et al</i> <sup>[82]</sup>	PEI	62 (59 LT)	MC	3 (4.8)	3 (5.1)	64.4% at 3 yr	67.7% at 3 yr
DuBay <i>et al</i> <sup>[78]</sup>	RFA	77 (51 LT)	MC	19 (25)	1 (2)	NA	> 80% at 3 yr
				16 (21)			
Ashoori <i>et al</i> <sup>[96]</sup>	TACE + RFA	36 (16 LT)	MC	6 (16.7)	0 (0)	NA	11/16 alive (median FU 29.9 mo)
				4 (11.1)			
Tsochatzis <i>et al</i> <sup>[67]</sup>	TACE, TAE	67 (67 LT)	MC	NA	4 (6)	NA	NA

HCC: Hepatocellular carcinoma; LT: Liver transplantation; RFA: Radiofrequency ablation; MC: Milan criteria; NA: Not available; TACE: Transarterial chemoembolization; PEI: Percutaneous ethanol injection; UNOS: United Network for Organ Sharing; TARE: Transarterial radio embolization; TAE: Transarterial embolization.

LT. HCC recurrence after LT was significantly lower in the TAE-TACE group (6%) than in the no TAE-TACE group (18.1%) (Table 2). Furthermore, post-transplant HCC recurrence was independently associated with no neo-adjuvant transarterial therapy and the total radiological size of the HCC nodules.

HCC recurrence after LT has been evaluated in 7 studies of patients who underwent RFA as the only bridging treatment. In total, 231 patients were evaluated over follow-up periods of 15-41 mo (mean 28 mo). Overall, HCC recurrence was detected in 8 patients (3.5%), and the rate of recurrence ranged between 0% and 13%<sup>[73-78,99]</sup> (Table 2).

Some recent single-center studies appear to confirm a positive impact of bridging treatments on HCC recurrence after LT. In a series of 147 HCC candidates (38% outside the MC) who underwent RFA, TACE, or multimodal treatment before LT, a complete or partial response was observed in 57.8% of cases. Transplanted patients with stable disease or no response to pre-LT HCC treatment had a significant 6-fold increase in tumor recurrence after LT compared with patients with a complete or partial response (13% *vs* 2%)<sup>[10]</sup>. In another study that included 315 HCC patients who were candidates for LT (17% outside the MC) and underwent TACE, RFA, PEI, or surgical resection, a

complete response to treatment was observed in 49.1% of cases; transplanted patients with a partial or no response to bridging treatments showed a significantly higher risk of HCC recurrence compared with patients with a complete response (19.4% *vs* 5.5%)<sup>[14]</sup>. Among 137 transplanted patients (42 outside the MC) who underwent locoregional bridging treatments such as resection, TACE, RFA, and PEI before LT, AFP > 400 ng/mL was the only significant pre-transplant factor linked to HCC recurrence after LT. Conversely, the use of locoregional treatments was a significant protective factor, and the best 5-year tumor-free survival was observed in patients within the MC who underwent locoregional treatment<sup>[100]</sup>. Lastly, within a group of 93 consecutive HCC patients (36 beyond MC) who underwent LT, 59 underwent pre-transplant TACE or RFA. The 5-year tumor-free survival did not significantly differ between treated and untreated patients (78% *vs* 68%). However, among the treated patients, the presence of more than 50% necrosis of the target lesions at explant analysis was associated with a significantly better 5-year tumor-free survival rate (96% *vs* 21%)<sup>[101]</sup>.

Overall, a trend toward a decreased recurrence rate after LT appears to emerge in patients achieving a total or subtotal response to the treatment administered before LT.

## IMPACT OF BRIDGING TREATMENTS ON SURVIVAL AFTER LIVER TRANSPLANTATION

Independent of the treatment administered, a key question remains to be answered: do bridging treatments improve survival in HCC patients who undergo LT? There is insufficient evidence of a beneficial effect of TACE because data obtained from prospective randomized studies are lacking<sup>[97]</sup>. A multicenter retrospective case control study from France compared 100 HCC patients who underwent TACE before transplantation and 100 HCC patients transplanted without any prior treatment. The 5-year survival (59% in both groups) and 5-year disease free survival (69% *vs* 64%) rates were not significantly different. At explant analysis, greater than 80% total or subtotal HCC necrosis was found in 30% of treated patients, and this subgroup showed a non-significant trend toward a better 5-year survival compared with a matched untreated control group (63% *vs* 54%)<sup>[62]</sup>. It can be reasonably argued that patients with total/subtotal tumor necrosis might receive a significant survival benefit from TACE before LT, perhaps due to a decreased risk of post-transplant HCC recurrence. This hypothesis appears to have been confirmed in a study by Millonig *et al*<sup>[63]</sup> that included 116 HCC patients who underwent TACE before transplantation. Most of the patients were within the MC, and complete tumor necrosis was found in 27% of the cases. The 5-year survival rate was higher in patients with completely necrotic tumors than in patients with partial necrosis (86% *vs* 66%), although this difference did not reach statistical significance.

The influence of neo-adjuvant treatments on post-LT survival should be analyzed independent of the treatment used; however, the only available data come from single-center retrospective series and provide contradictory results. In a study by Bharat *et al*<sup>[102]</sup>, 46 HCC patients undergoing various bridging treatments before LT were compared to 46 matched HCC patients transplanted without any treatment. The 5-year survival rate was significantly higher in the treated group (82% *vs* 52%), although the survival advantage was evident only for patients with T2-T4 tumors, not for patients with T0-T1 tumors. Even the 5-year disease-free survival rate was slightly higher in the treated group (84% *vs* 76%), although this difference was not significant. In a study by Lao *et al*<sup>[103]</sup>, 91 untreated HCC patients who underwent LT were compared to 33 patients with HCC who underwent TACE, RFA, or PEI before LT. HCC recurred only in 9 untreated patients, and the only factors significantly linked to tumor recurrence were a MELD score < 14, AFP > 1000 ng/mL, and the absence of pre-LT bridging treatment. The disease-free survival showed a non-significant trend toward a better outcome in treated patients, whereas the cumulative survival did not differ. Heckman *et al*<sup>[104]</sup> compared the outcomes of 50 HCC patients undergoing bridging therapy before LT to those of 73 HCC patients transplanted without any

prior treatment; they found a non-significant trend toward improved 5-year survival in treated patients (81% *vs* 71%). Porrett *et al*<sup>[79]</sup> compared 30 treated patients to 33 untreated patients before transplant. Their study failed to show any survival difference between the groups, but it should be noted that only 20% of the treated patients had complete HCC necrosis at explant analysis. Lastly, in the previously cited study by DuBay *et al*<sup>[78]</sup>, no differences in 5-year overall or tumor-free survival from the list date or transplant were identified when comparing 77 patients treated with RFA to 93 matched untreated patients. No data were provided about the achievement of complete necrosis in the ablated tumors at explant analysis.

Although a definitive conclusion cannot be made, a positive impact of pre-LT treatments on post-LT survival could be present. Indeed, in the United States, data on liver transplant activity for HCC from 1997 to 2006 demonstrated a higher 3-year post-LT survival in patients who underwent ablative procedures compared with patients who did not<sup>[105]</sup>. Moreover, studies reporting no difference between treated and untreated patients also tend to report shorter waiting times for LT<sup>[29]</sup>.

## DOWNSTAGING OF HCC BEYOND THE CONVENTIONAL LIVER TRANSPLANTATION CRITERIA

Downstaging of HCC to within the MC or the UCSF criteria is an attractive alternative to expanding the tumor size limits for LT. Theoretically, the downstaging process allows the selection of tumors with a more favorable biology that will likely respond to downstaging treatments and will also do well following LT<sup>[106]</sup>.

In recent years, several papers have been published defining successful downstaging as fulfilling the MC<sup>[107-114]</sup> or the UCSF criteria<sup>[106]</sup>. However, different criteria for successful treatment have been used in other studies, including fulfilling the MC without a serum AFP level higher than 400 ng/mL<sup>[115]</sup>, a 30%-50% decrease in the size of treated nodules<sup>[60,116]</sup>, or no tumor progression during the downstaging treatment in patients with well or moderately differentiated HCC<sup>[4,117]</sup> (Table 3). In some of these studies, only TACE<sup>[60,108,110,112-114]</sup> or transarterial chemoinfusion<sup>[107]</sup> was used as the downstaging procedure, whereas in other studies, a multimodal approach was used, including TACE, RFA, PEI, or surgical resection<sup>[4,106,114,115,117]</sup>. TARE as a single downstaging procedure was retrospectively compared to TACE in a study by Lewandowski *et al*<sup>[109]</sup>. Better performance was observed for TARE in terms of the downstaging success rate and 3-year intention-to-treat post-HCC treatment survival.

Significant factors for unsuccessful downstaging related to biological tumor features have been reported by some of these papers. In the study by Yao *et al*<sup>[106]</sup>, AFP > 1000 ng/mL was the only significant negative prognostic factor. Barakat *et al*<sup>[111]</sup> showed that the mean



**Table 3** Selected studies on downstaging therapy for hepatocellular carcinoma before liver transplantation *n* (%)

Ref.	Treatment	Pts	Inclusion criteria <sup>1</sup>	Successful downstage -Criteria -Rate	Transplanted pts	Recurrence free survival after LT	Intention to treat survival	Survival after LT
Graziadei <i>et al</i> <sup>[60]</sup>	TACE	36	HCC > 5 cm	Decreased size > 50% 11/36 (31)	10	Recurrent HCC: 3 pts (30)	31% at 5 yr	41% at 4 yr
Otto <i>et al</i> <sup>[116]</sup>	TACE	62	Beyond MC	Decreased size ≥ 30% 34/62 (55)	27	68% at 5 yr	NA	73.2% at 5 yr
Cillo <i>et al</i> <sup>[4]</sup>	TACE, RFA, PEI, Resection	40	Beyond MC WD or MD HCC	Maintenance of selection criteria NA	31	Recurrent HCC: 0 pts	79% at 5 yr	> 90% at 3 yr
Chapman <i>et al</i> <sup>[108]</sup>	TACE	76	Beyond MC	MC 18/76 (24)	17	50% at 5 yr	NA	93.8% at 5 yr
Yao <i>et al</i> <sup>[106]</sup>	TACE, RFA, Resection	61	1 HCC 5-8 cm 2-3 HCCs 3-5 cm, total diameter ≤ 8 cm 4-5 HCCs ≤ 3 cm total diameter ≤ 8 cm	UCSF 43/61 (71)	35	92% at 2 yr	69% at 4 yr	92% at 2 yr
Ravaioli <i>et al</i> <sup>[115]</sup>	Multimodal (TACE, PEI, RFA, Resection)	48	1 HCC 5-8 cm 2 HCCs 3-5 cm, total diameter ≤ 8 cm 3-5 HCCs ≤ 4 cm to- tal diameter ≤ 12 cm	MC and AFP < 400 ng/mL 32/48 (67)	32	71% at 3 yr	62% at 3 yr	NA
Lewandowski <i>et al</i> <sup>[109]</sup>	TACE (43 patients) TARE (43 patients)	86	UNOS T3	MC TACE 11/35 (31) TARE 25/43 (58)	TACE 11 TARE 9	TACE 73% at 1 yr TARE 89% at 1 yr	TACE 19% at 3 yr TARE 59% at 3 yr	NA
De Luna <i>et al</i> <sup>[107]</sup>	TACE	27	Beyond MC	MC 17/27 (63)	15	NA	84.1% at 3 yr	78.8% at 3 yr
Jang <i>et al</i> <sup>[110]</sup>	TACE	386	Beyond MC	MC or complete tumor necrosis 160/386 (41.5)	37	66.3% at 5 yr	NA	54.6% at 5 yr
Barakat <i>et al</i> <sup>[111]</sup>	TACE, TARE, RFA, Resection	32	Beyond UCSF (18 pts) Beyond MC (14 pts)	UNOS T2 18/32 (56.3)	13	Recurrent HCC: 2 pts (15.4%)	NA	75% at 2 yr
Bargellini <i>et al</i> <sup>[112]</sup>	TACE	33	Beyond MC	Complete or partial response, or stable disease according to mRECIST criteria NA	33	74.4% at 5 yr	NA	72.5% at 5 yr
Bova <i>et al</i> <sup>[113]</sup>	TACE, TAE	48	Beyond MC	MC AFP < 100 ng/mL 19/48 (39)	9	Recurrent HCC: 1 pt (11.1%)	NA	NA
Lei <i>et al</i> <sup>[114]</sup>	TACE, RFA, Resection, HIFU	58	Beyond MC Within UCSF	MC NA	58	63.8% at 5 yr	NA	74.1% at 5 yr

<sup>1</sup>Patients with vascular invasion or extrahepatic tumor spread at baseline excluded in all series. HCC: Hepatocellular carcinoma; LT: Liver transplantation; TACE: Transarterial chemoembolization; MC: Milan criteria; NA: Not available; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; WD: Well differentiated; MD: Moderately differentiated; UCSF: University of California San Francisco; AFP:  $\alpha$ -fetoprotein; TARE: Transarterial radio embolization; UNOS: United Network for Organ Sharing; TACE: Transarterial chemoembolization; mRECIST: Modified Response Evaluation Criteria in Solid Tumors; TAE: Transarterial embolization; HIFU: High-intensity focused ultrasound; pts: Patients.

AFP level and the rate of infiltrative tumors were significantly higher in patients who did not achieve successful downstaging. An AFP level lower than 100 ng/mL and the 3-year survival probability calculated using the Metroticket calculator<sup>[33]</sup> were the only independent predictors of successful downstaging in the study by Bova *et al*<sup>[113]</sup>. An AFP slope > 15 ng/mL per month and tumor progression according to the Modified Response Evaluation Criteria in Solid Tumors (mRECIST)<sup>[118]</sup> were independent risk factors for HCC recurrence and patient death in an international retrospective multicenter European study performed by Lai *et al*<sup>[25]</sup> that included

MC-within (316 cases) and MC-outside (116 cases) patients who underwent LT after locoregional therapy. We should also highlight that after successful downstaging, some authors have recommended that patients undergo a 3-mo observation period before listing to assess the stability of neoplastic disease<sup>[4,106,115]</sup>. This “test of time” will identify rapidly recurring lesions, vascular invasion, and distant metastasis, thereby decreasing the risk of tumor recurrence and poor overall results after LT<sup>[34]</sup>.

Overall, according to the presently available data, the successful downstaging rate ranges between 24% and 71% (Table 3). The proportion of patients transplanted

ranges between 10% and 67%, and the average waiting time to LT ranges between 2 and 10.9 mo<sup>[29]</sup>. Additionally, the reported survival rates range from 78.8% to more than 90% and from 54.6% to 93.8% at 3 and 5 years, respectively<sup>[119]</sup>. Two prospective studies have demonstrated that survival after LT in patients with large tumors successfully downstaged within the MC<sup>[115]</sup> or the UCSF criteria<sup>[106]</sup> is similar to that of patients who initially met the criteria for transplantation. Six studies<sup>[4,60,107,108,114,116]</sup> compared patients who were downstaged successfully within the MC with those who initially met the MC. Five of these studies<sup>[4,107,108,114,116]</sup> reported no significant difference in absolute or disease-free survival between groups, whereas one study<sup>[60]</sup> reported that patients who were downstaged successfully had significantly worse survival at 1, 2, and 5 years after LT. Lastly, in a recent study, no significant differences in postoperative complications, tumor recurrence, or survival rate were reported between two groups of patients with advanced HCC who underwent deceased donor LT (52 patients) or living donor LT (31 patients) after successful downstaging therapy<sup>[42]</sup>.

## CONCLUSION

Currently, locoregional therapies play a crucial role in the treatment of patients awaiting LT. For patients listed within the MC (stage T2-HCC), a delay of LT over 6-12 mo without bridging treatment is a well-recognized risk factor for tumor progression and dropout from the list or interval dissemination with post-transplant tumor recurrence<sup>[2,3,16]</sup>. For this reason, the optimal strategy for T2-HCC patients awaiting LT should be to transplant within 6 mo without pre-transplant therapy<sup>[120]</sup>. However, if a longer waiting time is needed, following the current guidelines of the American Association for the Study of Liver Disease and the European Association for the Study of the Liver for the treatment of HCC<sup>[121,122]</sup> and the recommendations of a recent international consensus conference on the management of HCC patients who are LT candidates<sup>[123]</sup>, the use of bridging treatments is recommended, as several studies in recent years have documented their usefulness in preventing tumor progression. There is, however, no evidence that bridging treatments are useful in patients with T1-HCC<sup>[123]</sup>.

In patients who underwent previous liver resection and experienced tumor recurrence but are within the currently accepted transplant criteria or those with liver function failure, salvage LT using deceased donor livers yields an acceptable long-term survival rate and can be considered<sup>[6,25]</sup>. Salvage LT using living donors has also been successfully performed in centers with high-volume living donor programs, and they appear to provide long-term results comparable to those obtained using deceased donor grafts<sup>[48]</sup>.

Regarding non-surgical bridging therapies, no recommendation can be made for one type of locoregional therapy over others<sup>[123]</sup>. However, RFA could be the first-

line treatment for lesions up to 3 cm, in which complete tumor necrosis has been shown in more than 50% of cases at explant analysis. The risk of major complications related to RFA in this patient setting appears to be quite low, but it is good clinical practice to limit needle insertions and to avoid the treatment of superficially located lesions. PEI appears to show lower efficacy and can be reserved for small lesions located in sites considered “dangerous” for RFA (*e.g.*, near the gallbladder or bowel loops). TACE should be preferred for treating lesions > 3 cm because its effectiveness appears to be better in well-vascularized tumors with large feeding arteries; selective and superselective TACE should be preferred, and the possible advantage of DEBs-TACE over lipiodol-TACE should be investigated in future studies. Multimodal treatment strategies, including sequentially applied TACE and RFA, appear to be promising, although the role of alternative treatments such as PLA, MWA, TARE, and CRT needs to be investigated in a larger number of patients. Regardless, all ablation procedures should be better evaluated with caution in patients with decompensated liver function to avoid irreversible liver failure and severe complications precluding LT.

The response of HCC to neoadjuvant treatments should be evaluated using the mRECIST criteria<sup>[118]</sup>. The RECIST criteria<sup>[124]</sup> were amended to the mRECIST in 2008<sup>[125]</sup> in the setting of HCC based on the concept that the evaluation of the treatment response should consider the amount of necrosis when estimating the decreased tumor load, not only the reduction in tumor size. However, it should also be considered that computed tomography and magnetic resonance imaging, which are currently used to assess the results of the ablation bridging procedures, tend to overestimate treatment effectiveness. In several studies, the concordance in the diagnosis of complete necrosis between the last imaging evaluation before LT and the pathological assessment at explant analysis of the target lesions has been reported to range between 50% and 83%; this is primarily due to the persistence of microscopic avascular neoplastic foci that are primarily located peripherally and cannot be detected by contrast-enhanced imaging techniques<sup>[74,75,77,85,106,115,126,127]</sup>.

Although no solid conclusions may be drawn due to the absence of prospective comparative studies, it appears reasonable to state that bridging treatments decrease the dropout rate from the waiting list of T2-HCC patients and could have a positive impact on post-LT HCC recurrence and overall survival, at least in patients with complete or subtotal necrosis of the targeted lesions and a longer waiting period<sup>[29,105]</sup>. Furthermore, the response to pre-LT treatments may represent a surrogate marker of tumor biology and should be considered in the selection and prioritization of candidates for LT. That is, the transplant priority of T2-HCC candidates could be reduced after successful bridging therapy and a 3-6 mo period of observation confirming inactive neoplastic disease, and patients showing stable or progressive disease after treatment could then be prioritized.

However, if an advantage is given on the waiting list to non-responding patients, a worsening in the outcome of LT in terms of overall survival, primarily due to an increased incidence of HCC recurrence after transplantation, should be considered. Whether this risk is acceptable is a matter of debate, and this issue should be further addressed in future studies<sup>[14,29,128]</sup>.

HCC downstaging using exclusively TACE or multimodal sequential therapies to meet the conventional criteria for LT among carefully selected patients yields promising results in terms of overall and disease-free survival. In particular, some recent papers have demonstrated that patients successfully downstaged within the MC or the UCSF criteria can achieve a 5-year survival rate comparable to that of patients meeting the above-mentioned criteria without requiring downstaging<sup>[123]</sup>. A follow-up period of 3 mo demonstrating stable disease after successful downstaging is suggested before inclusion on the waiting list for transplantation.

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## WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

# Enteric bacterial proteases in inflammatory bowel disease-pathophysiology and clinical implications

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## Abstract

Numerous reports have identified a dysbiosis in the intestinal microbiota in patients suffering from inflammatory bowel diseases (IBD), yet the mechanism(s) in which this complex microbial community initiates or perpetuates inflammation remains unclear. The purpose of this review is to present evidence for one such mechanism that implicates enteric microbial derived proteases in the pathogenesis of IBD. We highlight and discuss studies demonstrating that proteases and protease receptors are abundant in the digestive system. Additionally, we investigate studies demonstrating an association between increased luminal protease activity and activation of protease receptors, ultimately resulting in increased intestinal permeability and exacerbation of colitis in animal models as well as in human IBD. Proteases are essential for the normal functioning of

bacteria and in some cases can serve as virulence factors for pathogenic bacteria. Although not classified as traditional virulence factors, proteases originating from commensal enteric bacteria also have a potential association with intestinal inflammation *via* increased enteric permeability. Reports of increased protease activity in stools from IBD patients support a possible mechanism for a dysbiotic enteric microbiota in IBD. A better understanding of these pathways and characterization of the enteric bacteria involved, their proteases, and protease receptors may pave the way for new therapeutic approaches for these diseases.

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**Key words:** Protease; Proteinase; Protease associated receptor; Enteric microbiota; Epithelial barrier

**Core tip:** It is currently accepted that an enteric dysbiosis (alteration of the normal bacterial flora) is involved in the pathophysiology of inflammatory bowel diseases (IBD). One of the suggested mechanisms that ties an intestinal dysbiosis to the pathophysiology of IBD involves the release of enteric bacterial proteases that interact with protease activated receptors on epithelial cells, resulting in intestinal barrier dysfunction and exposure of the enteric immune system to luminal antigens. We have reviewed the literature that examined the role of microbial proteases and their enteric receptors in the pathogenesis of IBD, their suggested pathways of action, and discuss future therapeutic implications.

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## INTRODUCTION

Inflammatory bowel diseases (IBD), collectively known as Crohn's disease (CD) and ulcerative colitis (UC), are caused by dysregulated immune responses towards microbial antigens in a genetically predisposed host. The incidence of UC and CD has been increasing worldwide in developed and in developing countries<sup>[1,2]</sup>. These diseases are highly prevalent in the United States affecting 1.4 million individuals<sup>[3]</sup> and are associated with reduced quality of life<sup>[4,5]</sup>, and psychological co-morbidity<sup>[6]</sup>. Current estimates for IBD associated treatment costs in the US are \$6.3 billion<sup>[7]</sup>, but the initiating events of IBD and causes of disease exacerbation remain unclear. It is postulated that one potential mechanism involves disruption of the epithelial barrier, and exposure of a genetically defective immune system to enteric microbial antigens. Consistent with this hypothesis are animal models of colitis that use chemical disruption of the epithelial barrier with trinitrobenzene sulphonic acid (TNBS), dextran sodium sulfate or non-steroidal anti-inflammatory drugs (NSAIDs). Additionally, disruption of the intestinal epithelial barrier by exposure of susceptible patients to NSAIDs (blockers of prostaglandins synthesis) is a known risk factor that can trigger intestinal inflammation<sup>[8]</sup>. In line with this observation, in animal studies, the use of a prostaglandin receptor agonist preserved the intestinal epithelial barrier structure and function, maintained mucous secretion by goblet cells, and prevented the development of colitis<sup>[9]</sup>.

Proteases, peptidases, or proteolytic enzymes, are a class of enzyme that catalyze the cleavage of peptide bonds in other proteins in the presence of H<sub>2</sub>O (hydrolysis). Proteases act as both positive and negative effectors of several biological processes either broadly as catalysts of protein degradation or specifically as selective agents that control physiological processes<sup>[10]</sup>. The importance of proteases is highlighted in the human genome where 2%-4% of genes encompass the *degradome*<sup>[11]</sup>. In bacteria, proteases are involved in numerous biological processes, such as those associated with metabolism, development, and virulence. Additionally, these enzymes can disrupt mucosal barriers, provide a metabolic advantage, and modulate the host immune response. The high prevalence of proteases in enterobacteria suggests that proteases play important roles in pathogenesis<sup>[12]</sup>. Both mammalian and bacterial proteases have been implicated in the pathogenesis of IBD, usually through disruption of the epithelial barrier. In pathogenic bacteria, many proteases are virulence factors that aid in bacterial invasion into host cells and cause infectious colitis. However, accumulating evidence shows that commensal enteric microorganisms also produce proteases that possess the ability to disrupt the epithelial barrier<sup>[13,14]</sup>. These commensal proteases may be involved in the pathogenesis of IBD in the context of a genetically predisposed host and/or when an intestinal microbial dysbiosis occurs. Our aim in this review is to provide an overview of current studies that suggest potential mechanisms in which microbial proteases may play a role in the pathogenesis of IBD.

## PROTEASE CLASSIFICATION

Proteases frequently exist as multi-domain proteins, with catalytic activity restricted to a single structural domain. Although these enzymes appear to have a specific function (*i.e.*, hydrolysis of proteins), they exhibit vast diversity in their action and structure and are not easily categorized by general systems of enzyme nomenclature. Thus, proteases are broadly subdivided into two major groups, exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whilst endopeptidases cleave peptide bonds distant from the termini of the substrate. Proteases are further classified into five distinct groups on the basis of the chemical nature of the groups responsible for their catalytic activity, namely; aspartic, cysteine/thiol, metallo-, serine, and unidentified proteases<sup>[15]</sup>. In order to generate a comprehensive classification system for proteases, Rawlings and Barrett<sup>[16]</sup> developed a method to classify this group of enzymes based on the type of reaction they catalyze, the chemical nature of their catalytic site, and their evolutionary structure. This approach is a hierarchical system where classification levels were summarized as peptidases (*i.e.*, serine proteases), families and clans. This system initially recognized 84 families of proteases; however the subsequent massive accumulation of amino acid sequence data and three-dimensional structures of proteases from the scientific community warranted an updated classification system that was easily accessed for academic studies. Thus, based on the system outlined by Rawlings and Barrett<sup>[16]</sup> the MEROPS database was developed<sup>[17]</sup>. Along with data regarding protease classification, the MEROPS database also provides information regarding classification of protein inhibitors of peptidases<sup>[18]</sup>, small-molecule inhibitors<sup>[19]</sup>, and a collection of known protease cleavage sites and substrates<sup>[20]</sup>.

### Microbial proteases

Proteases are found in all forms of life suggesting that they are vital for the survival of all organisms. Microorganisms produce a vast array of aspartic, cysteine, metallo-, and serine proteases. Microbial aspartic proteases are specific for aromatic or bulky amino acid residues on both sides of a peptide bond. They are broadly divided into two groups: pepsin- and rennin-like enzymes. Cysteine proteases generally are only active in the presence of reducing agents. Some bacterial cysteine proteases are notable for their role in virulence and the inflammatory response they illicit<sup>[21]</sup>. Metalloproteases are characterized by the requirement for a divalent metal ion for their activity. These proteases are summarized into neutral and alkaline groups based on their specificity of action<sup>[22]</sup>. Serine proteases are characterized by the presence of a serine group in their active site and have broad substrate specificity. The complex microbial community in the human gut (referred to as the intestinal microbiota) is a substantial source of serine, cysteine, and metallo-proteases<sup>[23-25]</sup>. This is exemplified by the reduction of colonic bacteria

densities and protease activity by oral administration of antibiotics to mice<sup>[26]</sup>. By analyzing the protease activity of representative enteric bacterial strains and human fecal samples it has previously been suggested that the activity of specific classes of proteases present in human feces are likely to originate from *Bacteroides*, *Streptococcus*, and *Clostridium* species<sup>[27]</sup>. However, to date only one study has reported the correlation between specific groups of proteases and the abundance of enteric bacterial taxa using modern molecular methods. Carroll *et al.*<sup>[28]</sup> used high throughput sequencing of the 16S rRNA gene and correlated the abundances of specific bacterial families with fecal tryptic activity in stool samples from healthy individuals and IBS patients. This study found positive associations between *Lachnospiraceae*, *Streptococcaceae* and *Lactobacillales* with fecal protease activity, and a negative correlation with *Ruminococcaceae*. However, to date microbial proteases have been mainly exploited for commercial purposes. For example, bacterial alkaline proteases are characterized by their high activity at an alkaline pH and their broad substrate specificity, thus, making them ideal for use in the detergent industry<sup>[29]</sup>. In addition, most academic studies have focused on bacterial proteases as potential virulence factors in pathogenic bacteria<sup>[30]</sup>. However, little is known regarding the relationship between microbial proteases, found in or on the body, and the health of the host. Examples of such microbial proteases that are produced by enteric commensals are specified in Table 1.

## MICROBIAL PROTEASES IN THE PATHOGENESIS OF IBD

The antigenic contents of the intestinal lumen are separated from underlying intestinal tissues by an epithelial barrier that is one cell thick. Pathogenic bacteria have acquired virulence factors, many of which are proteases, that disrupt this barrier and cause infection<sup>[31]</sup>. For example, the serine protease autotransporter of *Enterobacteriaceae* family are generally secreted into the external milieu and are highly prevalent among enteropathogens, including *Shigella* species and all *Escherichia coli* (*E. coli*) pathotypes<sup>[12]</sup>. As there is an established genetic component to IBD<sup>[32]</sup>, it is difficult to identify microbial proteases that are potentially involved in the pathogenesis of these diseases as they would not be categorized in the same manner as traditional virulence factors. Indeed, an overproduction of microbial proteases originating for enteric commensal microbes may not have an effect on a healthy individual, but may play a role in the pathogenesis or perturbation of intestinal inflammation in a population with a genetic predisposition to IBD. Here we discuss four potential mechanisms in which microbial proteases from a non-pathogenic source (the intestinal microbiota) could contribute to the pathogenesis of IBD.

**Table 1 Commensal enteric microbial protease classification and origin**

Protease category	Microbial origin	Protease
Aspartic	<i>Candida albicans</i>	Secreted aspartic proteases <sup>[119]</sup>
	<i>Pseudomonas aeruginosa</i>	Type 4 prepilin peptidase <sup>[120]</sup>
Cysteine	<i>Methanococcus voltae</i>	Preflagellin <sup>[121]</sup>
	Gram positive bacteria	Sortases <sup>[122]</sup>
	<i>Porphyromonas gingivalis</i>	Gingipain <sup>[21]</sup>
	<i>Staphylococcus aureus</i>	Staphopain <sup>[123]</sup>
Metalloprotease	<i>Bacteroides fragilis</i>	Fragilysin <sup>[124]</sup>
	<i>Enterococcus faecalis</i>	Gelatinase <sup>[87]</sup>
	<i>Staphylococcus epidermidis</i>	Elastase <sup>[125]</sup>
	<i>Clostridium perfringens</i>	Collagenase <sup>[13]</sup>
Serine	<i>Helicobacter pylori</i>	High temperature requirement A <sup>[125]</sup>
	<i>Bacillus subtilis</i>	Subtilisin <sup>[126]</sup>

## MICROBIAL PROTEASES AND ADHERENCE AND INVASION TO THE INTESTINAL EPITHELIUM

Bacterial adhesion to intestinal epithelial cells is believed to be one of the first steps used in the pathogenicity of many enteric pathogens. Adhesion enables a microbe to colonize the intestinal epithelium and resist exclusion from the intestine by the mechanical movement of the gut. Adherent and invasive *E. coli* (AIEC) are a group of enteric microbes that are capable of adhering to and invading intestinal epithelial cells<sup>[33]</sup>. AIECs are not classified as enteric pathogens, but exhibit some pathogenic traits in the context of IBD. For example, AIECs isolated from CD patients are able to replicate within macrophages without escaping from the phagosome and without inducing macrophage death<sup>[34]</sup>. Proteases for pathogenic bacteria play a fundamental role in adherence and invasion virulence traits. For example, enteroaggregative *E. coli* (EAEC) expresses a factor referred to as “protease involved in colonization” or Pic. Pic catalyzes gelatin degradation which can be abolished by disruption of the predicted proteolytic active site. This protease is involved in the early stages of pathogenesis and most probably promotes intestinal colonization<sup>[30,35]</sup>. Pic is also essential for biofilm formation in EAEC. The first step of biofilm formation is bacterial adherence to a surface and then intercellular aggregation. In general, intercellular aggregation is mediated *via* the proteolytic processing of bacterial aggregation proteins by means of host or bacterial proteases<sup>[36,37]</sup> ultimately resulting in a biofilm. To date the role of microbial proteases involved in the formation of biofilms in members of the intestinal microbiota have not been investigated in the context of IBD. However, the role of biofilms in AIEC virulence in IBD has begun to emerge. It was reported that biofilm formation indi-

ces were higher amongst AIEC than non-AIEC strains isolated from the intestinal mucosa of CD, UC, and non-IBD controls<sup>[38]</sup>. Additionally, the adhesion and invasion properties of AIECs correlated positively with higher biofilm formation indices. Furthermore, the  $\sigma^E$  factor, which up-regulates genes that encode proteases, periplasmic foldases, and chaperones in response to environmental stresses, plays a pivotal role in biofilm formation in AIECs in the context of CD<sup>[39]</sup>. Thus, proteases may be important in biofilm formation and colonization of commensal enteric bacteria and related to IBD pathogenesis.

## PROTEASE RECEPTORS

Proteases can mediate their activity on mammalian cells through activation of protease receptors. Protease activated receptors (PARs) are a family of 7 transmembrane domain G-protein-coupled receptors (GPCRs) that mediate multiple responses to external stimuli, such as hemostasis, thrombosis and inflammation, and exist in four isoforms (PARs 1-4)<sup>[40-44]</sup>. PARs are activated through proteolytic cleavage of the extracellular N-terminal component of the receptor unmasking a tethered peptide ligand residue that binds with another region of the receptor causing a conformational change<sup>[45]</sup>. The result is an initiation of an intracellular signaling cascade that is diverse and includes calcium mobilization, phospholipase C-dependent production of inositol phosphates and diacylglycerol, Rho and Rac activation, mitogen-activated protein kinase signaling, and gene transcription<sup>[46]</sup>. Alternatively, PARs can be activated through peptide sequences that are homologues to the intrinsic tethered ligand. These synthetic peptides activate PARs without proteolysis of the N-terminal of the receptor in PAR1, PAR2 and PAR4 but not in PAR3<sup>[47]</sup>. The outcome of PAR activation is dependent on the type of ligand (*e.g.*, serine protease, matrix metalloprotease, plasmin, coagulation factors *etc.*), receptor type (PAR1, 2, 3 or 4) and on the type of cell which the PAR is expressed (*e.g.*, epithelial cells, platelets, nerve cells, or leukocytes). PAR activation, signaling and degradation are highly regulated by post translational modifications such as phosphorylation, glycosylation and ubiquitination (for review- Grimsey *et al*<sup>[48]</sup>). In the gastrointestinal (GI) tract, PARs are activated by endogenous proteases secreted by the pancreas (such as trypsin), by cells of the enteric wall (such as mast cells), or by the luminal enteric microbiota. Moreover, PAR expression on the gut epithelium differs between IBD patients and healthy individuals. This may be a result of the type of micro-organisms present in the GI tract and other receptors [such as toll-like receptors (TLRs)] they interact with. For example, on polymorphonuclear (PMN) cells, *Candida albicans* promoted a TLR2-dependent PAR1 activation and expression in contrast to *Aspergillus fumigatus* that suppressed TLR4-dependent PAR2 activation and expression<sup>[49]</sup>. In this regard it is important to note that endogenous host proteases are also PAR specific, *e.g.*, - thrombin activates PAR1<sup>[50]</sup>, PAR3<sup>[40,43]</sup> and PAR4<sup>[44]</sup>, while trypsin activates PAR2<sup>[51]</sup> and PAR4<sup>[52]</sup>.

While the majority of research relating to the relationship between PARs and colitis has examined the role of endogenous activation of PARs by mammalian proteases, the interaction between the enteric microbes, PAR expression and activation and the pathophysiology of colitis have not been extensively studied. The evidence that supports these associations is summarized below.

### PAR1

PAR1 has been implicated in hemostasis, platelet signaling, systemic pro-inflammatory responses (such as vasodilatation, increased vascular permeability and chemotaxis) and induction of analgesia<sup>[53,54]</sup>. PAR1 agonists induce apoptosis of intestinal epithelial cells in a caspase-3-dependent manner, with a concomitant loss of the epithelial barrier function and a consequent increase of permeability to macromolecules and bacteria<sup>[55]</sup>. PAR1 is expressed by enterocytes as well as by other cell types such as endothelial cells, enteric neurons, myocytes and immune cells<sup>[52]</sup>. The expression of PAR1 on the intestinal epithelium is linked to the presence of enteric microbiota<sup>[56]</sup>, and activation of this receptor in the mouse colon leads to colitis<sup>[57,58]</sup>. In addition, PAR1 expression has been reported to be increased in colonic biopsies from IBD patients<sup>[54]</sup>. Altogether, these reports support a role for PAR1 in the pathogenesis of IBD, however it is not clear if the enteric microbiota directly activate PAR1 through release of bacterial proteases. Nonetheless, this mechanism is supported by a study investigating oral epithelial cells, where PAR1 activation by a cysteine protease released by the oral pathogen *Porphyromonas gingivalis* (*P. gingivalis*) caused an up-regulation of pro-inflammatory cytokines<sup>[59]</sup>.

### PAR2

The majority of evidence that points towards an association between PARs and intestinal inflammation involves PAR2. This receptor is localized to the apical and basolateral membrane<sup>[60-62]</sup> of the intestinal epithelium and can be activated by trypsin, tryptase, and bacterial proteases<sup>[63]</sup>. PAR2 is expressed in immune, stromal, endothelial, and intestinal epithelial cells and thus, PAR2-associated inflammation may be a result of multiple, systemic and local pathways. Systemically, this receptor impacts leukocytes by mediating rolling, adhesion, and extravasation<sup>[64]</sup>. When activated on sensory neurons PAR2 mediates pain and edema<sup>[65]</sup>. In the mouse colon, activation of this receptor results in colitis<sup>[60]</sup> that is significantly ameliorated in PAR2-deficient mice<sup>[60,66]</sup>. Additionally, antagonism of PAR2 (by GB88) results in amelioration of colitis in rats that is induced by either TNBS or a PAR2 agonist (SLI-GRL-NH<sub>2</sub>)<sup>[67]</sup>. Thus, most studies indicate that activation of PAR2 leads to an inflammatory response. However, a single study has reported a protective effect of daily intra colonic administration of PAR2 agonist in a TNBS colitis model in rats<sup>[68]</sup>. It is not entirely clear why PAR2 exhibits anti-inflammatory properties in this model; however it may be the result of a chronic PAR2 activation and lo-



cal desensitization, or *via* anti-inflammatory effects on macrophages<sup>[69]</sup>. Additionally, it is not clear which of the various mechanisms that have been implicated in PAR2 activation in the gut is responsible for PAR2-dependent colitis. However, it has been speculated that PAR2-mediated intestinal inflammation is a result of increased levels of PAR2 ligands in the colon of IBD patients. Indeed, in the colon of human IBD patients the natural PAR2 ligands, trypsin<sup>[70]</sup> and tryptase<sup>[71,72]</sup> are elevated compared to healthy controls. Moreover, in human IBD, PAR2 is overexpressed on mast cells<sup>[73]</sup> which have also been implicated in the pathogenesis of PAR2-mediated colitis. In non-IBD patients permeability was found to be proportional to the concentration of tryptase (naturally secreted by mast cells) added to the basolateral surface and not to the mucosal surface of mucosal biopsies<sup>[74]</sup>. These studies support the importance of mast cells in colitis *via* PAR2 activation, however enteric bacteria may also play a role in PAR2 activation in the colon through release of bacterial proteases in the gut lumen<sup>[24]</sup>. Róka *et al.*<sup>[26]</sup> demonstrated increased levels of serine proteases in fecal samples from UC patients and hypothesized that these enzymes originated from luminal bacteria as it was reported that increased fecal protease activity was neither of a mast cells nor pancreatic origin. PAR2 can be activated by enteric bacteria either directly by bacterial proteases, as demonstrated in the oral epithelium by proteases of *P. gingivalis*<sup>[63]</sup> and in infectious colitis by the Toxin A of *Clostridium difficile*<sup>[75]</sup>, or indirectly by bacterial-dependent induction of host proteases<sup>[76]</sup>, as discussed above. Finally, it has been reported that antibiotic treatment directed at the gut microbiota resulted in reduced PAR2 expression suggesting that PAR2 is not only activated by enteric bacteria but its expression is also regulated by the presence of these microbes<sup>[77]</sup>.

### PAR3

The biological significance of PAR3 has not been fully delineated. Structurally, this PAR isotype does not have a C-terminal intra cytoplasmic tail and thus cannot signal through GPCRs. However, PAR3 may serve as a cofactor or co-receptor of other PARs. In mouse platelets, PAR3 functions as a cofactor for PAR4 by presenting thrombin to low-affinity PAR4, thereby resulting in efficient receptor cleavage<sup>[78]</sup>. On endothelial cells PAR3 can regulate PAR activity by forming a heterodimer with PAR1<sup>[79]</sup>. Despite evidence of PAR3 mRNA expression in the small intestine, this receptor's relationship with intestinal inflammation and bacterial proteases are unknown<sup>[40]</sup>.

### PAR4

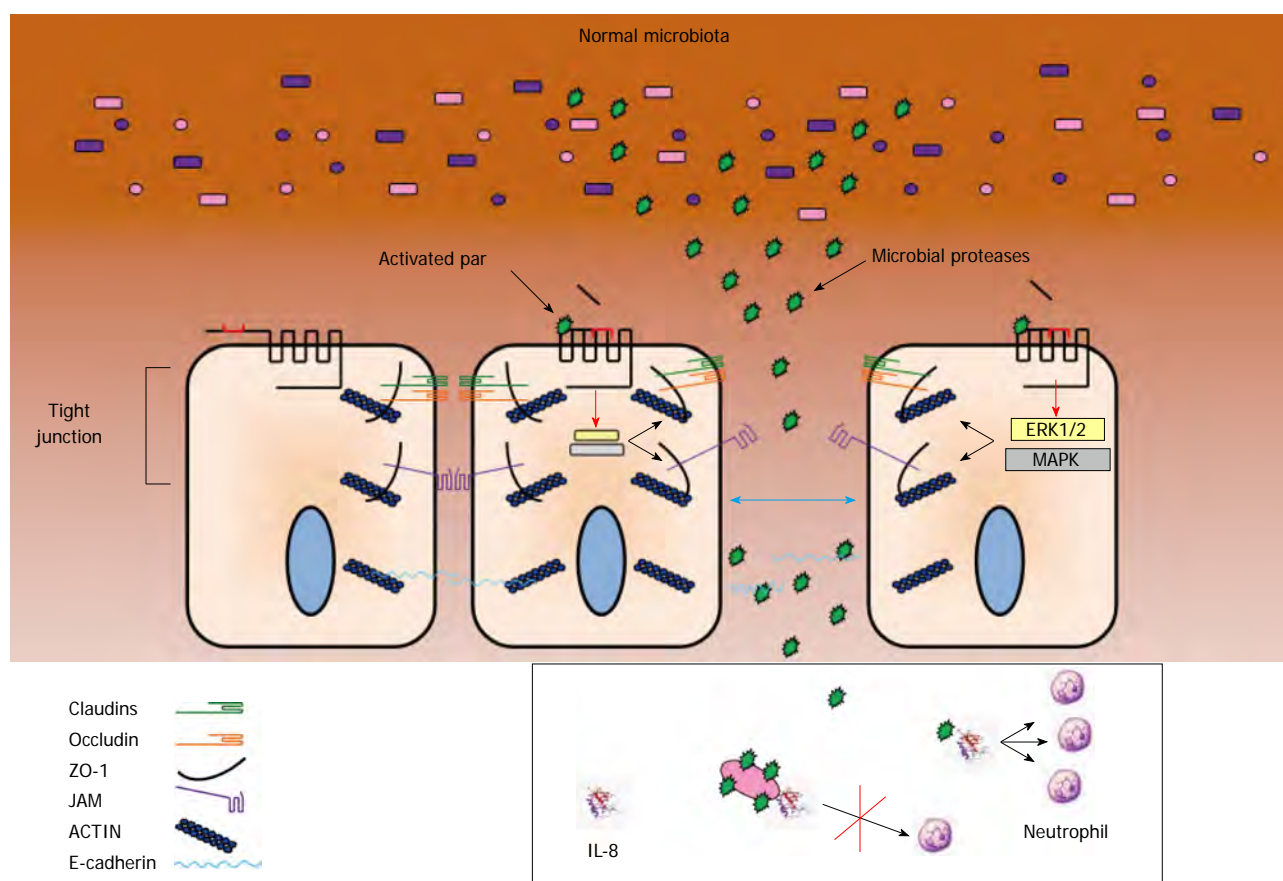
PAR4 is expressed in the small and large intestine<sup>[44]</sup> and is localized to colonocytes in rats<sup>[80]</sup>. It can be proteolytically activated by thrombin, trypsin and by the neutrophil granule protease cathepsin G<sup>[81]</sup>. Its activation induces leukocyte rolling and adherence, suggesting a pro-inflammatory role for this receptor<sup>[45,82-84]</sup>. Exposure of mouse colons to PAR4 agonists results in increased paracellular

colonic permeability, suggesting that this receptor may be involved in the pathophysiology of IBD<sup>[85]</sup>. In the human colon, expression of PAR4 on epithelial cells is negligible in non-IBD patients but is significantly higher in UC patients. Interestingly, the activity of cathepsin G was increased in the feces of UC patients compared to controls and inhibition of its activity resulted in ameliorated enteric permeability<sup>[85]</sup>. Thus, cathepsin G may mediate PAR4-dependent enteric permeability in UC patients. Nevertheless, a direct effect of bacterial proteases was not examined; therefore it is still unknown whether proteases released by the enteric microbiota contribute to enteric permeability and colitis in a PAR4 dependent manner.

## PROTEASES AND INTESTINAL BARRIER DISRUPTION

The intestinal epithelial barrier is made up of a single layer of cells that are tethered together *via* tight junctions and cell adhesion molecules. Enteric microbes can circumvent the defense of the intestinal epithelial barrier either directly through proteolytic degradation of cell adhesion molecules (such as E-cadherin) or indirectly by regulation of paracellular permeability *via* tight junctions. Intestinal epithelial tight junctions are composed of different protein complexes which consist of trans-membrane and intracellular scaffold proteins (Figure 1).

The trans-membrane proteins include occludin, claudins, and junctional adhesion molecules whose extracellular loops are bound together and intracellular domains interact with scaffold proteins such as zonula occludens (ZO), which in turn are anchored to the actin cytoskeleton. In the intestine the adherence junction protein, E-cadherin, cements epithelial cells together and is a significant factor in maintenance of the epithelial barrier function. The enteric commensal *Enterococcus faecalis* (*E. faecalis*) can induce inflammation in a gnotobiotic *IL-10*<sup>-/-</sup> mouse<sup>[86]</sup> and secretes a protease (gelatinase) which has the capacity to degrade collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a<sup>[87-92]</sup>. The potential of *E. faecalis* gelatinase to damage the intestinal epithelial barrier and cause inflammation in the *IL-10*<sup>-/-</sup> mouse was recently investigated<sup>[14]</sup>. Steck and associates created an *E. faecalis* mutant lacking the *gelE* gene ( $\Delta$ *gelE*). *IL-10*<sup>-/-</sup> mice mono-associated with *E. faecalis*  $\Delta$ *gelE* exhibited significantly lower colonic inflammation when compared to mice mono-associated with wild-type *E. faecalis*. The reduction in colonic inflammation was independent of colonization densities of *E. faecalis* strains. Interestingly, the expression of E-cadherin on epithelial cells in *IL-10*<sup>-/-</sup> mice was reduced in the presence of *gelE* (wild-type *E. faecalis*) but not when *gelE* was absent from *E. faecalis* ( $\Delta$ *gelE*). It was further demonstrated that *E. faecalis* *gelE* can degrade recombinant mouse E-cadherin. These data strongly suggest a mechanism in which a bacterial protease can disrupt the intestinal barrier function and lead



**Figure 1 Model for enteric microbial protease-dependent increased intestinal permeability.** Enteric microbial proteases activate epithelial protease activated receptors (PARs) through release of the tethered ligand. This results in intra-cellular signal transduction and activation of ERK 1, 2 and MAPK. These signaling molecules mediate disruption of tight junctions and consequently cause increased intestinal permeability that enables penetration of microbes and their proteases which can act upon cytokines. Further possible effects of bacterial proteases on the immune response are illustrated in the black box. These mechanisms have been demonstrated for *Porphyromonas gingivalis* in the oral cavity (and not in the gut) where gingipain proteases can enhance Interleukin (IL)-8-dependent attraction of neutrophils (when in their soluble forms) by partially degrading the N-terminal of this cytokine, or inhibit neutrophil activity via complete degradation of IL-8 when associated with the microbial membrane.

to inflammation. This finding is specifically significant to CD pathogenesis where a greater diversity of microbes with gelatinolytic activity was reported when compared to healthy controls<sup>[13]</sup>.

The intestinal microbiota has long been thought of as a significant contributor to the proteolytic activity of stool<sup>[24,27]</sup>. Specifically, Macfarlane *et al.*<sup>[24]</sup> found that the proteolytic activity in the stool from a patient that had undergone a pancreatectomy was comparable to that of the protease activities in stools from individuals that had not undergone surgery to remove their pancreas. This indicates that a source other than the pancreas (*i.e.*, enteric microbes) significantly contributes to the protease activity of the intestine. These observations have been more recently demonstrated by the reduction of colonic bacteria densities and protease activity by oral administration of antibiotics to mice<sup>[26]</sup>. As previously mentioned, increased protease activity has been reported in fecal samples obtained from subgroups of patients suffering from irritable bowel syndrome (IBS) and IBD<sup>[25,93,94]</sup>. Róka *et al.*<sup>[26]</sup> initially saw a four-fold increase in trypsin-like activity in diarrhea-predominant IBS (D-IBS) and UC patients.

Subsequently, it was found that fecal supernatants from D-IBS patients could increase colonic paracellular permeability in the mouse gut<sup>[94]</sup>. The application of D-IBS supernatants to the mouse colon resulted in an increase in phosphorylation of myosin light chain kinase and delayed redistribution of the tight junction-associated molecule ZO-1. Further investigations demonstrated that fecal supernatants from UC patients can affect visceral sensitivity and colonic permeability in mice that was mediated *via* differing protease receptors (see protease receptors in this review). Together these studies suggest a mechanism in which microbial proteases can alter intestinal barrier function by regulating tight-junctions.

## ENTERIC MICROBIAL PROTEASES AND IMMUNE CELL REGULATION

Once the intestinal epithelial barrier has been breached microbes or microbial antigens can potentially traverse into the underlying tissues of the intestine and interact with immune cells, ultimately leading to inflammation. Although enteric microbes are essential environmental

factors for immune cell development, as evidenced by an under established immune system found in germ-free mice<sup>[95]</sup>, the immune system can also be subverted by enteric microorganisms *via* microbial proteases. Bacterial proteases capable of disrupting cytokine signaling can potentially affect the pathogenesis of disease. For example, cysteine protease gingipains K (Kgp) and R (RgpA and RgpB) are produced by *P. gingivalis* and are significant factors in this oral microbe's pathogenesis<sup>[96]</sup>. Soluble gingipains secreted by *P. gingivalis* are capable of cleaving the N-terminus of IL-8 and enhancing this cytokine's activity of attracting neutrophils<sup>[97]</sup>. Additionally, Kgp, RgpA, and RgpB can also instantly degrade IL-8 when these enzymes are associated with membrane vesicles of *P. gingivalis*. This dual role of enhancing and inhibiting immune cell activity by the soluble and membrane-bound forms of these microbial proteases, respectively, may explain the pro- and anti-inflammatory sites found in periodontitis infections. The massive infiltration of neutrophils at periodontitis sites without the elimination of infection may also be explained by the dual roles of these microbial proteases. Another example is that of necrotizing fasciitis caused by *Streptococcus pyogenes* (*S. pyogenes*) that is characterized by an absence of neutrophils within lesions. It has been reported that the relative absence of neutrophils in necrotizing fasciitis lesions were due to restricted proteolysis of the C-terminal of IL-8 by the *S. pyogenes* protease *SlyCEP*<sup>[98]</sup>. Further investigations revealed that cleavage of the IL-8 C-terminal by *SlyCEP* from *S. pyogenes* is sufficient to reduce neutrophil endothelial trans-migration and is fundamental in the promotion of resistance of this microbe to neutrophil killing<sup>[99]</sup>. Given that a homologue of *SlyCEP* has been found in another *Streptococcus* species and no substrates other than cytokines have been identified, it is likely that this microbial protease is an effective weapon used by streptococci to impair bacterial clearance by neutrophils. Enteric microbial proteases can not only affect cytokines that are responsible for attracting the cellular branch of the innate immune system, but can also directly act upon neutrophils, macrophages, monocytes, and natural killer cells. SpeB from *S. pyogenes* has been shown to cause mitochondrial damage and prevent phagocytosis by granulocytes<sup>[100]</sup>. Additionally, a cysteine protease from *Staphylococcus aureus* (SspB) has been shown to selectively cleave CD11b on phagocytes which undergo apoptosis and are subsequently cleared by macrophages<sup>[101]</sup>. Taken together these studies identify microbial proteases from pathogenic and potentially commensal sources important molecules that have the ability to regulate the host immune system *via* specific mechanisms.

## FUTURE FOR MICROBIAL PROTEASES AND IBD

The importance of the enteric microbiota in IBD has been established during the last decade<sup>[102]</sup>. Currently, efforts are being made to decipher the pathways through which bacteria and their products cross-talk with various

cell types in the digestive tract that can potentially mediate inflammatory responses, pain or protection from chronic inflammation. The diversity of bacterial proteases and their effect on the intestinal epithelial, immune cells, and the enteric nervous system through various receptors open new avenues for research and potential therapeutic targets. Characterization of pathogenic proteases in IBD, the bacterial species that produce them and their mechanism of action are required to enhance our capability to understand the pathogenesis of these diseases and therapeutically intervene. Potential targets for therapeutic intervention include the following.

### Specific bacterial groups that carry potentially pathogenic bacterial proteases

The list of specific enteric bacteria that carry bacterial proteases that can disrupt epithelial barrier function and cause colitis in animal models is small and has been discussed earlier in this review. In humans there is even less information. However, the beneficial effects of antibiotics and probiotics in pouchitis<sup>[103,104]</sup> and IBS<sup>[105-107]</sup>, and antibiotics in CD<sup>[108]</sup> are well established. Although the proposed mechanisms for antibiotic and probiotic action are beyond the scope of this review, it is conceivable that one of the mechanisms involves action against protease-producing bacteria that cause increased permeability, pain and activation of the immune response. Future research characterizing these bacteria using high throughput sequencing, proteomics and metabolomics will potentially identify microbial targets for treatment of IBD.

### Bacterial proteases

Production of proteases is not restricted to bacteria. Host derived proteases have an important role in normal physiology of the digestion, immune response, signaling *etc.* Therefore, strategies that target bacterial derived, intraluminal, colonic proteases without harming the host may prove to be beneficial. Novel drugs for IBD could potentially target bacterial protease production or secretion, such as the serine protease autotransporters from *Enterobacteriaceae*<sup>[12]</sup>. This approach was recently demonstrated by Löwer *et al*<sup>[109]</sup> who investigated a specific inhibitor for the *Helicobacter pylori* serine protease. High temperature requirement A (HtrA) is a secreted serine protease that cleaves E-cadherin on the surface of host cells and disrupts the epithelial barrier. Through a receptor-based virtual screening method, they found a specific inhibitor of HtrA activity that was able to prevent *in vitro* cleavage of E-cadherin, without cross reactivity to mammalian proteases. HtrA is a virulence factor for other enteric bacteria, such as *E. coli*, *Shigella flexneri* and *Campylobacter jejuni*<sup>[110]</sup>. Thus, examining the ability of this inhibitor to reduce HtrA activity and its effect on intestinal inflammation and permeability in models of colitis is warranted.

An alternative approach is to use probiotics that can be beneficial through various mechanisms such as favorable metabolic effects on the epithelial cells, anti-bacterial activity or directly through production of protease inhibi-



tors. For example, *Bifidobacterium longum* and *Bifidobacterium breve* produce serine protease inhibitors (serpins)<sup>[111,112]</sup> that may antagonize potentially pathogenic bacteria proteases and may exert at least part of its favorable effects on the colon through this mechanism. Another probiotic micro-organism, the yeast *Saccharomyces boulardii*, produces a serine protease that is beneficial to the host through its activity against *Clostridium difficile* adherence to the gut wall and against its toxin, and thus suppresses bacterial overgrowth and infectious colitis<sup>[113]</sup>. These examples demonstrate that the potential favorable effects of the enteric microbiota on gut inflammation are vast and involve multiple mechanisms that are not yet fully understood.

### Protease activated receptors

PARs can be activated or antagonized by synthetic peptides that are analogous to the tethered ligand, irrespective of proteolytic cleavage of the receptor. Design of new, selective and potent drugs that correspond to the tethered ligand but also contain non-peptidic moieties may become useful in selective activation or inhibition of specific PARs. Activation of PAR2 is associated with colitis in animal models and has been used as a colitis model in rats<sup>[67]</sup> while oral administration of PAR2 antagonist resulted in amelioration of colitis. Although it is not clear if this action antagonizes host or bacterial derived proteases, the advantage of this approach is that it targets the final common receptor of the proteases, regardless of their source (bacterial or mammalian).

An additional approach would be to block PAR associated receptors. There is evidence that PAR signaling by *Candida* or *Aspergillus* on PMNs depends on the presence of TLR 2 and 4<sup>[49]</sup>. Although, similar studies regarding enteric epithelial cells is lacking, it is conceivable that such mechanisms are also required to induce PAR signaling on epithelial cells, and thus may serve as additional potential targets against activation by microbial proteases.

### Inhibitors of downstream molecular pathways

Activation of PARs by bacterial proteases results in diverse and complex signaling pathways. Characterization of specific pathways that may be inhibited to block the pathogenic effect of bacterial proteases without harming host homeostatic pathways, are required. Pepducins are such an evolving therapy<sup>[114]</sup>. Pepducins are lipoprotein molecules, composed of a synthetic peptide sequence (10-20 amino acids) that relates to the GPCR intracellular sequences and of a lipid hydrophobic moiety. The lipid component tethers the pepducin in the lipid bilayer membrane of the cells and enables these molecules to interact with specific and stabilize GPCRs (for review, Dimond *et al.*<sup>[115]</sup>). Specific pepducins that act as antagonists of PAR1 GPCR (P1pal-7) have shown favorable results in pre-clinical trials for lung cancer<sup>[116]</sup>. Additionally, PAR2 GPCR specific pepducin (P2pal-18S) ameliorates experimental pancreatitis through inhibition of PAR2 action that is expressed on pancreatic acinar cells<sup>[117]</sup> and ameliorates inflammation in additional mouse models<sup>[118]</sup>.

## CONCLUSION

In this review we have discussed the putative role and evidence of microbial proteases in inflammatory bowel disease pathogenesis. Proteases are essential for normal physiological development and are involved in numerous processes in our body. They are secreted by various cell types and their receptors are abundant in the gut wall, on immune cells, epithelial cells, and on neuronal cells. A growing amount of evidence supports a role for proteases and their receptors to IBD pathophysiology. The understanding that the enteric microbiota are crucial to disease initiation, and the fact that proteases are secreted by most bacteria and are considered virulence factors in infectious colitis, suggest that perhaps commensal bacterial proteases can also damage epithelial barrier function and may be involved in the initiation and perpetuation of IBD in genetically predisposed patients. Indeed, in this review we have summarized the current evidence that support this notion, the mechanisms through which bacterial proteases can impact the mucosal barrier function (through activation of PAR receptors), and the downstream signal pathways that result in increased epithelial permeability and perhaps in colitis.

However, it is not clear whether the proteolytic activity found in the gut lumen is exclusively of mammalian or bacterial origin. This is complicated by the fact that mammalian proteases, such as pancreatic digestive enzymes, are abundant in the gut lumen, and proteases secreted within the gut wall by leukocytes, such as neutrophils (cathepsin G) or mast cells (tryptase), “spill” into the inflamed gut. These factors may account for some of the discrepancies found between various studies investigating the origin of luminal proteolytic activity and the receptors they activate. Moreover, it is currently difficult to characterize luminal proteolytic activity, and while some research studies examine tryptic activity or gelatinase activity (each of which represents only a portion of the total luminal proteolytic activity) other studies have sought to characterize total luminal protease activity *via* functional assays and through inhibition of specific protease activity. These challenges may also explain why it is not fully clear which PAR isotype mediates increased enteric permeability and inflammation. For example, PAR1 and PAR2 have been implicated in mediating enteric inflammation or permeability by bacterial proteases or mammalian proteases while activation of PAR4 can equally result in increased enteric permeability. It is not improbable to hypothesize that for increased intestinal permeability and colitis to occur there is a multi-factorial hit process that results in activation of multiple PARs simultaneously by different proteases.

Only now we begin to unravel the effects of alterations in the normal enteric microbiota (dysbiosis), and how these “normal” bacteria can potentially induce colitis. The current challenge is to explore which commensal bacteria can secrete proteases that result in damage of the mucosal barrier. Additionally, we need to understand



which microbes are associated with colitis and what the genetically predisposing factors are that “allow” these events to happen. For example, genetic mutations associated with the reduction of mucus production and increased mucosal bacterial adherence, immune abnormalities that result in dysbiosis, and innate immune response defects that cause dysregulated immune responses once the mucosal barrier is breached.

Investigating these aspects through cell lines, mono-associated gnotobiotic animals, Ussing chambers, high-throughput sequencing of microbial DNA, metabolomics and genome wide association studies will enable us to understand the role of enteric microbial proteases in the pathogenesis of IBD and to develop effective targeted therapies that will involve specific enteric bacteria, PARs, and the downstream regulation and host immune response.

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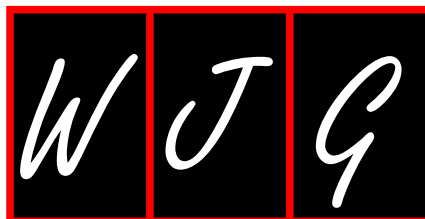


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## WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

# Beyond white light endoscopy: The role of optical biopsy in inflammatory bowel disease

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light active research areas with respect to the pathogenesis of IBD. Clinical indications for optical biopsies in IBD include assessment of mucosal inflammation, dysplasia detection and evaluation of cell shedding for disease relapse. Research application in the area of barrier dysfunction will also be discussed.

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**Key words:** Optical biopsy; Confocal endomicroscopy; Endocytoscopy; Dysplasia; Mucosal inflammation; Disease relapse; Mucosal healing; Barrier function

**Core tip:** This is a review of the latest advances in the applications of optical biopsy (either with confocal laser endomicroscopy of endocytoscopy) in inflammatory bowel disease. Clinical indications including assessment of mucosal inflammation, detection of dysplasia and predictors for disease relapse are discussed in detail. Novel research use of optical biopsy for functional mucosal assessment is also discussed.

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## Abstract

In this review, we will discuss the use of two optical biopsy modalities in inflammatory bowel disease (IBD). The two techniques reviewed here are confocal laser endomicroscopy and endocytoscopy. We will describe the technical performance of the procedure, discuss the clinical indications for optical biopsy in IBD, and high-

## INTRODUCTION

In the past decade, several advanced endoscopic imaging technologies that enable clinicians to examine the luminal gastrointestinal tract at a microscopic level were introduced. These techniques are called optical biopsies, as they are real-time histologic biopsies of the tissue. These include confocal laser endomicroscopy (CLE) and

endocytoscopy (EC). The CLE and EC systems come as either probe-based (pCLE and pEC) or endoscope-based (iCLE and iEC). In this review, we will describe the technical performance of the procedure, and discuss the clinical indications for optical biopsy in relation to inflammatory bowel diseases (IBD). We will also highlight some active research applications for optical biopsy in our understanding of the pathogenesis of IBD.

## TECHNICAL ASPECTS OF OPTICAL BIOPSY

CLE was introduced in 2003 and allows *in vivo* microscopic imaging of cellular and subcellular structures at approximately 1000-fold magnification<sup>[1]</sup>. The technique is based on tissue illumination with a low power laser after application of fluorescence agents, which can either be applied systemically (*i.e.*, fluorescein sodium) or topically (*e.g.*, acriflavine hydrochloride, cresyl violet). The laser light is reflected from the tissue and then refocused onto the detection system by the same lens, so that only returning light refocused through the pinhole is detected. Therefore, this process decreases the effect of scattered light resulting in the construction of two-dimensional grey-scale images.

Currently, two CE certified and Food and Drug Administration approved CLE-devices are available<sup>[1]</sup>. One is integrated into the distal tip of a standard, high-resolution endoscope (iCLE, Pentax, Tokyo, Japan). The other one is probe based, capable of passage through the accessory channel of any standard endoscope (pCLE, Cellvizio, Mauna Kea Technologies, Paris, France). Both systems use an incident 488 nm wavelength laser (blue laser light) enabling the detection of fluorescence between 205 nm and 585 nm wavelengths.

The iCLE-system collects images at a manually adjustable scan rate of 1.6 frames per second with a resolution of  $1024 \times 512$  pixels, or at 0.8 frames per second with a resolution of  $1024 \times 1024$  pixels with dynamically adjustable depth of scanning ranging from 0 to 250  $\mu\text{m}$ . The examiner can manually adjust the laser power between 0 and 1000  $\mu\text{W}$  and the optical slice thickness is 7  $\mu\text{m}$ , with lateral and axial resolution of 0.7  $\mu\text{m}$  and a confocal image field of view of  $475 \times 475 \mu\text{m}$ . The pCLE-system is a stand-alone confocal probe which is advanced through the working channel of any endoscope and could thereby also being used with high-definition video endoscopes in combination with dye-less chromoendoscopy (*e.g.*, Narrow Band Imaging, Fuji Intelligent Chromo Endoscopy, i-scan) as red-flag techniques.

pCLE-systems are available for different indications throughout the entire gastrointestinal tract and use a fixed laser power and a fixed image plane depth which is dependent on the probe type used. Confocal images are streamed at a frame rate of 12 frames per second. CLE in IBD is mostly being performed by using the ColoFlex Ultra-High Definition probe which requires a 2.8 mm working channel to be advanced through the scope. Lat-

eral resolution is 1  $\mu\text{m}$  and field of view is 240  $\mu\text{m}$  with an imaging plane depth of 65  $\mu\text{m}$ . In addition, a special computer algorithm ("mosaicing") allows reconstruction of single video frames with an increased field of view ( $4 \text{ mm} \times 2 \text{ mm}$ ). Costs for single probes vary and are approximately 100-200 Euro per procedure. Like any other endoscopic technique CLE requires special training in performing the procedure and interpretation of images. Therefore, especially at the beginning, extensive close collaboration with an expert histopathologist is strictly recommended. In addition, when starting with CLE optical biopsies should always be compared with physical biopsies. After an appropriate learning phase, CLE interpretation shows high inter- and intraobserver variabilities as compared to standard histology. Both CLE-systems have unique advantages. Advantages of the integrated system are its higher resolution and the possibility to alter the laser power and imaging plane depth. Advantages of the probe-based system include the possibility of an *ad hoc* usage and a greater versatility of the pCLE probes, which can be used with nearly any endoscopes.

In contrast to CLE, endocytoscopy (EC; Olympus, Tokyo, Japan) is based on the principle of contact light microscopy<sup>[2]</sup>. EC-systems are either integrated into the distal tip of a standard endoscope (iEC) or probe-based (pEC). Through-the-scope pEC-systems require a working channel of at least 3.2 mm. Similar to contact light microscopy, EC requires thorough mucolysis with N-acetyl cysteine followed by staining of the mucosa with absorptive staining agents, like methylene blue, toluidine blue, or cresyl violet. In fact, a combination of different dye agents is often used to acquire optimal tissue contrast<sup>[3]</sup>. After an appropriate time of exposure to the dye (approximately 60 s), repeat washing of the mucosa is necessary to remove the excess contrast dye before endocytoscopic imaging. Repeat staining is mostly necessary while using absorptive contrast agents. Depending on the system used (iEC or pEC), EC visualizes architectural details (*e.g.*, epithelial structure), cellular features (*e.g.*, size and arrangement of cells), and vascular pattern morphology (*e.g.*, size and tortuosity) at a magnification of up to 1390-fold<sup>[2,3]</sup>. Representative image of normal colon mucosa is shown in Figure 1.

## CLINICAL APPLICATIONS: ASSESSMENT OF INFLAMMATION IN IBD

### Endomicroscopy

Accurate assessment of mucosal inflammation in patients with IBD is of crucial importance as mucosal healing has emerged as an important treatment goal and appears to be of paramount importance for optimized medical therapy<sup>[4]</sup>. Various studies and one recent systematic review has shown that mucosal healing as assessed by endoscopy is predictive of reduced disease activity, a decreased need for active treatment, reduced rates of hospitalizations/surgical resections, and is associated with sustained clinical remission<sup>[4-7]</sup>. While standard white-light endoscopy is

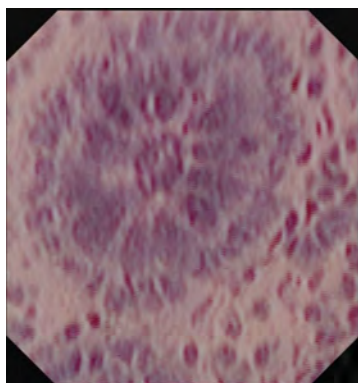


Figure 1 Endocytoscopic image at magnification  $\times 1390$  showing one single colonic crypt. Goblet and epithelial cells are clearly evident.

likely an insensitive test for assessment of mucosal healing, being false negative in up to fifty percent of patients, there is an urgent need for new endoscopic imaging techniques allowing assessment of microscopic inflammation even in case of macroscopic non-inflamed mucosa<sup>[8,9]</sup>. In this context CLE was proven to be efficient for real-time *in vivo* assessment of mucosal inflammation by requiring only a short learning curve<sup>[10]</sup>.

One early study investigated the features of CLE in inflamed and non-inflamed rectal mucosa and compared these results to standard histology<sup>[11]</sup>. On CLE, colonic crypts of normal colonic mucosa were small, round and regularly arranged, and the crypt lumens of the colonic glands were small and round. In contrast colonic crypts in non-active ulcerative colitis were small, round and slightly irregular in arrangement and the crypt lumens of the colonic glands were small and round. Inflammatory cells and capillaries were visible in the lamina propria. The colonic crypts in active ulcerative colitis were large, variously shaped and irregular in arrangement and in addition numerous inflammatory cells and capillaries were visible in the lamina propria. Li *et al*<sup>[12]</sup> confirmed these early results in a study including 73 consecutive patients. CLE-assessment of crypt architecture and fluorescein leakage showed good correlation with the corresponding histology results. Of note, more than half of the patients with normal mucosa seen on conventional white-light endoscopy revealed acute inflammation on histology, whereas no patients with normal mucosa or with chronic inflammation seen on CLE showed acute inflammation on histology. Therefore, CLE appears to be a sensitive tool for real-time assessment of inflammatory activity in patients with ulcerative colitis.

For Crohn's disease, our group evaluated in a case-control study whether CLE is feasible for *in vivo* diagnosis of Crohn's disease associated histological changes<sup>[13]</sup>. It was shown that a significantly higher proportion of patients with active Crohn's disease had increased colonic crypt tortuosity, enlarged crypt lumen, microerosions, augmented vascularization, and increased cellular infiltrates within the lamina propria. In quiescent Crohn's disease, a significant increase in crypt and goblet cell number was detected compared with controls. Based on these find-

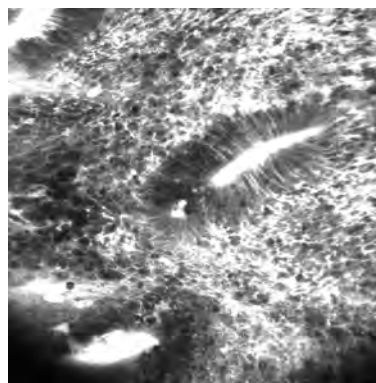
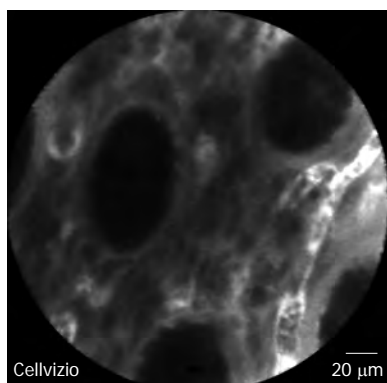


Figure 2 Endoscope-based confocal laser endomicroscopy of Crohn's disease shows remarkable cell infiltration, disturbed colonic architecture and leakage indicating severe inflammation.

ings, the Crohn's Disease Endomicroscopic Activity Score was proposed, allowing the assessment of Crohn's disease activity *in vivo*, even in macroscopically non-inflamed mucosa. Representative iCLE and pCLE images of Crohn's disease are shown in Figures 2 and 3. Taken these and the above mentioned results into account, CLE is reliable for real time *in vivo* assessment of microscopic inflammation in patients with IBD and macroscopically non-inflamed mucosa.

As most commonly used drugs for treatment of IBD are systemically bioavailable, they cover a potential risk of severe side effects. Therefore, a targeted, individualized approach to inflamed areas of the intestine with specific drugs is highly desirable. In this context, one recent study evaluated the potential of nanoparticle and microparticle uptake into the rectal mucosa of human IBD patients<sup>[14]</sup>. CLE was performed two hours after rectal application of fluorescent-labeled placebo nanoparticles and microparticles to 33 patients with IBD and healthy controls in order to visualize the particles in inflamed mucosal areas. A significantly enhanced accumulation of microparticles was observed in ulcerated areas, whereas nanoparticles were only visible in trace amount on mucosal surfaces of normal patients. Therefore, nanoparticles may enable local drug delivery to intestinal lesions in humans, thereby minimizing the risk of unintended translocation into the blood system. Very recently, our group created a fluorescent labeled antibody for molecular membrane-bound tumor necrosis factor (mTNF) imaging in Crohn's disease patients<sup>[15]</sup>. Topical antibody administration led to detection of intestinal mTNF positive immune cells during CLE. Interestingly, patients with high amounts of mTNF positive cells showed significantly higher short-term response rates at week 12 (92%) upon subsequent anti-TNF therapy as compared to patients with low amounts of mTNF positive cells (15%). This clinical response in the former patients was sustained over a follow-up period of one year and associated with mucosal healing on follow-up endoscopy. These results are promising and offer the exciting potential for individualized therapy for IBD patients using molecular imaging with fluorescent labeled antibodies.





**Figure 3** Probe-based confocal laser endomicroscopy in patient with Crohn's disease without histologic activity. Colonic crypts are regularly arranged with normal shape and size. Micro-vessels within the lamina propria are easily visible. Bar = 20  $\mu$ m.

### Endocytoscopy

Only limited data are currently available on the potential of EC in patients with IBD. One recent article correlated the efficacy between endocytoscopy and conventional histopathology for assessment of microscopic features in patients with ulcerative colitis<sup>[16]</sup>. Fifty-five patients were included and mucosal patterns were evaluated by using EC with  $\times 450$  magnification. Based on EC-findings a scoring system was developed that showed a strong correlation with Matts' histopathological grades. In addition, there was a strong correlation between the conventional Matts' endoscopic grade and Matts' histopathological grade. Furthermore, the newly developed EC-score showed high reproducibility among investigators with a  $\kappa$  value of 0.79. Another recent study determined the reliability of EC for the discrimination of mucosal inflammatory cells and intestinal inflammatory disease activity in patients with IBD<sup>[17]</sup>. In total, 40 patients were included and EC was reliable to distinguish single inflammatory cells, including neutrophilic, basophilic, and eosinophilic granulocytes and lymphocytes. Sensitivity and specificity ranges among different cell types between 60% and 89% and 90% and 95%, respectively. Interobserver agreement among investigators was substantial whereas intraobserver agreement was almost perfect. Moreover, concordance between endocytoscopy and histopathology for grading of intestinal disease activity was 100%.

In conclusion, EC holds significant potential in identifying early signs of mucosal inflammation in real-time by identifying single mucosal inflammatory cells in conjunction with architectural details. Large, prospective multicenter trials evaluating EC for prediction of disease course in IBD are thus highly warranted and anticipated.

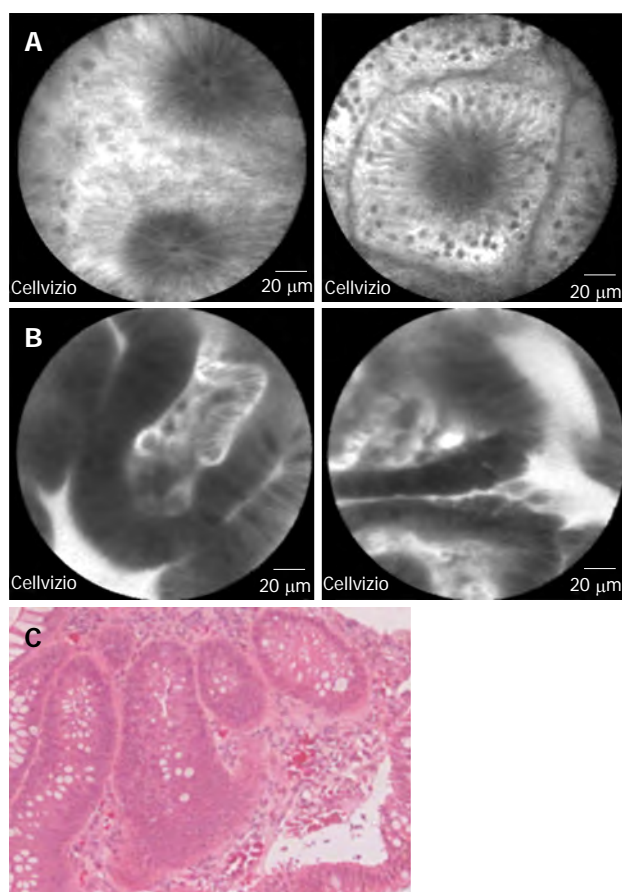
## CLINICAL APPLICATIONS: DYSPLASIA DETECTION IN IBD

Patients with IBD are at an increased risk for the development of dysplasia (also known as intraepithelial neoplasia) and colitis associated cancer (CAC). The risk of

developing CAC is comparable for Crohn's colitis and ulcerative colitis patients. In both cases the risk is increased with: duration of colitis, early age of IBD onset, extent of colonic involvement, severity of inflammation, family history of colorectal cancer and particularly the presence of primary sclerosing cholangitis<sup>[18]</sup>. The cumulative risk for developing CAC in primary sclerosing cholangitis associated IBD (PSC-IBD) patients is 33% at 20 years and 40% at 30 years compared to 8% and 18% in IBD without concomitant liver disease<sup>[19]</sup>. Although IBDs contribute only 1%-2% to all cases of colorectal cancer, the cancer-related mortality rate in IBD patients is approximately 15%<sup>[20,21]</sup>.

Colonoscopy with random biopsies, which is the gold standard for CAC screening in long standing IBD, can significantly reduce CAC-related mortality<sup>[22]</sup> and IBD patients undergoing surveillance colonoscopy had detection of neoplasia at an earlier stage, resulting in a better corresponding prognosis<sup>[23]</sup>. The principal limitation with this modality is that neoplasia may not be appreciated in up to a third of colonoscopies<sup>[24,25]</sup>. Taking four-quadrant biopsies is time-consuming and has only moderate sensitivity for neoplasia detection. The efficacy of surveillance for detection of early malignancies could also be questioned from the standpoint of cost and adherence. A population-based analysis of the cost and practice of colonic surveillance of patients with PSC-IBD in Alberta, Canada revealed that only 1/3 of the colonoscopies expected were actually performed, but despite suboptimal surveillance, the incidence of colorectal neoplasia was high. The study also found that the cost of finding one additional case of dysplasia was substantial<sup>[26]</sup>. Moreover, dysplasia in IBD can be found at distant sites from the cancer itself or before the cancer develops and is difficult to recognize on colonoscopy, as it often arises from flat, normal-appearing mucosa<sup>[20]</sup>. Dysplasia can also occur within or near plaque-like lesions or raised polypoid masses, defined as dysplasia-associated lesion or mass (DALM).

Targeted biopsies are an attractive alternative to random biopsies to increase the yield of dysplasia detection. CLE allows evaluation of suspicious lesions at the subcellular level with great detail prior to performing targeted biopsies, thus facilitating earlier diagnosis of CAC. While CLE only covers a limited field of view within the mucosa, pan-CLE of the whole colon is not feasible. Therefore, macroscopic visualization of suspected areas with chromoendoscopy (red flag technique) is useful before performing targeted endomicroscopy<sup>[27,28]</sup>. Prospective evaluation of CLE with concurrent chromoendoscopy predicted neoplasia in a randomized controlled trial of ulcerative colitis patients ( $n = 153$ ) with great accuracy (98%), sensitivity (95%) and specificity (98%). By using methylene-blue aided chromoendoscopy with CLE, the diagnostic yield of neoplasia was increased by 4.75-fold compared with conventional colonoscopy with random biopsies ( $P = 0.005$ ), though 50% fewer biopsy specimens were required<sup>[29]</sup>. Hurlstone *et al.*<sup>[30]</sup> demonstrated



**Figure 4** Probe-based confocal laser endomicroscopy images of (A) normal colonic mucosa; B: Adenomatous polyp with low grade dysplasia. Bar = 20  $\mu$ m; and C: The corresponding histologic image for the adenomatous polyp with dysplasia (magnification  $\times 40$ ).

remarkable results in a smaller ulcerative colitis cohort ( $n = 36$ ), where CLE enabled differentiation of DALM and adenoma-like mass with 97% accuracy and an excellent agreement between endomicroscopy and histopathological diagnosis was found ( $P = 0.91$ ). CLE based, targeted biopsies increased the diagnostic yield of intraepithelial neoplasia by 2.5-fold compared with chromoendoscopy-guided biopsies alone. One recent pilot study of pCLE during colonoscopic surveillance of patients with long-standing ulcerative colitis ( $n = 22$ ) demonstrated that the method is feasible with reasonable diagnostic accuracy<sup>[31]</sup>. A recent meta-analysis of 15 studies of confocal endomicroscopy for dysplasia detection<sup>[32]</sup> showed CLE could distinguish neoplasms from non-neoplasms in IBD patients for surveillance with a sensitivity of 0.83 (95%CI: 0.70-0.92) and specificity of 0.90 (95%CI: 0.87-0.93). Representative pCLE images of adenomatous polyp with low grade dysplasia and corresponding histologic images are shown in Figure 4.

CLE seems to be a particularly promising method for dysplasia detection in PSC-IBD patients. PSC-IBD is recently suggested to represent a specific IBD phenotype characterized by extensive colitis, low inflammatory activity, right-sided colonic inflammation and a high risk

of CAC. Jørgensen *et al*<sup>[33]</sup> showed a difference between the macroscopic and microscopic picture in PSC-IBD: in general the inflammatory activity in these patients was low and was not always visible endoscopically, though it could be seen histologically. Since proximal cancers are more common in PSC-IBD patients<sup>[34,35]</sup> and inflammatory activity is not always visible endoscopically the use of CLE may increase surveillance efficiency particularly in the right colon. One ongoing study evaluates the efficacy of pCLE as a complementary tool to high definition white-light endoscopy (HD-WLE) for the detection of colonic dysplasia in patients with PSC-IBD. Preliminary results ( $n = 25$ ) showed excellent accuracy (99%), sensitivity (93%) and specificity (100%) of pCLE in dysplasia detection that was superior to HD-WLE alone<sup>[36]</sup>. Low-grade intraepithelial neoplasia was found in 20% of patients and 60% of confirmed dysplastic lesions were localized in the right colon. These preliminary results suggest that careful CLE examination of at least the right colon in PSC-IBD patients may be warranted.

## CLINICAL APPLICATIONS: ASSESSMENT OF CELL EXTRUSION AND BARRIER FUNCTION FOR DISEASE RELAPSE

Mucosal healing has emerged as the most important endoscopic predictor of disease relapse in IBD patients. In the pre-biologic era, mucosal healing was associated with lower rate of relapse in ulcerative colitis but not Crohn's disease patients<sup>[6]</sup>. In the biologic era, mucosal healing is predictive of clinical and endoscopic remission for both Crohn's<sup>[7,37]</sup> and ulcerative colitis patients<sup>[38,39]</sup>. The use of optical biopsy in the small bowel in IBD patients have been studied by several groups<sup>[1,40,41]</sup>. The principal finding on CLE that appears to predict disease relapse is epithelial cell extrusion and associated barrier dysfunction<sup>[42,43]</sup>. Using CLE, Kiesslich *et al*<sup>[40]</sup> first reported unambiguous identification of epithelial gaps or extrusion zones in the intestine of patients. Epithelial cell extrusion occurs as part of a normal physiological renewal process of the intestine. Therefore, qualitative description of the presence or absence of epithelial gaps is not sufficient to discern a diseased from a healthy state. We have therefore developed a quantitative measure called epithelial gap density, defined as the total number of epithelial gaps counted normalized to the total number of epithelial cells counted on pCLE images<sup>[41]</sup>. Gap density was validated against conventional multi-photon confocal microscopy and conventional white-light microscopy<sup>[44]</sup>. The gap density was found to be increased in IBD patient<sup>[45]</sup>. More recently, increased gap density and certain types of epithelial gaps were found to be predictive of aggressive disease<sup>[43]</sup> and relapse in IBD patients<sup>[42]</sup>. Thus, qualitative and quantitative studies of epithelial gaps in the small intestine may have a role in defining new management algorithms for IBD patients.

The clinical applications of CLE (both pCLE and

iCLE) in the small intestine of IBD patients have been largely in the evaluation of the terminal ileum<sup>[40,42,43,45]</sup>. The terminal ileum is the preferred site of intestinal evaluation in IBD for a number of reasons: (1) it is within the reach of a standard colonoscope; (2) it is an active site of immunologic activity; (3) it is often the first site of disease in Crohn's patients; and (4) it is usually possible to find endoscopically normal appearing areas, even in patients with active Crohn's ileitis. We have devised a quantitative determination of the density of epithelial gaps or extrusion zone called the epithelial gap density in the terminal ileum<sup>[41,45]</sup>. The epithelial gap density derived using CLE could be validated against multi-photon confocal microscopy and light microscopy<sup>[46]</sup>. Increased epithelial gap density could identify patients at high risk for major events such as hospitalization or surgery in follow up<sup>[43]</sup>. In another recent study, certain types of epithelial gaps were found to be predictive of disease relapse in IBD patients<sup>[42]</sup>. The current evidence seems to suggest that quantification and evaluation of extrusion zones in the terminal ileum of IBD patients have predictive values for disease relapse. However, these are single-centered studies, and there are no multicenter trials to determine the role of CLE in the management of IBD patients.

## RESEARCH APPLICATIONS: FUNCTIONAL MUCOSAL ASSESSMENT IN IBD

The intestinal epithelium functions as a selective barrier to the luminal contents<sup>[47,48]</sup>. The epithelium separates the immune and neural networks of the human intestine from the intestinal microbiota, which comprises an estimated  $10^{13}$  to  $10^{14}$  microorganisms<sup>[49]</sup>. Compromised epithelial barrier expose the subepithelial immune system to resident microbes which induces secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ <sup>[50]</sup>, which in turn induces shedding of epithelial cells from the intestine which then further contributes to barrier dysfunction and promotes inflammation<sup>[51]</sup>. Other proinflammatory cytokines, including interleukin (IL)-1 $\beta$ , IL-6, IL-18 and TNF- $\alpha$  have been reported to be increased in active IBD and correlate with endoscopic severity of inflammation<sup>[52,53]</sup>.

Until advances of optical biopsy, either *via* CLE or EC, it was not possible to investigate the interactions between the intestinal epithelium and resident microbiota *in vivo*. Histologic evaluation of the intestinal mucosa is limited by fixation and processing artefacts, including contamination with luminal microbes. CLE was used to identify intramucosal bacteria, and IBD patients were found to have significantly higher distribution of involvement with intramucosal bacteria in the colon and terminal ileum<sup>[54]</sup>. CLE was recently used for molecular imaging to identify single bacteria species and *in vivo* diagnosis of bacteria associated colitis<sup>[55]</sup>.

Barrier dysfunction is another area of research interest for the past few years. Restoration of normal epithelial barrier function which prevent the translocation of

commensal bacteria is the structural basis of mucosal healing<sup>[4]</sup>. Intestinal permeability function as assessed with disaccharide solutions<sup>[56,57]</sup> has not been widely adopted for clinical use. Optical biopsy permits assessment of mucosal barrier function in the appropriate structural context. Studies have shown increased epithelial cell shedding may contribute to barrier dysfunction in the intestine<sup>[42,58]</sup>. The clinical relevance of cell shedding and barrier dysfunction is reflected in the ability of these measures to predict disease relapse and major events in follow up<sup>[42,43]</sup>.

## CONCLUSION

Optical biopsy of the intestine in IBD have been used for a variety of clinical and research indications, and appears to hold significant promise to improve the diagnosis and management of IBD patients. Future large, multi-centered studies are needed to validate these early study findings to facilitate clinical adaptation of this group of new advanced imaging technique.

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## WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

# Mucosal healing and deep remission: What does it mean?

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## Abstract

The use of specific terms under different meanings and varying definitions has always been a source of confusion in science. When we point our efforts towards an evidence based medicine for inflammatory bowel diseases (IBD) the same is true: Terms such as "mucosal healing" or "deep remission" as endpoints in clinical trials or treatment goals in daily patient care may contribute to misconceptions if meanings change over time or definitions are altered. It appears to be useful to first have a look at the development of terms and their definitions, to assess their intrinsic and context-independent problems and then to analyze the different relevance in

present-day clinical studies and trials. The purpose of such an attempt would be to gain clearer insights into the true impact of the clinical findings behind the terms. It may also lead to a better defined use of those terms for future studies. The terms "mucosal healing" and "deep remission" have been introduced in recent years as new therapeutic targets in the treatment of IBD patients. Several clinical trials, cohort studies or inception cohorts provided data that the long term disease course is better, when mucosal healing is achieved. However, it is still unclear whether continued or increased therapeutic measures will aid or improve mucosal healing for patients in clinical remission. Clinical trials are under way to answer this question. Attention should be paid to clearly address what levels of IBD activity are looked at. In the present review article authors aim to summarize the current evidence available on mucosal healing and deep remission and try to highlight their value and position in the everyday decision making for gastroenterologists.

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**Key words:** Inflammatory bowel disease; Mucosal healing; Deep remission; Treatment targets; Clinical activity

**Core tip:** "Mucosal healing" and "deep remission" have been discussed heavily as "new" treatment goals in inflammatory bowel diseases patients in recent years. This was based on evidence that the long term disease behaviour appears to be better, when mucosal healing is achieved. Unfortunately, a definite proof that therapy escalation for patients in clinical remission not achieving mucosal healing will be beneficial is still lacking. Clinical trials are under way to answer this question. At the moment it appears to be helpful to summarize the current evidence available on mucosal healing and deep remission to support the everyday decision making for gastroenterologists.

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## INTRODUCTION

Assessing the activity of inflammatory bowel disease (IBD) is important for our daily practice treating patients with these chronic inflammatory diseases. The assessment of disease activity will guide our therapeutic decision and our choice of medication. Furthermore it is most important for clinical investigations of new treatment options and new drugs. The reduction of disease activity remains the most important endpoint in clinical trials.

However, the discussion on which parameters are most useful for this purpose is still ongoing and unresolved.

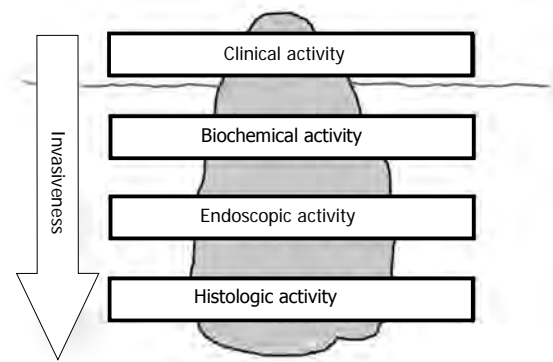
Assessment of activity of IBD can be performed on different levels such as clinical activity, biochemical activity (*e.g.* by measuring CRP or fecal calprotectin), endoscopy, and histology. Clinical remission in a given IBD patient does not necessarily imply biochemical, endoscopic, or histologic remission. To evaluate biochemical, endoscopic, and histologic activity, an increasing degree of invasive measures (blood sample, endoscopy, biopsies) is required. Assessing activity in IBD has thereby analogies to the iceberg phenomenon where the clinical assessment on the surface may show clinical remission, but inflammatory activity may still be present on biochemical, endoscopic, and histologic level (Figure 1).

## HISTOLOGICAL REMISSION AS INITIAL DEFINITION OF MUCOSAL HEALING

One of the first scientists and clinicians that used the term “healing” or “mucosal healing” within the field of IBD was Burton I. Korelitz, past chief of the Division of Gastroenterology at Lenox Hill Hospital in New York<sup>[1]</sup>. However, he used this term exclusively with respect to histological changes of the mucosa<sup>[1]</sup>. So when the term “mucosal healing” was introduced into IBD clinic it meant the absence of histological alterations of the mucosa. Korelitz was well aware that healing of IBD is not regarded to be possible as both Crohn’s disease (CD) and ulcerative colitis (UC) are regarded to be chronic diseases without spontaneous healing<sup>[2]</sup>. There may be an absence of symptoms and flares over years but mucosal inflammation may re-occur after remission for years or even decades (Figure 1).

Histological healing is difficult to determine especially in Crohn’s disease as the inflammation may be patchy and a biopsy could miss an inflammatory infiltrate only a few millimeters away<sup>[3]</sup>. Similarly, in UC the histological evaluation of a biopsy may be misleading<sup>[4]</sup>. Histological alterations may be absent from the rectum and sigmoid due to effective topical therapy despite the presence of

Activity assessment in IBD: the iceberg phenomenon



**Figure 1** Activity assessment in inflammatory bowel disease: The iceberg phenomenon. IBD: inflammatory bowel disease.

inflammation further proximal in the colon that may not be obvious to the endoscopist<sup>[4,5]</sup>. Histological healing would mean that we have to be sure that there had been an inflammatory infiltrate at a specific localization that completely disappeared upon therapy (or spontaneously). As is obvious this is hard or even impossible to prove as this would require frequent endoscopies with many biopsy samples and a labeling of former biopsy locations. Due to the impracticability of this approach the overall acceptance of the concept of “histological healing” was very limited<sup>[5]</sup>. Of note, newer techniques such as endomicroscopy suffer from the same shortcomings.

## ENDOSCOPIC REMISSION AS A NEW CONCEPT FOR MUCOSAL HEALING

In contrast to the initial concept of “mucosal healing” as a “disappearance of inflammatory infiltrate”<sup>[2]</sup> recent original manuscripts and reviews on the topic have used the term under different meanings. The “newer” meanings of “mucosal healing” have been summarized again by Korelitz in a critical review<sup>[2]</sup>. One of the “newer meanings” of mucosal healing would be the absence of inflammation (“healed mucosa”) to the eye of the endoscopist, a definition that now has been applied in many clinical trials<sup>[6-16]</sup>.

There is an obvious problem with this definition. One must assume the location of endoscopically normal mucosa has previously been inflamed<sup>[2]</sup>. Certainly this is easier to assess with endoscopy rather than histology as the area of evaluation is larger and small local differences and a patchy pattern would play a less important role. Nevertheless it requires that two endoscopic examinations are compared.

The definition also ignores that in endoscopically normal appearing mucosa there still may be histological inflammation. Another problem of this definition of course is that the inter-observer reproducibility of endoscopic IBD scores usually is very poor<sup>[17]</sup> and depends on the experience of the endoscopist<sup>[18]</sup> regardless of the technique used<sup>[19,20]</sup> (it may be discussed whether a kappa

between 0.7 and 0.8 is satisfying). Usually endoscopic findings are assessed on fixed point scales or described by dichotomous variables (present/absent)<sup>[18,21]</sup>. However, as outlined by de Lange and colleagues “endoscopic features of mucosal inflammation are continuous variables” for which dichotomous decisions are artificial and always require individual decisions<sup>[18]</sup>. The question arises how to interpret endoscopical findings indicating a clearly improved appearance of the mucosa in endoscopy with some or few remaining scattered erosions. A further important question arises with respect to endoscopical findings that cannot be interpreted as present inflammation but as residuals of former inflammation and a lack of complete normalization of the mucosa. Such findings would be pseudopolyps in an otherwise normal-appearing colon.

## BIOCHEMICAL (FECAL MARKERS) REMISSION AS MUCOSAL HEALING

Fecal markers such as calprotectin or lactoferrin correlate very well with the degree and extent of infiltration of the mucosa by leukocytes. A good correlation between fecal calprotectin and the Crohn's Disease Endoscopic Index of Severity (CDEIS) was reported in several studies<sup>[22,23]</sup>. There is also a good correlation of fecal calprotectin with the Simple Endoscopic Score for Crohn's disease (SES-CD) which itself has a strong correlation with the CDEIS (correlation coefficient  $r = 0.920$ ) and an excellent inter-observer reliability ( $\kappa$  coefficients 0.791-1.000)<sup>[24]</sup>.

In ulcerative colitis calprotectin correlates well with disease activity as determined by histology and endoscopy<sup>[25,26]</sup>.

It is a familiar experience to endoscopists that the mucosa may appear completely normal (healed) in patients that still have a markedly elevated fecal calprotectin. This would be an endoscopic remission but not biochemical remission, most likely reflecting a lack of histological remission with neutrophils still being present in the mucosal wall. It has been well established that calprotectin better correlates with histological findings (at least in UC) as compared to serum parameters or endoscopy<sup>[27-29]</sup>.

## MUCOSAL HEALING AND DEEP REMISSION: THE CONFUSED CLINICIAN

Surprisingly, some recent trials have reported a higher relative amount of patients with mucosal healing compared to the percentage of patients with clinical remission, especially in UC<sup>[30]</sup>. In those trials usually the endoscopist defined whether mucosal healing was present. How can this be explained? One reason could be that those patients had concomitant irritable bowel syndrome that was responsible for their complaints but no relevant remaining inflammation (“IBS superimposed on IBD”). The argument is straight forward and logical but it probably does not explain all cases. Firstly, little or no information is available on the

histological remission in those patients. Histological remission - if evaluated by biopsies - again may be patchy and the evaluated biopsies may not be representative. Damage to deeper layers of the mucosa may have occurred that are not visible to the endoscopist's eye. Therefore it has to be challenged whether healed mucosa to the eye of the endoscopist is indeed the “most satisfying objective confirmation to support the clinical response” as outlined by Korelitz<sup>[2]</sup>. As he states the endoscopic healing “might be satisfactory for comparison in time for response to therapy in an individual case, but not for mucosal healing as an entity and certainly not to be used as an index of response to therapy in trials.”<sup>[2]</sup>

To minimize the subjective component many clinical trials now apply the principle of a “central reader”. Not only does this make trials more complicated, more expensive and more time consuming. It substitutes the problem of a bias introduced by many subjective evaluations of the mucosal response to a bias introduced by one subjective interpretation of findings. The intra-observer agreement for many endoscopic scores is not satisfactory. It may well be argued that the subjective criteria used by a central reader may not be accepted by others and that there could be a reduction of bias by a “multi-subjective” view (as we assume is the case for multicenter trials as compared to monocentric studies). Of note, in a recent randomized-controlled trial in patients with UC the conclusion was significantly changed after blinded central review of endoscopic images, suggesting that central reading of endoscopy may be necessary for regulatory purposes<sup>[31]</sup>. However, the question about the best method of objective endoscopic assessment is far from being answered.

Korelitz<sup>[2]</sup> suggested that histological healing should be the “minimal criterion for mucosal healing and preferably this information should be derived from multiple biopsy sites of previous inflammation”. However, this would implicate that the evaluation of inflammation by a pathologist is objective. There have been studies on the inter-observer and intra-observer agreement of pathology findings<sup>[32]</sup>. Those results are not very encouraging. When a number of established criteria were used (excess of histiocytes in combination with a villous or irregular aspect of the mucosal surface and granulomas) experienced pathologists could correctly classify 70% of CD patients and 75% of UC patients<sup>[32]</sup>. Especially in mild disease, there is still dispute as to whether the presence of a “physiological (minor) inflammation” should be regarded as manifestation of IBD or not. Clinically unaffected siblings of IBD patients may show mild histological inflammation and increased cellular activation markers<sup>[33]</sup>. Cell counting will not solve the problem. The request for a “central pathology reader” also is not helpful as the same dilemma as for the central endoscopy reader will occur. Moreover, different pathologists have suggested different criteria to evaluate the presence or absence of “un-normal” inflammation (for an overview see<sup>[3,34-37]</sup>). There is no agreement on that. Geboes for ex-



ample suggested that the presence of neutrophils in the intestinal epithelium is an important discriminator for the presence or absence of inflammation. He therefore suggested that a combination of endoscopy and histology should be used to evaluate the presence of inflammation in IBD patients to finally judge whether mucosal healing has been achieved (see above).

## MUCOSAL HEALING AND DEEP REMISSION: THE CONFUSED SCIENTIST

CD and UC are regarded to be chronic diseases that never disappear. The concept of a healing of a part of the body affected by such a disease subsequently is surprising for scientists working on the elucidation of the pathophysiology of IBD.

However, there is another aspect that is disturbing. There have been reports that even in macroscopically and microscopically normal appearing mucosa specific changes can be found that are characteristic for inflammation or at least changes that could be associated with the pathophysiology<sup>[38-45]</sup>.

Changes of the microbiota in the lumen of the gut have been described in IBD patients despite the absence of detectable inflammation<sup>[46-51]</sup>. Could a “complete deep remission” be possible without normalization of the intestinal microbiome? The mucus layer of the mucosa may be changed also in normal appearing mucosa in endoscopy<sup>[52-56]</sup>. The normal fixation procedure of biopsies and the subsequent H&E staining does not allow evaluation of the mucus layer as it is destroyed during this procedure. A reduced thickness of the mucus layer in UC in remission has been described<sup>[54,56,57]</sup> as well as a reduced secretion of mucin<sup>[52,53,58-60]</sup> or defensins<sup>[61-64]</sup>. The question arises whether the mucosa can be termed as “normal” or “healed” if those changes are still present.

Epithelial cells may have an impaired barrier function despite a lack of inflammatory signs. Cytokine expression and cytokine secretion by immune cells may still be significantly increased despite a normal appearing histology. A normalization of those changes has been termed biochemical healing<sup>[65-68]</sup>. There are no data available with respect to the predictive value of “biochemical healing” and whether this would correlate to a more favorable disease outcome.

The confused scientist, however, is able to imagine a further level of “healing”. In macroscopically normal appearing mucosa with microscopically normal appearing cells that display normal cytokine expression and secretion levels, epigenetic changes may still be present that may trigger pathological responses upon minor stimuli<sup>[69-76]</sup>. Can a persistence of epigenetic changes in otherwise normal mucosa be termed “mucosal healing”? Or do we have to achieve “epigenetic healing” to finally achieve the best outcome possible for our patients? These questions will have to be answered in the future. Currently we are just at the start of investigations into these aspects with the first interesting pieces of the puzzle be-

ing put together.

## MUCOSAL HEALING AND DEEP REMISSION: THE CONFUSED “TRIALIST”

As mentioned above the terms “mucosal healing” and “deep remission” have been used in a number of trials with quite different meanings and definitions. The key confounder is the lack of unequivocal definition(s). Therefore, results and data from those trials with respect to mucosal healing cannot easily be compared. Nevertheless, this is done frequently. In most cases endoscopic investigation is used for the evaluation of “mucosal healing”. One crucial point is whether “mucosal healing” was defined simply as the absence of ulcers when ulcers had been seen previously or whether the absence of ulcerations and ulcers was investigated exactly at a place where those alterations had been found before.

The above is reflected in the way different trials have been reported. In the ACCENT 1 endoscopic sub-study the CDEIS was used for scoring and the complete absence of mucosal ulcerations that were observed at baseline was evaluated<sup>[77]</sup>. In the SONIC study in contrast no clearly defined score was used. Mucosal healing was defined as “complete absence of mucosal ulceration in the colon and terminal ileum”<sup>[78]</sup>. In the “Top-down versus step up” study by Gert D’Haens and coworkers SES-CD was used for the evaluation of mucosal healing which was a secondary endpoint<sup>[79,80]</sup>. Mucosal healing was defined as “absence of ulcers”. In the MUSIC trials again the CDEIS was applied. The definition of mucosal healing was “absence of ulcers and endoscopic remission defined as CDEIS < 6”. In the EXTEND study applying again SES-CD mucosal healing was seen as “absence of mucosal ulceration”<sup>[81]</sup>. As is obvious from those definitions, the question arises whether a few remaining aphthous lesions in a patient with severe and deep ulcers at the beginning of therapy also may be termed mucosal healing.

For UC the IOIBD attempted a consensus for mucosal healing in 2007: “absence of friability, blood, erosions and ulcers in all visualized segments of the gut mucosa”. According to the IOIBD experts the presence of an abnormal vascular pattern is still compatible with mucosal healing or “normal mucosa”. However, also in UC the definitions applied varied widely: In the ACT1 study mucosal healing was a secondary endpoint<sup>[82,83]</sup>. The Mayo endoscopic subscore was used and mucosal healing was defined as “absolute subscore for endoscopy of 0 or 1”<sup>[82,83]</sup>. The same definition was used for ULTRA 2<sup>[84]</sup>.

In studies on the outcome of therapy with 5-aminosalicylic acid the definition of mucosal healing largely defined the number of patients achieving this endpoint (Table 1). As an example, Vecchi *et al.*<sup>[85]</sup> compared mesalazine 4 g orally *vs* 2 + 2 g orally and enema in 2001 in patients with a clinical activity index (CAI) of 4-12 and used an endoscopic Rachmilewitz index < 4 as definition of mucosal healing leading to 58% *vs* 71% of patients

**Table 1 Association between the definitions of remission and mucosal healing and actual healing rates in patients with ulcerative colitis treated with mesalazine**

Author	Design	Study	Timing of endoscopy	Endoscopic index	Def. of MH	No of pat. Achieving MH
Vecchi (2001)	Mc, RCT	Mesalazine 4 g orally <i>vs</i> 2 + 2 g orally and enema	6 wk	Rachmilewitz	Rachmilewitz < 4	58% <i>vs</i> 71%
Malchow (2002)	Mc, db, RCT	Mesalazine 4 g enema <i>vs</i> 1 g foam	4 wk	Rachmilewitz	Rachmilewitz < 2	38% <i>vs</i> 37%
Mansfield (2002)	Mc, db, RCT	Balsalazide 6.75 g <i>vs</i> sulfasal. 3g	8 wk	4 point scale	Score of 0 = normal mucosa	27% <i>vs</i> 25%
Hanauer (2007) Ascend	Mc, db, RCT	Asacol 4.8 g <i>vs</i> 2.4 g	6 wk	Descriptive, no score	Normal endoscopic finding	25% <i>vs</i> 20%
Kamm (2007) MMX	Mc, db, RCT	MMX mes. 4.8 g <i>vs</i> 2.4 g <i>vs</i> placebo	8 wk	Mod. Sutherland index	Mod Sutherland index < 1	77% <i>vs</i> 69% <i>vs</i> 46%
Kruijs (2009)	Mc, db, RCT	Mesalazine 3 g <i>vs</i> 1g x 3	8 wk	Rachmilewitz	Rachmilewitz < 4	71% <i>vs</i> 70%

Mc: Multicenter; db: Double-blind; RCT: Randomized controlled trial; MH: Mesalazine.

**Table 2 One of the problems in endoscopic ulcerative colitis scores is the application of varying criteria**

	Truelove	Baron	Powell-T (St Mark's)	Levine	Rach-milewitz	Modified Baron	Mayo	Sutherland
Erythema						+	+	
Edema				+				
Granularity				+	+	+		
Vascular pattern		+		+	+	+	+	
Friability	+	+	+	+	+	+	+	+
Erosions				+	+		+	
Ulceration				+	+	+	+	
Exudate					+			+
Remission	0	0-1			0-2	0-1	0-1	0

achieving this endpoint<sup>[85]</sup>. In 2002 Malchow compared Mesalazine 4g enema *vs* 1g foam preparation in patients with a CAI > 4 for 4 wk and applied an endoscopic Rachmilewitz index < 2 as definition of mucosal healing leading to rates of 38% *vs* 37%<sup>[86]</sup>. As one would expect, the different definitions used cause huge variation in defined endoscopic mucosal healing rates in patients with UC, which makes the comparison of efficacy of different drugs or formulations extremely difficult.

One of the problems in endoscopic UC scores is the application of varying criteria (see Table 2). The reasons for such different definitions and endpoints may only be speculated. Unfortunately we lack an unequivocal definition; all of the scoring systems published so far have certain limitations, which have led to the introduction of several additional scoring systems. From a patient's and physician's perspective, however, the use of one single scoring system would be most desirable to enable valid comparisons among study outcomes.

## WHAT IS THE ADDITIVE VALUE OF DEEP REMISSION AS COMPARED TO MUCOSAL HEALING?

“Deep remission” is another term that has been discussed as a treatment target in recent years. The definition, however, is unfortunately not clearer than the one of mucosal healing. In the EXTEND study “deep

remission” was defined as clinical remission (CDAI < 150) and complete mucosal healing as defined according to CDEIS<sup>[13]</sup>. It is worthwhile to look a bit closer at this definition. If a patient with CD achieves mucosal healing but still has increased CDAI (no clinical remission) this may be due to superimposed IBS symptoms or the fact that without the presence of inflammation there is some bowel damage such as a fibrotic stricture or an internal fistula which might contribute to increased bowel frequency. Subsequently the lack of clinical remission is important for the patient and his/her clinical management (*e.g.* surgery of the stricture) but not for the medical (anti-inflammatory) management of the disease. Thus, the term “deep remission” in the definition outlined above is not useful and does not provide more information than mucosal healing. In fact - it contributes to confusion of scientists, clinicians and “trialists”.

## HOW CAN WE IMPROVE?

There should be standards on the definition of mucosal healing for clinical studies. It needs to be discussed - and finally decided - whether endoscopic mucosal healing, histologic mucosal healing or a combination of both can be standardized. Once agreement on definitions has been achieved, a given patient could be assessed by a -hopefully- simple binary coded tool that is oriented according to the TNM classification of oncology. A proposal for such a tool is illustrated in Table 3. The number “1”

**Table 3** Proposal of the CBEHI classification to assess Crohn’s disease activity

Activity level	Definition	Code
Clinical activity	Remission: CDAI < 150	C0
	Active: CDAI ≥ 150	C1
Biochemical activity	CRP normal	B0 (CRP)
	Elevated CRP	B1 (CRP)
	Calprotectin < 200 µg/g	B0 (Calpro)
	Calprotectin ≥ 200 µg/g	B1 (Calpro)
Endoscopic activity	Remission: SES-CD < 4	E0
	Active: SES-CD ≥ 4	E1
Histologic activity	Inactive	H0
	Active	H1
Imaging	Inactive: no fistulas, no stenoses	I0
	Active: presence of either fistula and/or stenosis	I1

Example: A Crohn’s disease (CD) patient with C0B0 (CRP) E1H1I0 would have clinical and biochemical remission, but endoscopic and histologic activity.

stands for “active”, “0” for “remission” and “x” for “not assessed”. Of note CD activity assessment would require, in contrast to UC, not only measuring clinical activity, biochemical, endoscopic and histologic activity, but also imaging modalities (presence of fistulas, strictures). This simple approach has the potential to reduce the amount of potentially confusing new definitions to describe different combinations of activities in IBD.

Other definitions of mucosal healing (such as “biological mucosal healing”, “epigenetic mucosal healing”, “mucus layer healing” or “microbiota mucosal healing”) require further studies and prospective trials. At this point they are purely investigational and should not be used in clinical trials.

What would happen if such an agreement cannot be achieved? Then it would not make sense to discuss mucosal healing as a treatment target for IBD any further as this would be a treatment target that lacks a definition and subsequently is blurry, vague and indistinct.

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## WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

# Has the risk of colorectal cancer in inflammatory bowel disease decreased?

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## Abstract

The association between inflammatory bowel disease (IBD) and colorectal cancer (CRC) has been acknowledged for almost a century and is assumedly promoted by a chronic inflammation-driven carcinogenic process in the intestine in combination with a genetic predisposition. The magnitude of the risk of CRC in IBD remains a continuing subject of debate. The early, high risk estimates for CRC in IBD were most likely overestimated due to selected patient populations originating from tertiary referral centers with a disproportional high percentage of patients with severe disease. Later population-based studies calculating risk estimates from a broad spectrum of IBD patients have found the risk to be significantly lower. At present, there is evidence that IBD patients with longstanding and extensive disease with uncontrolled inflammation are those at increased risk. Additional, other recognized risk factors include early age at onset, family history of CRC, and concomitant primary sclerosing cholangitis. A significant amount of effort is put into identifying potential preventive factors of CRC in IBD, including surveillance programs and chemopreventive agents but the individual effect of these remains uncertain. Interestingly, recent studies have reported a decline in risk of CRC over time. Sur-

veillance programs and the new treatment strategies, particular biological treatment might be part of the reason for the observed decline in risk of CRC in IBD over time but future studies will have investigate this assumption.

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**Key words:** Inflammatory bowel disease; Colorectal cancer; Risk; Ulcerative colitis; Crohn's disease

**Core tip:** The increased risk of colorectal cancer in inflammatory bowel disease is well established. But what is the true magnitude of this increased risk, does the risk of colorectal cancer differ between ulcerative colitis and Crohn's disease and what are the significant risk factors? Further, recent studies have indicated that the risk of colorectal cancer in patients with inflammatory bowel disease is decreasing over time, potentially due to improved treatment options that lower the inflammatory burden. These are some of the subjects that will be elucidated and discussed in this review.

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## INTRODUCTION

In almost a century it has been recognized that the risk of developing colorectal cancer (CRC) is increased in patients with longstanding inflammatory bowel disease (IBD), and it is estimated that one out of six deaths in ulcerative colitis (UC) patients and one out of 12 of all deaths in patients with Crohn's disease (CD)<sup>[1,2]</sup> is caused



by colorectal cancer. Together with the hereditary syndromes of familial adenomatous polyposis and hereditary non-polyposis colorectal cancer, IBD is in the top-3 high risk conditions for CRC. Both UC and CD carry an increased risk of CRC; however the risk is most extensively studied in UC. The augmented risk of CRC in IBD is assumedly promoted by a chronic, inflammation-driven carcinogenic process in the intestine in combination with a genetic predisposition<sup>[3]</sup>. The prognosis of sporadic CRC and IBD-related CRC is similar with a 5-year survival of 50%<sup>[4]</sup> whereas IBD-related CRC affect younger patients than sporadic CRC (60 years *vs* 70 years)<sup>[4,5]</sup>.

In 1925, Crohn and Rosenberg<sup>[6]</sup> were the first to elucidate the relation between CRC and UC and in 1928, Bagen<sup>[7]</sup> further described 20 cases of colorectal cancer in patients with UC from the Mayo clinic in the United States. In 1971, de Dombal<sup>[8]</sup> reported a cumulative risk of CRC in a population from Leeds with extensive UC to be 5% after 10 years and as high as 41.8% after 25 years. These findings led to the suggestion of cancer prophylactic colonic surgery in UC patients with extensive disease and a disease course of more than 10 years, but this proposal has never been carried out in practice. Since then, substantial effort has been made to elucidate the supposed risk of CRC in IBD and has presented a considerable variety in risk estimates, leading to an ongoing debate concerning the true magnitude of the risk of CRC in IBD. Additionally, novel population-based studies have suggested a decline in risk of IBD-related CRC over time, potentially due to a shift in treatment strategies from the era of sulfasalazine, 5-aminosalicylic acid and corticosteroids, to the era of immunomodulators, such as thiopurines and tumor necrosis factor (TNF)- $\alpha$  antagonists<sup>[5,9]</sup>.

## RISK OF COLORECTAL CANCER IN ULCERATIVE COLITIS

A landmark meta-analysis including 116 studies published by Eaden *et al*<sup>[1]</sup> in 2001, found that the cumulative risk of CRC for UC patients was 2% at 10 years, 8% at 20 years, and 18% at 30 years. However as an important weakness of the meta-analysis the underlying studies were of very different methodology and many factors may have biased results. A main subject, primarily in early studies, has been the selective collection of IBD patients from tertiary referral centers with a high percentage of patients with disproportionately severe disease, thereby potentially introducing referral bias with an overestimation of the risk. This is in line with the findings in a Dutch study, comparing a cohort of 121 IBD patients with CRC from referral centers with a cohort of 160 IBD patients with CRC from general hospitals and confirmed that IBD patients from referral centers represent a subgroup with a more severe IBD-phenotype<sup>[10]</sup>.

In order to approach a more unbiased risk estimate the use of population-based studies is essential with unselected cohorts of patients representing the complete and broad spectrum of disease. An early Swedish popu-

lation-based study by Ekblom *et al*<sup>[11]</sup> including a cohort of 3117 patients with UC and followed from 1922-1983 found 91 cases of colorectal cancer, corresponding to a standardized incidence ratio (SIR) of 5.7 (95%CI: 4.6-7.0). A matched population-based cohort study by Bernstein *et al*<sup>[12]</sup> from 2000 revealed an increased risk of CRC in 2672 UC patients (RR = 2.75; 95%CI: 1.91-3.97) compared with a non-IBD population. In accordance, Söderlund *et al*<sup>[13]</sup> conducted a population-based study of 7607 IBD patients from Sweden diagnosed between 1954 and 1989 and found, for UC patients, a more than 2-fold increased risk of CRC compared to the background population (SIR = 2.7; 95%CI: 2.3-3.2). A Hungarian, population-based study by Lakatos *et al*<sup>[14]</sup> followed 723 UC patients for 8564 person-year from 1974 to 2004 and revealed a cumulative risk of CRC in UC of 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years disease duration. Conversely, data from population-based studies originating from Scandinavia, Italy and the United States have reported lower risk estimates. A population-based study from Olmsted County, United States from 2006 found no overall increase in CRC in 378 UC patients (SIR = 1.1; 95%CI: 0.4-2.4), but in the subgroup of patients with extensive colitis the risk was increased 2-fold, although not reaching statistical significance (SIR = 2.4; 95%CI: 0.6-6.0)<sup>[15]</sup>. Winther *et al*<sup>[16]</sup> followed a population-based cohort of UC patients from Copenhagen County, for a median of 19 years and found no increased risk of CRC (standardized morbidity ratio: 1.05; 95%CI: 0.56-1.79). In accordance with this, a population-based study from Italy and a very recent population-based study from Northern Jutland, Denmark, did not find a significant increase in CRC in UC patients<sup>[17,18]</sup>. A recent meta-analysis<sup>[19]</sup>, solely including population-based studies in order to approach an unbiased, general estimate of CRC risk in UC, found that an average of 1.6% of UC patients was diagnosed with CRC during 14 years of follow-up. This corresponds to a 2.4 (95%CI: 2.1-2.7) fold increased risk of CRC in UC. Looking at absolute risk the cumulative risk of CRC was 1.15% after 15 years, 1.69% after 20 years and 2.61% after 25 years disease duration. With 5 out of 8 studies originating from the Nordic countries the low risk has been suggested to be explained by high surgery rates and a high percentage of patients with proctitis in these countries, but this is not supported by the fact that the 3 non-Scandinavian studies revealed similar or even lower risk estimates than the Scandinavian studies. Beaugerie *et al*<sup>[20]</sup> recently published a prospective cohort study on risk of colorectal high-grade dysplasia and CRC among nearly 20000 patients with IBD enrolled in the French observational cohort CESAME (Cancer et Surrisque Associé aux Maladies Inflammatoires Intestinales En France) between May 2004 and June 2005. The authors found a 2-fold higher risk of CRC in IBD patients compared to the general population; a risk that was similar for both UC and CD. Sub-analyses revealed that this increased risk was driven by the relatively small percentage (14.6%) of patients with long-standing extensive colitis (>



10 years disease and > 50% of colon affected) with a SIR of 7 (95%CI: 4.4-10.5) compared with a non-significant increased risk in patients without long-standing extensive colitis (SIR = 1.1; 95%CI: 0.6-1.8). These risk estimates are higher than those originating from population-based studies and it is of importance to notice that data from the CESAME cohort arise from a selected IBD population. The difference in risk estimates from selected population *vs* unselected populations was addressed in a novel meta-analysis stratifying between study design and revealed a 4-fold greater risk of CRC in IBD patients when data originated from referral centers with selected patients compared with data from unselected patients from population-based studies<sup>[9]</sup>.

## RISK OF COLORECTAL CANCER IN CD

In contrast to the risk of CRC in UC patients, which has been comprehensively investigated, the risk of CRC in CD patients remains less explored. As with the risk of CRC in UC, studies on risk of intestinal cancer in CD have revealed inconsistent results with a variation in reported relative risk estimates from 0.8 to 20.0<sup>[21]</sup>.

A meta-analysis from 2005 by Jess *et al*<sup>[22]</sup> exclusively including population-based studies and representing populations from North America, Scandinavia and Israel, estimated a pooled overall SIR for CRC in CD of 1.9 (95%CI: 1.4-2.5). Separate risk estimates for cancer in the colon and rectum resulted in a significant increased risk for colon cancer (SIR = 2.5; 95%CI: 1.7-3.5), whilst a slightly, non-significant increased pooled risk was estimated for rectum cancer (SIR = 1.4; 95%CI: 0.8-2.6). The risk of CRC cancer was significantly increased in CD patients with colonic involvement (SIR = 4.3; 95%CI: 2.0-9.4), non-statistically increased in patients with ileocolonic CD (SIR = 2.6; 95%CI: 0.8-8.2) and not increased in CD patients with pure ileal disease (SIR = 0.9; 95%CI: 0.2-4.1). Another meta-analysis from 2005, by Canavan *et al*<sup>[21]</sup> including both selected and unselected patient series studies, on risk of CRC in CD, reported similar results with an overall pooled RR of 2.5 (95%CI: 1.3-4.7) and only a significant increased risk for CD patients with colonic disease (RR = 4.5; 95%CI: 1.3-14.9). In subgroup analyses on site-specific CD the RR estimate increased for patients with colonic involvement whereas combined RR of CRC in CD patients with ileal disease was not increased (RR = 1.1; 95%CI: 0.8-1.5). A retrospective study by Herrinton *et al*<sup>[23]</sup> calculated risk of CRC cancer in a more recent IBD cohort from the Kaiser Permanente database from 1998 to 2010 and identified 29 incident CRC patients among persons with CD corresponding to a 1.6-fold higher risk of CRC compared with the general Kaiser Permanente population. In the up-dated meta-analysis from 2013 by Lutgens *et al*<sup>[9]</sup> the pooled risk estimate for CRC in CD was slightly increased (SIR = 1.6; 95%CI: 1.2-2.0) when data originated from population-based studies. Yet again, the risk was only increased in patients with colonic involvement, though not reaching statistical significance (pooled SIR = 2.0; 95%CI: 0.3-3.7).

## RISK FACTORS

It is essential, in a clinical aspect to obtain knowledge of potential cancer predictive factors in order to identify subgroups of patients who need closer surveillance or more intense treatment. Known risk factors for CRC in IBD patients include young age at diagnosis, duration and anatomic extent of disease, family history of CRC, concurrent primary sclerosing cholangitis and persisting inflammation of the colon.

## AGE AT ONSET

Young age at onset of colitis has been reported to be an independent risk factor for CRC. Interpretation of existing evidence is complicated as children tend to have more extensive and severe colitis compared with those diagnosed in adult age, and further have a potential for longer disease duration, a risk factor in itself.

The impact of early age at onset of IBD as a risk factor for CRC was assessed in a Danish cohort study by Jess *et al*<sup>[5]</sup>. They found that the risk of CRC varied markedly by age at diagnosis of UC; those diagnosed at childhood or adolescence (age 0-19 years) had the greatest risk (RR = 43.8; 95%CI: 27.2-70.7) followed by those diagnosed in young adulthood (age 20-39 years) with a RR of 2.65 (95%CI: 1.97-3.56). Those diagnosed with UC at the age period from 60-79 years had a risk of CRC that was below that of the background population (RR = 0.76; 95%CI: 0.62-0.92). However, as pointed out by the authors, the absolute risk of CRC was limited even in those diagnosed in young age<sup>[24]</sup>. Patients diagnosed with UC at age 0-19 had an absolute risk of CRC of 1.64% (95%CI: 0.25%-3.00%) after 25 years disease duration and for the group diagnosed at 20-39 years of age this risk was 0.80% (95%CI: 0.39%-1.20%). In matched controls the corresponding 25-year risk estimates were 0.05 (95%CI: 0.03%-0.07%) and 0.47% (95%CI: 0.43%-0.50%), respectively for the two age-groups. These data are supported by a population-based meta-analysis which showed that the standardized CRC incidence ratio was 4 times higher in IBD patients diagnosed at young age (< 30 years) compared with a non-significantly increased risk patients diagnosed at the age of 30 years or older<sup>[9]</sup>, but the meta-analysis did not report absolute risks.

## DISEASE EXTENT AND DURATION

UC patients with pancolitis are at highest risk, left-sided colitis carries a moderate risk, and patients with proctitis and protosigmoiditis are at similar risk of CRC as the background population. Ekblom *et al*<sup>[11]</sup> found that UC patients with pancolitis had a nearly 15-fold increased risk of CRC (SIR = 14.8; 95%CI: 11.4-18.9) as compared to the background population, in contrast to an increased risk of 2.8 for those with left-sided colitis and a non-significant increased risk of 1.7 for those with proctitis. A smaller risk was found in the population-based study by Söderlund *et al*<sup>[13]</sup> exploring the significance of site-

specific inflammation for both UC and CD, on risk of CRC. Within the cohort and compared with UC proctitis the risk of CRC cancer was 2.0 (95%CI: 1.3-3.0) for UC pancolitis, 1.2 (95%CI: 0.7-1.9) for left-sided UC, 0.9 for colonic CD (95%CI: 0.5-1.6) and 0.7 (95%CI: 0.4-1.1) for non-colonic CD. A risk of 5.6 (95%CI: 4.4-7.0) was found for UC pancolitis, compared to an overall risk for UC of 2.7 (95%CI: 2.3-3.2) when comparing with the general population. In accordance, the population-based meta-analysis by Jess *et al*<sup>[19]</sup> reported an overall risk of 4.8 (95%CI: 3.9-5.9) for UC patients with extensive disease. Backwash ileitis in UC, theoretically representing the greatest extent of disease due to ileal involvement, has been reported to carry an additional increased risk of CRC<sup>[25]</sup>, but this has not been confirmed by others<sup>[26]</sup>.

In addition to extent of disease, longer duration of disease is associated with an augmented risk of CRC in IBD. In a Danish, nationwide cohort study by Jess *et al*<sup>[5]</sup> the relative risk of CRC in IBD was low in the first years after diagnosis (except for an implausible high RR the first year after diagnosis, assumedly as a result of differential diagnostic problems or cases of coincidental detection of recent onset IBD in patients diagnosed with CRC), then progressively increasing with duration of IBD reaching the level of the non-IBD population after 8 years. After disease duration of 13 years the RR was significantly higher than the background population, reaching a level around 50% increase with longer follow-up. These results are consistent with the current surveillance guidelines defined by the American Gastroenterological Association<sup>[27]</sup> and the British Society for Gastroenterology<sup>[28]</sup> recommending initiation of surveillance after 8-10 years disease duration for CD and extensive UC and after 15-20 years for patients with left-sided UC. Nevertheless, studies have shown that up to a third of IBD patients develop CRC prior to the initial point of surveillance<sup>[5,20,29]</sup>, thus questioning the efficacy of the present surveillance strategy.

## PRIMARY SCLEROSING CHOLANGITIS

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease present in 3%-8% of patients with UC and 1%-3% of patients with CD<sup>[30-32]</sup>, whereas 60%-80% of patients with PSC have IBD<sup>[33]</sup>. In 1992, Broomé *et al*<sup>[34]</sup> were the first to suggest that IBD patients with PCS potentially had an increased risk of CRC. A later study by the same group revealed an absolute cumulative risk of CRC in patients with UC and PSC of 9% after 10 years disease duration, 31% after 20 years and as high as 50% after 25 years; compared with 2%, 5% and 10% in patients with UC alone<sup>[35]</sup>. A meta-analysis published in 2002, including 11 studies concerning risk of CRC in patients with concomitant UC and PSC, revealed that 21% of UC-PSC patients developed CRC compared to 4% of UC patients without PSC, thus giving an odds ratio of 4.8 (95%CI: 3.6-6.4)<sup>[36]</sup>. The risk of CRC in CD patients with PSC is uncertain. A British case control study by

Braden *et al*<sup>[37]</sup> studied risk of CRC in colonic CD/PSC patients and concluded that the presence of PSC did not increase the risk of CRC in patients with colonic CD. Thackeray *et al*<sup>[38]</sup> conducted a retrospective study in order to determine the time-interval between diagnosis of IBD, PSC and CRC and found that IBD-PSC patients developed cancer or dysplasia relatively soon after diagnosis of both IBD and PSC. Interestingly, patients with PSC and IBD typically have mild or asymptomatic pancolitis with prolonged remission and an inactive cause<sup>[39-41]</sup>. Further, studies have reported a more frequent location of cancer in the right colon in patients with IBD-PCS<sup>[42]</sup>. This could suggest a different pathogenesis in the subgroup of IBD patients with PSC compared to IBD patients in general, but these mechanisms remains unidentified.

Due to the high cumulative risk of CRC in IBD patients with PSC, the short time-interval between PSC diagnosis and CRC progression, and the predominately right-sided cancer location, it is recommended that patients with IBD-PSC should initiate an annual surveillance colonoscopy, rather than sigmoidoscopy, program already at time of PSC diagnosis<sup>[43]</sup>.

## FAMILY HISTORY OF COLORECTAL CANCER

Healthy individuals, with a family history of CRC, carry a near 2-fold increased risk of CRC. Few studies have assessed the significance of familial CRC, or IBD on risk of CRC in patients with IBD. A population-based study from Sweden found that a family history of CRC in IBD patients resulted in a doubling of the already increased risk of CRC in IBD, irrespectively of type and extent of IBD<sup>[44]</sup>. Further, sub-analyses revealed that IBD patients with a 1<sup>st</sup>-degree relative diagnosed with CRC before the age of 50 had an even higher risk (RR = 9.2; 95%CI: 3.7-23). A family history of IBD did not increase the risk of CRC.

## SEVERITY OF INFLAMMATION

Under the assumption that inflammation is the promoting factor in development and progression of CRC in IBD it seems evident that the relationship between degree of inflammation and risk of CRC would be comprehensively investigated. Paradoxically, data are sparse. Rutter *et al*<sup>[45]</sup> conducted a retrospective case-control study, using data on histological and endoscopic grade of inflammation from a well-established cancer surveillance program for patients with long-standing, extensive UC from the United Kingdom and found a highly significant correlation between severity of inflammation and the risk of CRC; both when using colonoscopic scores (OR = 2.5,  $P = 0.001$ ) and histological scores (OR = 5.1,  $P < 0.001$ ). These findings were replicated in another retrospective case-control study from Finland, concluding that the risk of dysplasia or CRC is strongly associated with the degree of inflammation in patients with UC<sup>[46]</sup>.

## IS THE RISK OF COLORECTAL CANCER DECREASING?

The management of IBD has changed markedly in the last decades<sup>[47]</sup> not only with advancement in medical treatments, *e.g.*, new biological therapies, surgical treatment options and improved diagnostic tools leading to early detection, but also with implementation of surveillance programs and awareness of the need of adherence to medication from a patient perspective. These factors could potentially reduce the long-term inflammation in IBD patients and thereby reduce the risk of CRC.

Eaden *et al*<sup>[1]</sup> reported in their meta-analysis an increase in incidence of IBD-related CRC over time from 1955 to 2001 by plotting cancer risk against the mid-point of 41 studies, but the result did not reach statistical significance (slope: 0.003,  $P = 0.80$ ). Another meta-analysis reported a decline in risk over time, by pooling results on risk estimates classified by the publication year. They found an incidence rate of 4.29/1000 pyrs in the 1950s compared to an incidence rate of 1.09/1000 pyrs from 2000-2011<sup>[48]</sup>. Several other studies have shown a declining trend in risk over time. Söderlund *et al*<sup>[13]</sup> conducted a population-based study showing time-trends in incidence and mortality of CRC from 1960 to 2004 in 7607 Swedish IBD patients and reported adjusted relative risks of 1.7 (95%CI: 0.6-4.4) from 1960 to 1969, 1.3 (95%CI: 0.7-2.6) from 1970 to 1979, 1.2 (95%CI: 0.7-2.2) from 1980 to 1989, 1.1 (95%CI: 0.7-1.8) from 1990 to 1999, and 1 (reference) from 2000 to 2004, revealing a non-significant, declining trend. Compared to the general population the relative risk declined from a 5-fold increased risk of CRC in IBD in the 1960s to a 2-fold increased risk in the period from 2000 to 2004 ( $P$  for trend = 0.06). The risk of death from CRC decreased significantly during the same time period, both when comparing patients within the cohort and with the general population. Whether this decline in mortality is due to surveillance, better surgical management, better follow-up, or other changes is unanswered. Results from other studies are diverse. Herrinton *et al*<sup>[23]</sup> used data on IBD and CRC from the United States health insurance Kaiser Permanente database to report time-trends over a 14.5 year study period from 1998 to 2010. Results showed no time-trend with incidence rates of CRC per 100000 pyrs, varying from 87.9 in 1998-2001, to 67.0 in 2002-2006 and to 73.9 in 2007-2010 ( $P$  trend = 0.98) but one could argue that this time-interval is too short to reveal any trend over time. In contrast to the results from the United States, a nation-wide cohort study from Denmark revealed a decrease in risk of CRC in IBD over 30 years from 1979 to 2008<sup>[5]</sup>. During 178 million person-years of follow-up, relative risk estimates of CRC in IBD were calculated, adjusted by sex, age and calendar period and subdivided into three time-periods of 10 years from 1979 to 2008. Compared to the general population the overall RR of CRC in UC decreased from 1.34 (95%CI: 1.13-1.58) in 1979-1988 to 1.09 (95%CI: 0.90-1.33) in 1989-1998 and further to 0.57 (95%CI:

0.41-0.80) in 1999-2008. It has been argued that the observed decreased risk could be explained by the initiation of screening of CRC in the general population but first of all there is no systematic screening program for CRC in Denmark before year 2014 and further relative risks compared within the cohort, using the RR in the intermediate period from 1989-1999 as reference (hence enabling adjustment for shorter length of follow-up in recent cohorts) the risk was still significantly reduced (RR = 0.59; 95%CI: 0.39-0.90) in the late period from 1999-2008. When analyzing time-trends in CD no significant changes were observed. Likewise, a study on mortality within the same population revealed a decrease in mortality in UC patients from 1982 to 2010, largely due to a reduction in mortality from gastrointestinal disorders and CRC<sup>[49]</sup>. In addition to the mentioned original studies, an updated meta-analysis by Lutgens *et al*<sup>[9]</sup> found a similar decreasing trend in risk of CRC in IBD over time in meta-regression analyses from 9 population-based studies, but the trend did not reach statistical significance, most likely due to a type II error as only few studies were available for analysis. Overall, there may be a declining risk of CRC in IBD over time and the reason for this observation needs to be studied further.

## CHEMOPREVENTION

Instead of focusing on early detection of neoplasia in IBD, the ideal would be to prevent neoplasia from ever developing. In light of the theory of an inflammation-driven carcinogenic process as a causative factor of IBD-related CRC, medical therapies reducing the inflammatory burden could potentially lower the risk of CRC in IBD. Hence, there has been an increasing interest in detecting chemopreventive agents that can reduce the overall risk of dysplasia and cancer and serve as a complement to current surveillance programs.

5-aminosalicylic acid (5-ASA) is the first line agent for maintenance therapy in mild to moderate UC and it has been shown in *in vitro* studies to have antineoplastic properties by inhibiting the nuclear kappa-B pathway which is involved in tumor progression<sup>[50]</sup>. However, the evidence of a potential chemoprophylactic effect of 5-ASA is contradictory. In 2005 Velayos *et al*<sup>[51]</sup> published a meta-analysis of 9 observational studies (3 cohort, 6 case-control) on effect of 5-ASA in preventing IBD related CRC. Pooled analysis revealed a protective effect of 5-ASA use on risk of IBD-related CRC with an odds ratio of 0.51 (95%CI: 0.37-0.69). Since then several case-control and population-based studies have not been able to detect any chemopreventive effect of 5-ASA<sup>[52-54]</sup>. Recently, Nguyen *et al*<sup>[55]</sup> published a meta-analysis solely including non-referral studies; thereby potentially reducing bias and presenting evidence that is more generalizable. The meta-analysis revealed a pooled adjusted OR of 0.95 (95%CI: 0.66-1.38) for CRC in patients with IBD treated with 5-ASA and based on these results the authors concluded that there does not seem to be a protective effect



of 5-ASA on risk of CRC in IBD.

In addition to 5-ASA, an increasing number of patients are treated with the thiopurine drugs, azathioprine and 6-mercaptopurine. Existing data on the potential chemopreventive effect of thiopurines in IBD are, however, conflicting. In the French CESAME clinical based cohort study the authors investigated the impact of thiopurines on risk of CRC in IBD<sup>[20]</sup>. Almost half of the 19,484 IBD patients had been exposed to thiopurines and among current users the adjusted hazard ratio for CRC was 0.57 (95%CI: 0.24-1.32) thereby revealing no significantly protective effect of thiopurine use on CRC risk in the general IBD population. However, in subanalysis confined to IBD patients with long-standing extensive colitis, current treatment with thiopurines reduced the risk of advanced colorectal neoplasia significantly (CRC and high grade dysplasia combined; HR = 0.28; 95%CI: 0.09-0.89). A Dutch cohort study by van Schaik *et al*<sup>[56]</sup> have further evaluated the effect of thiopurines on risk of CRC in an IBD cohort of 2578 IBD patients of whom 770 were exposed to thiopurines. They found that thiopurine exposure were associated with significantly decreased risk of developing advanced neoplasia (high grade dysplasia and colorectal cancer combined; adjusted HR = 0.10; 95%CI: 0.01-0.75). In a Danish nationwide population-based study by Pasternak *et al*<sup>[57]</sup> from 2013, the effect of thiopurine exposure on risk of CRC was assessed among 43969 IBD patients of whom 12% had been exposed to thiopurines. In contrast to the more selected French study, no difference in risk of CRC was observed among thiopurine exposed *vs* non-exposed patients (adjusted RR = 1.00; 95%CI: 0.61-1.63). Similar results were reported in another large population-based study from the United Kingdom<sup>[58]</sup>.

Recent data from models of experimental colitis have indicated that TNF- $\alpha$  has a tumor promoting effect<sup>[59]</sup>. Few studies have been able to evaluate the effect of the new biological treatments, as TNF- $\alpha$  blockers, on risk of CRC due to the relatively short existence of these agents relative to the latency of CRC. In a Dutch nested case-control study by Baars *et al*<sup>[60]</sup> risk factors for IBD-related CRC were identified by comparing 173 cases of IBD-related CRC (collected from 1990 to 2006) with 393 non-CRC IBD controls. The authors found that use of TNF- $\alpha$  antagonist was a protective factor for the development of CRC (OR = 0.09; 95%CI: 0.01-0.68). In a Danish, nationwide population-based cohort study, the risk of CRC was compared between IBD patients exposed to TNF- $\alpha$  antagonist *vs* non-exposed and revealed no impact of TNF- $\alpha$  antagonist on risk of CRC (adjusted RR = 1.06; 95%CI: 0.33-3.40)<sup>[61]</sup>. The association between TNF- $\alpha$  antagonists and cancer is two-edged with hypotheses on both a tumor-promoting and a tumor preventing effect and future studies are necessary to clarify this aspect.

## CONCLUSION

The absolute risk of CRC in IBD is limited. However,

subgroups of IBD patients with severe, long-standing active disease who do not undergo colectomy and patients with PSC carry a greater risk of CRC than the background population. Overall, it seems evident that to prevent CRC from occurring in IBD, the goal is to minimize severity and extent of inflammation, whereas the methods used to do this (regular follow-up, medical treatment, surgery, and surveillance) act in common and not as single cancer-preventive factors. Whether new treatment strategies, particular biological treatment might be part of the reason for the observed decrease in risk of CRC in IBD over time needs further investigation.

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## WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

# Autoantibodies and an immune-based rat model of inflammatory bowel disease

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## Abstract

The exact causes of inflammatory bowel disease (IBD) are not yet fully defined. From a vast body of literature, we know that the immune response has long been involved in the pathogenesis of IBD, including both ulcerative colitis and Crohn's disease. A variety of specific alterations can lead to immune activation and inflammation directed to the colon, as revealed by some animal models. Current research has focused on the role of antibodies in downstream events and mechanisms of autoimmunity and inflammation. It is not well known whether the production of antibodies is a serologic consequence of IBD, or if it is a result of barrier dysfunction induced by inflammation. Here, we present a new hypothesis to distinguish the complex links between genetic susceptibility, barrier dysfunction, commensal and pathologic microbial factors and inflammatory response (especially autoantibodies) in the pathogenesis of IBD. To ascertain the hypothesis, we developed a pilot model with the concept of the presence of antibodies against enteric bacterial antigens in IBD. Results confirmed our hypothesis. Our hypothesis suggests the possibility of subcutaneous vaccination of animals with

administration of all or specific enteric bacterial antigens.

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**Key words:** Inflammatory bowel disease rat model; Pathogenesis; Barrier dysfunction; Microbial factor

**Core tip:** We present a new hypothesis to distinguish the complex links between genetic susceptibility, barrier dysfunction, commensal and pathologic microbial factors and inflammatory response (especially autoantibodies) in the pathogenesis of inflammatory bowel disease (IBD). In our hypothesis, we suggest that prior activation of adaptive immunity against microbial flora antigens could initiate an IBD-like chronic inflammation if something like ethanol disturbs barrier function. If this hypothesis is supported with further experiments, it would illustrate unknown aspects of IBD pathogenesis. On this basis, we have developed a new immune-based model of IBD with the presence of antibodies against enteric bacterial antigens.

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## INTRODUCTION

### Etiology

Investigations have demonstrated that the pathophysiology of inflammatory bowel disease (IBD) is multifactorial, but briefly host (e.g., genetics, intestinal barrier and immune system function) and exogenous factors (e.g.,



normal luminal flora) are two basic themes<sup>[1]</sup>. The normal intestine contains a large number of immune cells in a chronic state of so-called physiologic inflammation to control the gut and to prepare it for any immunologic response. Lack of immune responsiveness to lumen antigens may be a result of oral tolerance<sup>[2]</sup>. Multiple mechanisms are involved in the induction of oral tolerance. For instance, deletion or anergy of antigen-reactive T cells or activation of regulatory CD4 T cells suppresses gut inflammation through secretion of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor  $\beta$  (TGF- $\beta$ ). In addition, a selectively permeable barrier prevents unwanted solutes, microorganisms, and luminal antigens from confronting the immune system in the internal mucosa<sup>[2-4]</sup>. In IBD, this tolerance is altered and leads to an uncontrolled inflammation; thus, IBD is considered as a breakdown in the regulatory constraints on mucosal immune response to the microbial flora or their products within the intestine. Most of this process is mediated through components of the autoimmune response to self-antigens<sup>[5]</sup>.

A variety of specific alterations can lead to immune activation and inflammation directed to the colon, as revealed in animal models demonstrating murine genetic models (transgenic models). These models showed us that deleting loci of specific cytokines (*e.g.*, IL-2, IL-10, TGF- $\beta$ ) or their receptors or T cell antigen recognition molecules (*e.g.*, T cell antigen receptors) or interfering with intestinal barrier integrity (*e.g.*, mucus glycoprotein, deleting N-cadherin or nuclear factor  $\kappa$ B) leads to inflammation<sup>[4]</sup>.

It has been suggested that the continuous penetration of luminal antigens and unremitting stimulation of the mucosal immune system due to an increased permeability of the intestine epithelial cells may be the primary defect in patients suffering from IBD<sup>[3]</sup>. Therefore, if we consider increased epithelial permeability as the trigger, the tragedy of IBD initiates after a disruption occurring in the mucosal integrity. Then lots of macromolecule antigens in the lumen penetrate into internal compartments of the mucosa and submucosa, and subsequently become recognized by the gut immune system. Later, interstitial macrophages and dendritic cells are locally activated and release cytokines to recruit more macrophages and monocytes from the systemic circulation<sup>[6]</sup>. In normal subjects, this acute response is subsided after controlling the invasion. In genetically susceptible subjects [defects in innate immunity response (*e.g.*, NOD2,  $\alpha$ -defensins mutations)] or in the long-term exposure to penetrated antigens in the situation of persistent integrity perturbations, or if the invader was a specific uncontrollable pathogen (*e.g.*, *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., *Clostridium difficile*), antigen presenting cells (APCs) secrete cytokines, which leads to induction of differentiation in various T-cells. In this way, the adaptive immune response is turned on<sup>[7-11]</sup>. Activation of both TH1 or TH2 leads to an inflammatory response<sup>[12]</sup>. This

ignition can be turned off by the regulatory systems. Generally, recovery is achieved after repair of the first alteration in intestinal permeability.

### Microbial factors

Microorganisms are a likely factor in the initiation of inflammation in IBD<sup>[13]</sup>. However, the unanswered question in this area is whether microorganisms involved in the pathogenesis of IBD are commensal flora or invasive microbial pathogens?

Normal intestinal microflora may contribute to the development of IBD in susceptible individuals. This finding has been demonstrated repeatedly in murine models of IBD<sup>[14,15]</sup>. As an example, animals which are genetically altered (*e.g.*, deficient in IL-2 and IL-10) to be susceptible to IBD do not develop the disease when raised under germ-free conditions<sup>[13]</sup>. Also, intestinal lesions in IBD typically predominate in areas of the highest bacterial exposure (*e.g.*, in distal ileum and colon with  $10^{12}$  organisms/g).

On the other hand, a number of studies have evaluated the possible role of specific infectious agents in the pathogenesis of IBD. This role has been evaluated in two ways: the relation between specific microorganisms and IBD (*e.g.*, presence of specific antibodies in serologic findings of IBD patients<sup>[16]</sup>), and the association between some acute gastroenteritis and IBD<sup>[17]</sup>.

Pathogens that could be directly responsible for initiating IBD are those that the mucosal immune system may fail to control in terms of the inflammatory response (*e.g.*, *Salmonella* sp., *Shigella* sp.). These bacteria are rich in peptides having chemotactic properties (*e.g.*, formyl-methionyl-leucyl-phenylalanine). The superantigens capable of global T-lymphocyte stimulation and subsequent inflammatory response, and those producing toxins (necrotoxins, hemolysins, and enterotoxins), cause mucosal damage<sup>[8,9,16,18]</sup>. In summary, an acute infection with specific pathogens leads to a permanent uncontrollable perturbation in intestinal integrity, even though after the acute phase there is perhaps mediation of some cytokines (*e.g.*, IFN- $\gamma$ ), and permeability changes across the epithelium are induced. This results in continuous exposure and stimulation of the mucosal immune system with commensal flora antigens<sup>[3,19-21]</sup>.

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## IMMUNE REGULATION AND INFLAMMATORY CASCADE DEFECTS IN IBD

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As discussed later, the mucosal immune system is normally nonresponsive to luminal contents due to oral tolerance. Once inflammation is initiated, the immune inflammatory response is propagated by T cell activation in the lamina propria. CD4 T cells are of three major types: TH1, TH2, and TH17 cells. The TH1 cells secrete predominantly IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-12, which



activate cell-mediated immunity by CD8 T cells (cytotoxic) resulting in transmural granulomatous inflammation resembling CD. Meanwhile, the TH2 cells can induce B-cell differentiation and humoral immunity by secreting predominantly IL-4, IL-5, and IL-13 with superficial mucosal inflammation features resembling UC<sup>[22]</sup>. TH17 cells secrete predominantly IL-17, IL-6, and granulocyte colony-stimulating factor and seem responsible for neutrophilic recruitment<sup>[5,8,23]</sup>. After activation of these cells, they produce specific cytokines and, consequently, the epithelial barrier permeability (*e.g.*, IFN- $\gamma$ ) is increased. Some of these cytokines have destructive and apoptotic effects on mucosal cells, which eventually allow more antigens to pass and produce more agitation of immune cells amplifying the inflammatory cascade<sup>[3,8]</sup>. In normal situations, an activated response is subsided with regulatory T cells, including designated TH3, Tr1, and CD4, and CD25 cells<sup>[23]</sup>. Their function is blocking or down-regulating the response of TH1 and TH2 either by producing specific cytokines (IL-10 and TGF- $\beta$ ) or *via* cell-cell contact. There is evidence which demonstrates some defects in this regulatory system in IBD-susceptible subjects<sup>[24,25]</sup>.

## INTESTINAL BARRIER DYSFUNCTION

The intestine is covered by a monolayer of simple columnar and non-ciliated epithelial cells that are a type of brush border cells. These are joined together by intercellular and circumferential tight junctions to form a selectively permeable membrane. This barrier prevents unwanted solutes, microorganisms, and luminal antigens from entering the internal parts. They are also part of the immune system, acting as a first-line pathogen-recognition system because they present antigens similar to classical APC. They also express toll-like receptor (TLR) 4 and, furthermore, secrete antimicrobial peptides (*e.g.*, cryptidins and defensins)<sup>[2-4,26]</sup>. However, the epithelial barrier has some guards of the innate immune system to ensure permanent immune responsiveness (*e.g.*, DC, interstitial macrophages)<sup>[27]</sup>. If anything alters the barrier function, lots of luminal antigens could pass through the submucosal layer resulting in recruitment of neutrophils and macrophages. If these cells can control the invasion, it is not necessary to call adaptive immunity components, but if the invasion takes long then adaptive immune response component will be activated. In this process, if the regulatory systems are not able to overcome the inflammatory cascade, the secreted cytokines will deteriorate and amplify the first defect in the epithelial barrier by inducing apoptosis and necrosis in the epithelial cells. In addition, a number of studies have shown that inflammatory cytokines like TNF- $\kappa$  and IFN- $\gamma$  may have a role in increasing intestinal barrier permeability<sup>[3,4,8]</sup>. Some animal models of IBD have shown alterations in barrier function as the first trigger contributing to pathogenesis of IBD. Furthermore, abundant evidence indi-

cates an increased intestinal permeability in IBD patients suggesting the permanent stimulation of the mucosal immune system as the primary defect in the pathogenesis of IBD<sup>[3,28,29]</sup>.

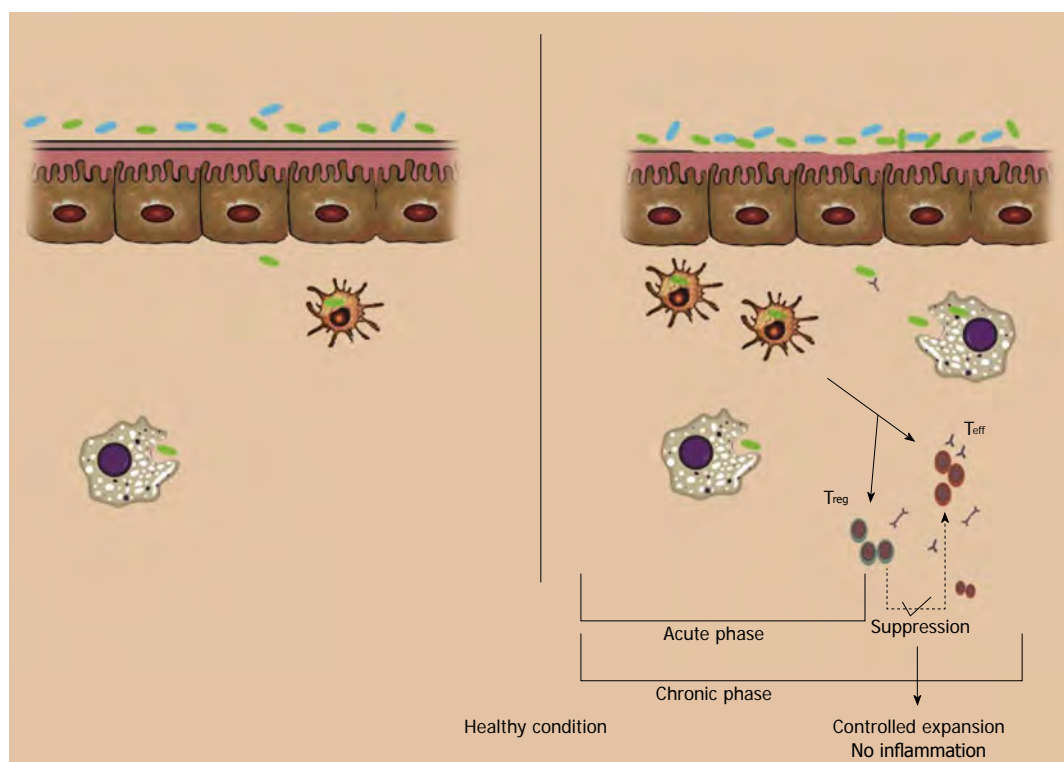
## STEPS OF AUTOIMMUNITY IN IBD

The pathogenesis of IBD and most of its extra-intestinal manifestations is immunologically mediated and appears to be mainly due to an autoimmune-related process<sup>[30,31]</sup>. As discussed, after a permanent alteration in barrier function, various antigens pass through the interstitial space which finally activates T cells. In normal subjects, the response is directed definitively against the specific epitope of antigens, but commensal organisms in the lumen have adhesive antigens (*e.g.*, flagellar antigens) which adhere to the surface proteins of mucosal cells. If there are some predisposing factors, then there is a chance for APCs to process epitopes of these antigens, with parts of the mucosal surface proteins, which activate T lymphocytes against mucosal cell surface protein<sup>[8,31]</sup>. Another scenario happens when the response to the specific epitopes of antigens is cross-reactive to auto-antigens. There is evidence demonstrating relations between precise human leukocyte antigen (HLA) molecules and cross-reactive cellular antigens<sup>[32,33]</sup>. However, in the TH2-mediated immune response in UC, it is thought that perhaps development of self-reactive B cells, which are triggered to produce mucosal IgG autoantibodies, results in an inflammatory response. Meanwhile, TH1 cell-mediated immunity and auto-reactive T cells (CD4 or CD8) may be primed by microbial antigens that are cross-reactive to autoantigens<sup>[34]</sup>.

A long series of studies demonstrated that IBD patients possess autoantibodies, some of which became serologic biomarkers to diagnose or distinguish subtypes of this disease, such as anti-lymphocyte, anti-goblet cell, pancreatic autoantibodies, the autoantibody against tropomyosin isoform 5 (a cytoskeletal protein found in colon epithelial cells), and antibodies against red blood cell membrane antigens that cross-react with enteropathogens such as *Campylobacter* sp.<sup>[31,34,35]</sup>

We will now discuss some of the known autoantibodies in IBD pathogenesis. There is a form of perinuclear antineutrophil cytoplasmic antibody (pANCA) which is non-reactive to myeloperoxidase. It is well defined that 60%-70% of UC patients and 5%-15% of their first-degree relatives are pANCA-positive, whereas this applies to only 2%-3% of the general population. There is a relation between positive pANCA antibody status and severity of UC disease and other complications. Interestingly, pANCA in CD is associated with colonic disease that resembles UC<sup>[31,34,35]</sup>. The definite antigens to which these antibodies are directed have not been identified, but they have cross-reactions with enteric bacterial antigens.

Other studies demonstrated the presence of another



**Figure 1 intestinal barrier dysfunction.** Left (normal conditions): no or few commensal bacteria can pass the normal epithelial barrier and those that pass will be swallowed by interstitial macrophages and dendritic cells; it is not necessary to call for adaptive immune cells. Right: in normal situations, if something breaks the barrier (e.g. pathogens and barrier breaker chemicals like ethanol 30%) lots of commensal bacteria in the lumen will pass through the epithelial layer. This acute invasion will be controlled with recruiting of neutrophils and lymphocytes. Even after activation of B cells or T cells, if the defect in barrier function is resolved, apoptotic pathways will control the activated colonies of lymphocytes.

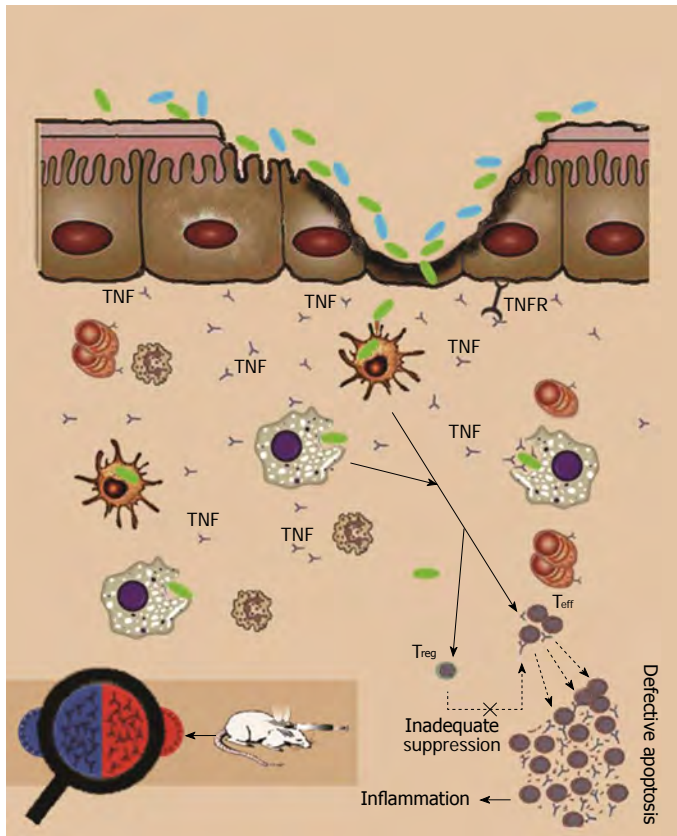
autoantibody, which is specific to patients with UC; it is an IgG autoantibody bound to a subtype of tropomyosin of colonic epithelial cell antigen. The capability of this antibody to initiate extracellular signal-regulated kinase (ERK) 1/2 signaling and up-regulating of the TLR and production of cytokines, and also the correlation between the titers of this antibody and the severity of colitis, suggest the possibility that such a protein could represent autoantigen- or complement-mediated responses<sup>[13,31]</sup>.

Although the presence of antibodies directed against microbial antigens has been illustrated in the serum of CD patients, a shared epitope among the host antigens is not clearly defined. For example, 55% of CD patients have antibodies against outer membrane porin C of *Escherichia coli*, and 50% have immunoglobulins that are reactive to a homologue of the bacterial transcription-factor families from a *Pseudomonas fluorescens*-associated sequence (I<sub>2</sub>). Around 50% of CD patients have serum reactivity to Cbir1, an immunodominant antigen of the enteric microbial flora. This antigen can strongly induce B cells and CD4<sup>+</sup> T cell responses. Transferring of Cbir1-specific CD4<sup>+</sup> TH1 T cells to C3H/SCID mice generates a severe colitis dependent on exogenous expression of Cbir1 flagellin in the colon. In 60%-70% of CD patients, anti-*Saccharomyces cerevisiae* antibodies have been found. A mannose sequence in the cell wall of this

commensal flora has been defined<sup>[35,36]</sup>.

## HYPOTHESIS

Although the above-mentioned studies support the concept of the presence of antibodies against enteric bacterial antigens in IBD, we propose a model to investigate whether the production of antibodies is a result of barrier dysfunction induced by inflammation or a serologic finding secondary to IBD. The hypothesis would result in a reliable model of IBD studies in animals. Our hypothesis suggests the possibility of subcutaneous vaccination of animals with administration of all or specific enteric bacterial antigens. In this way, production of immunoglobulin against these antigens would prevent intestinal inflammation. Anything that alters the function of this barrier and increases barrier permeability would result in inflammatory responses. To test this hypothesis, we have designed a pilot study and examined the model in male Wistar rats, which were immunized with anaerobic and aerobic enteric bacteria with and without an adjuvant. After assessing the IgG titers in the rats' plasma, well-immunized rats were anesthetized and then chitosan and ethanol were instilled intrarectally as a tight junction opener and a barrier breaker, respectively. This protocol induced a chronic inflammatory response with inflammatory features in the ethanol group with persistent le-



**Figure 2 Steps of autoimmunity.** If anything alters the barrier function, lots of luminal antigens can pass to the submucosal layer. If these cells can control the invasion, it is not necessary to call adaptive immunity components, but if the invasion take longer (e.g., in altered tight junction structure) or there are some defects in innate immunity response (e.g., mutations in Toll-like receptors), T cells are activated. If the regulatory system cannot overcome the inflammatory systems, cytokines and reactive destructive mediators further deteriorate the first defect *via* inducing apoptosis and necrosis in epithelial cells. Activation of T helper type 2 cells leads to a humoral response; also administration of a vaccine of commensal bacteria leads to a humoral immune response to their antigens, so after a disruption in barrier integrity with ethanol, the inflammatory cascade will turn on and induce inflammatory bowel disease in the animal as shown by histopathological findings. TNF: Tumor necrosis factor; TNFR: TNF receptor.

sions. We propose that this model of chronic intestinal inflammation would be a reliable model of human IBD. Of course, further studies would need to prove immunization with specific bacteria (Figures 1 and 2).

## CONCLUSION

In this article, we addressed some known immune derangements involved in the initiation and pathogenesis of IBD. The following general principles are highlighted for better understanding of the possible mechanisms involved in the IBD pathogenesis.

Increased barrier permeability secondary to a genetic susceptibility, a specific infectious pathogen or their toxins and activation of T cells create a positive feedback to amplify the first barrier dysfunction and initiate an inflammatory cascade.

Two common features of autoimmunity processes may differ in activation of autoreactive T or B cells, involving a variety of imbalances in cytokine production and the development of autoantibodies. In IBD, these antibodies are directed against shared enteric flora antigens and epithelial cell-surface proteins.

In this study, we focused on autoantibodies. It is not well defined whether various autoantibodies found in the serologic assessment of IBD patients are destructive or involved in pathogenesis of the disease, or whether they are produced after tissue damage due to releasing of sequestered antigens<sup>[31]</sup>. We suggest that antibodies which

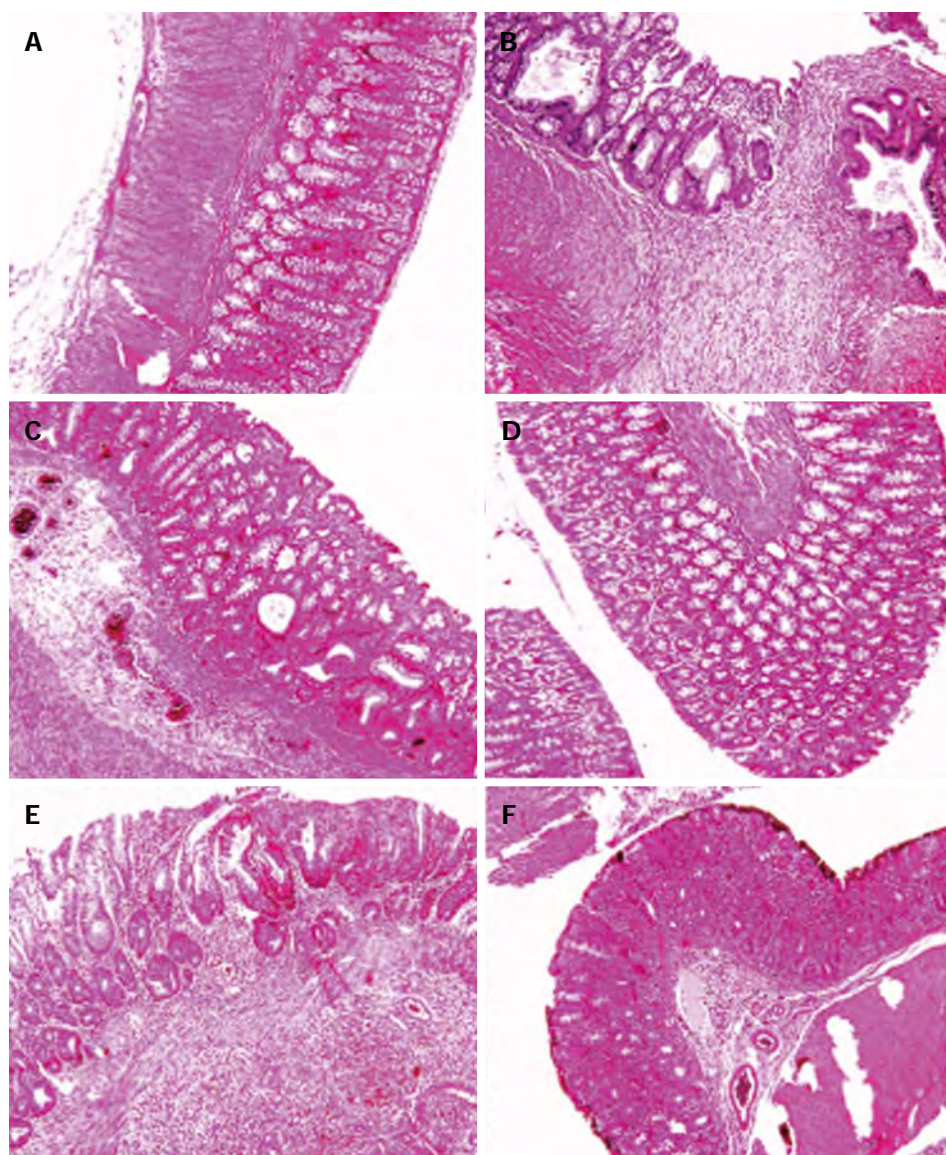
are secreted in UC are catastrophic and are involved in the inflammatory response, but antibodies which are produced in CD are not involved in the pathogenesis and are secreted post-release of sequestered antigens. However, antibodies in both UC and CD patients are involved in extraintestinal complications, while there are various overlaps between these two subtypes.

In our hypothesis, we suggest that prior activation of adaptive immunity against microbial flora antigens in the way described could initiate an IBD-like chronic inflammation (especially in UC). Further experiments are essential to test various aspects of the method and unknown points of IBD pathogenesis.

## Empirical data

After developing the hypothesis, we designed a pilot study. Six groups of male rats containing three rats in each group were considered. An extemporaneous vaccine was prepared with a mixture of heat-treated colonic commensal bacteria, which were obtained from cultured samples, and complete Freund's adjuvant. This vaccine was injected subcutaneously into nine rats on days 0 and day 14. On day 28, a blood sample was taken from each rat to assess immunoglobulin titers. All of the test animals showed an elevated titer. Then these rats were divided into three groups; intra-colonic ethanol 30% was instilled in two groups, and in the third group, normal saline was instilled instead of ethanol and this group was assigned as the vaccine group. The two groups which





**Figure 3** Histological images of colon tissues obtained from different groups. Microscopic evaluation of the trinitrobenzene sulfonic acid (TNBS) group shows villus atrophy, extensive severe transmural inflammation, granuloma with necrosis and crypt destruction, whereas features in the Sham group were normal. Histological examination of the Ethanol group showed a mild crypt distortion and some crypt abscess, whereas features in the Vaccine group were normal. Microscopic evaluation of the Model group showed mucosal inflammation and crypt distortion, branching and some ulceration with moderate to severe crypt destruction in ulcerated regions. Mild focal inflammation, minimal inflammatory cell infiltration and slight crypt branching were observed in the Infliximab group. A: Sham; B: TNBS; C: Ethanol; D: Vaccine; E: Model; F: Infliximab.

received ethanol were: the model group that received no treatment and an infliximab-treated group that received 5 mg/kg per day of infliximab for 10 consecutive days after ethanol instillation. The first of the other three groups consisted of an ethanol group that received intracolonic ethanol 30% with no pre- or post-treatment. An established colitis model was induced with instillation of 10 mg of trinitrobenzene sulfonic acid dissolved in 30% ethanol as the vehicle in another group. And the last group was normal rats (sham group), which received normal saline intracolonic. The animals were sacrificed, and colon samples were removed for histopathological assays. Details of microscopic assessments are described in Figure 3.

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## WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

# ***Clostridium difficile* and inflammatory bowel disease: Role in pathogenesis and implications in treatment**

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IBD exacerbation, and the prognostic implications of CDI in these patients, it is recommended to test all IBD patients hospitalized with a disease flare for *C. difficile*. Treatment includes general measures such as supportive care and infection control measures. Antibiotic therapy with either oral metronidazole, vancomycin, or the novel antibiotic-fidaxomicin, should be initiated as soon as possible. Fecal microbiota transplantation constitutes another optional treatment for severe/recurrent CDI. The aim of this paper is to review recent data on CDI in IBD: role in pathogenesis, diagnostic methods, optional treatments, and outcomes of these patients.

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**Key words:** *Clostridium difficile*; Diarrhea; Inflammatory bowel disease; Pathogenesis; Treatment

## Abstract

*Clostridium difficile* (*C. difficile*) is the leading cause of antibiotic associated colitis and nosocomial diarrhea. Patients with inflammatory bowel disease (IBD) are at increased risk of developing *C. difficile* infection (CDI), have worse outcomes of CDI-including higher rates of colectomy and death, and experience higher rates of recurrence. However, it is still not clear whether *C. difficile* is a cause of IBD or a consequence of the inflammatory state in the intestinal environment. The burden of CDI has increased dramatically over the past decade, with severe outbreaks described in many countries, which have been attributed to a new and more virulent strain. A parallel rise in the incidence of CDI has been noted in patients with IBD. IBD patients with CDI tend to be younger, have less prior antibiotic exposure, and most cases of CDI in these patients represent outpatient acquired infections. The clinical presentation of CDI in these patients can be unique-including diversion colitis, enteritis and pouchitis, and typical findings on colonoscopy are often absent. Due to the high prevalence of CDI in patients hospitalized with an

**Core tip:** In this review we focus on the role of *Clostridium difficile* (*C. difficile*) in inflammatory bowel disease pathogenesis, the unique clinical aspects of *C. difficile* infections and prognosis in patients with inflammatory bowel disease. We also present the implications of *C. difficile* infections in these patients and review the most recent literature concerning diagnostic methods and treatment.

Nitzan O, Elias M, Chazan B, Raz R, Saliba W. *Clostridium difficile* and inflammatory bowel disease: Role in pathogenesis and implications in treatment. *World J Gastroenterol* 2013; 19(43): 7577-7585 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7577.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7577>

## INTRODUCTION

The human gut microbiota contains about 10<sup>14</sup> bacterial

cells from more than 1000 different bacterial species<sup>[1,2]</sup> that play an important role in conservation of mucosal innate and adaptive immune function, integrity of the epithelial barrier and nutrient absorption<sup>[3-6]</sup>. Disruption of the gut microbiota (dysbiosis) has been linked with many gastrointestinal conditions<sup>[7,8]</sup>. Accumulating evidence suggests that inflammatory bowel disease (IBD) results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host<sup>[9-11]</sup>. Dysbiosis in IBD may also contribute to disease severity, and is correlated with the occurrence of abscesses in patients with Crohn's disease (CD) and need for surgery at a younger age<sup>[12,13]</sup>.

*Clostridium difficile* (*C. difficile*) is an anaerobic gram-positive, spore-forming, toxin-producing bacillus that causes intestinal disease varying from a mild diarrheal illness to severe colitis<sup>[14-16]</sup>. The burden of *C. difficile* infection (CDI) has increased dramatically over the past decade and it is now recognized that *C. difficile* is responsible for 20%-30% of cases of antibiotic associated diarrhea and 50%-75% of cases of antibiotic associated colitis<sup>[17,18]</sup>. *C. difficile* is also the leading cause of nosocomial diarrhea, with incidence ranging from 1:100-1:1000 hospitalized patients<sup>[19,20]</sup>. Loss of intestinal microbial equilibrium, most commonly following antibiotic use, creates an environment susceptible to colonization of *C. difficile* and subsequent CDI<sup>[21,22]</sup>.

IBD has been found to be associated with *C. difficile*<sup>[23-26]</sup>. Patients with IBD are at increased risk of developing CDI, have worse outcomes of CDI-including higher rates of colectomy and death, and experience higher rates of recurrence<sup>[27-30]</sup>. However, it is still not clear whether *C. difficile* is a cause of IBD or a consequence of the inflammatory state in the intestinal environment. The association between IBD and *C. difficile* may be due to different factors, such as drugs that are used for the treatment of IBD that might alter the intestinal flora and promote colonization (including repeat courses of antibiotics), altered immune and nutritional status, frequent hospitalizations, and even genetic predisposition<sup>[31,32]</sup>.

In this review we will try to focus on the role of *C. difficile* in IBD pathogenesis, the unique aspects of *C. difficile* infections in patients with IBD, and the implications for testing and treatment.

### The role of *C. difficile* in IBD

The initial trigger responsible for the onset of IBD is not yet known. A complex interplay between the immune system, environmental factors, such as stress and diet, enteric infections, and genetic factors play a role in the pathogenesis of IBD<sup>[33-35]</sup>. Gut microbiota interacts with both the innate and adaptive immune systems, playing a pivotal role in maintenance and disruption of gut immune quiescence<sup>[36]</sup>. Different bacteria have been implicated in the pathogenesis of IBD, including *Mycobacterium avium paratuberculosis*, enterotoxigenic *Bacteroides fragilis*, adherent/invasive *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Chlamydia* sp., *Aeromonas hydrophila*, *Salmonella typhi*, and *C. difficile*<sup>[37-39]</sup>. However, to date there is no con-

clusive evidence that a specific pathogen is responsible for IBD onset or relapse.

*C. difficile* has been found to be associated with IBD. Different studies found that patients with IBD, including ulcerative colitis (UC) and CD, are at increased risk of developing CDI. A study based on a large cohort of IBD patients in the United States found that CDI was more common in UC patients (2.8%) as compared to the general inpatient population (0.4%), and another study reported an adjusted odds ratios for CDI in all IBD, CD, and UC admissions from 1998-2004 to be 2.9, 4.0, and 2.1 respectively<sup>[40,41]</sup>. Since 2003 there has been a dramatic rise in the incidence of CDI with severe outbreaks described in Canada, United States and England, which have been attributed to a new and more virulent strain designated BI/NAP1/027, that has also been found in patients with IBD<sup>[18,42,43]</sup>. A parallel rise in the incidence of CDI in patients with IBD has also been noted. During 1998-2004 CDI rates approximately doubled in CD (9.5 to 22.3/1000 admissions) and tripled in UC (18.4 to 57.6/1000)<sup>[41]</sup>. A retrospective observational study found that the rate of CDI in IBD patients increased from 1.8% in 2004 to 4.6% in 2005, with the majority of patients having colonic IBD<sup>[29]</sup>. More recent studies, found that 5.5%-19% of patients with an IBD exacerbation, tested positive for *C. difficile* infection, and as many as 3.5% of children hospitalized due to IBD, were diagnosed with CDI<sup>[44,45]</sup>. Furthermore, analysis of a registry database suggests that 10% of IBD patients will develop a *C. difficile* infection at some point, and approximately 10% of CDI occur at the time of IBD diagnosis<sup>[46]</sup>. Patients with IBD also have higher rates of asymptomatic carriage of *C. difficile* 8.2% (9.4% in patients with UC and 6.9% in patients with CD), versus 1% in healthy volunteers<sup>[47]</sup>. It is possible, though, that the seemingly increased risk of CDI in patients with IBD is due to increased surveillance of this population for CDI. There are studies that question the role of CDI in IBD. A recent prospective Dutch study found a low prevalence of *C. difficile* in IBD patients and did not find any association of *C. difficile* with disease activity, disease subtype (CD or UC), gender, antibiotic, and immunosuppressive therapy<sup>[48]</sup>.

Though it is still not clear if *C. difficile* causes IBD, it is understood that *C. difficile* can cause an infectious colitis superimposed on IBD, or may precipitate an IBD flare leading to simultaneous inflammatory processes, and it is nowadays considered a risk factor for IBD exacerbation. The association between *C. difficile* and IBD is mediated by a chain of events, including- recurrent hospitalizations, that are a known risk factor for acquisition of *C. difficile* and CDI, medications administered to patients with IBD (including immunomodulatory and antimicrobial agents) that disturb the intestinal flora, thus allowing for *C. difficile* colonization and adherence, and a decreased nutritional status that promotes *C. difficile* infection<sup>[31,32]</sup>. Thus, *C. difficile* can colonize the intestines of these patients and produce its two potent exotoxins: toxin A ("enterotoxin") and toxin B ("cytotoxin") that bind to receptors on intestinal epithelial cells. This activates a cascade of proinflam-



**Table 1 Unique features and clinical implications of *Clostridium difficile* infection in patients with inflammatory bowel disease**

Risk factors
Colonic IBD
Immunomodulatory drugs
In comparison to patients with no IBD: younger age, more community acquired cases, less prior antibiotic exposure
Clinical characteristics
Diarrhea (can be bloody), often mimics a flare of IBD
In patients with ileostomies: acute enteritis( an increase in ileostomy output, nausea, fever and leukocytosis)
In patients with ileal pouch anal anastomosis: pouchitis
Often no pseudomembranes on colonoscopy
Outcomes and complications
Higher rates of toxic megacolon and colonic perforation
Higher rates of colectomies
Longer length of hospital stay
Increased mortality
Diagnosis:
Test for CDI in all IBD patients hospitalized with a disease flare
As in patients with no IBD-one step molecular assays or two step algorithms: screening with EIA for GDH, followed by EIA for toxins and/or a molecular assay
Treatment:
1 Escalation of immunosuppression should be avoided during CDI
2 Antibiotics treatment as in non IBD patients
Mild to moderate disease: oral metronidazole
Severe disease: oral vancomycin + intravenous metronidazole
Fidaxomicin-less recurrences (no data in IBD patients)
3 Fecal microbiota transplantation
Limited data in IBD patients though seems to be effective

IBD: Inflammatory bowel disease; CDI: *Clostridium difficile* infection.

matory cytokines and leukotrienes such as tumor necrosis factor (TNF), interleukin (IL)-6, IL-8, IL-1 $\beta$ , leukotrienes B<sub>4</sub>, and interferon- $\gamma$  leading to apoptosis of gut epithelial cells and increased permeability of the intestinal mucosa, which in turn can play a role in the pathogenesis of IBD<sup>[49,50]</sup>.

So, as elaborated above, there is a possible association between IBD and *C. difficile*, though there are many issues to be resolved, such as: whether CDI is a risk factor for the development of IBD, or the active inflammatory process in patients with IBD predisposes them to CDI, and what are the exact mechanisms that are responsible for this association.

## CDI IN PATIENTS WITH IBD

The unique features and clinical implications of CDI in patients with IBD are summarized in table 1.

### Risk factors

As noted above, patients with IBD are at increased risk for CDI, but this risk varies among different subsets of these patients. One of the major risk factors for CDI in patients with IBD is colonic IBD, either UC or CD with colitis-with 91% of patients with IBD suffering from CDI, reported to have colonic IBD, and a higher incidence of CDI was found in patients with left sided and extensive disease as compared to distal disease<sup>[29]</sup>. Other

risk factors for CDI in patients with IBD are similar to those in the general population, such as- older age, medications (antibiotic/immunosuppressive agents), hospitalization, residence at a long term facility and comorbidities<sup>[21,51,52]</sup>. However, there are a few differences in risk factors for CDI in patients with IBD. IBD patients with CDI tend be younger, and 76% of *C. difficile* infections in these patients represent outpatient acquired infections, as opposed to patients without IBD were the most part of *C. difficile* infections are hospital acquired<sup>[29,53]</sup>. IBD has been found to be a risk factor for outpatient acquired CDI. In a study done in our medical center, on 115 patients with CDI, we found a trend towards a higher rate of IBD in community acquired CDI versus hospital acquired CDI (20.2% *vs* 9.7% , unpublished data).

Also, up to 40% of IBD patients do not have documented antibiotic exposure prior to presentation with CDI<sup>[54]</sup>. Patients with IBD receive various types of immunosuppressive drugs that might predispose to CDI, and steroid treatment has been found to increase the risk of CDI 3 fold in these patients, though other immunomodulatory drugs such as purine analogs, methotrexate and biological agents, have not been consistently found to increase the risk of CDI<sup>[29,41,55,56]</sup>. Combination treatment with different immunomodulatory agents can increase the risk of CDI as was found in pediatric patients receiving concomitant therapy of methotrexate and anti-TNF- $\alpha$ , where 28% of patients developed CDI<sup>[57]</sup>.

### Clinical characteristics

Patients with IBD can have different and unique clinical presentations of CDI. To begin with, the similarity in symptoms between CDI and a flare of IBD (diarrhea, abdominal pain, fever and leukocytosis) make it extremely difficult to distinguish between the two<sup>[29,41]</sup>. *C. difficile* in IBD may also show atypical features such as frequent bloody stools, as opposed to watery stools in patients without IBD. Diarrhea may even be absent in postoperative patients who receive narcotics for pain control and develop paralytic ileus. In IBD patients with ileostomies, *C. difficile* can cause acute enteritis, which can manifest as an increase in ileostomy output, nausea, fever and leukocytosis<sup>[53]</sup>. In patients who have undergone ileal pouch anal anastomosis as treatment for IBD, *C. difficile* infection might be a triggering factor for pouchitis, which presents as an increase of the number of stools per day, with or without constitutional symptoms such as weight loss<sup>[58,59]</sup>. In one study 10.7% of patients with ileal pouch anal anastomosis, presenting with pouchitis, were found to have CDI<sup>[60]</sup>. Another study demonstrated that 18.3% of cases of pouchitis were positive for *C. difficile* toxin, with men 3.5 times more likely than women to develop *C. difficile* pouchitis<sup>[61]</sup>.

Typical findings of CDI on colonoscopy (such as pseudomembranous exudates, which are found in up to 60% of patients with CDI) are often absent in patients with IBD (0%-13% of cases)<sup>[62]</sup>. This might be due to a weakened inflammatory response in the colonic epithelial

environment in patients with chronic active IBD or due to immunosuppressive drugs that hamper the development of pseudomembranes, which are caused by disruption of cellular cytoskeleton by toxins, ulcer formation and leakage of serum proteins, inflammatory cells and mucus.

## Outcomes

*C. difficile* infections have a different and often more severe clinical course in patients with IBD. These patients have higher rates of endoscopies, higher rates of complications such as toxic megacolon and colonic perforation, higher rates of colectomies, longer length of hospital stay, and increased mortality<sup>[29,30]</sup>. Different studies found high rates of colectomies in these patients, ranging from 20% to 45%<sup>[28,29]</sup>, with one study finding a 6 fold increase in bowel surgery in patients with CDI with and without IBD<sup>[30]</sup>. Patients with IBD also experience more recurrences of CDI, than patients without IBD<sup>[29]</sup>. Mortality is also increased in these patients with one study demonstrating a 6%-18% case fatality rate in patients with IBD and CDI *vs* 1.4%-2.1% fatality rate in patients with CDI alone<sup>[40]</sup>. A large study of the inpatient care database in the United States found that hospitalized patients with concurrent CDI and IBD had a 4 times higher mortality rate than those admitted for IBD or CDI alone<sup>[63]</sup>.

All of these special aspects of CDI in patients with IBD should cause physicians to be alert to the possibility of CDI in a patient with an IBD exacerbation and prompt rapid diagnosis and treatment.

## Diagnosis

CDI is a clinical diagnosis supported by laboratory findings. As mentioned before, it is often difficult to distinguish between CDI and an exacerbation of IBD, because of the similarity in symptoms, and moreover, IBD patients may have a different clinical presentation of CDI. Laboratory findings in both CDI and IBD are also similar, including: leukocytosis, hypoalbuminemia, and fecal leukocytosis<sup>[53]</sup>. Endoscopic findings that are typical for CDI, such as colonic pseudomembranes, are also lacking in most patients with IBD that present with CDI<sup>[28]</sup>. As noted above, patients with IBD and CDI often acquire the infection in the outpatient setting and in many there is no previous documented antibiotic exposure<sup>[29,53]</sup>.

Due to the high prevalence of CDI in patients hospitalized with an IBD exacerbation, the suspected causal association between CDI and flare of IBD, and the prognostic implications of CDI in these patients, it is recommended by the American college of gastroenterology CDI Guidelines Task Force, to test all IBD patients hospitalized with a disease flare for *C. difficile*<sup>[64]</sup>. Also, the European Crohn's and Colitis Organization guidelines recommend testing for *Clostridium difficile* infection in patients with severe or refractory UC<sup>[65]</sup>. Patients should be tested even in the absence of traditional risk factors such as antibiotic exposure.

There are various laboratory tests used in the diag-

nosis of CDI. Only loose, watery, or semi-formed stool should be tested for *C. difficile* and specimens should be kept at 4 °C if delay in testing is anticipated due to degradation of *C. difficile* toxin at room temperature<sup>[66,67]</sup>. The different tests that have been used to date are<sup>[68-70]</sup>: (1) Selective anaerobic culture: the most sensitive diagnostic method, cannot distinguish toxin-producing strains from non-toxin producing strains, time and labor consuming and thus reserved for epidemiologic studies<sup>[71]</sup>; (2) Cell culture cytotoxicity neutralization assay: detects the presence of toxin B in stool by its cytopathic effects in a cell or tissue culture, is time consuming, with a sensitivity of 65%-90%, and is rarely performed today<sup>[71]</sup>; (3) enzyme immunoassay (EIA) for *C. difficile* toxins A and B: sensitivity for toxins A and B is 60%-75% and specificity is higher (up to 99%)<sup>[72,73]</sup>. Was the routine diagnostic assay for CDI in most microbiology laboratories in recent years, but due to its low sensitivity, is not recommended today as the initial diagnostic assay<sup>[70]</sup>; (4) EIA for *C. difficile* glutamate dehydrogenase (GDH), an enzyme produced in all *C. difficile* strains: sensitivity of 75% > 90%, but cannot differentiate between toxin positive and toxin negative strains<sup>[74]</sup>; and (5) Polymerase chain reaction (PCR): detect toxin A and B genes, are highly sensitive and specific<sup>[75,76]</sup>, and provide rapid results (within as little as 1 h).

Current recommendation for CDI diagnosis implement either one step molecular assays or two step algorithms with screening with EIA for GDH, followed by EIA for toxins and/or a molecular assay<sup>[67,68]</sup>. In patients with IBD, there is no evidence to date, that testing for CDI should be done differently. A recent retrospective study that compared the frequency and clinical outcomes of IBD inpatients with CDI, found that a greater percentage of patients tested positive by PCR for toxin B as compared with ELISA for toxins A + B, but the clinical outcomes were the same, regardless of method of testing<sup>[77]</sup>. More research is needed to determine the optimal diagnostic test for CDI in patients with IBD. Due to high rates of asymptomatic colonization of *C. difficile* in patients with IBD, only patients with significant diarrhea should be tested for CDI.

## Treatment

Treatment of CDI includes general measures such as supportive care with attention to correction of fluid losses and electrolyte imbalances, cessation of the inciting antibiotic as soon as possible (if possible), implementation of infection control policies-including hand hygiene with soap and water which is more effective than alcohol-based hand sanitizers in eradication of *C. difficile* spores<sup>[67,78]</sup>. Antimotility agents such as loperamide and opiates have traditionally been avoided in CDI for fear of decreasing toxin clearance and increasing the risk of ileus and/or megacolon, but the evidence that they cause harm is equivocal<sup>[79]</sup>.

Specific antibiotic therapy should be started as soon as possible, and empiric therapy is indicated pending results of diagnostic testing if the clinical suspicion is high and

when severe or complicated CDI is suspected. Currently, there are several drugs in use for treatment of CDI including: metronidazole (oral or intravenous), vancomycin (oral or per rectum), oral rifaximin, and a newer drug- oral fidaxomicin. A Cochrane systematic review from 2011 found no statistically significant difference in efficacy between vancomycin and other antibiotics including metronidazole, fusidic acid, nitazoxanide or rifaximin<sup>[80]</sup>. The updated guidelines for the treatment of CDI released by the Infectious Diseases Society of America and the Society for Healthcare epidemiology of America suggest that the initial choice of treatment should be determined based on the severity of illness and depending if it is a first episode of CDI or a recurrence<sup>[69]</sup>. There are different scoring systems to assess the severity of illness, including the severity score index that consists of 9 criteria, each accounting for one point: altered mental status, white blood cell count > 20000 or < 1500, albumin < 2.5 mg/dL, ascites or colitis by imaging, mean arterial pressure < 65 mmHg, fever > 38.3 °C, tachycardia > 110 bpm and admission to intensive care unit. 1-3 points indicates mild disease, 4-6 points moderate disease, and ≥ 7 points severe disease<sup>[81]</sup>. For an initial episode of mild to moderate CDI-metronidazole, at a dose of 500 mg orally 3 times per day for 10-14 d, is considered the drug of choice. Vancomycin at a dose of 125 mg orally 4 times per day for 10-14 d is the drug of choice for an initial episode of severe CDI. Vancomycin, administered 500 mg orally 4 times per day (and 500 mg in approximately 100 mL normal saline per rectum every 6 h as a retention enema, if ileus is present) with or without intravenously administered metronidazole 500 mg intravenously every 8 h, is the regimen of choice for the treatment of severe, complicated CDI. Treatment of the first recurrence of CDI is usually with the same regimen as for the initial episode but should be stratified by disease severity. Treatment of the second or later recurrence of CDI is with vancomycin therapy using a tapered and/or pulse regimen<sup>[67]</sup>.

Recent studies of fidaxomicin, 200 mg orally twice daily, compared with oral vancomycin, demonstrated non inferiority of clinical response after 10 d of treatment and superior sustained responses with a decrease in recurrences (13% *vs* 24% with vancomycin treatment)<sup>[82]</sup>. Among patients who received concomitant antibiotics, treatment with fidaxomicin resulted in higher cure rates (90% *vs* 79.4%) and lower recurrence rates (16.9% *vs* 29.2% with vancomycin)<sup>[83]</sup>. Due to these and other findings, fidaxomicin might be a promising treatment for patients with risk factors known to portend relapse and severe infection<sup>[84]</sup>, though two different economical analyses reported conflicting results of the cost effectiveness of using fidaxomicin as first-line treatment for CDI<sup>[85,86]</sup>.

In patients with IBD and CDI, there are no guidelines or evidence from prospective studies to suggest that one antibiotic regimen is better than another. Failure rates of up to 50% have been reported in IBD patients treated with metronidazole<sup>[87]</sup>. Considering the worse outcomes of patients with IBD and CDI, some institutions use

vancomycin as first line therapy in these patients. In a single center study, where vancomycin was adopted as first line therapy in IBD patients with CDI, colectomy rates decreased from 45.5% to 25% within 1 year after the change of policy<sup>[88]</sup>. There are no data as of yet regarding the use of fidaxomicin in the IBD patient population, though in another group of immune suppressed patients-recipients of solid organ and hematopoietic stem cell transplantation, fidaxomicin achieved over all cure rates in 86% of episodes and recurrence rate was 7%<sup>[89]</sup>.

Concomitant use of immunomodulators is another unresolved issue in patients with IBD and CDI. A retrospective multicenter European study comparing hospitalized IBD patients with CDI treated with antibiotics and immunomodulators or antibiotics alone, found that the primary outcome of complications including colectomy or death within 3 mo occurred in 12% of patients treated with both, as compared to none of the patients treated with antibiotics alone<sup>[90]</sup>. The use of 2 or more immunomodulators further increased the risk of complications. In a survey of North American gastroenterologists, there was significant disagreement on whether combination antibiotics and immunomodulators or antibiotics alone should be given in patients with an IBD flare and CDI<sup>[91]</sup>. The American College of Gastroenterology CDI task force, has given a conditional recommendation, with low quality supporting evidence, that ongoing immunosuppression can be maintained in patients with CDI, although escalation of immunosuppression should be avoided<sup>[64]</sup>.

Fecal microbiota transplantation (FMT) through retention enemas, rectal tube, colonoscopy, nasogastric and nasoduodenal tubes, or upper endoscopy is another option for treating recurrent CDI through restoration of a healthy microbiome in the lower gastrointestinal tract. Different studies have reported success rates of FMT approaching 90% in patients with recurrent CDI<sup>[92,93]</sup>. A randomized prospective trial, found that duodenal infusion of donor feces following vancomycin treatment was significantly more effective for the treatment of recurrent CDI than the use of vancomycin alone<sup>[94]</sup>. Data on the use of FMT among IBD patients is limited, though a recent systematic review found that out of 12 patients with IBD and CDI treated with FMT, all became toxin negative, with symptomatic resolution in 11 out of 12 patients<sup>[95]</sup>. A recent review of FMT<sup>[96]</sup> notes that though there are no guidelines concerning FMT for treatment of CDI in patients with IBD, after FMT and eradication of *C. difficile*, the severity of IBD is gradually reduced with improved responses to medications for IBD. FMT is considered a safe treatment, though a recent paper reported a case of a flare of UC in a patient who received FMT for CDI<sup>[97]</sup>.

In patients after restorative proctocolectomy and ileal pouch anal anastomosis that present with *C. difficile* pouchitis, treatment is empirical because there are no published prospective trials. Studies suggest that metronidazole is not completely protective against CDI of



the pouch, as this infection has developed in patients on metronidazole therapy, thus in these patients vancomycin might be considered as first line therapy<sup>[59]</sup>.

## CONCLUSION

Patients with IBD are at increased risk of developing CDI and having worse outcomes, including higher rates of colectomy and death. There has also been a rise in the percentage of patients with IBD that suffer from CDI during recent years, even in those lacking classic risk factors for CDI. Patients with IBD often present with unique and more severe symptoms of CDI. Diagnosis of CDI in patients with IBD warrants a high index of suspicion and physicians should be alert to the possibility of CDI in any patient with an IBD exacerbation. All hospitalized patients with a flare of IBD should be tested for CDI and antibiotic treatment should be initiated rapidly, especially in severe cases, where vancomycin is the treatment of choice. More studies are needed to better understand the pathogenetic role of CDI in IBD exacerbations, to define what are the best diagnostic methods for CDI in these patients, to assess the efficacy of newer treatments such as fidaxomicin in patients with CDI and IBD, and to better address the question of concurrent treatment with immunomodulatory agents.

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## Extravascular use of drug-eluting beads: A promising approach in compartment-based tumor therapy

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### Abstract

Intraperitoneal carcinomatosis (PC) may occur with several tumor entities. The prognosis of patients suffering from PC is usually poor. Present treatment depends on the cancer entity and includes systemic chemotherapy, radiation therapy, hormonal therapy and surgical resection. Only few patients may also benefit from hyperthermic intraperitoneal chemotherapy with a complete tumor remission. These therapies are often accompanied by severe systemic side-effects. One approach to reduce side effects is to target chemotherapeutic agents to the tumor with carrier devices. Promising experimental results have been achieved using drug-eluting beads (DEBs). A series of *in vitro* and *in vivo* experiments has been conducted to determine the suitability of their extravascular use. These encapsulation devices were able to harbor CYP2B1 producing cells and to shield them from the hosts im-

mune system when injected intratumorally. In this way ifosfamide - which is transformed into its active metabolites by CYP2B1 - could be successfully targeted into pancreatic tumor growths. Furthermore DEBs can be used to target chemotherapeutics into the abdominal cavity for treatment of PC. If CYP2B1 producing cells are proven to be safe for usage in man and if local toxic effects of chemotherapeutics can be controlled, DEBs will become promising tools in compartment-based anticancer treatment.

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**Key words:** Compartment based therapy; Intraperitoneal; Drug-eluting beads; Carcinomatosis; Hyperthermic intraperitoneal chemotherapy; Glioblastoma; Pancreatic cancer; CYP2B1; Ifosfamide

**Core tip:** Intraperitoneal carcinomatosis occurs with several tumor entities and prognosis is usually poor. Besides standard therapy, only few patients may benefit from hyperthermic intraperitoneal chemotherapy. The treatment may cause severe systemic side-effects. One different approach to target chemotherapeutic agents to the tumor employs carrier devices. Contemplable carriers are drug-eluting beads (DEBs). DEBs can be used to transfer drugs or pro-drug converting enzymes directly to the tumor. Furthermore, DEBs can successfully target chemotherapeutics into the abdominal cavity for *ip* treatment. When local toxic effects are controlled, DEBs are effective tools in compartment-based therapy.

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## CLINICAL BACKGROUND

Peritoneal carcinomatosis (PC) is a disseminated tumor stage, which is observed in patients with ovarian, pancreatic, gastric and colorectal cancer. With median survival rates of 3.1 mo for gastric cancer and 5.2 mo for colorectal cancer, respectively<sup>[1]</sup>, the prognosis is usually poor<sup>[2]</sup>.

Survival is prolonged by new agents used in palliative chemotherapy. With the availability of oxaliplatin, irinotecan, bevacizumab, and cetuximab the 5-year survival has significantly increased over the last decade<sup>[3-7]</sup>.

Although some patients seem to benefit from these drugs, the physical and psychological strain for patients suffering from PC remains high. In addition to the commonly known side effects of chemotherapy<sup>[8]</sup>, patients show a variety of symptoms originating from PC itself, ranging from abdominal pain, nausea and obstipation up to bowel obstruction and obstructive uropathy<sup>[9]</sup>.

## EVALUATION OF PRESENT TREATMENT

The treatment of peritoneal carcinomatosis requires an interdisciplinary and multimodal approach. Modern therapy combines cytoreductive surgery (CRS), radiation therapy and systemic chemotherapy, depending on the origin of the tumor<sup>[10-12]</sup>. Unfortunately survival rates remain low<sup>[1,13]</sup> at the cost of frequently observed dose limiting side-effects<sup>[8]</sup>.

Since tumor spread into the abdominal cavity may also be considered as an early step of dissemination - comparable to liver metastasis in colorectal carcinoma - and not as a state of generalized systemic disease<sup>[14-16]</sup>, one approach may be to resect all detectable tumor nodules and target drugs directly to the peritoneal cavity<sup>[17]</sup>.

The surgical procedure removes all macroscopic tumor manifestations by combination of different peritonectomy procedures, including greater omentectomy, splenectomy, left upper quadrant peritonectomy, right upper quadrant peritonectomy, lesser omentectomy, cholecystectomy with stripping of the omental bursa, pelvic peritonectomy with sleeve resection of the sigmoid colon, and antrectomy<sup>[18-21]</sup>, as well as parietal peritonectomy<sup>[22,23]</sup>. After the resection, the dissolved chemotherapeutic agent and carrier solution are heated up to 42 °C and pumped through the abdominal cavity for 40-90 min<sup>[24]</sup>. Since the abdomen remains opened, it is possible for the surgeon to support the circulation in the abdominal cavity manually<sup>[25]</sup>. This procedure is followed by thorough lavage, anastomosis of resected bowel segments and closure of the abdominal wall<sup>[24]</sup>.

A survival benefit using CRS and hyperthermic intraperitoneal chemotherapy (HIPEC) has been shown<sup>[26]</sup>. Verwaal *et al.*<sup>[27]</sup> reported a 3-year survival of 38% in their patients. A more recent follow up of the same cohort shows similar survival rates<sup>[28]</sup>. Median progression-free survival was 7.7 mo in the control arm and 12.6 mo in the HIPEC arm. A 5-year survival of 45% was found in patients, in which R1 resection could be achieved. This

indicates that CRS combined with HIPEC is superior to systemic chemotherapy alone. Nevertheless the findings of Franko *et al.*<sup>[29]</sup> suggest, that CRS combined with HIPEC as well as systemic chemotherapy alone have their roles in the multidisciplinary approach treating peritoneally disseminated cancer.

In selected patients even a long term survival may be possible, with CRS and HIPEC being a curative approach in disseminated colorectal carcinoma<sup>[28,30]</sup>.

The HIPEC procedure itself is demanding for most of the patients. Even though the median survival rates increased, the 30-d mortality rate of 4.8% and a morbidity rate reaching up to 55% are high<sup>[31]</sup>. The surgery itself and severe systemic side-effects may lead to deterioration of health or death<sup>[32,33]</sup>. Given that, the inclusion criteria to receive CRS and HIPEC remain strict. The peritoneal surface has to be the only site of disease dissemination<sup>[27]</sup> and the preoperative assessment<sup>[34]</sup> should suggest a high likelihood of achieving complete cytoreduction (CC-0)<sup>[35]</sup>. Therefore only patients with medium-sized intraperitoneal tumor nodules and a limited distribution within the abdomen are selected<sup>[36]</sup>. Patients have to be physically fit to endure this extensive procedure. Considering that peritoneal carcinomatosis only becomes symptomatic in advanced stages, where CC-0 or CC-1 can rarely be achieved, only few highly selected patients have access to this approach<sup>[37]</sup>. Excluded patients are left with systemic chemotherapy.

Alternative techniques have been investigated to target chemotherapeutic agents to the body cavities without the strain of surgery. These are promising approaches to circumvent both the systemic side effects and the hazard of an extensive surgical procedure.

## DRUG-ELUTING BEADS

### Bead characteristics

Promising carriers for contemplanable agents such as doxorubicin, irinotecan or mitoxantrone are drug-eluting beads (DEB).

By far the most commonly used product in clinic is DC Bead™, which are microspheres comprised of a sulphonate-modified polyvinyl-alcohol hydrogel. They are available in sizes from 70-700 µm<sup>[38]</sup> and can be loaded with doxorubicin (DOX), irinotecan (IRI) or mitoxantrone (MTX)<sup>[39]</sup>. When drug-loaded, the product provides an accurate dosage of drug per unit volume of beads *in vitro*<sup>[38]</sup>, which they release *via* ion exchange constantly over weeks<sup>[40,41]</sup>. *In vitro*, the beads are robust and maintain their size and shape after drug loading<sup>[42]</sup>. This is a prerequisite for DEBs, since damage of the beads may cause rapid liberation and significant systemic distribution of the encapsulated drug or adverse effects by the debris itself.

The surface of the DEBs itself is inert and did not cause any immune reaction in control groups treated with unloaded beads<sup>[39,43]</sup>. Furthermore the biomechanical engineered material is able to shield its content from the immune system<sup>[44,45]</sup>.

### Present field of application

DEBs are used in clinical practice for trans-arterial chemoembolisation (TACE) of hypervascularized tumors<sup>[46]</sup>, such as hepatocellular carcinoma (HCC) and liver metastasis. By administering them selectively into the tumor-feeding vessels, the route for essential nutrients is obstructed and high levels of antineoplastic drugs can be reached within the tumor<sup>[47]</sup>.

As the procedure itself can be carried out under local anesthesia, morbidity and complication rates are low<sup>[48]</sup>, TACE has become the standard palliative approach in patients with unresectable HCC<sup>[49-51]</sup>. The objective response rates range from 70%-75%<sup>[52,53]</sup> at a low rate of complications<sup>[53]</sup>. This suggests a good risk-benefit ratio.

For both associated side effects<sup>[54]</sup> and progression free survival<sup>[55]</sup> as well as overall disease control<sup>[54,55]</sup>, doxorubicin-loaded DC beads (DOXDEB™) produced the most promising results.

## DEBS IN EXTRAVASCULAR USE

Since DEBs are able to liberate agents continuously *in vitro*<sup>[56]</sup> they can also serve as drug carriers for extravascular application if the beads are directly instilled into the compartments.

### Intracerebral therapy

The median survival of rats with experimental glioblastoma multiforme (GBM) could be successfully prolonged using doxorubicin polymers<sup>[57]</sup>. This demonstrated a superior effect of chemotherapeutic carriers as compared to *iv* administration of the free drug. The most efficient drug - namely doxorubicin - caused the most severe side effects. Intracerebral hemorrhage and edema as well as hemiparesis were observed<sup>[57]</sup>. A significantly longer median survival could be achieved in patients with GBM using carmustine warfers<sup>[58]</sup>, but they did not affect the recurrence-free survival times<sup>[59]</sup>. These findings justified the idea of compartment-based therapy, but also called for new delivery systems and alternative antineoplastic drugs in return.

Baltes *et al*<sup>[60]</sup> showed that the intracerebral administration of DEBs is safe for use depending on the loaded drug. Both doxorubicin- and irinotecan-loaded DEBs significantly improved survival time in a rat BT4Ca GBM model. Doxorubicin again caused severe side effects<sup>[57]</sup> whereas irinotecan seemed to selectively affect only the cancer cells and not healthy brain tissue<sup>[61,62]</sup>. These findings could be confirmed in follow-up experiments where alginate was used as a viscosity modifier to secure the administration of the beads into the tissue<sup>[63]</sup>.

### Intrapancreatic therapy

The efficacy of irinotecan- and topotecan-loaded DEBs have been evaluated by use of a modified MTS assay and in a PSN-1 mouse xenograft model of pancreatic cancer by direct injection at the tumor site. Topotecan was shown to be more potent than irinotecan in the *in*

*vitro* cell assay, had reasonable efficacy and tolerability at 0.2-0.4 mg doses but was lethal at doses of 0.83-1.2 mg. Irinotecan however, was well tolerated even with repeated injections of doses from 3.3-6.6 mg and displayed good efficacy<sup>[64]</sup>. A similar study evaluated combinations of doxorubicin, irinotecan, topotecan and rapamycin DEBs and demonstrated synergistic activity for certain drug combinations, in particular doxorubicin and rapamycin<sup>[65]</sup>.

Feasibility for the clinical application of the direct intratumoral delivery of a compartment-based therapy was first demonstrated by delivery of a reservoir of a thermosensitive gel containing paclitaxel (Oncogel®) into the pancreas by use of ultrasound-guided endoscopic needle injection<sup>[66,67]</sup>. This approach has been subsequently adapted for the administration of irinotecan-loaded DEBs suspended in alginate into the tail of the pancreas of a healthy pig. The therapy was well tolerated up to doses of 300 mg of irinotecan, with only localized pancreatic tissue reactions on histopathologic review<sup>[68]</sup>.

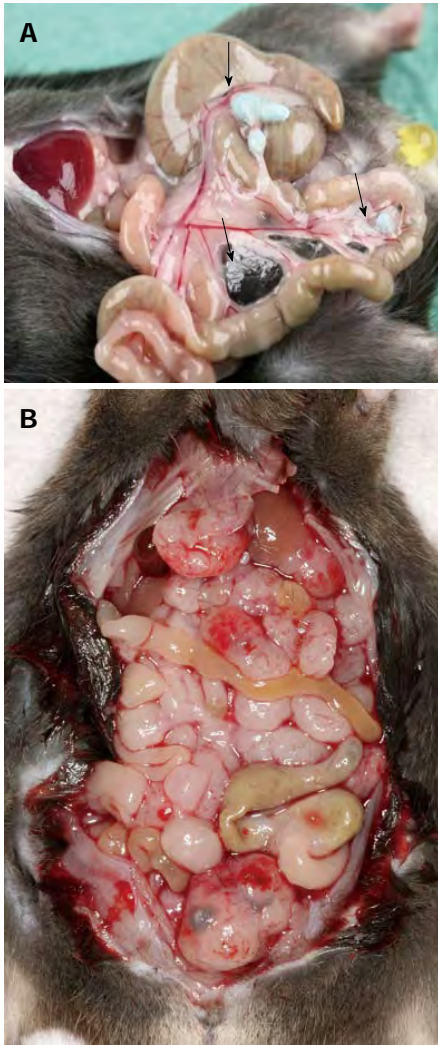
### Intraperitoneal therapy of peritoneal carcinomatosis

An elegant approach to target drugs to a tumor is to administer them as pro-drugs and activate them intratumorally. The active metabolites are formed by enzymes which are selectively injected into the tumor.

Routinely, ifosfamide has been used *via iv* application in pancreatic cancer treatment<sup>[69,70]</sup>. After administration cytochrome P450 2B1 (CYP2B1) produced by hepatocytes, transforms ifosfamide into 4-OH-ifosfamide, which results in the active compounds phosphoramidate mustard and acrolein<sup>[71]</sup>. *In vitro* preparation and direct administration of the active compounds are limited due to their short half life (45 min)<sup>[72]</sup>.

Löhr *et al*<sup>[73]</sup> used encapsulated feline kidney cells, engineered to produce CYP2B1<sup>[74]</sup>, to target activated ifosfamide into pancreatic carcinoma<sup>[73]</sup>. Therefore, cells were encapsulated in cellulose sulphate<sup>[75]</sup> for immobilization and to protect them from the immune system when injected into the tumor. To model a pancreatic cell-like carcinoma PaCa-44 human pancreatic tumor cells were injected subcutaneously into nude mice. All mice received ifosfamide *iv*, one group received intra-tumorous injection of encapsulated CYP2B1 producing cells and one group received nonencapsulated cells. Tumor growth was impaired in all mice receiving ifosfamide. However, the most significant tumor reduction was detectable in the group that had received encapsulated cells. Complete macro- and microscopic tumor remission could be achieved in 20% of the animals. Although the same dosages of ifosfamide were used in both groups, the apoptotic rate of tumor cells was three times higher in the group receiving encapsulated cells. Furthermore these animals appeared healthier than the ones receiving nonencapsulated cells. Müller *et al*<sup>[43]</sup> were able to reproduce these results using a CYP2B1 producing cell line of human origin. This cell line did not produce potentially harmful retroviruses<sup>[76]</sup> and is immune resistant<sup>[77]</sup>.

The approach worked for other tumor entities as



**Figure 1** Peritoneal metastasis. A: Beads accumulate in the mesentery of the small bowel (arrows). Animals show a complete tumor remission; B: Control animal with disseminated peritoneal carcinomatosis induced by Panc02 cells.

well. Samel *et al.*<sup>[78]</sup> showed, that similar results could be achieved in Balb/c mice carrying peritoneal tumor nodules, induced by syngenic C-26 cells injected into the abdominal cavity. This cell line is highly malignant and rapidly forms tumor nodules on the peritoneum. Again, in some animals a complete response was achieved. One major drawback of this approach is the use of genetically engineered cells. These cells may maintain a malignant potential. It remains to be shown if they can be safely applied to patients.

Therefore, an easier approach directly employs encapsulated chemo agents. *In vitro* tests with wild-type C-26 murine colon-carcinoma cells showed potent tumor toxicity for free DOX, IRI and MTX and the encapsulated drugs when combinations of the chemotherapeutic agents and DEBs were tested<sup>[39]</sup>. For free IRI and MTX the inhibition of cell growth was superior to their encapsulated forms. The proportion of apoptotic cells was significantly higher for free DOX as well as for DOXDEB™ when compared to the other two agents. Both DOX and MTX showed a dose-depending induction of apop-

tosis, whereas IRI did not show any significant effect.

*In vivo* tests followed after determining appropriate concentration levels<sup>[39]</sup>. For better detection of micrometastases and for tumor load quantification, C-26 cells had been transfected with the marker protein enhanced green fluorescent protein as described<sup>[78,79]</sup>. All animals developed disseminated PC. Thereafter, animals were treated with free and encapsulated DOX and MTX. Best tumor reduction was obtained when splitting the DEB application into three sessions. Complete tumor remission could be obtained. Weight loss and mortality of the subjects was significantly higher in the groups which were treated with the corresponding free drugs, suggesting a lower toxicity in the DEB groups.

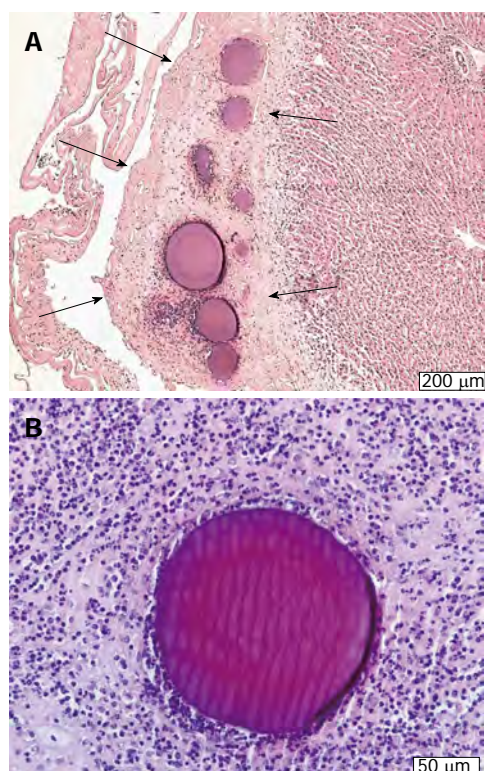
The results obtained in this model of colorectal tumor, could be reproduced for pancreatic carcinoma dissemination (Figure 1). Yagublu *et al.*<sup>[80]</sup> used a model of peritoneally metastasized panc02 pancreatic carcinoma cells in C57 black6 mice. Treatment was performed with free and encapsulated DOX, IRI and MTX. The free drug was more potent in decreasing tumor cell growth and inducing apoptosis than the encapsulated drugs *in vitro*. Again, *in vivo* free drug administration caused more weight loss and significantly higher lethality than the encapsulated drug, while no relevant differences in antitumoral activity could be observed.

To test the safety of the intraperitoneal injection and therapy using the DOXDEB™ a large animal trial was carried out<sup>[81]</sup>. Black-headed meat-sheep received an application of DOXDEB™ into the abdominal cavity. Up to 50% of the maximal cumulative dose suggested for male humans were used in one single intraperitoneal injection<sup>[82]</sup>. DEBs were injected using a verres needle. Upon autopsy, no DEBs were distributed *via* blood or lymphatic vessels. Beads remained on the peritoneum, immobilized by a fibrin layer (Figure 2A and B). No evidence for organ-related damage or systemic toxicity was observed. This is remarkable, as cardio toxicity<sup>[83-87]</sup> and myelosuppression<sup>[88-91]</sup> are frequently described with the systemic use of doxorubicin, along with less severe side effects such as stomatitis, alopecia, nausea and vomiting<sup>[90]</sup>. The systemic distribution of DOX followed a three-compartment-model omitting a rapid and high peak, in comparison to *iv* administration. Serum levels reached a steady-state 360 min after application with a half-life of 615 h. Some sheep did not reach the end point and developed a chemical peritonitis<sup>[82,92,93]</sup> (Figure 3). By circumventing the systemic administration and its accompanying side effects, local toxicity was the only limiting factor. This underlines the importance of drug choice when it comes to DEB therapy within the intraperitoneal compartment.

## CONCLUSION

There is convincing evidence that drug-eluting beads can be employed in an extravascular environment for a compartment-based therapy. In several tumor models, the carrier devices showed convincing tumor control and





**Figure 2** Doxorubicin-eluting beads. A: HE-stained, magnification  $\times 50$ : A layer of fibrin (between arrows) immobilizing the doxorubicin-loaded DC beads (DOXDEB™) on the livers surface; B: HE-stained, magnification  $\times 100$ : DOXDEB™ in layer of fibrin and surrounded by lymphocytes immobilizing it on mesenteric connective tissue.



**Figure 3** Chemical peritonitis. Autopsy of an animal 28 d after installation of *ip* doxorubicin-loaded drug-eluting beads: Situs with greater omentum and intestinal loops with fibrinous adhesions, amber-colored ascitic fluid.

side-effects were less likely to occur. Also, encapsulation devices can be used to transform pro-drugs into their active metabolites within or in vicinity of the tumor. Here, drug-eluting beads successfully immobilized transforming-enzyme producing cells and protected them from the host immune system. However, the application of genetically engineered cell lines remains a major safety concern.

Intraperitoneal application of DEBs is a small procedure which can be safely performed under local anesthesia. Within the abdominal cavity DEBs show predictable liberation characteristics, remain inert and do not distrib-

ute *via* blood or lymphatic vessels.

Compartment-based therapy could be considered as a favorable treatment option for palliative patients with a deteriorated general condition, who are not eligible for HIPEC. Local toxicity is a limiting factor. Other drugs - for example irinotecan - have to be tested in a large animal model to further investigate local reactions.

If the adverse effects of the loaded substances are controlled, the extravascular use of drug-eluting beads is a promising future approach in compartment-based tumor therapy.

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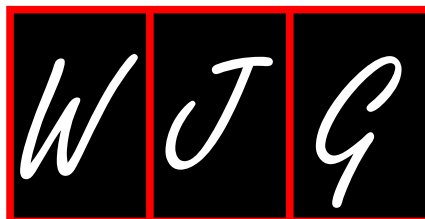
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## Role of sirtuins in ischemia-reperfusion injury

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### Abstract

Ischemia-reperfusion injury (IRI) remains an unresolved and complicated situation in clinical practice, especially in the case of organ transplantation. Several factors contribute to its complexity; the depletion of energy during ischemia and the induction of oxidative stress during reperfusion initiate a cascade of pathways that lead to cell death and finally to severe organ injury. Recently, the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases has gained increasing attention from researchers, due to their involvement in the modulation of a wide variety of cellular functions. There are seven mammalian sirtuins and,

among them, the nuclear/cytoplasmic sirtuin 1 (SIRT1) and the mitochondrial sirtuin 3 (SIRT3) are ubiquitously expressed in many tissue types. Sirtuins are known to play major roles in protecting against cellular stress and in controlling metabolic pathways, which are key processes during IRI. In this review, we mainly focus on SIRT1 and SIRT3 and examine their role in modulating pathways against energy depletion during ischemia and their involvement in oxidative stress, apoptosis, micro-circulatory stress and inflammation during reperfusion. We present evidence of the beneficial effects of sirtuins against IRI and emphasize the importance of developing new strategies by enhancing their action.

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**Key words:** Sirtuin 1; Sirtuin 3; Ischemia-reperfusion injury; Oxidative stress; Apoptosis

**Core tip:** Sirtuins are responsible for the regulation of protein activation by deacetylating a range of proteins that play important roles in the pathophysiology of various diseases. The present review summarizes the beneficial effects of sirtuins 1 and 3, the two most prominent sirtuins involved in mammalian energy homeostasis and oxidative stress. We conclude that both sirtuins might be attractive targets for counteracting the detrimental effects of ischemia-reperfusion injury.

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### INTRODUCTION

Sirtuins belong to the highly conserved class III histone



deacetylases with homology to the yeast silent information regulator 2. To date, seven sirtuins have been described in mammals. They possess nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent deacetylase activity, with the exception of sirtuin 4 (SIRT4) which has only ADP-ribosyltransferase activity, and SIRT1 and SIRT6 which have not only deacetylase activity but also relatively weak ADP-ribosyltransferase activity<sup>[1]</sup>. Their enzymatic activity depends on their protein expression levels, the availability of NAD<sup>+</sup> and the presence of proteins that modulate sirtuin enzymatic activity. For instance, SIRT1 expression increases during starvation or when cells are exposed to conditions of oxidative stress and DNA damage<sup>[2,3]</sup>.

Sirtuins are found in several subcellular locations, including the nucleus (SIRT1, SIRT6, and SIRT7), cytosol (SIRT2), and mitochondria (SIRT3-SIRT5). In some studies, however, SIRT1 has been found to possess cytosolic activity, and SIRT2 has been found to be associated with nuclear proteins<sup>[4]</sup>.

Several recent studies have shown that sirtuins regulate a wide variety of cellular processes, such as gene transcription, metabolism and cellular stress response<sup>[5-7]</sup>. SIRT1, the most studied member of the family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis<sup>[8,9]</sup>. SIRT3 has recently been reported to have a considerable impact on mitochondrial energy metabolism and function<sup>[10,11]</sup>. In this review, we will focus mainly on SIRT1 and SIRT3 functions in ischemia-reperfusion injury (IRI).

IRI is one of the most significant problems in graft injury, contributing to primary graft dysfunction or non-function after organ transplantation<sup>[12-14]</sup>. Many factors contribute to IRI. First of all, the loss of oxygen supply during ischemia results in the reduction of adenosine triphosphate (ATP) synthesis and subsequent changes in ion influx, acidosis and cell swelling which may eventually lead to cell death. The restoration of blood flow is followed by an excessive acute inflammatory response triggering the reperfusion injury. Although the ischemic insult causes significant damage in cells, the tissue injury generated during reperfusion is much more severe. On reperfusion, oxygen is suddenly available, and metabolism proceeds rapidly, resulting in a sudden production of reactive oxygen species (ROS), cytokines and chemokines which increase the accumulation of inflammatory cells (monocytes, dendritic cells and granulocytes). In combination with excessive nitric oxide (NO), ROS are able to induce DNA damage and activate various types of cell death pathways<sup>[15-17]</sup>.

Understanding the mechanisms involved in the pathogenesis of IRI is the first step to mitigate its adverse effects. Sirtuins are known to regulate many important processes in cell physiology, including those affecting IRI, such as cellular metabolism and stress response. This makes them potentially appealing targets for therapeutic interventions against IR-induced injury.

## ROLE OF SIRTUINS IN ISCHEMIA

The low energy state during ischemia results in activation

of adenosine monophosphate protein kinase (AMPK), a fuel-sensing enzyme that is positively regulated by an increased ratio of adenosine monophosphate to ATP. When AMPK is activated, it stimulates processes that restore ATP levels (*e.g.*, fatty acid oxidation) and inhibits other processes that consume ATP (*e.g.*, protein synthesis)<sup>[18]</sup>. The activity of sirtuins is directly related to the metabolic state of the cell due to their dependence on NAD<sup>+</sup>. Suchankova and collaborators found that glucose-induced changes in AMPK are linked to alterations in the NAD<sup>+</sup>/reduced nicotinamide adenine dinucleotide ratio and SIRT1 abundance and activity<sup>[19]</sup>. These results may suggest a possible interaction between AMPK and SIRT1 in ischemic conditions. Indeed, an activator of AMPK, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside, has been found to improve IRI and increase SIRT1 expression in the rat kidney<sup>[20]</sup>. Furthermore, enhancing the activity of SIRT1 through the application of resveratrol, a SIRT1 activator, has been demonstrated to protect against cerebral ischemia<sup>[21]</sup>.

Another element that plays an essential role in triggering cellular protection and preventing metabolic alterations caused by oxygen deprivation is hypoxia-inducible factors (HIFs). Mammals possess three isoforms of HIF $\alpha$ , of which HIF1 $\alpha$  and HIF2 $\alpha$  are the most structurally similar and the best characterized. During hypoxia, protein levels of HIF2 $\alpha$  increase slightly, but it presents significant activation, which suggests that its activity is regulated by additional post-translational mechanisms. One of these post-translational modulations may be deacetylation, since in hypoxic Hep3B cells SIRT1 deacetylates lysine residues in the HIF2 $\alpha$  protein, enhancing its transcriptional activity<sup>[22]</sup>.

Additionally, SIRT1 interacts with HIF1 $\alpha$ , but in this case SIRT1 represses HIF1 $\alpha$  transcriptional activity<sup>[23]</sup>. Under hypoxic stress, decreased cellular NAD<sup>+</sup> downregulates SIRT1, increases HIF1 $\alpha$  acetylation, and thereby promotes the expression of HIF1 $\alpha$  target genes<sup>[23]</sup>. Interestingly, other studies have shown that HIF2 $\alpha$  compete with HIF1 $\alpha$  for binding to SIRT1<sup>[24]</sup>. Moreover, it has been demonstrated that SIRT6 is also linked to HIF1 $\alpha$  by repressing the transcription of HIF1 $\alpha$  target genes<sup>[25]</sup>.

Likewise, the effects of SIRT3 appear to be protective in the context of hypoxic stress in human cancer cells. SIRT3 overexpression resulted in decreased ROS production, impediment of HIF1 $\alpha$  stabilization and subsequent suppression of tumorigenesis<sup>[26,27]</sup>. However, the effect of SIRT3 in HIF1 $\alpha$  stabilization in IRI has not been reported to date.

One of the most important factors involved in the metabolic control regulated by SIRT1 is peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ), a transcriptional co-activator of many nuclear receptors and transcriptional factors. SIRT1 functionally interacts with PGC1 $\alpha$  and deacetylates it, thus inducing the expression of mitochondrial proteins involved in ATP-generating pathways<sup>[28]</sup>. Increased PGC1 $\alpha$  activity is also associated with lower levels of oxidative damage during

ischemia, as shown by the decrease ROS scavenging in rodents lacking PGC1 $\alpha$  subjected to global ischemia<sup>[29]</sup>. Furthermore, the uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, regulates the proton electrochemical gradient and in neuronal cells PGC1 $\alpha$  is required for the induction of UCP2 and subsequent protection against oxidative stress<sup>[30]</sup>. It has also been shown that enhanced activity of SIRT1 during ischemic preconditioning (IPC) or resveratrol preconditioning confers protection against cerebral ischemia by reducing UCP2 levels, which results in increased ATP levels<sup>[21]</sup>. However, a more recent study associated the protective effect of resveratrol against oxidative stress in cerebral ischemia with increased levels of SIRT1/PGC1 $\alpha$  and UCP2<sup>[31]</sup>. Moreover, the exact role of UCP2 during ischemia is not fully understood, as studies of its effects have produced conflicting results<sup>[32-35]</sup>.

## ROLE OF SIRTUINS IN REPERFUSION

Deprivation of oxygen to the grafts during ischemia induces severe lesions, but the most important damage is caused during reperfusion, when oxygen entry to the organ is restored. During reperfusion, the cellular metabolism returns to aerobic pathways, which results in the generation of a wide variety of ROS, including superoxide, hydrogen peroxide and reactive nitrogen species, such as peroxynitrite. ROS are mainly produced in mitochondria and trigger several phenomena, including accumulation of Ca<sup>2+</sup>, caspase activation, cytokine upregulation, lipid, protein and DNA damage<sup>[36-38]</sup>. ROS can be eliminated by enzymatic pathways including manganese superoxide dismutase (MnSOD), catalase (Cat) and peroxidases. Imbalance between ROS generation and elimination produces oxidative stress<sup>[15,16]</sup>.

Various reports in cardiomyocytes have demonstrated the protective role of SIRT1 against oxidative stress<sup>[39,40]</sup>. Hearts overexpressing SIRT1 were more resistant to oxidative stress in response to IRI, as SIRT1 upregulated the expression of anti-oxidants like MnSOD and thioredoxin 1<sup>[41]</sup>. SIRT1 also deacetylated Forkhead box-containing protein O (FoxO) 1 transcription factor, inducing its nuclear translocation and subsequent transcription of anti-oxidant molecules<sup>[41,42]</sup>. Moreover, the question of whether SIRT1 can induce the transcription of other FoxO transcription factors, like FoxO3 $\alpha$ , has not yet been investigated. However, the levels of SIRT1 activation are decisive for its protective role, as very high cardiac SIRT1 expression induces mitochondrial dysfunction and increases oxidative stress<sup>[39]</sup>. Furthermore, in a model of kidney IRI, the protective effect of SIRT1 against oxidative stress has also been demonstrated since SIRT1 upregulated Cat levels and maintained peroxisome number and function<sup>[43]</sup>.

Although mitochondrial sirtuins (SIRT3-SIRT5) have not been studied as extensively as SIRT1, an increasing body of evidence indicates the importance of SIRT3 in mitochondrial biology and function. Lombard *et al*<sup>[44]</sup>

demonstrated that SIRT3 is the dominant mitochondrial deacetylase, as a significant number of mitochondrial proteins are hyperacetylated in SIRT3<sup>-/-</sup> mice. SIRT3 deacetylates and thus enhances the activity of various proteins that appear to be an important part of the anti-oxidative defense mechanisms of mitochondria, such as MnSOD<sup>[45,46]</sup>, regulatory proteins of the glutathione and thioredoxin system<sup>[50]</sup>.

Transcriptional upregulation of the antioxidant enzymes MnSOD, Cat and peroxiredoxin can also be achieved by FoxO3 $\alpha$  transcription factor, which is translocated to the nucleus after being deacetylated by SIRT3<sup>[51,52]</sup>. Furthermore, SIRT3 is necessary for the enhanced expression of cytochrome c, which presents peroxidase- and superoxidase-scavenging capacity<sup>[47,49,53]</sup>. However, a similar anti-oxidant effect of SIRT3 in models of IRI has not yet been established.

A wide array of functional alterations develop in mitochondria during reperfusion injury<sup>[36,54]</sup>. In healthy cells, their primary function is the provision of ATP through oxidative phosphorylation in order to meet the high energy demands. There is increasing evidence of the involvement of a multi-protein complex called the mitochondrial permeability transition pore (mPTP) in the decline in mitochondrial function, which is a common finding during reperfusion injury<sup>[55-57]</sup>. SIRT3 is known to deacetylate the regulatory component of the mPTP, cyclophilin D, and thereby reduce its activity and the subsequent mitochondrial swelling in the heart<sup>[58]</sup>. It has also been shown that SIRT4 interacts with the adenine nucleotide translocator, another component of mPTP, and that SIRT5 deacetylates cytochrome c, but the physiological importance of these interactions has not yet been established<sup>[59,60]</sup>, especially in models of IRI.

Microcirculatory alterations play an important part in IRI. During the ischemic period, vascular hypoxia can cause increased vascular permeability. After reperfusion, complement system activation, leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation further aggravate microvascular dysfunction<sup>[61]</sup>.

NO produced by endothelial NO synthase (eNOS) is a key regulator of endothelial function, as it opposes the vasoconstrictive actions of endothelins and provokes vasodilatation. Thus, it can abrogate the microcirculatory stress generated during reperfusion<sup>[62]</sup>. However, NO produced by inducible NO synthase (iNOS) exacerbates IRI through the NOS-derived superoxide production or the generation of peroxynitrite<sup>[12]</sup>. There is a large body of evidence in favor of the relationship between eNOS and SIRT1; SIRT1 interacts and modifies the acetylation state of eNOS, resulting in the activation of the enzyme<sup>[63-65]</sup>. In SIRT1<sup>+/-</sup> hearts subjected to IRI SIRT1 was associated with eNOS activation<sup>[66]</sup>. SIRT1 activation by resveratrol protected against subacute intestinal IRI by reducing the NO production through iNOS<sup>[67]</sup>. Moreover, various experimental models showed that resveratrol inhibits endothelin-1 levels, providing better regulation of vascular tone<sup>[68-70]</sup>. However, a recent study in human umbilical vein endothelial cells

has shown that the inhibitory effects of resveratrol on endothelin-1 levels are SIRT1-independent<sup>[71]</sup>.

## ROLE OF SIRTUINS IN IRI-ASSOCIATED INFLAMMATION

IRI results in a profound inflammatory tissue reaction with immune cells infiltrating the tissue. The damage is mediated by various cytokines, chemokines, adhesion molecules, and compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factors with nuclear factor kappa B (NF- $\kappa$ B) being one of the key modulators of inflammation. After activation, the transcription factor migrates to the nucleus and enhances the transcription of pro-inflammatory genes potentiating the inflammatory response. This is followed by an infiltration of lymphocytes, mononuclear cells/macrophages, and granulocytes into the injured tissue<sup>[72-74]</sup>.

In this way, SIRT1 plays an important role in neuro-protection against brain ischemia by deacetylation and subsequent inhibition of p53 and NF- $\kappa$ B pathways<sup>[75]</sup>. In SIRT1<sup>+/+</sup> hearts subjected to IRI SIRT1 was correlated with decreased acetylation of NF- $\kappa$ B and possible prevention of inflammation<sup>[66]</sup>. Moreover, the anti-inflammatory action of SIRT1 by deacetylating NF- $\kappa$ B and thus inhibiting the expression of endothelial adhesion molecules has also been demonstrated in human aortic endothelial cells<sup>[74]</sup>.

## SIRTUINS: CELL SURVIVAL OR DEATH?

Apoptotic cell death is a well known mechanism involved in IRI which occurs *via* activation of caspases that cleave DNA and other cellular components<sup>[16,17,76]</sup>. There is evidence that SIRT1 is associated with longevity in mammals and enhances mammalian cell survival under stress conditions *via* regulating the specific substrates<sup>[77-79]</sup>. In fact, several studies have mentioned the anti-apoptotic effect of SIRT1 in IRI. SIRT1 deacetylates known mediators of apoptosis, such as the tumor-suppressor p53, resulting in inhibition of its transcriptional activity<sup>[80,81]</sup>. SIRT1 also deacetylates the DNA repair factor Ku70<sup>[2,82,83]</sup>, thus Ku70 prevents the translocation of Bax, a pro-apoptotic B cell lymphoma-2 (Bcl-2) family protein, to the mitochondria. In ischemic kidney and brain SIRT1 has been identified as an important survival mediator, given that increased SIRT1 was associated with reduced p53 expression and apoptosis<sup>[75,84]</sup>. SIRT1 also modulates apoptosis-related molecules through the deacetylation of the FoxO family of transcription factors. During IRI in heart-specific SIRT1<sup>+/+</sup> transgenic mice, SIRT1 induces nuclear translocation of FoxO1, which upregulates the anti-apoptotic factors Bcl-2 and Bcl-like X and down-regulates Bax<sup>[41]</sup>. As regards other members of the FoxO family, Brunet *et al.*<sup>[85]</sup> revealed a dual role of SIRT1 in the cell cycle depending on stress conditions; SIRT1 inhibited the ability of FoxO3 to induce cell death, thus promoting cell survival and, surprisingly, it also increased the

ability of FoxO3 to induce cell cycle arrest and resistance to oxidative stress.

A possible pro-apoptotic role of SIRT1 in IRI has not been reported previously. However, studies in human embryonic kidney cells have revealed that SIRT1 can promote cell death by inhibiting NF- $\kappa$ B in response to tumor necrosis factor alpha<sup>[86]</sup>. Further investigation is required to define the conditions under which SIRT1 may promote apoptosis.

Apoptotic pathways are known to be initiated during reperfusion upon the opening of the mPTP which leads to the release of caspase-activating molecules<sup>[87,88]</sup>. Since SIRT3 is located in the mitochondria, it may be involved in anti-apoptotic pathways. In this regard, SIRT3 protects various types of cells from apoptotic cell death triggered by genotoxic or oxidative stress<sup>[89-92]</sup>. The pro-apoptotic role of SIRT3 has also been associated with tumor suppression and restraint of ROS<sup>[93]</sup>. However, SIRT3 has also been reported to contribute to Bcl-2- and JNK-related apoptotic pathways in human colorectal carcinoma cells<sup>[94]</sup>. In any case, the potential anti-apoptotic mechanisms of SIRT3 during IRI are yet to be elucidated.

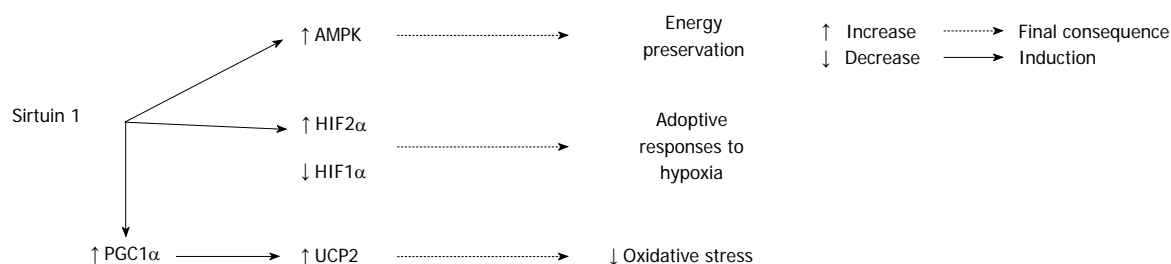
## CONCLUSIONS AND PERSPECTIVES

A wide range of pathological processes contribute to IRI. Particularly during organ transplantation, IRI contributes to early graft dysfunction. For this reason, it is important to gain additional mechanistic insight into the molecular mechanisms underlying this injury. In the past few years, sirtuins have emerged as critical modulators of various cellular processes, including those that contribute to the pathogenesis of IRI.

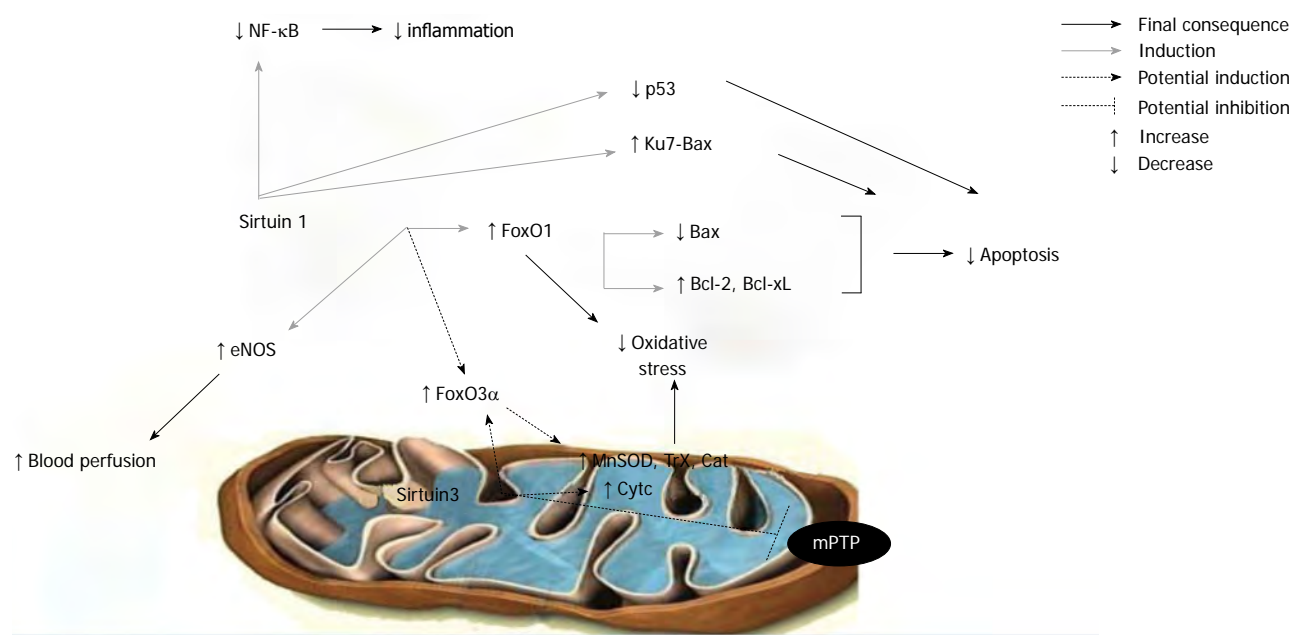
In this paper, we have reviewed the signaling pathways of SIRT1 and SIRT3 protection in IRI. SIRT1 has been shown to exert its beneficial effect against oxidative stress, hypoxic injury or inflammation associated with IRI by activating FoxO1, PGC1 $\alpha$  and HIF2 $\alpha$  or by inhibiting NF- $\kappa$ B transcription factors (Figures 1 and 2). SIRT3's protective role in IRI is mainly mediated by activating FoxO3 $\alpha$  and mitochondrial anti-oxidant enzymes (Figure 2). Investigations that can further determine other intracellular signaling, trafficking and post-translational modifications by SIRT1 and SIRT3 in a variety of cell systems and environments will allow us to translate this knowledge into effective treatment strategies that will be applicable in multiple disorders.

Numerous studies have demonstrated key roles for SIRT1 and SIRT3 in brain, heart and kidney IRI. However, the protective effect of these sirtuins against ischemic processes in other organs such as the liver has not yet been demonstrated. The relevance of SIRT3 in the hepatic metabolism has been confirmed in a study showing that its overexpression in hepatocytes decreased the accumulation of lipids *via* AMPK activation<sup>[95]</sup>. Furthermore, deletion of hepatic SIRT1 resulted in hepatic steatosis, hepatic inflammation and endoplasmic reticulum stress<sup>[96]</sup>. Since SIRT1 and SIRT3 have been shown





**Figure 1** Protective role of sirtuin 1 during ischemia. Sirtuin 1 (SIRT1) activates adenosine monophosphate protein kinase (AMPK) as a cell response to counteract the energy deficiency. SIRT1 upregulates hypoxia-inducible factor 2 $\alpha$  (HIF2 $\alpha$ ) and downregulates HIF1 $\alpha$  to increase their transcriptional activity. SIRT1 upregulates peroxisome proliferator-activated receptor- $\gamma$  coactivator, leading to enhancement of anti-oxidant capacity of uncoupling protein 2 (UCP2). PGC1 $\alpha$ : Peroxisome proliferator-activated receptor- $\gamma$  coactivator.



**Figure 2** Protective role of sirtuin 1 and suggestive role of sirtuin 3 during reperfusion. Sirtuin 1 (SIRT1) inhibits inflammation through inhibition of nuclear factor kappa B and activates endothelial nitric oxide synthase for a better microcirculation. SIRT1 downregulates apoptosis through multiple pathways, for example, inhibiting p53 transcriptional activity or favoring the binding between Ku70 and Bax. SIRT1 also enhances forkhead box-containing protein O 1 (FoxO1) transcriptional activity, resulting in Bax downregulation and in the upregulation of B cell lymphoma-2 and Bcl-like X. Deacetylation of FoxO1 by SIRT1 also results in lessening oxidative stress, whereas the same effect may be achieved by deacetylation of forkhead box-containing protein 3 alpha (FoxO3 $\alpha$ ). Sirtuin 3 (SIRT3) is suggested to contribute to decrease in oxidative stress either by a direct interaction with mitochondrial anti-oxidant enzymes [manganese superoxide dismutase (MnSOD), thioredoxin system (Trx), cytochrome (Cyt)] or by enhancing FoxO3 $\alpha$  to transcribe MnSOD and Cat. Mitochondrial permeability transition pore (mPTP) may also be inhibited by SIRT3 and result in less production of oxidative stress. NF- $\kappa$ B: Nuclear factor kappa B; eNOS: Endothelial nitric oxide synthase; Bcl-2: B cell lymphoma-2; Bcl-xL: Bcl-like X; Bax: Bcl-2-associated X; Cat: Catalase.

to exert a beneficial effect in regulating hepatic fatty acid metabolism, it would be interesting to investigate their role in the context of liver transplantation. Currently, the shortage of organs for transplantation has obliged physicians to utilize marginal grafts, including grafts with moderate steatosis. Steatotic livers exhibit a more severe inflammatory reaction and more exacerbated oxidative stress and consequently a higher vulnerability to IRI<sup>[12]</sup>. Thus, activating SIRT1 and SIRT3 might be a potential strategy to protect steatotic livers from IRI as well as to expand the donor pool for liver transplantation. In fact, in preliminary studies our group observed that SIRT1 is involved in the protective mechanisms against IRI elicited by IPC in fatty livers.

For this reason, both surgical and pharmacological

strategies should be developed to enhance the activity of sirtuins and thus mitigate the detrimental effect of IRI. Recent studies have highlighted the important role of SIRT1 in IPC-mediated protection in the heart and brain; in IPC brain, SIRT1 prevents neuronal death<sup>[97]</sup>, whereas during cardiac IPC, SIRT1 regulates HIF1 $\alpha$  protein levels<sup>[98,99]</sup>. A recent review has also associated SIRT1 with the protective effects of hyperbaric oxygen preconditioning against apoptosis in the rat brain<sup>[100]</sup>. However, it is still to be established whether SIRT1 contributes to the protective effects of preconditioning through the regulation of other signalling pathways. Furthermore, its possible implication in IPC related mechanisms in other organs, including the liver or kidney, remains to be elucidated.

Nor has the potential role of sirtuins in cold ischemia



and reperfusion yet been established. In the context of liver IRI, a previous study by our group demonstrated that during normoxic reperfusion, after cold ischemia, the presence of NO favors HIF1 $\alpha$  accumulation, also promoting the activation of other cytoprotective proteins, such as heme oxygenase-1<sup>[101]</sup>. Among these cytoprotective proteins, SIRT1 may be ideally suited to enhance the protective effect.

This review summarizes the basic mediators of IRI influenced by the action of SIRT1 and SIRT3 and highlights the importance of their regulation. Future research should aim to elucidate the complete action of all members of the sirtuins family in IRI, and to develop pharmacological strategies that can allow their action to be modulated.

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## Rare cystic liver lesions: A diagnostic and managing challenge

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### Abstract

Cystic formations within the liver are a frequent finding among populations. Besides the common cystic lesions, like simple liver cysts, rare cystic liver lesions like cystadenocarcinoma should also be considered in the differential diagnosis. Thorough knowledge of each entity's nature and course are key elements to successful treatment. Detailed search in PubMed, Cochrane Database, and international published literature regarding rare cystic liver lesions was carried out. In our research are included not only primary rare lesions like cystadenoma, hydatid cyst, and polycystic liver disease, but also secondary ones like metastasis from gastrointestinal stromal tumors lesions. Up-to date knowledge regarding diagnosis and management of rare cystic liver lesions is provided. A diagnostic and therapeutic algorithm is also proposed. The need for a multidisciplinary approach by a team including radiologists and surgeons familiar with liver cystic entities, diagnostic tools, and treatment modalities is stressed. Patients with cystic

liver lesions must be carefully evaluated by a multidisciplinary team, in order to receive the most appropriate treatment, since many cystic liver lesions have a malignant potential and evolution.

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**Key words:** Liver cyst; Cystic tumor; Hepatic lesion; Gastrointestinal stromal tumors; Metastases; Cystadenoma; Cystadenocarcinoma; Hydatid cyst; Polycystic liver disease; Caroli; Echinococcus

**Core tip:** This paper reviews diagnosis differential diagnosis and management of rare cystic liver lesions which should be considered when a cystic hepatic lesion is identified. A diagnostic and therapeutic algorithm is provided. Patients with cystic liver lesions must be carefully evaluated by a multidisciplinary team, in order to receive the most appropriate treatment, since many cystic liver lesions have a malignant potential and evolution.

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### INTRODUCTION

Cystic lesions within the liver have been reported to occur in up to 5% of the population<sup>[1]</sup>. Most of them are common and benign, but the possibility of a rarer cystic liver lesion, such as hepatobiliary cystadenoma (HC) or hepatobiliary cystadenocarcinoma (HCA), should not be overlooked. They can present with general or specific symptoms depending of the nature of the lesion, or they

can be silent and discovered accidentally<sup>[2]</sup>. In fact, most are found incidentally on imaging studies and tend to have a benign course, but a minority may cause symptoms, and rarely may be associated with serious morbidity and mortality<sup>[2]</sup>. The aim of our review is to focus upon the diagnostic and therapeutic algorithm of rare cystic lesions, including cystadenomas/cystadenocarcinomas, hydatid disease, polycystic liver disease, and metastatic neoplasms from the view of surgeons specialized in hepatobiliary surgery.

## CYSTADENOMA AND CYSTADENOCARCINOMA

It is estimated that cystic neoplasms constitute approximately 5% of liver cysts, among which the malignancy is about 5%<sup>[2,3]</sup>. The overall incidence among hepatic malignant tumors is lower than 0.41%<sup>[2,3]</sup>. About 200 cases of HC, and a little more than half as many HCa, have been reported in the literature<sup>[4]</sup>.

More than 85% of HC are reported in women, and typically in middle-aged persons in the fifth decade of life. HC is an unusual cystic lesion accounting for less than 5% of all biliary neoplasms<sup>[2,4]</sup>. The incidence of HCa is approximately 1 per 10 million patients. Malignant transformation is known to occur from HC to HCa. Older patients in the sixth decade of life are more likely to present with malignant tumors<sup>[2,4]</sup>.

The histogenesis of HC is unknown, although a congenital origin is generally favored. A reactive process to some focal injury is still debated<sup>[5,6]</sup>. Pathologically, HC are multiloculated cysts with a stratified or pseudo-stratified non-ciliated columnar or cuboidal epithelium that contains mucous-producing cells. Papillary infolding is frequently present, and the mesenchyma underlying the tumor is usually hyper cellular, often with ovarian-appearing cells (85%-90%)<sup>[7-9]</sup>. The pre-malignant progression of HC is based on the histologic presence of intestinal metaplasia (IM), characterized by the presence of numerous goblet cells<sup>[10,11]</sup>. HC can easily be distinguished histologically from HCa, where a loss of epithelial nuclear stratification and a tubulo-papillary architecture with nuclear pleomorphism and atypia predominates. The malignant epithelium is multilayered with numerous papillary projections, and the confirmation of an invasion of the stroma confirms the diagnosis of HCa.

Regardless of the various diagnostic modalities, such a lesion (HC) may be difficult to distinguish preoperatively from an HCa<sup>[12]</sup>.

The majority of HC is asymptomatic and discovered incidentally during radiographic studies, or they can present with symptoms related to tumor compression of adjacent organs due to their large size<sup>[2]</sup>. Patients presenting with symptoms generally complain of abdominal pain, abdominal distension, or a palpable mass. Less common presentations include intra-cystic hemorrhage, rupture, and fever from secondary bacterial infection. Any patient presenting with recurrence of liver cysts after treatment

should be suspected of having a neoplastic cyst until proven otherwise<sup>[12]</sup>.

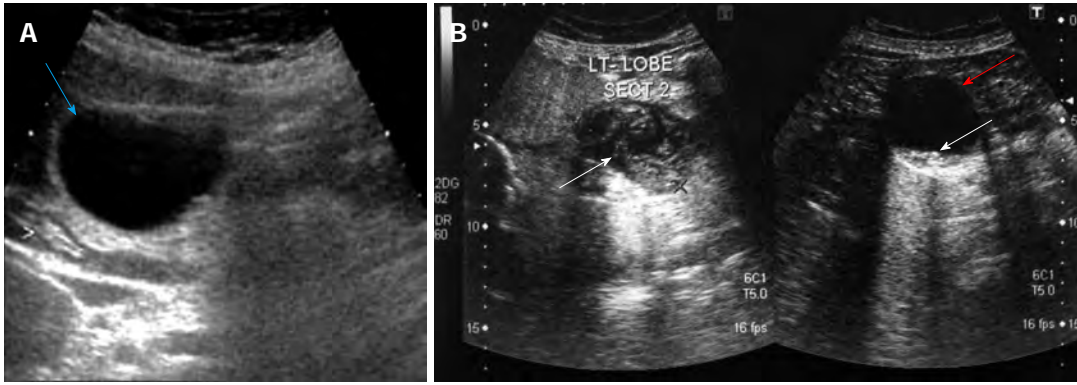
HC and HCa should be differentiated from benign cystic hepatic lesions, including simple hepatic cyst, hepatic abscess, and echinococcal (hydatid) cyst. Simple hepatic cysts usually lack septa. Though hepatic abscesses and echinococcal cysts may appear similar to cystadenocarcinoma on diagnostic imaging, both infectious diseases are easily diagnosed through clinical and laboratory findings. Improvements in imaging techniques have helped to identify HC and HCa.

Ultrasound is an excellent modality that may delineate a simple cyst from other cystic lesions. Additionally, needle aspirates can be performed under ultrasound guidance. Simple cysts appear as anechoic unilocular fluid-filled space with imperceptible walls and posterior acoustic enhancement. A simple cyst is defined as a well-demarcated water attenuation lesion that does not enhance after the administration of intravenous contrast<sup>[2,4]</sup>.

Contrast enhanced ultrasound (CEUS) is useful in assessing the vascularity of a mural nodule and making a distinction between a mural or septal nodule and intracystic debris<sup>[13]</sup>. In conventional ultrasound cystic lesions with solid components (septae, wall, mural nodule), this represents a wide range of rare entities like HC and HCa, as well as more common entities like simple liver cysts (after bleeding or with cell detritus), liver abscesses, or necrotic liver tumors<sup>[13]</sup>. CEUS can be informative regarding the vascularity of solid parts of a cystic lesion. Simple cysts, which are unclear in conventional ultrasound, might be identified in CEUS<sup>[13]</sup>. A cystic liver lesion without vascularization is most probably benign. CEUS is helpful in evaluating nodule vascularity and facilitates the final diagnosis<sup>[13]</sup>.

On conventional ultrasound, a HC typically appears hypoechoic, with thickened irregular walls and occasional internal echoes. Xu *et al*<sup>[13]</sup>, Lin *et al*<sup>[14]</sup>, and Anderson *et al*<sup>[15]</sup> describe it as a well-defined unilocular, or more typically multilocular, cystic mass with mural or septal nodules in rare cases. On CEUS, a HC presents with septa enhancement during the arterial phase and hypo-enhancement during the portal and late phases<sup>[13,14]</sup>. Cystadenocarcinoma, on the other hand, appears as a multilocular cystic mass with mural or septal nodules with thick and coarse calcifications on the septa on conventional ultrasound, while appearing on CEUS with septa enhancement during the arterial phase, mural or septal nodules enhancement and hypo-enhancement during the portal or late phase<sup>[13,14]</sup>. Xu *et al*<sup>[13]</sup> reported that on CEUS there is no significant difference between cystadenoma and cystadenocarcinoma regarding enhancement pattern and extent. Simple cysts, unlike HC, are virtually never septated<sup>[2,4]</sup>. Ultrasonography (US) is a very useful initial investigation in these patients as it demonstrates cystic lesions with thin internal septations, debris, projections, or mural nodes, and it can in most cases accurately distinguish simple from neoplastic cysts (Figure 1A).

Differential diagnosis between HC and HCa is dif-



**Figure 1** Ultrasound image. A: Showing an anechoic mass in the liver (light blue arrow), with a rather thin capsule (cystadenoma); B: Showing two echinococcal cysts. The first on the right (red arrow-right image) appears as an anechoic mass with hydatid sand (type CE1) (white arrow-right image), while in the second (on the left), the detached and folded endocyst membrane is obvious (type CE3) (white arrow-left image).

ficult. Although the presence of mural nodularity is not pathognomonic for cystadenocarcinoma, the absence of mural nodularity is suggestive of cystadenoma<sup>[15,16]</sup>. The diameter of the mural nodule (when it exists) in cystadenomas is much smaller (less than 1.0 cm) than mural or septal nodules in cystadenocarcinomas (larger than 1.0 cm)<sup>[13]</sup>. It seems that the presence of the internal septations and a mural or septal nodule, as well as the nodule diameter, might be diagnostic clues for differentiation between cystadenoma and cystadenocarcinoma<sup>[13]</sup>. The other differential-diagnostic characteristic between HC and HCa is that cystadenomas are more typically multilocular cystic lesions and cystadenocarcinomas more typically unilocular cystic or solid lesions<sup>[13]</sup>.

Computed tomography (CT) is another useful modality to evaluate a cystic lesion of the liver. On a CT scan, a cystadenoma may be unilocular, multilocular, or may have septations. In a study from Vogt *et al*<sup>[3]</sup>, all patients demonstrated septations within the cyst at the CT scan. The cyst wall is usually thickened or irregular, in contrast to a simple cyst. A cystadenoma may also have a smooth external surface and a thin wall (Figure 2A).

Magnetic resonance imaging (MRI) is very useful, as it demonstrates a well-defined lesion that does not enhance after the administration of intravenous gadolinium. On T1 images, the cyst shows a low signal; conversely on T2 weighted images, a very high intensity signal is observed. However, no specific information is gained towards pseudo-ovarian stroma detection<sup>[17]</sup>.

Despite the various diagnostic modalities, it remains difficult to distinguish HCs from HCa on preoperative imaging; however, a significant solid component on the cystic wall suggests invasive malignant disease. Furthermore, HC can evolve into HCa after long periods lasting more than 10 years<sup>[18,19]</sup>.

Liver enzymes and bilirubin are usually normal unless the biliary tree is compressed. The elevation of alkaline phosphate and bilirubin occurs in cases of bile duct obstruction. Carbohydrate antigen 19-9 may be elevated, but CEA and  $\alpha$ -fetoprotein are usually normal<sup>[3,20]</sup>. It has been reported that most patients with cystadenocarcino-

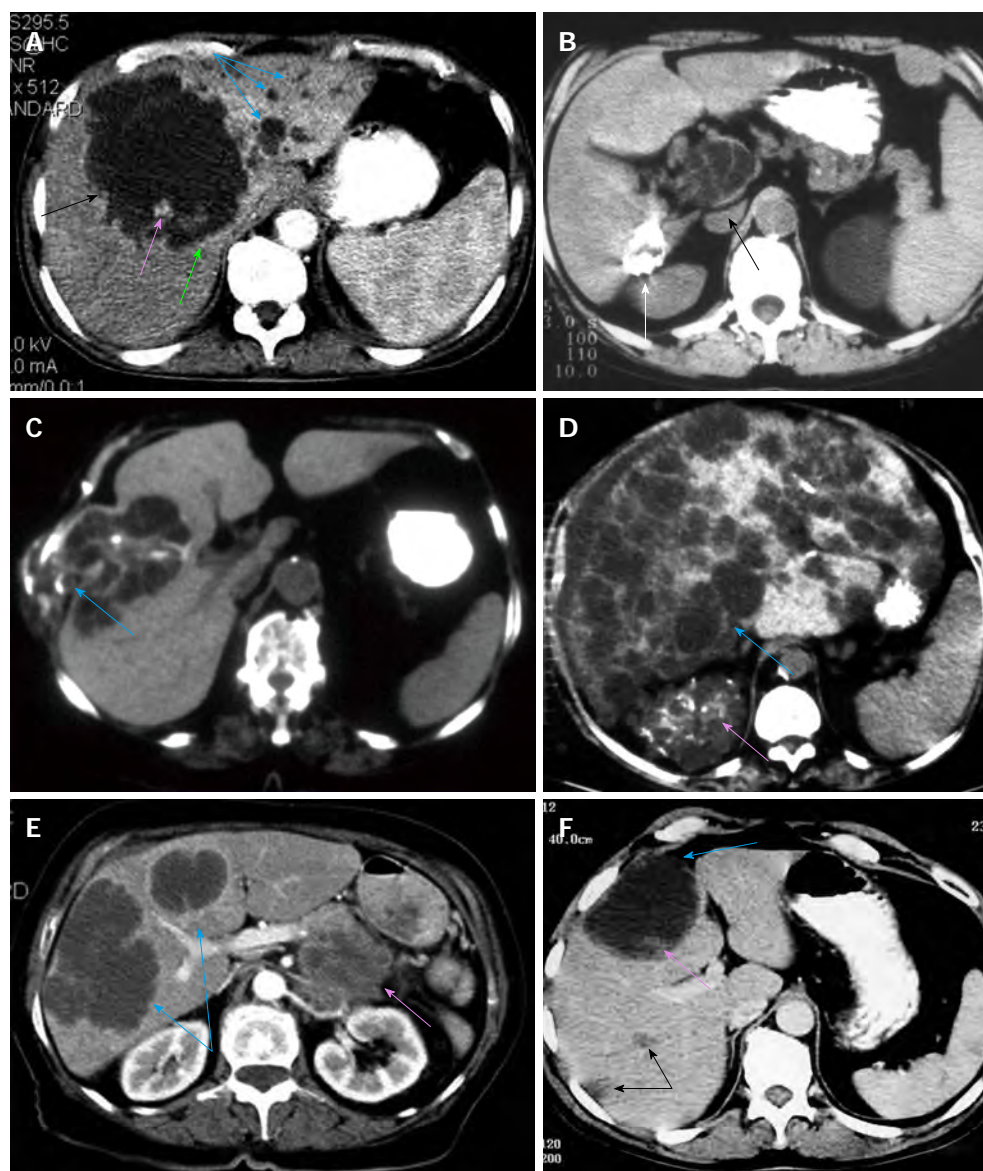
ma have normal serum concentrations of CEA and CA 19-9. Moreover, the serum concentrations of these tumor markers can be elevated in patients with HCa as well. Therefore, these serum tumor markers cannot distinguish HCa from HC.

Some authors have reported that fine needle aspiration cytology of the cyst contents is a good method for diagnosing cystic lesions<sup>[5,16]</sup>. In many studies, however, no malignant cells were recovered in patients with HCa who underwent intraoperative cytology examination. Thus, this procedure rarely generates a definitive diagnosis and carries the risk of pleural or peritoneal dissemination, and should therefore be avoided, especially when surgery is planned. The fluid of the cystic cavity often consists of a high-molecular-weight glycoprotein called mucin. However, hemorrhagic, bilious, clear, and mixed fluid contents have also been observed<sup>[5,16]</sup>. Aspiration and cyst fluid analysis for CEA and Ca 19-9 has been proved more useful than serum analysis<sup>[5,16]</sup>. Cyst fluid demonstrates marked, but variable, elevation in Ca 19-9 and moderate elevation of CEA<sup>[5,16]</sup>. Elevation of these cyst fluid tumor markers has high specificity and sensitivity in distinguishing HC from simple and echinococcal cysts.

HC has been treated by marsupialization, internal Roux-en-Y drainage, repeated needle aspirations, fenestration, or partial resection. All these methods have been associated with high rates of recurrence and complications including sepsis, continued growth, and progress to malignancy. Although the rate of malignant transformation is relatively low (5%-10%), all suspected HCs must be excised<sup>[16,21]</sup>. Liver resection with clear margins is strongly indicated due to the possibility of synchronous appearance of HCa at the borders of the cyst<sup>[16,21]</sup>. Enucleation is also acceptable. Reports supporting resection cite the low associated mortality of the procedure and the permanent relief of symptoms<sup>[16,21]</sup>.

The majority of HC can be completely and safely excised by enucleation, including those that are centrally located. Once the cyst has been decompressed and the proper plane identified, enucleation can proceed without





**Figure 2** Computed tomography image. A: In liver segment IV there is a large cystic lesion (black arrow) causing compression with dilatation of biliary ducts (light blue arrows) in the left liver lobe. Peripheral contrast enhancement (light green arrow) as well as a nodule (pink arrow) is evident in this case of cystadenoma; B: Echinococcal disease evaluated. Two lesions are evident. The first in liver segment I appears as a multilocular cystic lesion (black arrow) and the second in liver segment VI as a calcified mass with irregular margins (white arrow); C: Demonstrates direct infiltration of a liver hydatid cyst in the adjacent peritoneal surface and abdominal wall (light blue arrow); D: A typical case of multicystic disease with liver (light blue arrow) and kidney (pink arrow) involvement were very well depicted; E: Showing two large cystic-appearing liver lesions (light blue arrows) in a case of a metastatic pancreatic cystadenocarcinoma that is also evident (pink arrow); F: A large cystic lesion (light blue arrow) with a small solid component at the periphery (pink arrow), as well as two small hypodense liver lesions (black arrows), are seen on this image, in a case of proven gastrointestinal stromal tumors metastatic lesions.

significant blood loss. If the possibility of hemorrhage is high due to adjacent major venous vascular structures, enucleation can be completed with either inflow occlusion (Pringle maneuver) or total vascular exclusion. In the era of laparoscopic surgery, a laparoscopic frozen section biopsy of the cyst wall is feasible. If the frozen section is consistent with a simple, benign cyst, laparoscopic partial excision is adequate. If the biopsy demonstrates HC, then complete excision is necessary. However, frozen section biopsies are not always accurate due to inconsistency and discontinuity of the pathological epithelium<sup>[3,7,22]</sup>. Frozen sections cannot definitely exclude or confirm the diagno-

sis of HC, especially in the case of HCa<sup>[3,7]</sup>.

The only potentially curative treatment for HCa is complete removal, usually by a major liver resection with 1-cm margins. Reported survival rates for HCa range from 25% to 100% (87% disease free) at 5 years<sup>[4]</sup>. It has been reported that patients with HCa who have invasion of the liver parenchyma or neighboring organs have a poor prognosis<sup>[4]</sup>. Asahara *et al* have reported that the prognosis of patients with HCa is poor when the tumor lacks mesenchymal stroma<sup>[2,4]</sup>. Absence of mesenchymal stroma in HCa appears to be associated with aggressive disease behavior (*i.e.*, rapid dissemination or distant metastasis)<sup>[2,4]</sup>.



## HYDATID DISEASE

Human cystic echinococcosis, or hydatid cyst disease, is a zoonosis caused by the larval cestode *Echinococcus granulosus*, *Echinococcus multilocularis*, or *Echinococcus vogeli*. *E. granulosus* produces unilocular cystic lesions, whereas *E. multilocularis* and *E. vogeli* produce multilocular alveolar cysts<sup>[23,24]</sup>. Dogs are the definitive hosts for *E. Granulosus*, with sheep being the major intermediate host (yaks, goats, and camels are other relevant intermediate hosts). Man is only incidentally infected when ingesting tapeworm eggs<sup>[24]</sup>. The eggs penetrate the intestinal wall, with the resulting larvae infiltrating the blood and lymphatic circulation system. Then, through the portal vein into the liver, lungs, and other tissues, the larvae develop into hydatid cysts<sup>[25,26]</sup>.

The liver is the most frequent site for the cystic lesions (52%-77%) seen in hydatid disease, followed by the lung (10%-40%), brain, and other viscera<sup>[24,26,27]</sup>. The disease may remain silent for many years before coming into medical attention as an incidental imaging finding, or it may present with complications.

The diagnosis of uncomplicated hepatic hydatid disease is based on clinical suspicion, with special attention paid to factors such as the patient's residence, place of origin and occupation in order to identify high-risk patients. The symptoms depend on the size, location, and development stage of the cyst<sup>[26,28]</sup>. Pain in the right upper quadrant or the epigastrium is the most common symptom, whereas hepatomegaly and a palpable mass are the most common signs. Nonspecific symptoms such as fatigue, fever, nausea, or dyspepsia may also be present. Patients with complicated hepatic hydatid disease may present with fever, jaundice, or anaphylactic symptoms, depending on the complication<sup>[26,29]</sup>.

Acute cholangitis is the most common syndrome when the hydatid cysts rupture in the biliary tract. Rupture of a cyst may produce fever, pruritus, eosinophilia, or fatal anaphylaxis<sup>[23]</sup>. Lower chest pain, a productive cough, and hemoptysis are the most frequent symptoms when there is thoracic involvement. Biliptysis is diagnostic of a biliobronchial fistula<sup>[25]</sup>.

General blood tests are not specific except in complicated disease, whereas a high white blood cell count with eosinophilia are possible findings. Hepatic parameters are normal except in the case of biliary compression<sup>[30]</sup>. Serologic tests such as hemagglutination, latex agglutination, and enzyme-linked immunosorbent assay (ELISA), are associated with a high incidence of false-negative and false-positive results<sup>[28]</sup>. Nevertheless, the detection of specific antigens and immune complexes of the cyst with ELISA yields a positive result in more than 90% of patients. Specific IgE antibodies are demonstrated with ELISA and the radioallergosorbent test is positive in the presence of active disease. Confirmatory tests such as arc-5 immunoelectrophoresis and immunoblotting use parasite-specific antigens. The positivity rate with arc-5 immunoelectrophoresis is as high as 91.1%<sup>[26,29]</sup>. The Casoni and Weinberg tests are no longer used for the diag-

nostic workup, mainly due to their low sensitivity<sup>[29]</sup>.

The indirect immunofluorescence assay (IFA) first reported by Coudert *et al* is specific and sensitive, especially in cases of hepatic cystic hydatidosis. This easy-to-do assay can be achieved in less than 2.5 h and is the most sensitive test in more than 95% of patients with hepatic cystic hydatidosis<sup>[30]</sup>. The diagnosis of hydatidosis by molecular biology is based on the polymerase chain reaction and the technique needs to be evaluated. Based on the choice of primers and probes, molecular biology can differentiate *E. granulosus* from *E. multilocularis* in clinical samples<sup>[30]</sup>.

False-positive results have been described in some patients with tumors, for which there is no explanation as yet, whereas false-negative results are observed when cysts are calcified, even if fertile and corresponding to the lack of antigenic stimulation. Serologic tests do not supplant clinical or imaging investigations but they can, however, confirm the hydatid origin of a cyst. Specific antibodies increase 4-6 wk after surgery, after which they decrease slowly for the next 12-18 mo. The decrease in specific antibodies is too irregular to be a good witness of recovery or relapse, however. Persistently high specific antibody titers or a secondary increase in the antibody titers 6 to 12 mo after surgery indicate a relapse<sup>[30]</sup>.

Standard chest and abdomen radiographs may reveal an elevated diaphragm and concentric calcifications in the cyst wall. Liver scanning was an important diagnostic tool during the 1970s. Since then, US and CT have replaced scanning and are considered the first choice in the diagnostic armamentarium. These methods are helpful for determining complications as well<sup>[29]</sup>. MRI and endoscopic retrograde cholangiopancreatography (ERCP) can prove helpful during the diagnostic approach.

US, a noninvasive, readily available, sensitive, and cost-effective imaging technique, should be the diagnostic method of choice. US is helpful for defining the internal structure, number, and location of the cysts and the presence of complications (Figure 1B). The specificity of US is in the range of 90%. Several authors have proposed an ultrasonographic classification of hepatic hydatid disease (Table 1)<sup>[26,31]</sup>. Classification was standardized by the World Health Organization-International Working Group on Echinococcosis (WHO-IWGE) in 2001 (Table 2)<sup>[26]</sup>. According to the five categories noted in the classification of Gharbi types: II and III are characteristic of hydatid cysts, types I and V are suggestive of hydatid cysts in endemic areas, and type IV simulates a pseudotumor<sup>[25]</sup>.

CT is a helpful tool for confirming the diagnosis, essentially when an ultrasound examination shows a type IV sonographic pattern<sup>[25]</sup>. It provides information equivalent to that derived by US, but it shows the location and depth of the cyst within the liver more accurately (Figure 2B and C). Moreover it can reveal calcified cystic walls<sup>[28]</sup>, daughter cysts, and exogenous cysts, as well as evaluate their volume and density. CT is essential for planning surgical treatment, especially when a minimally invasive approach is to be used<sup>[26,29]</sup>. Imaging findings on CT depend on the stage of cyst growth and the *Echinococcus* species

Table 1 Gharbi classification of cystic hydatid disease

Type	Ultrasonographic features and patterns
I	Pure fluid collection
II	Fluid collection with a split wall (water-lily sign)
III	Fluid collection with septa (honeycomb sign)
IV	Heterogeneous echographic patterns
V	Reflecting thick walls

Table 2 World Health Organization-Informal Working Group on Echinococcosis

Type	Ultrasonographic features and patterns
CL	Unilocular cystic lesion with uniform anechoic content, cyst wall not visible
CE1	Unilocular cystic lesion with uniform anechoic content, cyst wall visible, snowflake sign
CE2	Multivesicular, multiseptated cysts, daughter cysts present, honeycomb sign
CE3	Unilocular cyst containing liquid with a floating membrane inside, daughter cysts may be present, water-lily sign
CE4	Cysts with heterogeneous hypoechoic or hyperechoic degenerative contents, no daughter cysts
CE5	Cysts characterized by a thick calcified wall, which is arch-shaped, producing a cone-shaped shadow; degree of calcification varies from partial to complete

involved. Hepatic involvement by *E. multilocularis* is characterized by a different appearance than *E. granulosus*, consisting of an infiltrating solid mass composed of multiple cysts and indistinct margins. Infection by *E. granulosus* usually forms a single cyst, with or without daughter cysts<sup>[23]</sup>.

Although MRI can be helpful for demonstrating the lesion in the liver (Figure 3), it does not provide additional information in hepatic lesions and is not cost-effective when compared with either US or CT<sup>[26,29]</sup>. However, both CT and MRI have high specificity and sensitivity in the detection and differential diagnosis of hepatic cysts and extracapsular (satellite) cysts<sup>[28]</sup>.

The ideal treatment for hepatic hydatid disease should completely eliminate the parasite and prevent recurrence of the disease with minimum morbidity and mortality. There are three available therapeutic modalities for hepatic hydatid cysts; systemic chemotherapy, surgery, and the treatment known as “puncture, aspiration, injection, reaspiration” (PAIR). Chemotherapy and PAIR are recommended as alternatives to surgery, especially for patients who cannot tolerate or refuse surgery. However, surgery is still the first choice of treatment for hepatic hydatid cysts. Selection of the most appropriate treatment depends on the patient’s health status, the nature of the cyst(s) (considering number, size, location, and presence of complications), and the available resources and expertise<sup>[26]</sup>.

Mebendazole (MBZ) was the first benzimidazole carbamate agent found to have *in vivo* activity in hydatid disease. The drug interferes with mechanisms of glucose absorption through the wall of the parasite, leading to

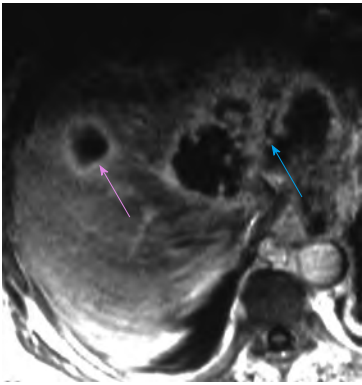


Figure 3 Magnetic resonance T1-w image shows an echinococcal cyst as a multiloculated cystic liver lesion, indicative of the presence of daughter cysts (light blue arrow). A second smaller unilocular lesion with peripheral contrast enhancement is also seen (pink arrow).

glycogen depletion and subsequent degenerative changes in the mitochondria and endoplasmic reticulum of the germinal cells<sup>[32]</sup>. Albendazole (ABZ) is more active *in vitro* than MBZ and has improved gastrointestinal absorption and bioavailability, as well as reports of better clinical results<sup>[32]</sup>. Although orally administered, ABZ results in high serum concentrations and penetration into cyst contents is erratic. Currently, ABZ chemotherapy as the primary treatment may be considered for patients who are not acceptable candidates for surgery, have inoperable, recurrent, peritoneal or multiple liver cysts within the whole liver, have multiple cysts in several organs, refuse surgery or percutaneous drainage, and perhaps, for asymptomatic individuals<sup>[32]</sup>.

Both drugs may decrease the size of hydatid cysts and lead to the sterilization of cyst contents in some cases; however, without concomitant drainage, clinical and radiographic resolution is unpredictable and occurs in less than half of treated patients<sup>[24]</sup>. Hepatic and hematologic toxicities are the most frequent serious adverse effects of ABZ and MBZ. Treatment of hepatic cystic echinococcosis with MBZ or ABZ alone is not as effective as a combined chemotherapy-drainage approach<sup>[24,33]</sup>. Clinical and radiographic improvement (in most studies defined as a > 25% reduction in cyst size, membrane separation, or cyst calcification) is seen frequently, but complete cure (*i.e.*, cyst disappearance) generally occurs in less than half of patients treated with anti-parasitic monotherapy<sup>[24,33]</sup>.

According to the WHO guidelines, chemotherapy is indicated for inoperable primary liver or lung echinococcosis, for patients with multiple cysts in two or more organs, and for peritoneal cysts. Another important indication for chemotherapy is the prevention of secondary echinococcosis. Preoperative use of ABZ or MBZ can reduce the risk of recurrence of cystic echinococcosis and facilitate the operation. Concomitant chemotherapy is also recommended for PAIR. Chemotherapy is contraindicated for large cysts that are at risk of rupture (superficially situated, infected cysts) and for inactive or calcified cysts<sup>[34]</sup>.

The usually recommended oral dosage of ABZ is

10-15 mg/kg per day in two divided doses for several 1-mo courses separated by 14-d intervals. The usual oral dosage of MBZ is given as 500 mg tablets in daily doses of 40-50 mg/kg (in three divided doses) for at least 3-6 mo. Better intestinal absorption of benzimidazole compounds is gained by administering it with a fat-rich meal or by combining it with cimetidine. Medical and laboratory examinations for adverse reactions are initially necessary every 2 wk and then monthly<sup>[35]</sup>.

A third antiparasitic agent, praziquantel, has had limited use in the treatment of hydatid cysts of the liver. The drug increases the permeability of the parasite's cell membrane to calcium, resulting in strong contractions and paralysis of the musculature leading to detachment from host tissue. In humans, it has favorable pharmacokinetics when given in a dose of 50 mg/kg either once weekly or every two weeks. There are few clinical studies documenting the efficacy of praziquantel in humans, however several of these have suggested that the use of praziquantel in combination with MBZ or ABZ is more effective and perhaps, more rapid than with benzimidazole alone (47.4% *vs* 36.4%) after only 2-6 mo of drug therapy<sup>[24]</sup>.

Surgery was defined as the only definitive and curative modality by the WHO-IWGE in 1996<sup>[33]</sup>. The goals of surgery in hydatid disease are to inactivate the cestode parasites, evacuate the cyst cavity, remove the germinal layer, and obliterate the residual cavity. Surgical interventions consist of open conservative, radical, and laparoscopic approaches<sup>[24]</sup>. Conservative techniques involve drainage, marsupialization, capitonnage, deroofting, partial simple cystectomy, and open or closed total cystectomy with or without omentoplasty<sup>[24]</sup>. The conservative procedures are safer and easier to perform<sup>[25]</sup>. Radical procedures include total pericystectomy, partial hepatectomy, or lobectomy<sup>[24]</sup>. Although it seems logical that radical operations would be associated with higher intra- and postoperative morbidity but less frequent recurrence, recent studies have shown that radical surgery is not associated with a high complication rate<sup>[26]</sup>.

Laparoscopic drainage of hepatic hydatid cysts is a "minimally invasive" surgical technique that appears safe and effective. It has the theoretic advantages of a shorter hospital stay, lower incidence of wound infection, and less postoperative pain, but the disadvantages of difficult accessibility to the various locations, increased risk of cyst content spillage, and the difficulty of aspirating the cyst content of the thick, degenerated cyst contents, especially in some WHO-IWGE CE3 and CE4 cysts. Thus, choosing the best candidates for the laparoscopic approach requires careful evaluation of the cystic disease<sup>[25,26]</sup>. Whichever technique is used, a benzimidazole agent is best administered before any surgery in an attempt to sterilize the cyst contents and reduce the risk of anaphylaxis and dissemination<sup>[24]</sup>.

Meticulous packing of the operative field is necessary irrespective of the surgical technique employed, as is the use of solutions that kill the infective scoleces and

protoscolices of the parasite residing within the hydatid cyst, or potentially leaking from the cyst during surgical manipulation. Various scolicidal solutions used in surgical (and percutaneous) approaches include: hypertonic saline (3%-20%), povidone-iodine, hydrogen peroxide, iodine, formalin, silver nitrate, ethyl alcohol, and ABZ. These scolicides can be used alone or in combination<sup>[24]</sup>.

Potential major complications associated with the surgical treatment of hepatic hydatid cysts include postoperative hemorrhage, bile exudation from the residual cyst cavity, incisional fistula formation, cholangitis, wound infection, sepsis, incisional fistulae, pulmonary complications such as pneumonia and pulmonary embolization, complications of anesthesia, and death<sup>[24]</sup>.

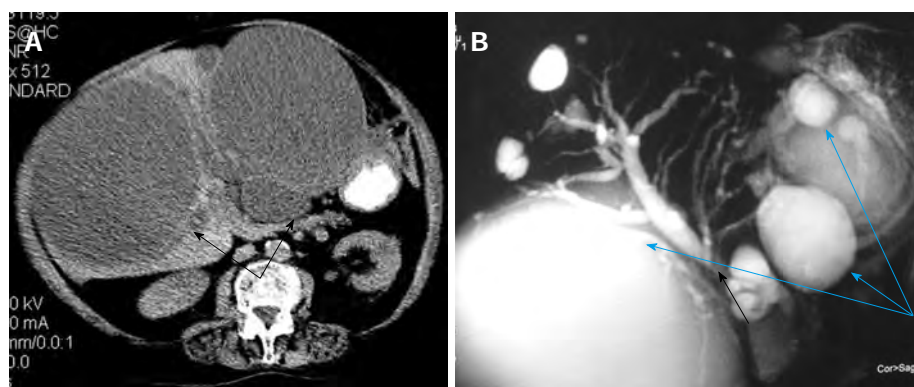
ERCP is used as a diagnostic and therapeutic tool in the management of biliary tract-complicated hepatic hydatid cysts. Preoperatively, ERCP defines biliary tract-related complications and allows the assessment and management of acute conditions, including acute cholangitis and biliary obstruction, so that elective surgery can be performed later. When combined with sphincterotomy, this drains the cyst cavity and helps prevent postoperative biliary fistula. Postoperatively, ERCP allows visualization of distorted anatomy in recurrent cases, helps clarify the etiology of ongoing or recurrent biliopancreatic symptoms and biochemical abnormalities, allows endoscopic management of a biliary fistula, and enables treatment of secondary biliary strictures by stenting<sup>[26]</sup>.

The treatment modality that we prefer using in our department with optimal results is the evacuation of the cavity with careful removal of the laminated membrane and the daughter cysts in order to avoid spillage. The cyst cavity is obliterated by omentoplasty or capitonnage, and the site is drained externally by suction catheter. Partial cystectomy and internal drainage with a Roux-en-Y intracystic hepaticojunostomy is performed when large ducts had been disrupted due to large cysts. Preoperative ERCP is performed when communication between the cyst cavity and biliary tree is suspected, and endoscopic sphincterotomy is performed in cases of obstruction.

The minimally-invasive technique of puncture, aspiration of cyst, injection of hypertonic saline and/or absolute alcohol, and re-aspiration (PAIR), described initially by Voros *et al*<sup>[28]</sup> and Falagas *et al*<sup>[32]</sup>, is an alternative to major interventional procedures. PAIR treatment satisfies all the goals of surgery in hydatid disease, but substitutes germinal membrane sclerosing and separation using scolicides for surgical removal. PAIR drainage is best performed under continuous ultrasonographic or CT guidance.

Patients undergoing PAIR typically receive oral ABZ that is administered 24-4 h before intervention and 15-30 d after intervention according to cyst size<sup>[34]</sup>. Different scolicidal solutions can be used in PAIR, although hypertonic saline is most commonly employed. Hypertonic saline (in 5%-30% concentrations) exerts its scolicidal effect by creating a strong osmotic gradient across the outer cuticular membrane of the protoscolex, which





**Figure 4** Image. A: Computed tomography image in a case of multicystic disease, showing two large dominant cysts causing mild intrahepatic biliary dilatation (black arrows); B: Magnetic resonance cholangiopancreatography image shows multiple hepatic cysts (light blue arrows) while the common bile duct seems compressed between the two larger cysts (black arrow).

causes its lysis. For multiseptate Type III cysts or large cysts over 6 cm in size, some authors advocate the use of absolute alcohol because it is a more effective sclerosing agent than hypertonic saline, may destroy daughter cysts not killed by saline, and may also result in a more rapid involution of the cyst cavity. Alcohol should not be used, however, if pre-existing biliary communication is suspected or documented, as the agent may cause a chemical cholangitis<sup>[33]</sup>.

With PAIR, cyst fluid or operative tissue specimens are immediately subjected to cytologic, histopathologic, and parasitologic examinations after aspiration or catheter drainage in order to confirm the diagnosis and assess the success of the drainage procedure.

Complications after PAIR therapy, such as infections, are generally well tolerated and can be managed with systemic antimicrobial therapy. Leakage during drainage may lead to fever, urticaria, transient hypotension, or anaphylaxis, but these can be anticipated and effectively managed with antipyretics, IV fluids, antihistamines, and subcutaneous epinephrine. Cyst-biliary communications (biliary rupture and fistula formation) developing after PAIR and caused by cyst decompression, can usually be managed endoscopically<sup>[24]</sup>. For patients who underwent PAIR as a primary procedure, a total complication rate of 14.7% and a recurrence rate of 1.57% have been reported<sup>[28]</sup>.

In conclusion, compared to patients undergoing surgical intervention for cystic hepatic echinococcosis, PAIR plus ABZ is associated with greater clinical and parasitologic efficacy, less major and minor morbidity whenever it is indicated (*i.e.*, for non-echoic lesion  $\geq 5$  cm in diameter (CE1), cysts with daughter cysts (CE2), and/or with detachment of membranes (CE3). Surgery may be reserved for patients with hydatid cysts refractory to PAIR because of secondary bacterial infection or difficult-to-manage cyst-biliary communication or obstruction<sup>[24,34]</sup>.

## POLYCYSTIC LIVER DISEASE

Polycystic liver disease (PLD) is inherited as an autosomal

dominant trait presenting in adulthood and is more common in women<sup>[36]</sup>. Autosomal dominant polycystic disease is genetically heterogeneous, with mutations in two distinct genes predisposing to the combination of renal and liver cysts (AD-PKD1 and AD-PKD2)<sup>[36,37]</sup>. PLD is genetically linked to protein kinase C substrate 80K-H and SEC63<sup>[38]</sup>. The cysts in PLD can also increase in size and number during pregnancy or simultaneously with the use of exogenous female steroid hormones<sup>[39]</sup>.

Most patients are asymptomatic and do not require treatment. Some patients develop massive hepatic cystic disease and become clinically symptomatic, which is associated with increased liver volume and adjacent visceral compression. Usually patients suffer from chronic dull abdominal pain, satiety, weight loss, dyspnea, physical disability, and descensus<sup>[36,40]</sup>. Liver function tests are usually normal except for mild elevation in ALP or  $\gamma$ -GT<sup>[36,40]</sup>. Liver failure or complications of advanced liver disease, such as infection or intracystic hemorrhage, are rare. Less than 5% of patients have acute medical complications. These consist of cyst hemorrhage, rupture, infection, uterine prolapse due to displacement, obstructive jaundice, portal hypertension, ascites, and Budd-Chiari syndrome<sup>[19,36,40-42]</sup>. Even with marked hepatosplenomegaly and portal hypertension, liver function is well preserved in PLD. Ascites may be present and usually results from hepatic venous flow obstruction. Diagnosis is confirmed with US and CT imaging (Figure 2D), which along with MRI provides the surgeon with valuable preoperative information, such as the location of infected or hemorrhagic cysts that may be responsible for symptoms<sup>[40]</sup> (Figure 4). Treatment should be considered in cases of persistent symptoms or associated complications.

Cyst aspiration with sclerosis, open or laparoscopic cyst fenestration, combined hepatic resection and fenestration, liver transplantation, and recent medical treatment with somatostatin analogues, are possible therapeutic options based on the type of PLD<sup>[19,36,40,42-46]</sup>. Aspiration, combined with ethanol instillation to induce sclerosis of the cyst lining epithelium, can be effective in patients with a few dominant cysts (Type I PLD - few large cysts



greater than 7 cm). Open or laparoscopic cyst fenestration with omentoplasty is another modality of treatment that can be performed in patients with more diffuse PLD (Type II -multiple medium cysts 5-7 cm in diameter). Patients with small cysts throughout the liver have a greater risk of persistence and/or recurrence of symptoms<sup>[19,42]</sup>. Postoperative morbidity consists of temporary ascites, pleural effusion, and rarer biliary leakage<sup>[40]</sup>.

Combined hepatic resection and fenestration is more effective for reducing the hepatic mass and relieving gastric compression. This procedure has an advantage in the case of massive hepatomegaly with associated gastric compression<sup>[40,47,48]</sup>. Resection addresses the problem of liver mass, but poses significant risk of bile duct injury, vascular compromise, and liver insufficiency, as cysts markedly distort intrahepatic anatomy. In particular, ascites has been troublesome due to continued cyst secretion from residual fenestrated cysts, disruption of intrahepatic lymphatics, and partial venous outflow obstruction. Candidates for combined resection/fenestration should have at least two adjacent liver segments not affected by cysts and have normal liver function. Furthermore, these patients should be managed by experienced hepatobiliary surgeons at institutions with advanced intensive care, as well as interventional radiological and gastroenterology support.

Liver transplantation has been performed in rare cases, especially when the above-mentioned interventions are not an option. In patients who harbor diffuse PLD, orthotopic liver transplantation (OLT) is effective, but inherently assumes the risks of long-term immunosuppression and rejection. OLT is indicated for patients with progressive PLD after resection/fenestration, patients with concurrent liver dysfunction and renal failure, and patients with diffuse PLD without segmental sparing. Although symptomatic relief from hepatomegaly occurred in all surviving patients, long term follow up addressing quality of life, hepatorenal function, immunosuppressive complications, and survival is limited<sup>[42,49]</sup>.

Regarding the results of invasive methods, in case series it is noted that aspiration and sclerosis of individual liver cysts reduced liver volume by 19% in patients with 1 or more large dominant liver cysts<sup>[50]</sup>. Reduction of liver volume is reported to be as high as 12.5% when laparoscopic fenestration is used, but the complication rate reported is also high (0%-33%)<sup>[51-55]</sup>.

The drawbacks of invasive procedures in treating PLD are their partial effectiveness, their related morbidity and mortality and, most importantly, the fact that they do not change the natural course of the disease as symptoms recur due to growth of new cysts or re-growth of treated ones<sup>[41]</sup>.

Several studies have reported the positive effects of somatostatin analogues in decreasing liver and kidney growth in PKD and ADPLD over a treatment period of minimum 6 mo<sup>[43-46]</sup>.

Somatostatin may reduce cyst development through

several mechanisms<sup>[45]</sup>: (1) by inhibiting secretin release from the pancreas<sup>[56]</sup>; (2) by inhibiting secretin-induced cAMP generation and fluid secretion in cholangiocytes<sup>[57-59]</sup>; (3) by vasopressin-induced cAMP generation and water permeability in collecting ducts<sup>[60-63]</sup> by its effects on Gi protein-coupled receptors; and (4) by suppressing the expression of IGF-1, vascular endothelial growth factor, and other cystogenic growth factors and downstream signaling from their receptors<sup>[60-64]</sup>.

Ruggenti *et al.*<sup>[43]</sup> in 2005 showed that kidney volume increased by 2.2%-3.7% during active treatment with octreotide LAR compared with 5.9%-5.4% ( $P < 0.01$ ) while on placebo. Octreotide LAR (40 mg intramuscularly every 4 wk) was given for 6 mo in 12 adult polycystic kidney disease (ADPKD) patients with advanced renal disease (mean total kidney volume 2435 mL, mean serum creatinine 1.9 mg/dL)<sup>[43]</sup>.

In 2009, van Keimpema *et al.*<sup>[44]</sup>, tested lanreotide for treating PLD (120 mg subcutaneously every 4 wk) for 6 mo in 54 patients with PLD (32 ADPKD and 22 AD-PLD). He concluded that liver volume decreased by 2.9% (from 4606 to 4471 mL) in the lanreotide group, whereas it increased by 1.6% (from 4689 to 4895 mL) in the placebo group ( $P < 0.01$ )<sup>[44]</sup>. In the 32 patients with ADPKD, total kidney volume decreased by 1.5% (from 1000 to 983 mL) in the lanreotide group, whereas it increased by 3.4% (from 1115 to 1165 mL) in the placebo group ( $P < 0.02$ )<sup>[44]</sup>.

The results of the clinical trial reported in 2010 by Hogan *et al.*<sup>[45]</sup> showed that administration of octreotide LAR for 1 year induced a moderate but significant reduction in liver volume, inhibited the growth of polycystic kidneys, and improved quality of life in patients with ADPKD and/or ADPLD, with low toxicity and few side effects.

In 2012, Hogan *et al.*<sup>[46]</sup> reported their results in treating patients with PLD with Octreotide LAR for 2 years. He concluded that his study further substantiates the positive effects of somatostatin analogs in reducing TLV (6.08% overall reduction in TLV in the first year), demonstrating their safety and efficacious over a 2-year period in individuals with ADPKD or ADPLD, many of whom had chronic renal insufficiency<sup>[46]</sup>. While OctLAR inhibited renal enlargement within the first year of treatment, it appeared to lose effectiveness during Year 2. While the results of OctLAR therapy on TLV were clearly positive and results on TKV may show some benefit, he did not detect any positive effect of OctLAR on GFR<sup>[46]</sup>.

The administration of octreotide or lanreotide has been generally well tolerated in all studies, with mostly mild, predictable, and dose-dependent gastrointestinal side effects. Patients undergoing long-term octreotide treatment should be monitored for cholelithiasis symptoms or signs; a known complication<sup>[45,65]</sup>. Alopecia, symptomatic bradycardia, and steatorrhea are other known adverse events associated with somatostatin analogue treatment<sup>[66-73]</sup>.

## CYSTIC METASTASES

Many cystic tumors may metastasize to the liver (*e.g.*, pancreatic or ovary cystadenocarcinomas) (Figure 2E). Other liver metastatic lesions which can appear cystic usually originate from rapidly growing hypervascular tumors (sarcoma, melanoma, and neuroendocrine tumors) and appear so due to necrosis and cystic degeneration<sup>[74]</sup>. Despite the fact that these metastatic sites often show cystic characteristics, in most cases the differential diagnosis between them and benign liver cysts is easily made with the contribution of computed tomography.

Of particular interest are the liver metastases from gastrointestinal stromal tumors (GIST) that may appear cystic even before treatment (Figure 2F).

GIST is the most common mesenchymal tumor of the gastrointestinal tract, accounting for 1%-3% of all gastrointestinal malignancies<sup>[75-77]</sup>. GIST can arise anywhere along the GI tract, but is most common in the stomach (50%-70%) and small bowel (25%-35%)<sup>[78]</sup>.

Despite its less aggressive pathologic nature, GIST can metastasize after a long remission period. When GIST originates in the small bowel it behaves in a more aggressive manner. The most common site for metastases is the liver and the peritoneal cavity<sup>[76,77,79]</sup>, but it can also occur in bones, lungs, skin, and lymph nodes. Data from the MD Anderson Cancer Center report 18% of patients presented with metastatic disease<sup>[80]</sup>. Liver metastases are commonly multiple, distributed in both lobes, and more frequently detected synchronously with the primary tumor than metachronously. Case reports showed further metachronous liver metastases being detected after more than 10 years (13 years after gastrectomy for gastric GIST, 17 years after resection for retroperitoneum GIST, and 12 years after surgery for rectal GIST)<sup>[77]</sup>.

GIST mostly affects males between the ages 40 and 70 and are usually found incidentally. Features at clinical presentation depend on tumor size. Large or advanced lesions may present with a variety of clinical findings, including bleeding, abdominal pain, early satiety, bowel obstruction, or perforation. Bowel obstruction is reported in up to 30% of clinical series, but accounts for less than 10% of presentations in most reports<sup>[81]</sup>.

The initial workup should include history and physical examination, appropriate imaging (*i.e.*, chest, abdominal and pelvic CT with contrast and/or MRI), endoscopy in selected cases of primary gastric mass, endoscopic ultrasound, liver function tests, full blood counts, and surgical assessment of tumor resectability. On CT scans, metastases within the liver developed lower attenuation than that of the normal surrounding parenchyma. Liver metastases are hypervascular in 92% of patients, and rapidly become cystic following therapy with imatinib.

GIST and GIST liver metastases are soft and fragile, and biopsy may cause tumor hemorrhage or dissemination. The decision to obtain a biopsy should be based on data regarding the stage of the disease and the clinician's suspicion of other malignancies. If the diagnosis is in doubt, neo-adjuvant therapy using IM is put under con-

sideration, but if there is synchronous metastatic disease, then biopsy is essential<sup>[77]</sup>.

Before 2001, the only effective therapy was surgery alone. The development of clinically effective inhibitors targeting the trans-membrane receptor tyrosine kinase KIT radically changed the management of advanced (locally advanced and metastatic) disease. IM, a selective inhibitor of tyrosine kinase, has revolutionized the management of this disease in recent years. Laboratory studies revealed significant molecular heterogeneity among GIST<sup>[82-84]</sup>. In 2010, a meta-analysis showed that most patients with different genotypes of GIST and KIT exon 11-mutants benefit from the individualized treatment of imatinib<sup>[85]</sup>.

Imatinib has become the first line treatment for recurrent and/or metastatic disease<sup>[79]</sup>. Another meta-analysis comparing the efficacy of imatinib given either once (400 mg) or twice daily, revealed that the higher dose offers a progression-free survival advantage among patients with exon 9 mutations<sup>[86]</sup>, but that the overall survival was the same in the two groups of patients.

Nowadays imatinib therapy and surgical intervention are combined to give patients better disease free and survival rates. Surgical intervention in patients responding to medical therapy may provide a complete cure<sup>[87,88]</sup>. Complete pathological response to imatinib alone occurs in less than 5% of patients<sup>[87,88]</sup>. Surgery has the best results when offered to patients with lesions responsive to 6 mo imatinib therapy. CT with or without PET can be used to assess the therapeutic effect. MSKCC patients with lesions responsive to imatinib in one study had a 2-year progression free survival of 61% and a 2-year overall survival of 100% after surgical resection. In contrast, the 2-year survival was 36% in patients resistant to imatinib therapy<sup>[89]</sup>. Raut *et al.*<sup>[90]</sup> also reported that debulking surgery may prolong survival in patients who are either responsive to imatinib or have limited radiographic progression, but it has poor or no result in patients with progressive metastatic disease. Gronchi *et al.*<sup>[91]</sup> reported in 2007 that surgery may be of value to patients who develop responsive or stable disease while on preoperative imatinib therapy.

Regarding the timing of surgical resection, Suzuki *et al.*<sup>[92]</sup> reported a complete resection rate of 31.4% after IM therapy for a period of 6.9-37.5 mo (mean 10 mo). They also emphasized that surgical resection for IM- responsive recurrent or metastatic disease should be considered as early as possible before the development of progression and secondary resistance to IM<sup>[92]</sup>. Surgical resection 6-12 mo after the start of IM treatment is recommended among responders<sup>[79]</sup>. However a large tumor may prohibit resection because of the risk of postoperative liver failure. An option to counteract this phenomenon is the use of portal vein embolization (PVE). In general, the median time for detection of further metastases following resection of liver metastases, is 12 mo after the initial hepatectomy<sup>[77,79]</sup>. Therefore, careful evaluation of the liver is critical during the first year post-hepatectomy.

Radio-frequency ablation, microwave ablation, and

hepatic artery embolization are other treatment modalities that can normally be used in patients with unresectable disease or in those who cannot undergo surgical excision due to co-morbidities.

## CYSTIC HEPATOCELLULAR CARCINOMA

Rarely hepatocellular carcinoma (HCC) can have a cystic appearance, due to necrosis and cystic degeneration in cases of rapidly growing tumors. Co-existing liver cirrhosis and specific HCC imaging characteristics, such as hypervascularity of solid components and tumor invasion of the portal and hepatic veins, can help to reach the correct diagnosis<sup>[74]</sup>. Typically the conventional ultrasound would reveal a heterogeneous echogenic lesion with a hypoechoic rim and peripheral or internal arterial flow signals in a liver cirrhosis background. The CEUS would reveal a heterogeneous hyper-enhancement during the arterial phase and hypo-enhancement during the portal and late phases<sup>[14]</sup>.

## CAROLI DISEASE

Caroli disease (CD) is a benign congenital disorder, characterized by unilobular or bilobular segmental cystic dilatation of the intrahepatic biliary tract. The first report was by Todd in 1818, but Jacques Caroli in 1958 defined the disease precisely with its different types<sup>[93]</sup>. The estimated incidence of Caroli disease is 1 in 1000000, with males and females being equally affected and more than 80% of patients presenting before 30 years of age<sup>[94]</sup>.

There are two forms of the disease, one associated with congenital hepatic fibrosis, also called Caroli syndrome, and the other a simple form occurring alone. Recent studies suggest that the simple form may be as common as congenital hepatic fibrosis<sup>[95,96]</sup>. It is characterized by segmental cystic dilatation of the intrahepatic ducts, increased incidence of biliary lithiasis, cholangitis, and liver abscesses. Absence of cirrhosis and portal hypertension is typical<sup>[94]</sup>. Various renal disorders have been described in association with this liver disease, including autosomal polycystic kidney disease, medullary sponge kidney, and medullary cystic disease<sup>[93,95]</sup>.

Mode of inheritance is still unclear, but in the majority of cases it is transmitted in autosomal recessive fashion<sup>[93]</sup>. Caroli disease is associated with an increased risk of cholangiocarcinoma, with the reported incidence of malignancy ranging from 5% to 10%. The estimated risk is 100 times greater than that of the general population and is triggered by long-standing inflammation and chronic injury of the biliary epithelium<sup>[94,97]</sup>. Although present from birth, the disease usually remains asymptomatic during the first 20 years, and may also remain so throughout life<sup>[94]</sup>. However when symptomatic, a significant number of these patients present significant loss in their quality of life and clinical course.

The disease is frequently noted by recurrent fever, jaundice, and/or pain in the right hypochondrium<sup>[97]</sup>. A

literature review found recurrent acute cholangitis as the main mode of presentation in 64% of patients<sup>[94]</sup> and the most life-threatening complication of CD. Usually caused by gram-negative bacilli, it has a recurrent course and, despite different antibiotic associations, medical treatment is often not satisfactory<sup>[93]</sup>. Patients with Caroli syndrome, on the other hand, usually present early in life, with complications of portal hypertension, mainly variceal bleeding, hypersplenism, or portal hypertension in 20%-50% of cases<sup>[97]</sup>.

Laboratory studies typically show an elevation of serum alkaline phosphatase, direct bilirubin, and a leukocytosis with a predominance of neutrophils. Hepatic synthetic function is well-preserved initially, but may be affected by progressive liver damage due to recurrent cholangitis and biliary obstruction. Coagulopathy from vitamin K malabsorption may occur in cholestatic patients<sup>[94]</sup>.

Histologically, the main macroscopic and microscopic features of CD are: non-obstructive, localized dilatation of the bile ducts; intraluminal bulbar protrusions of the ductal wall and intra ductal vascular tracts containing patent portal venous; and hepatic arterial channels that traverse the true lumen and terminate within the lumen<sup>[94]</sup>. The diagnosis of CD therefore rests on demonstrating that the cystic lesions are in continuity with the biliary tree. This can be done by imaging studies such as isotope scan, MRCP, CT scan, US, ERCP, and PTC.

The classical finding of CD is finding that a cold area on 99mTc sulfur colloid scan becomes hot on 99mTc DISIDA scan<sup>[94]</sup>. MRCP presents advantages in depicting the entire biliary tree. Three main patterns of CD are identified in MRCP: (1) multiple cystic ectasias connected with fusiform dilatations; (2) isolated fusiform dilatations with multiple calculi; and (3) solitary dilatation of the left bile ducts with cysts and multiple calculi<sup>[93]</sup> (Figure 5).

A CT scan shows central dot signs in CD patients. The fibrovascular bundles containing portal vein radical and a branch of hepatic artery bridging the saccule appear as central dots or a linear streak. This central dot sign described on a CT scan is suggested as a pathognomonic finding in CD, and can also be demonstrated on US<sup>[94]</sup>. ERCP is the method with the highest sensitivity in the diagnosis of CD. The cholangiographic features of Caroli disease are well established as saccular or fusiform dilatation of the intrahepatic bile ducts. Irregular bile duct walls, strictures, and stones may be present. Therefore, direct cholangiography is considered the method of choice for an accurate diagnosis of CD<sup>[94]</sup>. With PTC, the diagnosis can be made confidently when the large intrahepatic branches have focal or segmental involvement with cystic outpouchings in which the contrast medium collects. False-positive findings are rare when PTC is used<sup>[93]</sup>.

The treatment of CD depends on the clinical features and location of the biliary abnormalities. It seems more than justified to advocate a rather aggressive surgical strategy in symptomatic patients who have had several

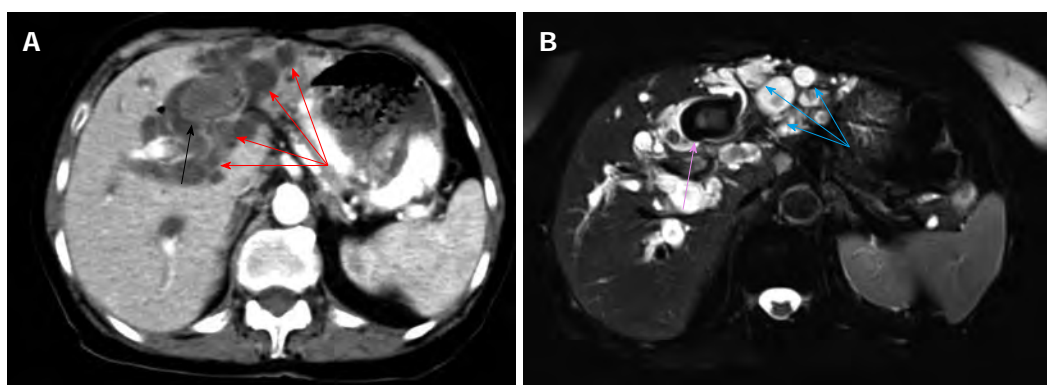


Figure 5 A case of Caroli disease. A: On computed tomography. A large intra-biliary stone (black arrow) is evident in the dilated ducts (red arrows); B: On magnetic resonance imaging. A large intra-biliary stone (pink arrow) is evident in the dilated ducts (blue arrows).

futile conservative treatment attempts<sup>[98]</sup>. The localized forms, which involve either the whole of the left or the right half of the liver, are curable by surgery. They should be treated by hemi-hepatectomy, left or right, with associated treatment of any problem affecting the common duct<sup>[94]</sup>. The procedure is associated with low morbidity and virtual no mortality. There is no report of malignant tumors arising after surgical resection<sup>[98]</sup>.

Diffuse involvement of both lobes can be treated with conservative management in asymptomatic patients, with appropriate antibiotics for cholangitis and ursodeoxycholic acid therapy for litholysis in cases of intrahepatic cholelithiasis, endoscopic therapy (sphincterotomy for clearance of intrahepatic stones), and internal biliary bypass procedure<sup>[94]</sup>. These patients in whom there is no indication for liver resection or transplantation should at least be followed up regularly on an outpatient basis to detect any kind of deterioration or malignant transformation as early as possible<sup>[98]</sup>.

Bilateral disease complicated by recurrent cholangitis, cirrhosis, or both, together with symptoms of associated hepatic fibrosis, do not find the same solution, and it is often difficult to manage. Emergency surgery in the presence of acute cholangitis and deteriorating liver function is associated with high mortality (20%–40%) and morbidity (44%–80%)<sup>[94]</sup>.

It seems that liver transplantation or living donor transplantation is an effective therapeutic option and possibly the only and ultimate management option for these patients with end-stage diffuse Caroli disease providing gratifying long-term results<sup>[94,97,98]</sup>. OLT has become a therapeutic option which, aside from the better long-term outcome, can prevent the development of cholangiocarcinoma<sup>[96]</sup>.

## DISCUSSION

Liver cystic lesions consist of a heterogeneous group of disorders. Rare liver cystic lesions such as cystadenoma, hydatid cyst, polycystic liver disease, Caroli disease, and cystic liver metastases pose several dilemmas to the practicing surgeon or physician. It is very important that

awareness and a high index of suspicion for rare diseases and HC are present, so as to provide as accurate a diagnosis as possible. Since our diagnostic tools have become more powerful and accurate, our adequate knowledge of the nature, evolution, confirmation, and treatment of all the possible pathological entities in the differential diagnosis becomes more necessary than ever.

The use of CEUS in diagnoses of liver lesions has shown promising results, providing more accurate images than conventional ultrasound<sup>[99–102]</sup>. The discrimination between malignant and benign lesions is easier and more accurate than in a conventional ultrasound.

Complete non-enhancement throughout three phases of CEUS or sustained enhancement in the portal and late phases is noticed in most benign lesions<sup>[14]</sup>. Conversely, hypoenhancement in the late phase is seen in malignancies<sup>[14]</sup>. Real time CEUS improves the capability of discrimination between benign and malignant complex cystic focal liver lesions<sup>[14]</sup>. It has been shown that CEUS can greatly improve the diagnostic accuracy of focal liver lesions compared with conventional ultrasound<sup>[14]</sup>.

Contrast-enhanced ultrasound has also been used for diagnostics of the biliary system. In 2009, Xu<sup>[100]</sup> summarized the methodology, image interpretation, enhancement pattern, clinical usefulness, and indications for CEUS in the biliary system.

The first important step, regarding liver cystic lesions, is to make a definitive diagnosis of the nature of the cystic lesion. The second is determining whether the patient's symptoms are related to the cystic lesion. The third is deciding whether and when to initiate therapy for the lesion. Finally, a number of treatment options are available, leading to the fourth issue, which is deciding the appropriate therapy for the patient.

Ideally the cystic liver lesions should be handled by a multidisciplinary team familiar with liver diseases, consisting of interventional radiologist, interventional gastroenterologist, surgeon, clinical oncologist, and pathologist. In our opinion this is the way that even rare entities can be identified and treated promptly. The algorithm used in our department for managing such cystic lesions is provided in Figure 6.



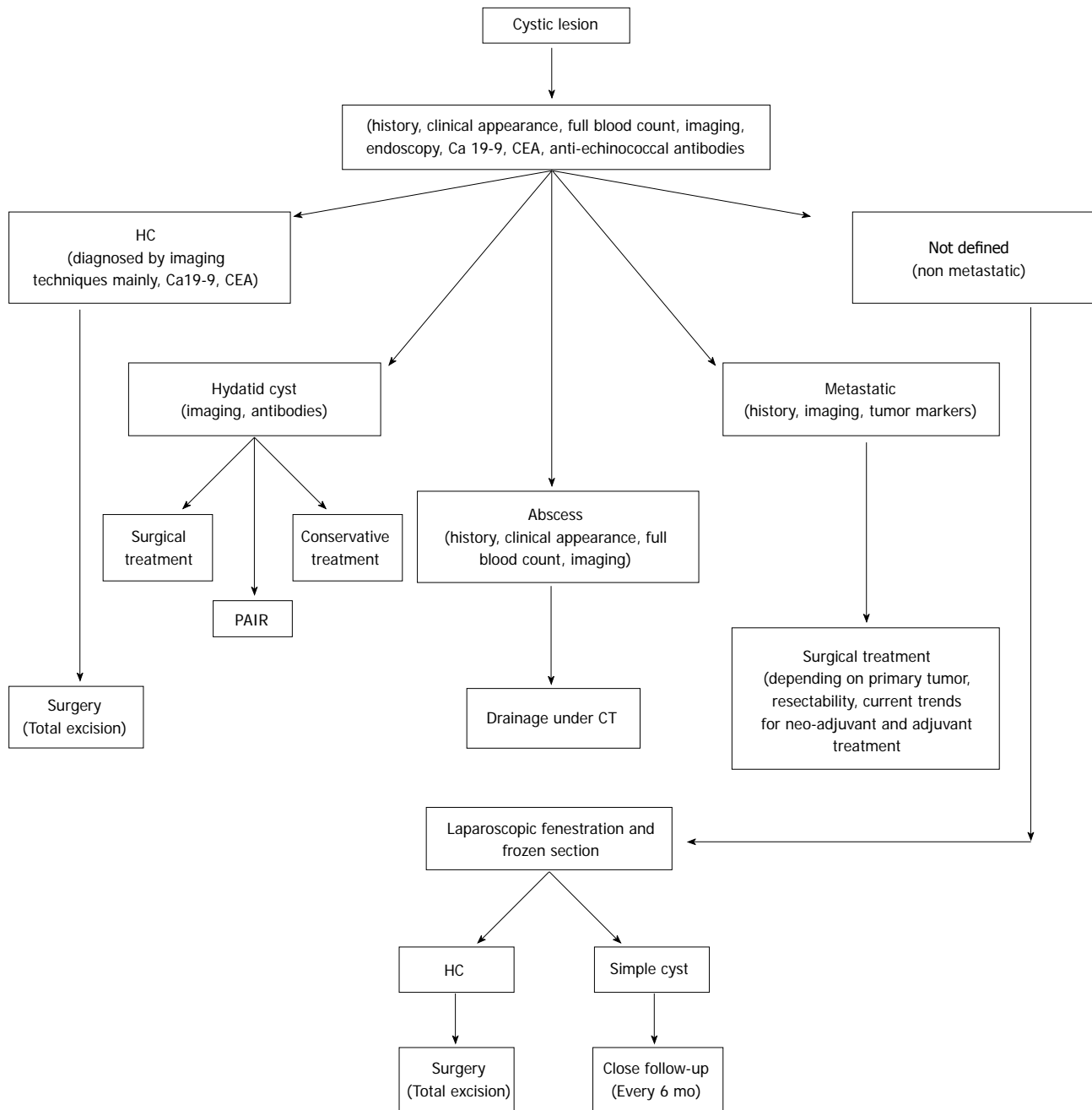


Figure 6 Liver cystic lesions management algorithm.

## CONCLUSION

Cystic liver lesions require accurate pre-treatment diagnosis in order to select the appropriate therapy for each patient, as they can represent benign or malignant formations. It is best that a specialized team deals with cystic liver lesions so that diagnosis and treatment are accurate and focused. Specifically, rare entities require accurate diagnosis and management, as they can pose a malignant impact.

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## Branched-chain amino acids in liver diseases

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chain amino acids (BCAAs) are decreased in patients with liver cirrhosis, and the amino acids imbalance could affect the clinical picture of the disease and the prognosis of the patients. However, there are few comprehensive reviews on the biological activities of BCAAs. In this review, we summarize the biological activities of BCAAs, and discuss possible applications of BCAAs for the management of patients with advanced liver diseases with a list of clinical trials of BCAA administration.

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### Abstract

Branched chain amino acids (BCAAs) have been shown to affect gene expression, protein metabolism, apoptosis and regeneration of hepatocytes, and insulin resistance. They have also been shown to inhibit the proliferation of liver cancer cells *in vitro*, and are essential for lymphocyte proliferation and dendritic cell maturation. In patients with advanced chronic liver disease, BCAA concentrations are low, whereas the concentrations of aromatic amino acids such as phenylalanine and tyrosine are high, conditions that may be closely associated with hepatic encephalopathy and the prognosis of these patients. Based on these basic observations, patients with advanced chronic liver disease have been treated clinically with BCAA-rich medicines, with positive effects.

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**Key words:** Liver disease; Branched chain amino acids; Gene expression; Hepatocyte apoptosis; Hepatocyte regeneration; Immunity; Treatment

**Core tip:** Advanced liver diseases are commonly accompanied by nutritional disturbances, which worsen the prognosis of the patients. Serum levels of branched-

### INTRODUCTION

The three branched chain amino acids (BCAAs), leucine, isoleucine and valine, are among the nine essential amino acids for humans. Recent studies have revealed the functions of these BCAAs, and they have been administered for the treatment of advanced liver diseases. In this review, we summarize current understanding of the biological properties of BCAAs and review the results of clinical application of BCAAs to treat patients with liver diseases.

### BASIC ASPECTS OF BCAAS IN LIVER

#### *Serum concentration of BCAAs in patients with chronic liver diseases and liver cirrhosis*

Serum concentrations of BCAAs are decreased, while the concentrations of the aromatic amino acids (AAAs) phenylalanine and tyrosine are increased, in patients with advanced liver diseases, resulting in a low ratio of BCAAs to AAAs, a ratio called the Fischer ratio<sup>[1]</sup>. A low Fischer ratio has been associated with hepatic encephalopathy (HE). The imbalance of amino acids tends to become more marked with the progression of liver diseases, and aminograms are useful for assessing the prognosis of cir-

rhotic patients with or without hepatocellular carcinoma (HCC)<sup>[2,3]</sup>. Moreover, a simplified Fischer ratio, the BCAA to tyrosine ratio (BTR), has been reported useful for predicting serum albumin concentration one year later<sup>[4]</sup>. These data indicate that amino acid imbalance, either low Fischer ratio or BTR, is a marker for progression of liver diseases, and that correcting this ratio may have therapeutic potential, not only for nutritional improvement, but also for HE, in patients with advanced liver diseases.

### **Gene expression and mitochondrial biogenesis**

In mice, BCAA-rich diets have shown to up-regulate the expression of peroxisome proliferator-activated receptor (PPAR)  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), a master regulator of mitochondrial biogenesis and the defense system against reactive oxygen species (ROS), and of sirtuin-1, a member of the sirtuin family linked to life span extension, enhanced mitochondrial biogenesis, and decreased ROS production, leading to the prolongation of the lifespan of male mice<sup>[5]</sup>. BCAAs have also been shown to induce the activation of genes involved in antioxidant defenses and inhibition of ROS production, as well as to induce the hepatic expression of mRNA encoding 8-oxoguanine DNA glycosylase 1, an enzyme involved in repair of oxidative DNA damage, in a rat model of liver injury, indicating that BCAAs are involved in the induction of antioxidant DNA repair<sup>[6]</sup>.

In various cell lines, BCAAs, especially leucine, have been shown to activate the mammalian target of rapamycin (mTOR) signals, stimulating the synthesis of proteins, including albumin, and of glycogen<sup>[7]</sup>. The ability of leucine to enhance glucose metabolism was confirmed in normal rats and in a rat cirrhosis model. BCAA activation of mTORC1 has also been associated with cell growth<sup>[8]</sup> and PGC-1 $\alpha$ -mediated mitochondrial gene expression<sup>[9]</sup>. BCAAs have been shown to up-regulate PPAR- $\gamma$  and uncouple (UCP) 2, reducing triglyceride concentrations in mouse livers<sup>[10]</sup>. These findings suggest that BCAAs may have a therapeutic effect on metabolic disorders and/or obesity.

### **Apoptosis and regeneration of hepatocytes**

BCAA supplementation was shown to delay the progression of CCl<sub>4</sub>-induced chronic liver injury in a rat model by reducing hepatic apoptosis<sup>[11]</sup>. On the other hand, BCAAs promoted hepatocyte regeneration in a rat model of hepatectomy<sup>[12]</sup>. Moreover, BCAAs were reported to stimulate the production of hepatocyte growth factor<sup>[13]</sup>. Taken together, these findings indicate that supplementation with BCAAs, by reducing hepatocyte apoptosis and promoting liver regeneration, may result in rapid recovery from liver injury.

### **Albumin synthesis**

BCAAs activate mTOR and subsequently increase the production of eukaryotic initiation factor 4E-binding protein-1 and ribosomal protein S6 kinase, which upregulate the synthesis of albumin<sup>[14-16]</sup>. Furthermore, leucine stimulates the nuclear importation of polypyrimidine-

tract-binding protein, which binds to albumin mRNA and increases its translation<sup>[17]</sup>.

### **Insulin resistance**

BCAAs were shown to improve homeostasis model assessment scores for insulin resistance (HOMA-IR) and beta cell function (HOMA-%B) in patients with chronic liver disease, indicating that BCAAs can ameliorate insulin resistance<sup>[18]</sup>. In mice lacking the gene encoding mitochondrial BCAA aminotransferase, an enzyme that catalyzes BCAAs, serum BCAA concentrations were elevated. In those mice, fasting blood glucose and insulin concentrations were decreased and HOMA-IR was significantly lower than in wild-type mice<sup>[19]</sup>. Furthermore, administration of leucine or isoleucine improved insulin sensitivity in mice with high-fat diets<sup>[20,21]</sup>. BCAAs were also shown to temporarily increase plasma insulin concentrations in healthy young men, although plasma glucose concentrations were not altered<sup>[22]</sup>.

Several organs are involved in the mechanism by which BCAAs improve insulin resistance. In the liver, BCAAs increase the liver X receptor/sterol regulatory element binding protein-1c pathway and subsequently activate liver-type glucokinase and glucose transporter. Furthermore, BCAAs suppress hepatic expression of glucose-6-phosphatase<sup>[23]</sup>. In adipose tissue, leucine increases insulin-induced phosphorylation of Akt and mTOR, increasing glucose uptake<sup>[24]</sup>. In skeletal muscle, BCAAs promote glucose uptake through activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase C and subsequent translocation of glucose transporter to the plasma membrane<sup>[25]</sup>. In addition, BCAAs increase PPAR- $\gamma$  and subsequent UCP2 in liver and UCP3 in muscle, stimulating oxidation of free fatty acids. Thus, BCAAs improve insulin resistance through interactions in organs targeted by insulin.

### **Liver cancer cells**

The direct effects of BCAAs on liver cancer cells have been analyzed in culture systems. Increased concentrations of BCAAs in culture medium were reported to suppress the proliferation of HCC cell lines<sup>[26]</sup>. Moreover, all three BCAAs were found to accelerate insulin-induced vascular endothelial growth factor (VEGF) mRNA degradation at the post transcriptional level, downregulating VEGF expression during the development of HCCs<sup>[27]</sup>. BCAAs were also shown to induce apoptosis of liver cancer cell lines by inhibiting insulin-induced PI3K/Akt and NF $\kappa$ B pathways through mTORC1- and mTORC2-dependent mechanisms<sup>[28]</sup>. Moreover, BCAAs may inhibit obesity-related hepatocarcinogenesis by suppressing the stimulatory effect of visfatin, an adipokine with a critical role in HCC proliferation<sup>[29]</sup>.

Insulin was found to induce cell proliferation through activation of the mitogen-activated protein kinase pathway<sup>[30]</sup>, and BCAAs inhibit insulin signals by suppressing the expression of insulin-like growth factor<sup>[31]</sup>. BCAAs have been reported to decrease insulin resistance-induced

expression of endothelial growth factor and to subsequently suppress tumor angiogenesis<sup>[32]</sup>. Collectively, these data suggest that BCAAs inhibit the proliferation of HCC cells or hepatocarcinogenesis through multiple mechanisms.

### Immunity

Immunity and nutrition are closely associated, and several studies have indicated the importance of BCAAs during lymphocyte proliferation or dendritic cell maturation. Depletion of any of the three BCAAs from the culture medium was shown to markedly inhibit phytohemagglutinin-induced lymphocyte proliferation<sup>[33]</sup>, with removal of valine from the culture medium completely abolishing lymphocyte proliferation. In contrast, increased concentrations of BCAAs in the culture medium did not significantly affect lymphocyte proliferation, indicating that, although the BCAAs are requisite for lymphocyte proliferation, there are optimal concentrations. On the other hand, BCAAs have little effect on macrophage functions.

*In vivo* studies have also shown the importance of BCAAs for immunity. We previously analyzed the effects of a BCAA-rich diet on immune system functions in the spleen and liver of rats<sup>[34]</sup>. We found that addition of BCAAs to the diet increased the numbers of intrahepatic lymphocytes and stimulated natural killer (NK) cell activity and lectin-dependent cytotoxic activities in the liver. Interestingly, the number of intrahepatic lymphocytes was positively correlated with valine concentrations in plasma and the liver. BCAAs, especially valine, are also involved in the maturation of dendritic cells. For example, valine was found to dose-dependently increase the allostimulatory capacity of IL-12 production by monocyte-derived dendritic cells (DCs) obtained from both healthy volunteers and cirrhotic patients with chronic hepatitis C virus (HCV) infection<sup>[35]</sup>. These findings suggest that valine may have therapeutic potential in HCV-infected cirrhotic patients by restoring immune system activities, which may lead to inhibit hepatocarcinogenesis<sup>[35,36]</sup>. In patients with cirrhosis, BCAA administration increases the numbers of hepatic lymphocytes and restores the phagocytic activity of neutrophils and the NK activity of lymphocytes<sup>[37]</sup>. In addition, BCAAs increased the number of blood lymphocytes in postsurgical patients<sup>[38,39]</sup>, and significant correlations were observed between the serum concentration of BCAAs and the survival rates of the patients with sepsis<sup>[40]</sup>. These data indicate that BCAAs are closely associated with the maturation and function of various immune cells.

## CLINICAL APPLICATION OF BCAAS IN LIVER DISEASES

### BCAAs for liver cirrhosis

The liver is a central organ for nutrient metabolism, and patients with chronic liver diseases may develop various metabolic and nutrition disorders<sup>[41]</sup>. Patients with cirrhosis frequently show protein and energy deficiency. Protein

deficiency leads to hypoalbuminemia, inducing ascites and edema, whereas energy deficiency decreases fat and muscle mass and causes muscle weakness, decreasing the quality of life of patients with cirrhosis<sup>[42]</sup>. Several clinical trials have suggested that BCAA supplementation improves the prognosis of cirrhotic patients<sup>[43,44]</sup>. For example, a multicenter randomized trial from Italy showed that oral BCAA supplementation in patients with advanced cirrhosis prevented progressive hepatic failure and improved surrogate markers and perceived health status<sup>[44]</sup>. Furthermore, a large scale post marketing clinical study in Japan showed that oral BCAA administration significantly reduced the occurrence of complications associated with poor prognosis, such as liver failure, ruptured esophageal varices, HCC, and death, compared with patients who received diet therapy with defined daily food intake (HR = 0.67, 95%CI: 0.49-0.93)<sup>[43]</sup>. Furthermore, BCAA supplementation in patients with advanced cirrhosis may improve abnormal glucose tolerance in addition to improving serum albumin concentration<sup>[45]</sup>, and a randomized study showed that oral BCAA was effective in patients with both compensated and decompensated cirrhosis, maintaining or increasing serum albumin concentrations<sup>[46]</sup>. Oral BCAA treatment has also been reported to improve protein malnutrition in patients, especially during the early stages of liver cirrhosis, increasing serum albumin level to 3.5-3.9 g/dL and increasing total hepatic parenchymal cell mass<sup>[47-49]</sup>. BCAA treatment also improved nutritional status and reduced the frequency of albumin infusion in children with end-stage liver disease<sup>[50]</sup>. Taken together, these findings indicate that BCAA supplementation is effective in improving nutritional status in cirrhotic patients, regardless of patient age or disease stage.

Furthermore, BCAA supplementation was reported to improve the quality of life in cirrhotic patients. Two randomized trials showed that BCAA supplementation improved the Short Form-36 scores of general health perception compared with control groups<sup>[43,44]</sup>. Another randomized study showed that BCAA-enriched supplements improved weakness and fatigue compared with ordinary foods<sup>[51]</sup>. BCAA-enriched supplementation has also been reported to improve sleep disturbance<sup>[52]</sup>.

Accelerated fat oxidation and a catabolic state after fasting, represented as a decreased respiratory quotient (RQ), are frequently observed in patients with cirrhosis<sup>[53]</sup>. Late evening snack supplementation with a BCAA mixture was found to improve RQ, nutritional state and glucose intolerance<sup>[53,54]</sup>. The energy efficiency of BCAAs is higher than that of glucose or fatty acids, suggesting that BCAAs may be the preferred energy substrate for patients with cirrhosis<sup>[55]</sup>. Others also reported that late evening snacks with BCAAs were useful in improving protein metabolism and lipolysis in cirrhotic patients<sup>[56]</sup>.

Thus, BCAA supplementation for advanced cirrhotic patients improves nutritional status and quality of life. The guidelines of the European Society for Clinical Nutrition and Metabolism and the Study Group for the Standardization of Treatment of Viral Hepatitis Includ-



ing Cirrhosis of the Ministry of Health, Labour and Welfare of Japan recommend BCAA supplementation in the treatment of patients with advanced cirrhosis<sup>[57,58]</sup>.

### BCAAs for hepatic encephalopathy

HE is a major complication of cirrhosis associated with poor prognosis and quality of life, and often occurs repeatedly. Elevated blood ammonia is seen in patients with HE, and ammonia is one of the pathogenic factors for the development of HE<sup>[59]</sup>. Unfortunately, infusion of BCAAs was reported to increase venous blood ammonia in most patients with liver failure<sup>[60]</sup>. Thus, the effects of BCAAs on HE may not be associated with blood ammonia levels, especially when administered intravenously. HE may also be caused by a decreased plasma ratio of BCAAs to AAAs. In patients with advanced cirrhosis, HE frequently occurs after gastrointestinal bleeding, perhaps due to an absence of isoleucine and an abundance of leucine in hemoglobin molecules, leading to HE by way of BCAA antagonism<sup>[61]</sup>. Treatment with BCAAs may therefore have a beneficial effect on patients with hepatic encephalopathy mainly by compensating decreased ratio of BCAAs to AAAs, but not by reducing serum ammonia levels. A systematic review reported that BCAAs appeared to have a modest effect in improving encephalopathy without adverse events, although convincing evidence was not supplied<sup>[62]</sup>. Two randomized studies also showed that BCAAs did not clearly prevent HE in patients with advanced cirrhosis, although BCAAs prevented the progression of hepatic failure<sup>[43,44]</sup>. Furthermore, postoperative BCAA treatment could not prevent postoperative hepatic encephalopathy<sup>[63]</sup>. A recent randomized, double-blind, multicenter study evaluating the effect of BCAAs on HE found that BCAAs did not decrease the recurrence of HE but improved minimal HE and muscle mass<sup>[64]</sup>. Moreover, a systematic review showed that oral (RR = 1.44; 95%CI: 1.07-1.94) but not intravenous (RR=1.12; 95%CI: 0.91-1.39) administration of BCAAs improved HE manifestations<sup>[65]</sup>. Non-absorbable disaccharides such as lactulose or lactitol also improved the manifestations of HE (RR = 1.99; 95%CI: 1.14-3.48) and prevented clinically overt HE (RR = 0.26; 95%CI: 0.17-0.41), suggesting that non-absorbable disaccharides be used as the first line treatment of HE and BCAAs may be considered as a second line treatment<sup>[65]</sup>.

Recently, a systematic review with meta-analyses on the effect of oral BCAAs for the treatment of HE was published<sup>[66]</sup>. The review has revealed that supplementation of oral BCAAs in cirrhotic patients inhibits the manifestation of HE, especially in patients with overt HE rather than those with minimal HE, but showed no effect on the survival of those patients<sup>[66]</sup>. Thus, oral administration of BCAAs is the treatment of choice in cirrhotic patients with HE, especially in combination with non-absorbable disaccharides.

### BCAAs for hepatocellular carcinoma

Clinical studies have suggested that BCAA supplementa-

tion can help in the management of HCC. Prolonged surgical stress and advanced malignancy can result in systemic catabolism and muscle wasting, with BCAA supplementation having the potential to improve these conditions<sup>[67]</sup>.

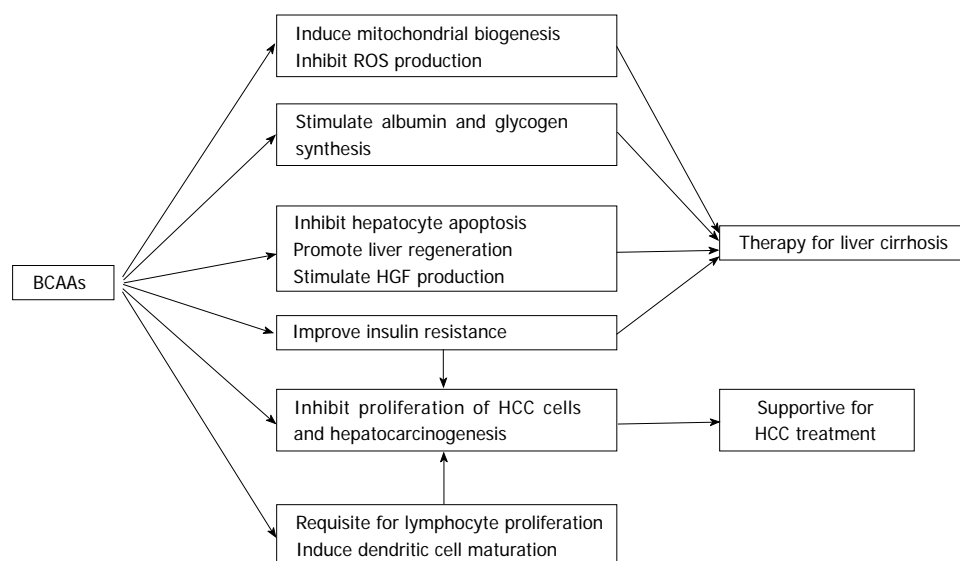
A randomized control trial in obese, HCV-infected patients with cirrhosis showed that BCAA supplementation reduced the frequency of development of HCC, by approximately 30% over 3 years<sup>[68]</sup>. In addition, a second randomized trial in patients with compensated liver cirrhosis due to HCV showed that oral BCAAs reduced the incidence of HCC (15.8% *vs* 25.0%)<sup>[69]</sup>. A retrospective analysis in patients with cirrhosis showed that the incidence of HCC was significantly lower in patients who did than did not receive BCAAs (HR = 0.416, 95%CI: 0.216-0.800, *P* = 0.0085)<sup>[70]</sup>. Furthermore, combinations of BCAAs and angiotensin-converting enzyme inhibitors may prevent the development of HCC in patients with insulin resistance<sup>[71]</sup>.

Perioperative nutritional support, especially enteral rather than parental nutrition, was found to improve the prognosis of cirrhotic patients by reducing complications following hepatectomy<sup>[72,73]</sup>. Recently, a randomized trial showed that BCAA supplementation after hepatectomy promoted rapid improvement in protein metabolism and inhibited progression to liver cirrhosis<sup>[74]</sup>. Furthermore, another randomized trial showed that oral BCAA supplementation after hepatectomy for HCC significantly reduced the 30 month recurrence of HCC (28.5% *vs* 55.7%, *P* = 0.044)<sup>[75]</sup>. Perioperative BCAA treatment in patients undergoing hepatectomy was also shown to contribute to shorter hospital stay and quicker improvement of liver function during the early postoperative period<sup>[76]</sup> and to improve postoperative quality of life by restoring and maintaining nutritional status and whole-body kinetics<sup>[77]</sup>.

The effect of BCAAs on HCC recurrence after radio-frequency ablation (RFA) remains unclear. Two prospective studies showed that BCAA supplementation improved nutritional state and liver function, but its effect on HCC recurrence was not determined<sup>[78,79]</sup>. However, a recent retrospective study showed that oral BCAA supplementation after RFA improved 1 year (61.8% *vs* 52.0%) and 3-year (28.0% *vs* 12.0%) progression-free survival rates compared with a control group after RFA (*P* = 0.013)<sup>[80]</sup>.

Oral BCAA supplementation after chemoembolization also prevents the decrease of liver function after treatment and improves the quality of life, although its ability to prevent HCC recurrence was not determined<sup>[81,82]</sup>. Oral BCAA treatment before chemoembolization was found useful in maintaining hepatic functional reserve<sup>[83]</sup>. A randomized trial also found that oral BCAA supplementation improved nutritional status by increasing BCAA concentration during radiotherapy for HCC<sup>[84]</sup>.

Thus, BCAA supplementation for patients with HCC is of clinical importance in the preservation of liver function and quality of life during treatment, although it is unclear whether BCAAs directly prevent HCC.



**Figure 1** Mechanism of action of branched chain amino acids in liver diseases. BCAAs: Branched chain amino acids; ROS: Reactive oxygen species; HGF: Hepatocyte growth factor; HCC: Hepatocellular carcinoma.

**Table 1** Prospective randomized trials of branched-chain amino acid administration for advanced liver diseases

Object	Time	No.	Major outcome	Ref.
Cirrhosis	2 yr	646	Improve event-free survival and QOL. Increase serum albumin levels.	[43]
Cirrhosis (advanced)	1 yr	174	Improve event-free survival. Lower hospital admission. Improve the Child-Pugh score and QOL.	[44]
Cirrhosis (decompensated)	24 wk	281	Increase serum albumin levels.	[45]
Cirrhosis	2 yr	65	Maintain serum albumin levels.	[46]
Cirrhosis (early)	2 yr	65	Maintain serum albumin levels.	[49]
Cirrhosis	3 mo	48	Increase serum albumin levels. Improve energy metabolism.	[51]
Cirrhosis (HCV)	168 wk	39	Reduce hepatic carcinogenesis in patients with compensated cirrhosis with a serum albumin level of < 4.0 g/dL.	[69]
Cirrhosis (HCV, obese)	2 yr	622	Reduce hepatic carcinogenesis in patients with BMI of 25 or higher and with an alpha-fetoprotein level of 20 ng/mL or higher.	[68]
Cirrhosis (pre liver transplant)	3.3 yr	50	Preserve hepatic reserve functions. Lower complications associated with cirrhosis.	[99]
Cirrhosis after an episode of HE	56 wk	116	Not decrease recurrence of HE. Improve minimal HE and muscle mass.	[64]
Cirrhosis after hepatectomy	1 yr	43	Improve hepatic metabolism after hepatectomy. Inhibit progression to cirrhosis.	[74]
HCC after hepatectomy	6.5 mo	56	Reduce early recurrence of HCC.	[75]
HCC after hepatectomy	12 wk	44	Shorten hospital stay. Quicker improvement of liver functions.	[76]
After hepatectomy	12 mo	76	Improve post operative QOL.	[77]
HCC after RFA	12 mo	35	Improve nutritional state and QOL.	[78]
HCC after RFA	12 wk	30	Improve liver functions.	[79]
HCC undergoing chemoembolization	12 mo	84	Increase serum albumin levels, reduce morbidity, and improve QOL.	[81]
HCC undergoing chemoembolization	2 wk	56	Prevent reduction of liver functions.	[82]
HCC during radiotherapy	10 wk	30	Increase serum albumin levels.	[84]

QOL: Quality of life; HCV: Hepatitis C virus; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma. RFA: Radiofrequency ablation.

### Acute liver injury

Although BCAAs have no proven benefit in patients with acute liver injury, enteric nutritional support is essential<sup>[85]</sup>. Several animal studies have shown that BCAAs may prevent acute liver injury<sup>[86-88]</sup>, although its effects in humans are as yet undetermined. BCAA concentrations have been reported to be increased, unaltered or decreased following acute liver injury<sup>[89,90]</sup>. In alcoholic hepatitis, parentally or enterally administered hyperalimentation with or without BCAAs did not show survival benefits<sup>[91]</sup>.

### HCV infection

Insulin resistance occurs frequently in patients infected with HCV and is associated with various complications, such as steatosis, disturbances in glucose metabolism, and carcinogenesis<sup>[92]</sup>. BCAAs, especially leucine or isoleucine, have been shown to have beneficial effects on glucose metabolism<sup>[93]</sup>. A randomized study showed that BCAA treatment of patients with chronic hepatitis C and insulin resistance improved HbA1c concentrations in patients with marked peripheral insulin resistance, although

BCAA did not significantly affect parameters of glucose metabolism or lipid profiles<sup>[94]</sup>. A multicenter randomized control trial showed that BCAAs prevented the development of HCC in obese, HCV-infected patients<sup>[68]</sup>. Furthermore, BCAA treatment can restore impaired interferon signaling caused by malnutrition through the mTOR and FoxO pathways in patients with chronic hepatitis C<sup>[95]</sup>. Interestingly, valine was reported to reduce HCV viral load, possibly by enhancing DC function or interferon signaling<sup>[96]</sup>. Thus, BCAA supplementation may be useful for adherence to interferon therapy in patients with chronic hepatitis C and may enhance the effects of interferon in these patients<sup>[97]</sup>.

### Liver transplantation

Protein-energy malnutrition is commonly found in patients with end-stage liver disease requiring liver transplantation and is a risk factor for posttransplant morbidity. A report of 50 recipients undergoing living donor liver transplantation (LDLT) showed that absence of preoperative BCAA treatment was an independent risk factor for postoperative severe infection and in-hospital death<sup>[98]</sup>. Kawamura *et al.*<sup>[99]</sup> reported that early interventional oral BCAAs might prolong the liver transplant waiting period by preserving hepatic reserve in patients with cirrhosis. A retrospective analysis also showed that BCAA treatment before LDLT may reduce the incidence of posttransplant bacteremia<sup>[100]</sup>.

### Other clinical problems related to management of liver diseases

**Insulin resistance:** Increased insulin resistance is found in patients with chronic liver diseases and is a therapeutic target associated with malnutrition and hepatocarcinogenesis. BCAAs are thought to act on insulin target organs, such as skeletal muscles, adipose tissue, and the liver<sup>[101]</sup>. BCAA infusion was reported to decrease plasma glucose concentrations in patients with advanced liver cirrhosis<sup>[102]</sup>, and oral BCAA administration was recently shown to reduce both blood glucose concentrations<sup>[103,104]</sup> and insulin resistance in patients with chronic liver diseases, especially in men<sup>[19,105]</sup>. More recently, long-term BCAA supplementation was shown to improve glucose tolerance in patients with nonalcoholic steatohepatitis (NASH)-related cirrhosis, and may be an alternative treatment for NASH<sup>[106]</sup>.

## CONCLUSION

BCAAs are involved in various biological activities (Figure 1), and prospective randomized clinical trials showing possible effectiveness of BCAAs in the management of chronic liver diseases are summarized in Table 1. Supplementation with BCAAs may be a promising therapeutic option for patients with chronic liver diseases, although more analyses are needed to determine their basic mechanisms of action.

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## Liver diseases in pregnancy: Diseases not unique to pregnancy

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### Abstract

Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. Few challenges arise in reaching an accurate diagnosis in light of such physiological changes. Laboratory test results should be carefully interpreted and the knowledge of what normal changes to expect is prudent to avoid clinical misjudgment. Other challenges entail the methods of treatment and their safety for both the mother and the baby. This review summarizes liver diseases that are not unique to pregnancy. We focus on viral hepatitis and its mode of transmission, diagnosis, effect on the pregnancy, the mother, the infant, treatment, and breast-feeding. Autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, Wilson's disease, Budd Chiari and portal vein thrombosis in pregnancy are also discussed. Pregnancy is rare in patients with cirrhosis because of the metabolic and hormonal changes associated with

cirrhosis. Variceal bleeding can happen in up to 38% of cirrhotic pregnant women. Management of portal hypertension during pregnancy is discussed. Pregnancy increases the pathogenicity leading to an increase in the rate of gallstones. We discuss some of the interventions for gallstones in pregnancy if symptoms arise. Finally, we provide an overview of some of the options in managing hepatic adenomas and hepatocellular carcinoma during pregnancy.

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**Key words:** Liver; Pregnancy; Viral hepatitis; Autoimmune; Cirrhosis; Gallstones; Adenoma

**Core tip:** Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. Challenges involve making the diagnosis and the methods of treatment and their safety for both the mother and the baby. This review summarizes liver diseases that are not unique to pregnancy.

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### INTRODUCTION

Although not unique to pregnancy, liver diseases reviewed here can have significant consequences on pregnant women and their infants.

Approach to the diagnosis of liver conditions in preg-



**Table 1** Normal physiological alterations in liver tests in pregnancy

Test	First trimester	Second/third trimesters
Albumin	↓	↓
ALT	N	N
AST	N	N
Total bilirubin	↓	↓
Alkaline phosphatase	N	↑
GGT	N	↓
5'-nucleotidase	N	May increase in second and third trimesters
Fasting total bile acids	N	N
Prothrombin time	N	N

N: No change; ↑: Increase; ↓: Decrease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase.

nant women should take into consideration the physiological changes during pregnancy that allow for normal fetal development. Sex hormones such as estrogen and progesterone increase progressively during pregnancy. This increase has an influence on hepatic metabolic, synthetic, and excretory functions<sup>[1]</sup>. During late pregnancy, biliary excretion of few compounds can be reduced. Furthermore, reduction in serum protein concentrations secondary to reversible hemodilution resulting from expanding plasma volume while pregnant is reflected by alterations in some liver function tests (Table 1).

Whereas nausea and vomiting are common in early pregnancy, those should not be considered normal in the second or third trimesters and ought to be investigated<sup>[2]</sup>. Jaundice and generalized pruritus are not normal features in pregnancy. Spider nevi and palmar erythema were found up to 66% and 63% respectively by the end of normal pregnancy in one study<sup>[3]</sup>. Most of those were reversible after delivery.

Unique aspects such as the effect of the disease on pregnancy, the effect of the pregnancy on disease progression, the use of specific therapies during pregnancy, and issues related to breast-feeding are discussed.

## VIRAL HEPATITIS AND PREGNANCY

### Hepatitis A virus

Hepatitis A virus (HAV) is an RNA virus that transmits through fecal-oral route, usually through contaminated water or food. Overall incidence is 9.1 per 100000 in the United States and less than 1:1000 pregnancies. Clinical presentation ensues within 2-4 wk of exposure. Generally, HAV does not result in chronic infection. Acute hepatitis A starts with prodromal symptoms including anorexia, malaise, nausea and vomiting, and progresses into jaundice and elevated liver transaminases. Presence of HAV immunoglobulin M (IgM) antibodies confirms the acute infection. Management is supportive care including optimizing hydration and nutrition. Rarely acute hepatitis A can lead to fulminant hepatic failure. Inactivated HAV vaccine and immunoglobulin prophylaxis are safe in pregnancy<sup>[4]</sup>. Although vertical transmission has been reported,

**Table 2** Interpretation of hepatitis B blood tests

Test	HBsAg	Anti-HBs	Total Anti-HBc	Anti-HBc IgM	HBV DNA
Acute infection	+	-	+	+	+
Resolved infection	-	+	+	-	-
with natural immunity	-	+	-	-	-
Immunity through vaccination	-	+	-	-	-
Chronic infection	+	-	+	-	+/-
Different possibilities <sup>1</sup>	-	-	+	-	-

<sup>1</sup>Could represent resolving acute infection, resolved infection (most likely), chronic infection with low viral load or false positive. HBsAg: Hepatitis B surface antigen; Anti-HBs: Hepatitis B surface antibody; Total anti-HBc: Total hepatitis B core antibody; Anti-HBc IgM: Hepatitis B core antibody immunoglobulin M; HBV: Hepatitis B virus.

intrauterine transmission is rare<sup>[5-7]</sup>. Fecal-oral transmission during birth is possible. No cases of teratogenicity were reported, but maternal complications such as preterm labor were described. Susceptible woman should receive vaccination. Breast-feeding is not contraindicated in acute hepatitis A with following appropriate hygiene measures.

### Hepatitis B virus

Hepatitis B virus (HBV) is a DNA virus that is highly infectious and transmits through intravenous route, sexual contact, and vertically from the mother to her fetus. It can present both as an acute or chronic infection. Pregnancy does not affect the course of infection directly. Fortunately, since universal children vaccination for hepatitis B was implemented in 1992, the numbers of vertically transmitted chronic hepatitis B cases, and its complications such as hepatocellular carcinoma have dropped<sup>[8-10]</sup>. Prenatal screening for HBV is standard of care in many countries including the United States. Those susceptible should be vaccinated. Pregnant women exposed to HBV should receive HBV immunoglobulins (HBIG) within 72 h of exposure in addition to the vaccination series. Infants with infected mothers should receive both immunoglobulins and vaccination series at the time of delivery. While acute infection can present with a viral syndrome and jaundice such as that of acute hepatitis A infection, chronic infection is usually asymptomatic and diagnosis can be made relying on serum serology testing. A summary of the tests used in hepatitis B diagnosis and their interpretation is displayed in Table 2.

Treatment should follow guidelines published by medical societies such as the American Association for the Study of Liver Disease (AASLD)<sup>[11]</sup>, the European Association for the Study of the Liver<sup>[12]</sup>, or the Asian Pacific Association for the Study of the Liver<sup>[13]</sup>. In the United States, we recommend referring infected pregnant women to the state's perinatal hepatitis B prevention program<sup>[14]</sup>, that is CDC-funded (centers for disease control and prevention), and to liver specialists for optimizing counseling and treatment.

There are seven Food and Drug Administration (FDA)-approved medications for the treatment of hepatic

**Table 3 Food and Drug Administration approved medications for hepatitis B treatment**

Generic name	Trade name	Company	Approved for HBV treatment
Interferons			
Interferon $\alpha$ -2b, recombinant	Intron® A	Schering Corporation/ Merck and Co	1992
Perinterferon $\alpha$ -2a	Pegasys®	Genentech/Roche group	2005
Nucleosides/nucleotides			
Lamivudine <sup>1</sup>	EPIVIR-HBV®	GlaxoSmithKline	1998
Adefovir dipivoxil	HEPSERA™	Gilead Sciences	2002
Entecavir	BARACLUDE™	Bristol-Myers Squibb	2005
Telbivudine <sup>2</sup>	TYZEKA™	Novartis	2006
Tenofovir <sup>2</sup>	Viread	Gilead Sciences	2008

<sup>1</sup>Pregnancy risk category C, can be used in the third trimester; <sup>2</sup>Pregnancy risk category B. HBV: Hepatitis B virus.

tis B (Table 3) in non-pregnant patients. Interferon use is contraindicated in pregnancy. Tenofovir and Telbivudine belong to pregnancy risk category B; all others belong to category C. The choice to treat or not should be weighed in light of benefits versus risks for both the mother and her fetus. Those with higher viral load (serum HBV DNA  $> 10^8$  copies/mL) were at higher risk for vertical transmission in one study<sup>[15]</sup>. Wen *et al*<sup>[16]</sup> showed recently that the adjusted odds ratio of transmission for each log<sub>10</sub> copy/mL increase, is 3.49 ( $P = 0.001$ ), with predictive rates of infection at maternal viral load levels of 7, 8, and 9-log<sub>10</sub> copies/mL of 6.6% ( $P = 0.033$ ), 14.6% ( $P = 0.001$ ), and 27.7% ( $P < 0.001$ ), respectively. Therefore, it is reasonable to treat those women or women with previous infected children, especially towards the end of pregnancy (from week 28 and up), with risk category B drugs or Lamivudine (increases birth defects if used in 1<sup>st</sup> trimester)<sup>[17,18]</sup>. In a meta-analysis, significant drop in the risk of vertical transmission was found in those who succeeded to lower HBV DNA below  $10^6$  copies/mL<sup>[18]</sup>. Telbivudine was used safely and with good efficacy in reducing transmission (0% *vs* 8%;  $P = 0.002$ ) in a recent study<sup>[19]</sup>.

Although cesarean section is proposed as a measure to lower the risk of transmission, particularly in women with high viral loads towards term, there is a conflicting evidence regarding choosing cesarean section versus vaginal delivery to lower the risk of vertical transmission<sup>[20,21]</sup>. Breast-feeding should be encouraged for infants receiving HBIG and vaccination<sup>[22-25]</sup>. On the other hand, no adequate evidence of the safety of breast-feeding in mothers receiving antiviral therapy is available and women on antiviral therapy with lamivudine, telbivudine or tenofovir should be discouraged from breast-feeding<sup>[26-28]</sup>.

### Hepatitis C virus

With prevalence around 1.6%, chronic hepatitis C infection continues to present a big public health concern in the United States. The majority of those patients, left untreated, will progress to cirrhosis with expected peak in prevalence around the year 2030, with expected medical cost exceeding \$85 billion<sup>[29]</sup>. Generally, all high-risk patients should be screened for hepatitis C virus (HCV) following CDC and AASLD guidelines. Those include

children born to HCV infected mothers. While there is no approved medicine to treat chronic hepatitis C in pregnant women, those should be referred to liver experts for education regarding options of treatment after delivery and preventive measures to slow the progression of the disease. HCV antibodies ELISA testing is a sensitive test and carries high positive predictive value in high-risk patients. Diagnosis can be confirmed using HCV RNA polymerase chain reaction (PCR). There are several therapies for hepatitis C that are under investigation currently. Some of those could prove safe to use in pregnancy in the future. Pregnant women with hepatitis C should be educated about the mode of transmission and how to reduce the risk, smoking cessation, alcohol abstinence, and vaccination for hepatitis A and hepatitis B. They should also be screened for hepatitis B and human immunodeficiency virus (HIV) infection. Women undergoing treatment for hepatitis C, or those with partners undergoing treatment for hepatitis C, should avoid pregnancy by using at least 2 forms of barrier contraception, for the period of treatment and 6 mo after.

Infants of hepatitis C infected- mothers were at higher risk for low birth weight, being small for gestational age, or requiring intensive care upon birth in one report<sup>[30]</sup>. The risk of vertical transmission is approximately 4%. This risk increased up to 19.4% when co-infected with HIV<sup>[31-35]</sup>. High viral load also increase the risk for vertical transmission. HCV transmission could occur through viral transcytosis across trophoblast cells mediated by HCV receptors expressed on trophoblasts or through some form of injury that influences the placental barrier<sup>[36]</sup>. Although there were few reports of increased risk of transmission with premature rupture of membrane, more than 6 h before delivery, mode of delivery was not found to change the risk of hepatitis C transmission<sup>[31,37-39]</sup>. As the new era of direct antiviral agents is evolving, treating hepatitis C during pregnancy may become an option and thus the possibility of reducing the risk of transmission<sup>[40]</sup>.

Breast-feeding is considered safe when nipples are not cracked or bleeding according to CDC recommendations.

### Hepatitis D virus

Hepatitis D virus (HDV) is an RNA virus that requires

hepatitis B surface antigen for replication. Anti-HDV antibodies establish the diagnosis. Although vertical transmission is possible, hepatitis D is preventable by preventing HBV transmission<sup>[41]</sup>.

### **Hepatitis E virus**

Hepatitis E virus (HEV) is an RNA virus that is usually transmitted through fecal-oral means, although transmission via infected blood products and vertical transmission has been reported<sup>[42]</sup>. It is usually a self-limiting disease in immunocompetent patients. Hepatitis E can cause significant disease in patients with chronic liver disease and can present in a chronic form leading to fibrosis in immunocompromised individuals<sup>[43]</sup>. Pregnant women in highly endemic areas are particularly at risk with up to 60% developing fulminant hepatic failure with a maternal death rate of up to 31%<sup>[44,45]</sup>. A review from Bangladesh suggests it is responsible for 9.8% of pregnancy-related deaths<sup>[46]</sup>. On the other hand, the severity of the disease was not different between pregnant and non-pregnant women in non-endemic places such as the United States and Europe. A report suggested that such variance in severity between endemic and non-endemic areas might be related to different genotypes of HEV<sup>[47]</sup>. Other studies suggested that pregnancy per se is not a poor prognostic factor for those who developed acute liver failure<sup>[48]</sup>. To a lesser extent, hepatitis E is prevalent in some western countries, particularly genotype 3.

Vertical transmission was described up to 78.9% with infant mortality of 40%<sup>[42]</sup>. The level of viremia appears to be associated with the severity of the disease during pregnancy<sup>[49]</sup>. Despite such high mortality, current treatment remains supportive. Pregnant woman seeking travel to endemic areas should be counseled about the risk of hepatitis E, and be advised to avoid unpurified water, uncooked fruit, vegetables, and shellfish.

Hepatitis E vaccines have been developed and evaluated in trials but has not been approved for commercial use yet. Their utility is yet to be determined<sup>[50-54]</sup>.

### **Herpes simplex virus**

32 out of 137 cases of herpes simplex virus (HSV) hepatitis were pregnant women in one report, suggesting their susceptibility<sup>[55]</sup>. Although rare, HSV hepatitis carries a very high mortality (39%) if inappropriately treated<sup>[56]</sup>. Providers should have high index of suspicion in this patient group in the appropriate clinical setting; elevated liver transaminases usually 100 times upper level of normal with typically normal or mildly elevated bilirubin (anicteric hepatitis)<sup>[57-60]</sup>. Serology testing including anti-HSV IgM should be ordered. HSV PCR can be ordered as well to confirm diagnosis. Recent study has revealed that HSV DNA load correlated with liver transaminase levels and disease severity<sup>[61]</sup>. Although no strong evidence to support starting Acyclovir in patients with indeterminate acute liver failure, clinicians should consider empirical therapy with acyclovir when HSV hepatitis is

suspected<sup>[59]</sup>. Liver biopsy with appropriate immunohistochemistry staining can be useful, but usually is avoided because of its invasive nature, coagulopathy and because of the potential delay in results/treatment.

## **AUTOIMMUNE HEPATITIS AND PREGNANCY**

Autoimmune hepatitis is a disease characterized by elevated liver aminotransferases, hypergammaglobulinemia, and positive serum autoantibodies. Autoimmune hepatitis and pregnancy (AIH) is more common in females, especially those in childbearing ages. It can happen during pregnancy and may not follow consistent pattern. Normalization of liver aminotransferases has been described in patients with no treatment<sup>[62]</sup>. This normalization could be related to the immunotolerant state that predominates pregnancy. On the other hand, flare-ups have been reported during and after pregnancy<sup>[63]</sup>. Prematurity and fetal-loss were described in those patients<sup>[64]</sup>. A link was observed between antibodies to soluble liver antigen/liver-pancreas and ribonucleoprotein/Sjögren's syndrome A and adverse outcomes<sup>[65]</sup>. Inadequate disease control in the year prior to pregnancy and the absence of treatment during pregnancy were associated with unfavorable outcomes in a recent study<sup>[66]</sup>.

Although the patients should be counseled about possible adverse outcomes, pregnancy appears to be safe in well-controlled AIH women<sup>[67]</sup>. Special considerations should be given to the postpartum period as flare-ups may occur frequently, and treatment should be resumed preemptively two weeks before delivery and maintained thereafter<sup>[68]</sup>. Immunosuppressive therapy with steroids and agents such as azathioprine is the mainstay for treatment of AIH. Azathioprine use during pregnancy is generally safe (despite reports of birth defects in animal models)<sup>[64]</sup>.

## **PRIMARY BILIARY CIRRHOSIS/PRIMARY SCLEROSING CHOLANGITIS AND PREGNANCY**

There is limited data about pregnancy in patients with primary biliary cirrhosis. Reports have ranged from normal course of pregnancy and good fetal outcomes to poor prognosis for both mother and fetus<sup>[69,70]</sup>. Earlier diagnosis and the use of ursodeoxycholic acid (UDCA) in treatment, which has been used safely in pregnancy, have been linked to favorable outcomes<sup>[71]</sup>. Primary sclerosing cholangitis did not appear to reduce fertility and resulted in good outcomes, in one report. UDCA was successfully used to control pruritus in this cohort<sup>[72]</sup>.

## **WILSON'S DISEASE AND PREGNANCY**

Wilson's disease is an autosomal recessive disease with

**Table 4 Options for portal hypertension management in pregnancy**

Esophageal varices	Nonselective $\beta$ -blockers Endoscopic and ligation and/or sclerotherapy TIPS: Data on TIPS and pregnancy is limited
Ascites	Sodium (salt) restriction, diuretics
Hepatic encephalopathy	Lactulose, rifaximin

TIPS: Transjugular portosystemic shunt.

prevalence of 1:30000 to 1:50000<sup>[73]</sup>. It affects hepatic copper transport with inhibition of biliary excretion, resulting in excess circulating copper and deposition in organs such as the liver and the brain. Cases of reduced fertility and recurrent spontaneous abortions in untreated women were reported<sup>[74]</sup>. Chelation therapy using *D*-penicillamine or trientine, or the use of zinc to reduce intestinal absorption of copper, have been the mainstay therapy for Wilson's disease. Zinc has been used with minimal teratogenicity during pregnancy<sup>[75]</sup>. Although teratogenic effects of *D*-penicillamine in humans and animals, and teratogenic effects of trientine in animals were described<sup>[76,77]</sup>, therapy should not be discontinued as this can result in severe hemolysis, worsening of liver function and even death. Even though zinc dosages can be maintained during pregnancy, AASLD recommends lowering *D*-penicillamine and trientine to the minimum needed (usually 25%-50% of the pre-pregnancy dose)<sup>[78]</sup>, particularly towards term to aid in wound healing. Baseline dosages can be resumed postnatal. The mother should be counseled, and both the mother and her fetus should be monitored closely during pregnancy. Breast-feeding is discouraged as *D*-penicillamine can be harmful to the infant and safety has not been established with trientine and zinc.

## GALLSTONES AND PREGNANCY

Physiological changes during pregnancy particularly hormonal changes lead to decrease in contractility of the gallbladder and changes in bile content, with increase in cholesterol saturation, resulting in increase in lithogenicity of the bile<sup>[79]</sup>. Incidence of gallstones is up to 12% in pregnant women<sup>[80]</sup>. Those typically remain asymptomatic. The patient can present with biliary pain, gallstone pancreatitis, or less likely acute cholecystitis. Other manifestations such as choledocholithiasis and cholangitis can also happen. Management is mostly conservative with hydration and antibiotics if indicated. In more severe cases, cholecystectomy can be indicated. Endoscopic retrograde cholangiopancreatography (ERCP) can also be used with taking precautions to minimize radiation exposure of the fetus. In general, surgical procedures are the safest in the second trimester. ERCP was reported to be associated with higher risk for preterm pregnancy and low birth-weight when performed in the first trimester. Post-ERCP pancreatitis rate was higher in pregnancy than general population<sup>[81-85]</sup>.

## CIRRHOSIS/ PORTAL HYPERTENSION AND PREGNANCY

Pregnancy in cirrhotic women is rare, probably because of low prevalence of cirrhosis in reproductive age group (45 in 100000) and also due to amenorrhea and anovulation, likely related to metabolic and hormonal derangements<sup>[86]</sup>. The physiological increase in plasma volume during pregnancy can worsen portal hypertension, resulting in increase risk of variceal bleeding. Variceal bleeding can happen in up to 38% of cirrhotic pregnant women. This is even higher in those with known portal hypertension. Those with known varices have a 78% chance of bleeding<sup>[87]</sup>. AASLD recommends screening for esophageal varices by the second trimester, as the risk of bleeding appears to be highest at that time. Women with cirrhosis planning to become pregnant should be screened before conception by endoscopy and prophylaxis (with nonselective beta blockers) should be started as recommended by AASLD guidelines. Complications of portal hypertension in pregnancy can be as high as 50% resulting in high mortality rate of up to 18%, and higher risk for fetal loss<sup>[88]</sup>. Pregnancy should be avoided in women with previous history of variceal bleeding and liver insufficiency. Means such as early forceps delivery or vacuum extraction should be considered to prevent excessive straining during vaginal delivery. Management options of complications of portal hypertension are summarized in Table 4. All medications used during pregnancy should be checked as of which risk category they fall under according to the FDA classification before prescribing (Tables 5 and 6).

## HEPATOCELLULAR ADENOMA AND PREGNANCY

The incidence of hepatocellular adenoma has increased since the introduction of oral contraceptives. There is a link between pregnancy and liver adenomas secondary to higher levels of hormones<sup>[89]</sup>. Rupture of adenomas has resulted in maternal mortality of a 44% and fetal loss of 38% in one study<sup>[90]</sup>. Adenoma rupture risk increases towards the end of pregnancy<sup>[91]</sup>. Women with adenomas > 5 cm or those with previous complications with adenomas, should avoid subsequent pregnancies. Those pregnant with smaller adenomas should be monitored closely with serial ultrasound imaging. If the lesion is progressively enlarging, or 5 cm in size or bigger, surgical resection should be considered<sup>[90]</sup>. Radiofrequency ablation is another modality that can be used in the treatment of hepatic adenomas<sup>[91-93]</sup>. Close monitoring of the lesion should continue in the postpartum period as well.

## HEPATOCELLULAR CARCINOMA AND PREGNANCY

Although rare, hepatocellular carcinoma has been reported during pregnancy. Fibrolamellar variant of hepatocel-



**Table 5** The Food and Drug Administration pregnancy risk categories of medicines

Pregnancy category	Definition
A	Controlled studies show no risk
B	Animal studies show no risk, and there are no human controlled studies. Or animal studies may have revealed an adverse effect that was not reproduced in human controlled studies
C	No human studies and either animal studies show an adverse effect or there are no studies available. Use if the risk is justified
D	Positive evidence of risk in human studies, only if the potential benefits outweigh the risk
X	Contraindicated in pregnancy: Risk is confirmed in animal and human studies and outweighs any advantage

**Table 6** Food and Drug Administration pregnancy risk categories of some liver disease medications

Medicine	Pregnancy category	Medicine	Pregnancy category
Nadolol	C	Ribavirin	X
Propranolol	C	Telaprevir	B
Rifaximin	C	Boceprevir	B
Lactulose	B	Tenofovir	B
Furosemide	C	Entecavir	C
Spironolactone	C	Telbuvudine	B
Corticosteroids	B	Adefovir	C
Azathioprine	D	Lamivudine	C
Cyclosporin	C	Acyclovir	B
Mycophenolate mofetil	D	Ursodeoxycholic acid	B
Tacrolimus	C	Penicillamine	D
Sirolimus	C	Trientine	C
Antithymocyte globulin	C	Zinc sulfate	C
Pegylated interferon	C (contraindicated in pregnancy)	Interferon alpha 2b	C (contraindicated in pregnancy)

lular carcinoma (HCC) was also reported<sup>[94-96]</sup>. Pregnant women with HCC can have shorter median survival than those non-pregnant. Higher levels of estrogen and immune suppression during pregnancy can play a role with HCC progression<sup>[97]</sup>. Modalities such as surgical resection and radiofrequency ablation can be used in selected patients. Limited data are available about the management of hepatocellular carcinoma in pregnancy.

## HEPATIC VEIN THROMBOSIS/PORTAL VEIN THROMBOSIS AND PREGNANCY

Budd-Chiari syndrome (BCS) is rare in pregnancy but can have grave consequences for both the mother and her fetus. The physiological hypercoagulable state can contribute in BCS development in pregnancy. Other predisposing factors are factor V Leiden mutation and prothrombin gene mutations. BCS entails thrombosis of the hepatic vein resulting in passive congestion of the hepatic sinusoids leading to ischemia and portal hypertension. Low molecular weight heparin should be started if no contraindications. Extreme measures such as portacaval shunting and liver transplantation during pregnancy were reported<sup>[98,99]</sup>. Subsequent pregnancies are not absolutely contraindicated with appropriately treated disease. The mother should be counseled about the possible maternal and fetal unfavorable outcomes.

Portal vein thrombosis (PVT) is rare and can also occur during pregnancy. Local causes such as cirrhosis, intra-abdominal infections, or malignancies may predispose to PVT. Systemic disorders resulting in hypercoagulable state

such as factor V Leiden mutation, anti-phospholipid syndrome, or myeloproliferative disorders should be also excluded. In acute portal vein thrombosis, anti-coagulation should be used for 3 mo at the least. Patients with chronic portal vein thrombosis should be screened for gastroesophageal varices and should be treated accordingly<sup>[100]</sup>.

## CONCLUSION

Pregnant women can have a variety of liver diseases with different incidences. Clinicians should be aware of the clinical presentations and be able to manage those conditions with special attention to the peculiarities in relation to the mother and her infant. In this review we have summarized several of the liver diseases that can happen during pregnancy and offered an overview of their management.

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## Liver diseases in pregnancy: Diseases unique to pregnancy

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### Abstract

Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. This review summarizes liver diseases that are unique to pregnancy. We discuss clinical conditions that are seen only in pregnant women and involve the liver; from Hyperemesis Gravidarum that happens in 1 out of 200 pregnancies and Intrahepatic Cholestasis of Pregnancy (0.5%-1.5% prevalence), to the more frequent condition of preeclampsia (10% prevalence) and its severe form; hemolysis, elevated liver enzymes, and a low platelet count syndrome (12% of pregnancies with preeclampsia), to the rare entity of Acute Fatty Liver of Pregnancy (incidence of 1 per 7270 to 13000 deliveries). Although pathogenesis behind the development of these ailments are not fully understood, theories have been proposed. Some propose the special physiological changes that accompany pregnancy as a precipitant. Others suggest a constellation of factors including both the mother and her fetus that come together to trigger those unique conditions. Reaching a

timely and accurate diagnosis of such conditions can be challenging. The timing of the condition in relation toward which trimester it starts at is a key. Accurate diagnosis can be made using specific clinical findings and blood tests. Some entities have well-defined criteria that help not only in making the diagnosis, but also in classifying the disease according to its severity. Management of these conditions range from simple medical remedies to measures such as immediate termination of the pregnancy. In specific conditions, it is prudent to have expert obstetric and medical specialists teaming up to help improve the outcomes.

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**Key words:** Liver; Pregnancy; Hyperemesis gravidarum; Intrahepatic cholestasis; Hemolysis, elevated liver enzymes, and a low platelet count; Preeclampsia; Eclampsia; Acute fatty liver

**Core tip:** Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. Challenges involve making the diagnosis and the methods of treatment and their safety for both the mother and the baby. This review summarizes liver diseases that are unique to pregnancy.

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### HYPEREMESIS GRAVIDARUM

Although nausea and vomiting of pregnancy affect up to 90% of pregnancies, hyperemesis gravidarum (HG) oc-

curs in approximately 1 out of every 200 pregnancies<sup>[1]</sup>. Women with HG present with severe and persistent vomiting in the first trimester that can cause dehydration, metabolic disturbances, and nutritional deficiencies. HG may result in weight loss and ketonuria. Risk factors for HG include multiple gestations, molar pregnancies, fetal anomalies such as hydrops fetalis and trisomy 21<sup>[2,3]</sup>. Not all women with HG develop liver disease. Half of the patients who require hospitalization for HG suffer from liver disease<sup>[4]</sup>. HG was the cause in up to 94% of pregnant women with elevated liver transaminases in their first trimester in one series<sup>[5]</sup>. Veenendaal *et al*<sup>[6]</sup> conducted a meta-analysis that showed women with HG are more likely to have low birthweight < 2500 kg (OR = 1.42; 95%CI: 1.27-1.58), small for gestational age (OR = 1.28; 95%CI: 1.02-1.60), and premature delivery (OR = 1.32; 95%CI: 1.04-1.68) than those with no HG. On the other hand, no correlations with Apgar scores, congenital anomalies or perinatal death were identified. Some of those poor outcomes were more likely in pregnant women with low gestational weight gain (< 7 kg)<sup>[7]</sup>.

### Pathogenesis

Despite several hypotheses, the pathogenesis of liver disease in HG is not well understood and likely multifactorial. Starvation injury was proposed as an etiology since 1968<sup>[8,9]</sup>. Over expression of cytokine-producing cells was implicated as a potential cause for pregnancy-related liver diseases such as preeclampsia and HG. Other hypotheses predicted damage to the liver resulting from impaired maternal or fetal mitochondrial fatty acid oxidation, implicating deficiency in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) as a reason for accumulation of fatty acids in the placenta and eventually causing liver damage<sup>[10]</sup>. Other report linked fetal deficiency of hepatic carnitine palmitoyltransferase I, the enzyme responsible for transporting long chain fatty acids from the cytoplasm of cells across the outer mitochondrial membrane, to HG<sup>[11-16]</sup>.

### Clinical presentation

The clinical presentation of HG with liver disease can range from mild aminotransferase elevation to rarely severe elevation. No fulminant hepatic failure has been reported with HG<sup>[17,18]</sup>. Patients usually are acutely ill with signs of dehydration. Rarely, it can present with jaundice and electrolyte disturbances such as hypokalemia and hyponatremia as well as metabolic alkalosis and erythrocytosis. It seems that the severity of nausea and vomiting correlates well with the degree of liver enzymes elevation<sup>[4]</sup>. No specific abdominal ultrasound findings are associated with HG. Liver biopsy may show necrosis, steatosis or bile plugs<sup>[19,20]</sup>, and usually is not indicated.

### Management

Patients with HG usually require hospitalization for intravenous fluid replacement, anti-emetics, bowel rest, and possible parenteral nutrition.

### Prognosis

Hyperemesis gravidarum is usually a reversible condition with no permanent damage to the liver and almost never fatal.

## INTRAHEPATIC CHOLESTASIS OF PREGNANCY

Intrahepatic cholestasis of pregnancy (ICP) is a reversible condition of cholestasis that happens usually in the third trimester. Findings such as pruritus, high serum bile acids levels, and abnormal liver function tests usually resolve after delivery. ICP is more prevalent in Scandinavian and South American countries<sup>[21,22]</sup>. Prevalence in Europe, United States, Canada and Australia is 0.1% to 1.5%<sup>[23]</sup>. In a recent review, although no causality effect can be claimed, ICP was associated with an increase in the risk of developing hepatobiliary diseases later in life, such as hepatitis C, cirrhosis, and gallstones. Having underlying chronic liver disease (hepatitis C or chronic hepatitis) increased the odds of developing ICP<sup>[24]</sup>.

### Pathogenesis

Genetic predisposition and hormonal factors have been implicated in the pathogenesis of ICP. The familial tendency and the observation of clustering of ICP in families led to the belief that genetics play a role in its development. Although some studies revealed results connecting MDR3 (*ABCB4*) gene with ICP, several other studies failed to demonstrate such relation<sup>[25-29]</sup>. Other genes such as *ABCB11* and *ATP8B1* were examined but showed weaker linkage to ICP<sup>[30-32]</sup>. Explaining ICP on a molecular basis in relation to sex hormones has gained interest<sup>[33]</sup>. The facts that ICP happens late in pregnancy and has a higher incidence in multiple gestation pregnancies, and that it resolves after delivery when sex hormones levels fall, make a logical connection between sex hormones and ICP. The estrogen metabolite estradiol-17 $\beta$ -glucuronide and differences in progesterone metabolites between pregnant women with and without ICP were also implicated<sup>[34-38]</sup>.

### Clinical presentation

ICP usually commences in the third trimester although earlier start in the second trimester has been reported<sup>[39]</sup>. The most common symptom is pruritus. Severity of pruritus increases at night and can involve the palms and soles. Other symptoms include steatorrhea, malabsorption of fat-soluble vitamins, and weight loss. ICP seems also to increase the incidence of gallstones and cholecystitis<sup>[40]</sup>. ICP tends to return in subsequent pregnancies with variable severity<sup>[41]</sup>. Elevated fasting serum bile acids level (> 10  $\mu$ mol/L) confirms the diagnosis. Aminotransferases can be elevated as well up to 2-10 folds<sup>[42]</sup>. Alkaline phosphatase levels might not be helpful due to higher physiological levels in late pregnancy. Clinical jaundice is detected in 10%-15% of the cases only and bilirubin levels rarely exceed 100  $\mu$ mol/L<sup>[23,43]</sup>. As in all

cholestatic patients, women with ICP tend to have higher low-density lipoprotein cholesterol and triglycerides<sup>[44]</sup>. Liver biopsy can reveal bland cholestasis (intrahepatic cholestasis without parenchymal inflammation). Liver biopsy is usually not indicated.

### Management

Bile acids sequestrants such as cholestyramine, antihistamines and opioid antagonists have been used to alleviate the pruritus. Cholestyramine is an exchange resin that binds bile acids and other anions in the intestine and increases their fecal excretion. Cholestyramine does not improve biochemical parameters or fetal outcomes in ICP<sup>[45]</sup>. *S*-adenosyl-methionine has shown limited efficacy in ICP<sup>[46,47]</sup>. Ursodeoxycholic acid (UDCA) is the first line therapy for ICP. UDCA has shown significant decrease in serum bile acids, serum aspartate aminotransferase and alanine aminotransferase, serum bilirubin, and was effective for pruritus<sup>[48-50]</sup>. Weekly non-stress testing did not prove to make a difference in ICP-related fetal deaths<sup>[51]</sup>. Some studies suggested 40  $\mu\text{mol/L}$  as a cutoff level of bile acids, after that fetal complications may happen<sup>[52,53]</sup>. Others did not observe such correlation until bile acids are  $> 100 \mu\text{mol/L}$ <sup>[54]</sup>. No evidence is strong enough to recommend early delivery (at 37 wk of gestation) for mothers with high bile acids levels, although this strategy is still used in some practices<sup>[55]</sup>.

### Prognosis

Although ICP is a benign condition for the mother, poor fetal outcomes can occur. In some studies ICP resulted in premature births up to 60%. Other complications such as fetal distress and intrauterine fetal death were reported at 61% and 1.6% respectively<sup>[23,39,56]</sup>. The onset of pruritus and higher maternal fasting serum bile acids were associated with higher risk for premature delivery<sup>[57]</sup>.

## ACUTE FATTY LIVER OF PREGNANCY

Acute fatty liver of pregnancy (AFLP) is a rare but a serious condition that is unique to pregnancy and happens in the third trimester. AFLP can lead to significant maternal and fetal morbidity and mortality<sup>[20,58]</sup>. Although rare, incidence of 1 per 7270 to 13000 deliveries, outcomes can be grave with acute liver failure and death<sup>[20,59-61]</sup>.

### Pathogenesis

Until recently the pathogenesis of AFLP was unknown and still has not been fully elucidated. However, molecular advances over the past decade suggest that AFLP may result from mitochondrial dysfunction. Defects in fetal mitochondrial fatty acid  $\beta$ -oxidation have been linked to development of maternal AFLP, particularly fetal defects in LCHAD, which is part of the mitochondrial trifunctional protein (MTP) complex<sup>[14,62-66]</sup>. In a retrospective study, Ibdah *et al.*<sup>[14]</sup> examined the association between MTP defects in children and liver disease in their mothers during pregnancy in 24 families with documented pe-

diatric defects in MTP. Fifteen of 24 women (62%) were diagnosed to have had maternal liver disease consistent with AFLP, although in two cases a clear distinction between AFLP and hemolysis, elevated liver enzymes, and a low platelet count (HELLP) syndrome was not possible. Nine of the 24 women had normal pregnancies. All 15 pregnancies with maternal liver disease were associated with fetal LCHAD deficiency. Molecular analysis revealed a common LCHAD mutation, G1528C in the offspring of women who developed AFLP. The results from this study show that when carrying a fetus that is LCHAD deficient, the mother has a high risk of developing AFLP. In a subsequent study, Ibdah *et al.* evaluated fetal genotypes and pregnancy outcomes in 83 pregnancies in 35 families with documented pediatric MTP defects<sup>[66]</sup>. This study provided further evidence that carrying a fetus with LCHAD deficiency is associated with a high risk for developing AFLP. With the growing evidence suggesting that carrying an LCHAD-deficient fetus is associated with AFLP, it was recommended that neonates born to pregnancies complicated by AFLP be tested for the common G1528C mutation and that this testing when done early after birth can be lifesaving as it may identify LCHAD-deficient children before they manifest the disease allowing early dietary intervention by institution of a diet low in fat, high in carbohydrate, and by substitution of the long chain fatty acids with medium chain fatty acids (for complete review on the association between AFLP and pediatric LCHAD deficiency<sup>[61]</sup>).

The precise mechanism by which an LCHAD deficient fetus causes AFLP in a heterozygote mother is still unclear. However, several factors appear to contribute to this fetal-maternal interaction. First, the heterozygosity of the mother for an MTP defect reduces her capacity to oxidize long chain fatty acids. Second, third trimester is accompanied by changes in metabolism, an increased lipolysis, and a reduction in mitochondrial fatty acid oxidation, all increase the susceptibility of the mother who carries a fetus with LCHAD deficiency. Thus it has been speculated that potentially hepatotoxic long-chain 3-hydroxyacyl fatty acid metabolites, produced by the affected fetus or placenta, accumulate in the maternal circulation<sup>[61]</sup>.

### Clinical presentation

Although there were few reports of AFLP starting in the second trimester, it usually presents in the third trimester between the 30<sup>th</sup> and 38<sup>th</sup> week of gestation<sup>[67-70]</sup>. It is more frequent in primiparous women and can return in subsequent pregnancies<sup>[12,62,71]</sup>. Nonspecific symptoms such as nausea, vomiting, headache, and fatigue can be the initial presentation. Right upper quadrant pain or epigastric pain can occur. Jaundice common and early jaundice may indicate severe disease<sup>[72]</sup>. Other features such as hypoglycemia, renal failure, coagulopathy, ascites, and encephalopathy were reported frequently. AFLP can result in maternal and fetal demise<sup>[73]</sup>. Although hypertension can be present, severe hypertension is likely

**Table 1 Proposed (Swansea) diagnostic criteria for acute fatty liver of pregnancy**

Vomiting	Abdominal pain
Polydipsia/polyuria	Encephalopathy
Elevated bilirubin	Hypoglycaemia
Elevated uric acid	Leucocytosis
Ascites or bright liver on US	Elevated transaminases
Elevated ammonia	Renal impairment
Coagulopathy	Microvesicular steatosis on liver biopsy

To meet the criteria the patient should have 6 or more of these clinical findings. Source: Ref. [80], with permission; US: Ultrasound scan.

secondary to the reduction in peripheral vascular resistance associated with liver failure. AFLP is a medical and obstetric emergency and diagnosis relying on clinical and laboratory findings should be prompt. Liver biopsy can be helpful in early and mild cases of AFLP especially if diagnosis is not clear<sup>[74]</sup>. Liver biopsy is not necessarily needed and should be avoided in more severe cases where the risk of bleeding is high and prompt therapeutic intervention is needed. Although elevated aminotransferases is expected, the severity of liver dysfunction is not always reflected by the degree of elevation. Alkaline phosphatase is usually elevated. Other findings such as leukocytosis, thrombocytopenia, disseminated intravascular coagulopathy (DIC), abnormal prothrombin time, partial thromboplastin time, and normal fibrinogen can occur<sup>[74-76]</sup>. Ketonuria and proteinuria can be present. Elevated blood urea nitrogen and creatinine indicate renal insufficiency. Low serum albumin and hypoglycemia can occur. Uric acid and ammonia levels can be increased. Hyperuricemia can be an early indicator and develop before hyperbilirubinemia<sup>[77,78]</sup>. In comparison with diffuse or microvesicular steatosis, Swansea criteria had a sensitivity of 100% (95%CI: 77-100) and specificity of 57% (95%CI: 20-88), with positive and negative predictive values of 85% and 100% in one report (Table 1)<sup>[79-81]</sup>. Ch'ng *et al*<sup>[80]</sup> proposed a set of clinical findings, known as Swansea criteria, to help reach the diagnosis of AFLP. Those diagnostic criteria have not been validated in different populations. Liver biopsy usually displays microvesicular steatosis<sup>[82]</sup>. Electron microscopy can show mitochondrial disruption. Imaging studies can be useful to exclude other pathologies; but have limited utility in the diagnosis of AFLP.

### Management

Stabilization of the mother and early recognition and delivery are the keys for successful management. Close monitoring and management of associated complications is necessary to improve outcomes. Plasmapheresis was used in few series in severe cases with reported success<sup>[83,84]</sup>.

### Prognosis

AFLP is severe disease with high maternal (18%) and fetal (23%) mortality. Prenatal diagnosis can provide

**Table 2 Hemolysis, elevated liver function tests, and low platelet counts syndrome diagnostic criteria**

HELLP class	Tennessee classification	Mississippi classification
1	Platelets $\leq 100 \times 10^9/L$ AST $\geq 70$ IU/L LDH $\geq 600$ IU/L	Platelets $\leq 50 \times 10^9/L$ AST or ALT $\geq 70$ IU/L LDH $\geq 600$ IU/L
2		Platelets $\leq 100 \times 10^9/L$ , $\geq 50 \times 10^9/L$ AST or ALT $\geq 70$ IU/L LDH $\geq 600$ IU/L
3		Platelets $\leq 150 \times 10^9/L$ , $\geq 100 \times 10^9/L$ AST or ALT $\geq 40$ IU/L LDH $\geq 600$ IU/L

AST: Aspartate aminotransferase; Source: Haram *et al.* BMC Pregnancy and Childbirth 2009 9:8 doi:10.1186/1471-2393-9-8; ALT: Alanine aminotransferase; HELLP: Hemolysis, elevated liver function tests, and low platelet counts; LDH: Lactate dehydrogenase.

benefit for both the mother and her fetus in subsequent pregnancies.

## PREECLAMPSIA/ECLAMPSIA AND HELLP SYNDROME

Preeclampsia is a syndrome that is unique to pregnancy. Manifestations include hypertension and proteinuria, and can result in fetal growth retardation. By far, preeclampsia is the most common serious medical disorder in pregnancy with prevalence up to 10%. It is associated with up to 20% of maternal mortality in developed countries<sup>[85,86]</sup>. Organ involvement such as liver, brain and kidneys signifies severe disease. Elevated aminotransferases occurs up to 10% of severe preeclampsia cases<sup>[86,87]</sup>. Although preeclampsia can start as early as the second trimester, liver involvement is mainly seen in the third trimester. Severe preeclampsia can be life threatening to the mother and can result in fetal morbidity and mortality. Eclampsia usually refers to preeclampsia with seizures. HELLP syndrome is a variant of severe preeclampsia that happens in up to 12% of patients with preeclampsia, and entails constellation of findings including hemolysis, elevated liver aminotransferases of and low platelet counts. Table 2 shows the diagnostic criteria of HELLP syndrome.

### Pathogenesis

In reviewing liver biopsies and autopsies of cases with preeclampsia, eclampsia and unclassified toxemia, from the Armed Forces Institute of Pathology between 1920 and 1984, Rolfes *et al*<sup>[88]</sup> reported that despite that large cerebral and midbrain hemorrhages, extensive thrombosis and infarction as well as cerebral edema with herniation were the major causes of deaths, liver disease contributed to 17 deaths out of the 102 cases reviewed. Extensive periportal lesions producing widespread parenchymal hemorrhage and necrosis were described. Large areas of infarction, wide bands of fibrin replacing liver cells, extravasation of red blood cells, and capillary



**Table 3** Preeclampsia associated liver diseases

	Severe preeclampsia and eclampsia	HELLP syndrome	Acute fatty liver of pregnancy
Time	After gestational week 22	Late second trimester to early postpartum	Third trimester
Prevalence	Increases in multiple gestation (5%-7%)	0.10%	Increases in male fetus, multiple gestations, primiparous women (0.01%)
Findings	High blood pressure; proteinuria; edema; seizure; renal failure; pulmonary edema	Abdominal pain, nausea/vomiting, overlap with findings in preeclampsia	Abdominal pain, nausea/ vomiting, jaundice, hypoglycemia and hepatic failure
Tests	Platelets > 70000; urine protein > 5 g/24 h; abnormal liver enzymes (10%)	Low platelets; hemolysis; elevated liver enzymes; prothrombin time may remain normal; normal fibrinogen	Platelets < 100000; AST and ALT 300-1000 U/L; low antithrombin III; high prothrombin time; low fibrinogen; high bilirubin; DIC
Management	Blood pressure control; beta-blockers, methyldopa, magnesium sulfate, early delivery	Prompt delivery 5% maternal death 1% hepatic rupture	Prompt delivery; liver transplant ≤ 10% maternal death
Outcome	1% maternal death	1%-30% fetal death	Up to 45% fetal death

HELLP: Hemolysis, elevated liver function tests, and low platelet counts; DIC: disseminated intravascular coagulation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

**Table 4** Complications of preeclampsia/hemolysis, elevated liver function tests, and low platelet counts syndrome

Maternal complications	Neonatal complications	Labor complications
Eclampsia	Fetal death	Preterm labor
HELLP syndrome	Prematurity	
Hepatic subcapsular hematoma, infarction or rupture	IUGR	
Acute renal failure	Respiratory distress syndrome	
Stroke, cerebral hemorrhage, edema and herniation	Intraventricular hemorrhage	
Pulmonary edema and acute respiratory distress syndrome	Sepsis	
Laryngeal edema		
Retinal detachment		

HELLP: Hemolysis, elevated liver function tests, and low platelet counts; IUGR: Intrauterine growth retardation.

thrombi were also seen. Histological changes of the liver in HELLP syndrome include periportal or focal parenchymal necrosis with hyaline deposits of fibrin-like material in the sinusoids<sup>[89]</sup>. Other molecular mechanisms such as vascular remodeling and placentation, immunological factors, and fatty acid oxidation defects were proposed as potential factors in the development of this spectrum of diseases<sup>[12,71,90-92]</sup>.

### Clinical presentation

Preeclampsia, HELLP syndrome, and acute fatty liver of pregnancy share similar presentations and differentiating between the three entities can be difficult. All present late in pregnancy and can have similar clinical features. Clinical presentation followed by typical laboratory findings can help in reaching the diagnosis. Although it may be reasonable to do an ultrasound of the liver for pregnant women with abnormal liver enzymes, imaging studies such as computed tomography and magnetic resonance imaging are rarely useful in making the diagnosis. Such

studies can have a role in diagnosing complications such as liver infarcts, hematomas, and liver rupture<sup>[93]</sup>. Table 3 presents a comparison between the three preeclampsia-associated liver diseases in pregnancy.

### Management

Successful management strategies rely on early diagnosis and prompt intervention. Women with severe preeclampsia or HELLP syndrome should be hospitalized and closely monitored in labor and delivery units, and placed on bed rest with good blood pressure control (systolic blood pressure < 155 and diastolic blood pressure < 100)<sup>[94]</sup>. The use of intravenous magnesium sulfate to prevent seizures is recommended. Close monitoring of mental status and appropriate use of imaging studies as indicated can help in identifying complications early. Prompt delivery can be the only effective therapy. Timing of delivery should be based on gestational age (reflecting the degree of fetal maturity) and the severity of the disease (maternal morbidity and mortality). Prompt delivery is indicated if the syndrome develops after 34 wk of gestation or earlier if complications occur, such as multi-organ dysfunction, liver infarction or hemorrhage, DIC, renal failure, suspected abruption of placenta, or fetal compromise<sup>[95-97]</sup>. Fetal lung maturity is not achieved before 34 wk of gestation. Therefore making a determination about terminating the pregnancy before 34 wk of gestation can be difficult<sup>[96,98-102]</sup>. Although a favorable effect on the platelet count and the aminotransferases levels has been observed, it's not clear if corticosteroids alter the course of the disease, and therefore their use remains controversial<sup>[101,103,104]</sup>. Betamethasone 12 mg intramuscularly every 24 h twice or four doses of intramuscular dexamethasone 6 mg every 12 h is recommended for enhancing fetal maturity<sup>[101]</sup>. Fetal and maternal complications are listed in Table 4.

### Prognosis

Although not very common, preeclampsia and HELLP syndrome remain a significant cause of morbidity and mortality for both pregnant women and their fetuses. With a maternal mortality of 1% in severe preeclampsia

sia, up to 5% in HELLP syndrome, and up to 30% fetal death rate, early diagnosis and prompt delivery remain the only effective treatment strategy.

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## Liver diseases in pregnancy: Liver transplantation in pregnancy

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### Abstract

Pregnancy in patients with advanced liver disease is uncommon as most women with decompensated cirrhosis are infertile and have high rate of anovulation. However, if gestation ensued; it is very challenging and carries high risks for both the mother and the baby such as higher rates of spontaneous abortion, prematurity, pulmonary hypertension, splenic artery aneurysm rupture, postpartum hemorrhage, and a potential for life-threatening variceal hemorrhage and hepatic decompensation. In contrary, with orthotopic liver transplantation, menstruation resumes and most women of childbearing age are able to conceive, give birth and lead a better quality of life. Women with orthotopic liver transplantation seeking pregnancy should be managed carefully by a team consultation with transplant hepatologist, maternal-fetal medicine specialist and other specialists. Pregnant liver transplant recipients need to stay on immunosuppression medication to prevent allograft rejection. Furthermore, these medications need to be monitored carefully and continued throughout pregnancy to avoid potential adverse effects to mother and baby. Thus delaying pregnancy 1 to 2 years after

transplantation minimizes fetal exposure to high doses of immunosuppressants. Pregnant female liver transplant patients have a high rate of cesarean delivery likely due to the high rate of prematurity in this population. Recent reports suggest that with close monitoring and multidisciplinary team approach, most female liver transplant recipient of childbearing age will lead a successful pregnancy.

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**Key words:** Liver; Pregnancy; Liver transplantation; Hemolysis elevated liver low platelets; Acute fatty liver; Cirrhosis

**Core tip:** This review provides an up-to-date summary of literature in the field of liver transplantation and pregnancy. It outlines the outcomes of pregnancy prior to and after orthotopic liver transplantation. Furthermore, it provides input on preconception counseling for mothers contemplating pregnancy after liver transplantation, risks of immunosuppression, and safety of breastfeeding.

Hammoud GM, Almashhrawi AA, Ahmed KT, Rahman R, Ibdah JA. Liver diseases in pregnancy: Liver transplantation in pregnancy. *World J Gastroenterol* 2013; 19(43): 7647-7651 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7647.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7647>

### INTRODUCTION

Liver transplantation is considered to be the treatment of choice for patients with advanced liver disease. Since the first pregnancy in a transplant recipient in 1958, pregnancy in recipients of solid organ transplants has become increasingly common. In the setting of decompensated

cirrhosis, pregnancy is very uncommon as most women are infertile and have anovulation and secondary amenorrhea. However, once liver transplantation is performed, liver transplant recipients possess an improved quality of life, their hormonal imbalance return to a normal state, ovulation resumes and pregnancy may ensue if contemplated. The first successful pregnancy in a liver graft recipient was reported in 1978<sup>[1]</sup>. Given the improving success of liver transplantation over the past two decades and decreasing levels of immunosuppression, most solid organ transplant recipients lead happy and healthy lives with an average 1-year survival rate of greater than 85% for most indications.

## **PATHOGENESIS**

Women with decompensated liver disease commonly have menstrual dysfunction. In fact, menstrual abnormalities may be the first signs of chronic liver disease in females with chronic liver disease. In cirrhotic state, hypothalamic-pituitary dysfunction is associated with an inadequate response to the gonadotropin-releasing hormone agonists and clomiphene citrates as well as diminished gonadotrophin release relative to the reduced levels of circulating sex steroids<sup>[2]</sup>. Furthermore, serum levels of estradiol and testosterone are increased in patients with portosystemic shunts. Thus pregnancy in decompensated cirrhosis is very uncommon. Obstetrical syndromes associated with transplantation may depend on several factors such as defective deep placentation, underlying maternal diseases, uterine vascular bed and effect of immunosuppressive therapy on uteroplacental arteries<sup>[3]</sup>. Reports from the the National Transplantation Pregnancy Registry (NTPR) revealed that immunosuppressive medication is associated with an increased risk of miscarriage, prematurity, intrauterine growth retardation, and low birth weight<sup>[4]</sup>.

## **MATERNAL AND FETAL OUTCOMES IN PREGNANT FEMALE PATIENTS WITH ADVANCED LIVER DISEASE**

Pregnancy is associated with increase in portal pressure. During pregnancy, a hypervolemic state develops leading to an increase in portal flow and elevation of portal venous pressure transmitted to the collateral veins with increased risk of esophageal variceal bleeding<sup>[5,6]</sup>. The outcome of pregnancy in 339 patients with cirrhosis was reported in a large population-based study from 1993 to 2005<sup>[7]</sup>. Maternal and fetal mortality were much higher than the general population (1.8% *vs* 0%,  $P < 0.0001$ ; 5.2% *vs* 2.1%,  $P < 0.0001$ ) respectively. The rate of hepatic decompensation occurred in 15% and patients with cirrhosis were more likely to deliver by cesarean delivery (42% *vs* 28%; adjusted OR = 1.41; 95%CI: 1.06-1.88). Similarly, the spontaneous abortion rate in cirrhotic patients is approximately 15%-20%.

## **MATERNAL AND FETAL OUTCOMES IN PREGNANT FEMALE LIVER TRANSPLANT RECIPIENTS**

Most outcome data on pregnancy during and after liver transplantation are obtained from the NTPR. The NTPR was established in 1991 at Thomas Jefferson University in Philadelphia, Pennsylvania, to study the outcomes of pregnancies in transplant recipients in North America, including female transplant recipients and those fathered by male transplant recipients. Since then many other reports and case series have been reported and published. A retrospective study from a single institution evaluated a total of 115 gestations in 37 women with liver transplant (LT) and in 34 women with kidney transplant. The authors found 81 (70%) of all gestations were successful, 15 (13%) were terminated, and there were 19 (17%) spontaneous abortions and 2 (2%) intrauterine deaths<sup>[8]</sup>. Deshpande *et al*<sup>[9]</sup> reported in a systematic review and meta-analysis outcome of 450 pregnancies in 306 LT recipients in comparisons with the general United States population as well as kidney transplant recipients. The post-LT live birth rate was higher than the live birth rate for the US general population (76.9% *vs* 66.7%, 95%CI: 72.7%-80.7%). The post-LT miscarriage rate was lower than the miscarriage rate for the general population (15.6% *vs* 17.1%, 95%CI: 12.3%-19.2%). Moreover, these rates were similar to the post-kidney transplant rates. The rates of pre-eclampsia, cesarean section delivery and preterm delivery were higher than the rates for the US general population (21.9% *vs* 3.8%, 95%CI: 17.7%-26.4%; 44.6% *vs* 31.9%, 95%CI: 39.2%-50.1%; and 39.4% *vs* 12.5%, 95%CI: 33.1%-46.0%) respectively. Moreover, these rates were lower than those for post-kidney transplant recipients. The overall mean birth weight for newborns of LT recipients was less than the birth weight for the United States general population (2866 g *vs* 3298 g). More notably, the authors found that the mean gestational age and mean birth weight seems significantly greater for liver transplant versus kidney transplant recipients and the risk of hypertension during pregnancy seems also lower for liver transplant than kidney transplant recipients<sup>[9]</sup>. In another recently published study by Alvaro *et al*<sup>[10]</sup> from a single center in Spain, the authors analyzed the impact of pregnancy among 1341 liver transplant recipients from April 1986 to April 2011. Thirty pregnancies commenced among 18 liver transplant recipients during the follow-up. Sixteen patients (88%) became pregnant beyond a year after orthotopic liver transplantation. The post-LT live birth was 66.6% and the post-LT abortions were 26.6%. There were no maternal deaths encountered during pregnancy or the postpartum period. However, fetal deaths were observed in 6% of LT recipients. The most common maternal complications during pregnancy were preeclampsia (15%), viral reactivation (15%), acute rejection episodes (10%), infections (10%), and high blood pressure (5%)<sup>[10]</sup>. Table 1 shows a summary of maternal and fetal outcomes in female liver transplant recipients from selected reports and studies<sup>[11]</sup>.

**Table 1** Summary of important fetal and maternal outcomes in liver transplant recipients from selected publications

Author, reference, number of pregnancies	Live birth rate (%)	Preterm (%)	Graft dysfunction (%)	Cesarean section rate (%)	Spontaneous abortions (%)	Low birth weight < 2500 g (%)	Maternal/neonatal deaths (%)
Nagy <i>et al</i> <sup>[12]</sup> , n = 38	63	29	17	46	NA	17	17/0
Jain <i>et al</i> <sup>[13]</sup> , n = 49	100	4	25	47	0	9	10/6
Armenti <i>et al</i> <sup>[14]</sup> , n = 205	73	35	7	35	19	34	/0
Christopher <i>et al</i> <sup>[15]</sup> , n = 71	71	NA	17	40	19	20	4/0
Dei Malatesta <i>et al</i> <sup>[16]</sup> , n = 285	78	31	10	43	NA	23	/4
Sibanda <i>et al</i> <sup>[17]</sup> , n = 16	69	50	NA	62	13	57	NA
Coffin <i>et al</i> <sup>[18]</sup> , n = 206	70	27	5	38	5	NA	0/6
Jabiry-Zieniewicz <i>et al</i> <sup>[19]</sup> , n = 39	100	31	8	80	0	20	/0
Dashpande <i>et al</i> <sup>[9]</sup> , n = 450	76.9	39.4	NA	44.6	6.2 (including intrauterine fetal death)	NA	NA
Alvaro <i>et al</i> <sup>[10]</sup> , n = 30	66.6	NA	10	42	26.6	NA	0/6

NA: Not available.

**Table 2** Food and drug administration pregnancy categories of common immunosuppressive therapy

Medicine	Pregnancy category
Corticosteroids	B
Azathioprine	D
Cyclosporin	C
Mycophenolate mofetil	D
Tacrolimus	C
Sirolimus	C

## PRECONCEPTION COUNSELING

Pregnancy after liver transplant should be considered as a high-risk pregnancy and should be monitored closely by a team of a transplant hepatologist and experts in obstetrics and fetal medicine. Female liver transplant recipients who are planning of becoming pregnant should be counseled on optimal timing of pregnancy, mode of delivery and risks associated with immunosuppressive therapy. Furthermore, they should also be counseled on methods of contraception if pregnancy is not contemplated. Immunosuppressive agents are at their nadir one-year post liver transplantation and thus risk of allograft rejection is low. Furthermore, renal and liver functions tend to be stabilized during that period and thus it is ideal to delay pregnancy till patient is on a maintenance immunosuppression 1 to 2 years after transplantation to minimize fetal exposure to high doses of immunosuppressants<sup>[14,20]</sup>.

As per mode of delivery, vaginal delivery appears to be safe. However, high rates of cesarean section have been reported in female liver transplant patients (45.8%<sup>[12]</sup>, 71%<sup>[21]</sup>, 35%<sup>[22]</sup>, 38%<sup>[18]</sup> and 44.6%<sup>[9]</sup>) signifying the high rates of prematurity in this population. It is not known whether a particular immunosuppressive therapy is associated with increased rate of cesarean section. If pregnancy is not contemplated in young females of childbearing age, contraceptive method is advised particularly in the first few months post liver transplantation. Barrier methods possess low risk of systemic side effects. Intrauterine devices are generally discouraged due to their

potential infection complications. Furthermore, oral contraceptives did not appear to impair liver function or glucose metabolism after its introduction within 6 mo to 7 years post transplantation<sup>[23]</sup>.

## IMMUNOSUPPRESSION IN LIVER TRANSPLANT PREGNANT RECIPIENTS

There is no consensus on the optimal maintenance regimen for transplant recipients. The use of immunosuppressive therapy after liver transplantation is unavoidable. Therefore, women planning to conceive after transplantation should be counseled about the risks such therapy may pose on them and their fetuses. All immunosuppressive medication cross the placenta and enter fetal circulation and could potentially have deleterious effects in utero. Despite the fact that immunosuppressive agents such as Azathioprine, Cyclosporine, and Mycophenolic acid, were teratogenic in animals, the risk of birth defects was not statistically different between those who received immunosuppressive medications and those who did not. Birth defects have been reported with Calcineurin inhibitors<sup>[8,12,19]</sup>. Renal dysfunction and rates of pre-eclampsia appears higher with cyclosporine therapy<sup>[12,24]</sup>. No matter what immunosuppressive therapy is chosen based on maternal allograft function and laboratory assay, patients treated with either calcineurin inhibitors cyclosporine or tacrolimus should have serial blood tests in pregnancy to follow medication levels and to assess hepatic and renal function while avoiding unnecessary toxicity. Table 2 shows the food and drug administration classification of risk of medication and their categories in pregnancy<sup>[25]</sup>.

## BREASTFEEDING IN FEMALE LIVER TRANSPLANT RECIPIENTS ON IMMUNOSUPPRESSIVE THERAPY

The American academy of pediatrics advises that breast-

feeding mothers can use prednisone and other glucocorticoids safely. Infant exposure to tacrolimus in milk is very low and that maternal tacrolimus therapy may be compatible with breastfeeding. Data collected from the NTPR indicated no adverse outcomes in infants who were breastfed during maternal cyclosporine use. There is insufficient evidence in the literature to suggest that women taking azathioprine should refrain from breastfeeding. Nevertheless, mothers may be discouraged to breast feed in the first few months post transplantation where immunosuppressive therapy is at high serum level.

## CONCLUSION

Pregnancy after liver transplantation, although considered a high risk pregnancy, has an acceptable outcome for both mother and baby. With the return of fertility following transplantation, accurate family planning advice is essential. To date there is no evidence of specific structural malformations among children born to female liver transplant recipients, but there appears to be increased risk of prematurity and low birth weight after solid organ transplantation. Multidisciplinary team approach is of utmost importance to ensure smooth pregnancy. The NTPR data and others have revolutionized our understanding of the outcomes of pregnancy in this high risk population. We encourage physicians in the field to continue to report their outcome to the transplant registry.

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## Use of exclusive enteral nutrition in adults with Crohn's disease: A review

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### Abstract

Exclusive enteral nutrition (EEN) is well-established as a first line therapy instead of corticosteroid (CS) therapy to treat active Crohn's disease (CD) in children. It also has been shown to have benefits over and above induction of disease remission in paediatric populations. However, other than in Japanese populations, this intervention is not routinely utilised in adults. To investigate potential reasons for variation in response between adult studies of EEN and CS therapy. The Ovid database was searched over a 6-mo period. Articles directly comparing EEN and CS therapy in adults were included. Eleven articles were identified. EEN therapy remission rates varied considerably. Poor compliance with EEN therapy due to unpalatable formula was an issue in half of the studies. Remission rates of studies that only included patients with previously untreated/new CD were higher than studies including patients with both existing and new disease. There was limited evidence to determine if disease location, duration of disease or age of diagnosis affected EEN therapy outcomes. There is some evidence to support the use of EEN as a treatment option for a select group of adults, namely those

motivated to adhere to an EEN regimen and possibly those newly diagnosed with CD. In addition, the use of more palatable formulas could improve treatment compliance.

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**Key words:** Exclusive enteral nutrition; Crohn's disease; Adults

**Core tip:** Exclusive enteral nutrition (EEN) is an established treatment for children with active Crohn's disease (CD). At present, this therapy is used sparingly in adult patients outside of Japan. In reviewing the published literature regarding the use of EEN in adult patients, this article highlights evidence supporting the use of EEN as a treatment option for selected patients: namely those motivated to adhere to an EEN regimen and those newly diagnosed with CD. The role of EEN in adult patients with CD should now be re-examined, with particular regard to treatment protocols and the use of more palatable polymeric formulae that may enhance compliance.

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### INTRODUCTION

Crohn's disease (CD) is an incurable inflammatory bowel disease (IBD) characterised by inflammation of the gastrointestinal tract, which leads to chronic symptoms such as diarrhoea, abdominal pain and rectal bleeding<sup>[1]</sup>. The peak age of diagnosis is between 15 and 30 years of age,

leading to many years of disease and associated morbidity. Standard first line treatment in adults newly diagnosed with CD is corticosteroid (CS) therapy, which is effective at inducing remission or response in approximately 85% of patients<sup>[2]</sup>. However, CS therapy has many well documented acute side-effects: furthermore there are numerous long term adverse effects due to repeated or continual use of CS<sup>[3]</sup>. Also, CS resistance can occur in 8%-22% of patients and CS dependency occurs in 15%-36% of patients<sup>[4]</sup>. Alternative therapies that can effectively induce and maintain disease remission without short and long term side effects are desirable.

Exclusive enteral nutrition (EEN) is the provision of 100% of a person's nutritional requirements from a liquid nutrition formula either orally or *via* a feeding tube. EEN is usually provided for 6-8 wk and then usual diet is gradually reintroduced<sup>[5]</sup>. In children with CD, EEN has been shown to be an effective and feasible alternative to CS<sup>[6]</sup>. In addition to avoiding the adverse effects of CS exposure, EEN provides additional benefits over and above those provided by CS. EEN therapy is associated with higher rates of mucosal healing<sup>[7]</sup>, altered intestinal flora<sup>[8]</sup>, greater weight gain<sup>[9]</sup>, improved vitamin D status<sup>[10]</sup>, enhanced bone turnover<sup>[11]</sup>, an early rise in Insulin-like growth factor 1<sup>[12]</sup>, and better quality of life after treatment<sup>[13]</sup>. There are few long term follow up studies post EEN, but those that have been conducted in children indicate that EEN may improve time to relapse<sup>[14]</sup>. The administration of supplementary enteral nutrition (SEN) once disease remission is achieved has been shown to be beneficial in maintaining remission compared with a free diet in Japanese adults<sup>[15]</sup> and children<sup>[16]</sup>.

However, in adult CD populations, EEN is generally not seen as a first line therapy for newly diagnosed or those with a flare of pre-existing CD. European<sup>[2]</sup> and North American<sup>[17]</sup> clinical guidelines only recommend EEN if a patient declines drug therapy or as an adjunctive therapy to support nutrition, rather than as a primary therapy. These recommendations are primarily based on the results of a Cochrane systematic review of six randomised controlled trials including 192 patients treated with EEN and 160 patients treated with CS<sup>[18]</sup>. The review found a pooled OR of 0.33 (95%CI: 0.21-0.53) in favour of CS and concluded that CS were superior to EEN in the induction of remission of disease. In contrast to these guidelines, recent Japanese experience demonstrates efficacy in that setting<sup>[15]</sup>.

It is not clear why the benefits of EEN therapy seen in paediatric populations are not achieved in adults. We aimed to review the published literature reporting the use of EEN as a primary therapy for active CD in adults and examine potential reasons for this apparent discrepancy.

## SEARCH

The Ovid database was searched from September 2012 to March 2013 for articles published between 1946 and now. Key search terms were: "CD", "Crohn disease",

"EEN" and "enteral nutrition". Abstracts were scanned and articles in English that compared enteral nutrition with CS treatment in adults were considered relevant. Studies were excluded if enteral nutrition was not the sole source of nutrition, enteral nutrition was provided as well as other medication (for example, antibiotics), the study included children, or the study did not compare CS and EEN. A manual search was also completed of reference lists of articles retrieved, relevant review articles and meta-analyses on the topic.

## RESEARCH

### Study characteristics

Eleven studies published between 1984 and 2002 were identified that compared EEN with CS treatment in adults (Table 1). Two were abstracts<sup>[19,20]</sup> and the rest were full articles. The studies were conducted in Europe, North America and Asia: three in England<sup>[19,21,22]</sup>, one in Spain<sup>[23]</sup>, one in Greece<sup>[20]</sup>, one in Italy<sup>[24]</sup>, one in the United States<sup>[25]</sup>, one in Japan<sup>[26]</sup> and three<sup>[27-29]</sup> were multi-centre European trials. All but two studies enrolled a mix of patients with newly diagnosed CD (naïve to prior treatment) and existing CD. All but one study compared one enteral nutrition formula with CS therapy.

The studies utilised a range of nutritional products, in varying regimens, as summarised in Table 2. Eight of the studies used elemental formula and three studies used polymeric formula. Most formulas were a 1 kcal/mL concentration apart from one which used a 1.5 kcal/mL formula. Duration of EEN treatment ranged from 2-6 weeks but most studies used EEN therapy for four weeks. Mode of delivery of the EN formula was either orally, or *via* a nasogastric tube (NGT) if not tolerated orally, or continuous feeding *via* an NGT or nasoduodenal tube. Nutritional composition of the formulas was quite different depending on the type and brand of formula used. All formulas had relatively similar amounts of protein (14%-22% of total energy), whereas fat content varied considerably (1%-35% of total energy). Carbohydrate content varied relative to fat content (49%-82% of total energy).

The only study that compared two different enteral formulas and CS was published by Gassull *et al*<sup>[27]</sup>. They compared two EEN formulas that were the same except for the predominant type of fat: one was high in oleic acid and the other was high in linoleic acid. Study recruitment was ended prematurely because less than 33% of the high oleic acid formula group had achieved disease remission and the remission rate was significantly different from that of the other treatments.

CS protocols also ranged between the evaluated studies. Usual initial CS dosage was between 0.5 mg/kg per day and 1.0 mg/kg per day, with subsequent weaning courses. CS were given orally in two studies<sup>[23,27]</sup> but the route of administration was not published in the majority of studies. Two studies administered CS and sulfasalazine concurrently<sup>[28,29]</sup>.

Table 1 Studies of adults that compared exclusive enteral nutrition with corticosteroid therapy

Ref.	Year	Country	Age (SD or range)	Received no previous CD treatment (% of EEN group)	Number of participants		% that achieved remission (intention to treat)		Significant difference ( <i>P</i> value)	Remission criteria	Number that did not complete EEN intervention, <i>n</i> (%)		% that achieved remission (treatment completed)	
					EEN	CS	EEN	CS			Formula un-palatable	Other reason	EEN	CS
Engelman <i>et al</i> <sup>[19]</sup>	1993	England	23-54	Not stated	7	4	100%	100%	NS	HBI < 6.0	0 (0)	0	100%	100%
Gassull <i>et al</i> <sup>[27]</sup>	2002	Europe	31.3 (3.3)	50%	20	19	20%	79%	0.0005	VHAI < 120	5 (25)	0	27%	79%
Gassull <i>et al</i> <sup>[27]</sup>	2002	Europe	30.8 (4.1)	43.50%	23	19	52%	79%	NS	VHAI < 120	4 (17)	0	63%	79%
González-Huix <i>et al</i> <sup>[23]</sup>	1993	Spain	31.1 (4.1)	47%	15	17	8%	88%	NS	VHAI < 120	0 (0)	0	80%	88%
Gorard <i>et al</i> <sup>[21]</sup>	1993	England	31.6 (3.0)	50%	22	20	45%	85%	< 0.05	HBI - remission not defined, mean < 2	9 (41)	2	91%	89%
Lindor <i>et al</i> <sup>[25]</sup>	1992	United States	34.7 (26-64)	33%	9	10	50%	33%	NS	CDAI decrease > 100 points	3 (33)	1	60%	63%
Lochs <i>et al</i> <sup>[28]</sup>	1991	Europe	27.5 (1.5)	Not stated	55	52	53%	79%	< 0.01	CDAI decrease > 100 points or > 40%	7 (13)	0	60%	85%
Malchow <i>et al</i> <sup>[29]</sup>	1990	Europe	30.1 (11.5)	20%	51	44	41%	71%	< 0.05	CDAI decrease > 100 points or > 40%	20 (39)	0	71%	91%
Mantzaris <i>et al</i> <sup>[20]</sup>	1996	Greece	Not stated	20%	10	10	40%	70%	NS	CDAI < 150 or decrease > 100 points	0 (0)	0	40%	70%
Okada <i>et al</i> <sup>[26]</sup>	1990	Japan	21.0 (3.3)	100%	10	10	80%	30%	< 0.01	HBI < 1	0 (0)	0	80%	30%
O'Moráin <i>et al</i> <sup>[22]</sup>	1984	England	31.9 (15-60)	100%	11	10	82%	80%	NS	HBI - remission not defined. Mean < 3	2 (18)	0	100%	100%
Zoli <i>et al</i> <sup>[24]</sup>	1997	Italy	33.5 (15.9)	Not stated	12	10	67%	50%	NS	HBI < 3	1 (8)	1	80%	50%

<sup>1,2</sup>Gassull *et al* had two EEN arms: <sup>1</sup>High oleic fatty acid formula; <sup>2</sup>High linoleic fatty acid formula. CD: Crohn's disease; CDAI: Crohn's disease activity index; CS: Corticosteroids; EEN: Exclusive enteral nutrition; HBI: Harvey Bradshaw Index; NS: Non-significant; VHAI: Van Hees activity index.

Disease remission criteria

Three remission criteria were used across the 11 studies - the CD Activity Index (CDAI), the Harvey Bradshaw Index (HBI), and the Van Hees Activity Index (VHAI). The CDAI score uses a seven day history of general well-being, abdominal pain, loose stools, presence of abdominal mass and CD complications, anti-diarrhoeal use, haematocrit and weight<sup>[30]</sup>. The HBI is based on a one day history of general well-being, abdominal pain, loose stools and presence of abdominal mass and CD complications<sup>[30]</sup>. It correlates well with the CDAI (*r* = 0.8)<sup>[30]</sup>. Clinical remission is usually defined as a CDAI of less than or equal to 150 points or a HBI of less than or equal to 4 points<sup>[30]</sup>. Of the four studies that used the CDAI to define remission one used this criteria, one used a decrease of more than 100 points and two used either a CDAI of less than 150 or a decrease of 40% or more. Five studies used the HBI to define disease remission, the cut-offs used by each study were different.

The VHAI is calculated using serum albumin and erythrocyte sedimentation rate, body mass index, abdominal mass, gender, fever, loose stools, bowel resection and CD complications. The VHAI correlates moderately (*r* = 0.67) with the CDAI<sup>[31]</sup>. Both studies that used the VHAI used the same cut-off of less than 120 to define disease remission.

Remission of disease

Remission was achieved with EEN therapy on an intention to treat basis in 20%-100% of patients and 30%-100% of patient on CS therapy (Table 1). Seven of the 11 studies found no significant difference between EEN and CS treatment to induce disease remission<sup>[19,20,22,25,27]</sup>. Of those patients who completed the course of EEN therapy disease remission was achieved in 23%-100% of patients and in 30%-100% of patients that completed CS treatment<sup>[19,20]</sup>. Those that did not complete the course of EEN therapy were



**Table 2** Characteristics of exclusive enteral nutrition regimens used in studies of adults that compared exclusive enteral nutrition with corticosteroid therapy

Ref.	Nutritional product	Type of feed	Duration of EEN (wk)	Calorie density (kcal/mL)	Nutritional composition (% TE)	Mode of delivery	Calorie intake per day
Engelman <i>et al</i> <sup>[19]</sup>	Peptamen	Peptide based elemental	2	1	Pro 16, CHO 51, Fat 33	Orally	30-35 kcal/kg per day
Gassull <i>et al</i> <sup>[27]</sup>	High oleic acid	Polymeric (powder)	4	1	Pro 22, CHO 46, Fat 32	Orally and NGT	Not stated
Gassull <i>et al</i> <sup>[27]</sup>	High linoleic acid	Polymeric (powder)	4	1	Pro 22, CHO 46, Fat 32	Orally and NGT	Not stated
González-Huix <i>et al</i> <sup>[23]</sup>	Edanec HN	Polymeric	4	1	Pro 22, CHO 46, Fat 32	NGT	Not stated
Gorard <i>et al</i> <sup>[21]</sup>	Vivonex TEN	Elemental	4	1	Pro 15, CHO 82, Fat 3	Orally, or NGT	2100 kcal per day
Lindor <i>et al</i> <sup>[25]</sup>	Vital HN	Peptide based elemental	4	1	Pro 17, CHO 74, Fat 9	Orally	40 kcal/kg per day
Lochs <i>et al</i> <sup>[28]</sup>	Peptisorb	Peptide based elemental	4-6	1	Pro 16, CHO 69, Fat 15	NGT or NDT	35 kcal/kg per day
Malchow <i>et al</i> <sup>[29]</sup>	Survimed	Peptide based elemental	3-6	1	Pro 14, CHO 76, Fat 10	Orally	33 kcal/kg per day
Mantzaris <i>et al</i> <sup>[20]</sup>	Nutrison HE	Polymeric	4	1.5	Pro 16, CHO 49, Fat 35	NDT	2250 kcal per day
Okada <i>et al</i> <sup>[26]</sup>	Elental	Elemental	6	1	Pro 19, CHO 81, Fat 1	NDT	40-60 kcal/kg per day
O'Moráin <i>et al</i> <sup>[22]</sup>	Vivonex	Elemental	4	1	Pro 15, CHO 82, Fat 3	Orally, or NGT	40-60 kcal/kg per day
Zoli <i>et al</i> <sup>[24]</sup>	Peptamen	Peptide based elemental	2	1	Pro 16, CHO 51, Fat 33	Orally	Not stated

CHO: Carbohydrate; EEN: Exclusive enteral nutrition; NDT: Nasoduodenal tube; NGT: Nasogastric tube; Pro: Protein; % TE: Percentage of total energy.

usually started on CS therapy.

### Withdrawal from treatment

Withdrawals from treatment varied between studies. EEN study group withdrawals were mostly due to unpalatable enteral nutrition formula. The number of withdrawals for this reason was as high as 41% of the EEN group in one study but 0% in other EEN study groups. Occasionally patients had to withdraw as they required urgent surgery. Withdrawals from CS groups were much lower. Common reasons cited for withdrawing were side effects, non-compliance with treatment or the patient needing urgent surgery.

### Disease location

All 11 of the studies recorded the disease location of patients. The majority of patients had ileocolonic disease and smaller numbers had ileal or isolated colonic disease. No studies found disease location to be associated with the likelihood of achieving disease remission using EEN or CS therapy.

### Age of participants

The age of the participants was recorded differently across the 11 studies. The mean age of patients enrolled in the studies was 27.5-34.7 years old. Inclusion of older adults aged more than 50 years of age was not uncommon. Only one study included mostly younger adults (mean 21.0 ± 3.3 years)<sup>[26]</sup>.

## DISCUSSION

EEN is rarely used in adults with active CD, apart from in Japan. Its use is usually reserved for those patients who

do not want to use CS therapy, as an adjunctive therapy or where other treatment options have failed. Since the first studies with adults in the 1980s and 1990s much more is known about the way in which EEN therapy induces disease remission in children and how SEN therapy can assist in maintenance of disease remission. It is timely to readdress the possible reasons for the discrepancy between results from adult and paediatric studies that have compared EEN and CS therapy.

### Disease remission criteria

The disease remission criteria used by researchers can have a profound impact on the study results. Comparison of disease remission rates between studies is challenging when disease remission is not universally defined. Five of the 11 studies used the HBI to measure disease remission<sup>[19,21,22,24,26]</sup>. Two of the studies that used the HBI did not describe their remission criteria<sup>[21,22]</sup>, however the mean HBI of participants after the EEN intervention was less than 4, which corresponds with standard interpretations of clinical remission. Another study used a HBI cut off of less than six points with 100% of participants in both the EEN and CS therapy groups achieving remission in this study<sup>[19]</sup>. The fourth study to use the HBI used a cut-off of 0-1 points to define disease remission<sup>[26]</sup>. Only 30% of patients in the CS group achieved remission using this criterion compared with 80% of the EEN group. It is unknown if a more liberal cut-off would have increased the number of patients achieving disease remission in the CS group. Regardless of the HBI cut-off used at least 80% of the EEN group participants (that completed the course of EEN) in each of the five studies achieved disease remission.

Four of the 11 studies used the CDAI to measure dis-

ease remission<sup>[20,25,28,29]</sup>. The remission rates of the EEN therapy group in all four studies were low (40%-53%), with the two larger studies concluding that, on an intention to treat basis, CS therapy induces disease remission in significantly more patients than EEN therapy<sup>[28,29]</sup>. In two of the studies at least one third of the patients withdrew from the EEN group due to unpalatable formula<sup>[25,29]</sup>. Withdrawals from the CS groups were much lower (20% or less). Of those that did complete the course of EEN therapy only 40%-71% of patients achieved disease remission, whereas remission was achieved in 62%-98% of those that completed the course of CS therapy.

The disease remission rates of the two studies that used the VHAI to define disease remission were quite different. Gassull *et al.*<sup>[27]</sup> hypothesised that the formula high in linoleic acid, an n-6 polyunsaturated fat, would be less effective than a high monounsaturated fatty acid formula because n-6 fatty acids are pro-inflammatory precursors. Of the 20 patients enrolled in the high oleic acid EEN group only 20% achieved disease remission after 4 wk of therapy, compared with 52% of the high linoleic acid group and 79% of those using CS therapy. It seems that the fat content of EEN formulae may affect the efficacy of EEN therapy. The other study that used the VHAI to define disease remission found that EEN therapy was as effective as CS therapy: 80% of those on EEN therapy achieved disease remission compared with 88% of those using CS therapy<sup>[23]</sup>.

The criteria used to define disease remission should not impact greatly on the results of the study; however, in this case, the studies can be grouped into three categories based on the remission criteria applied. The studies that used the HBI found that EEN therapy was at least as effective as CS therapy in inducing disease remission. The two larger studies that used the CDAI found that CS therapy was superior to EEN therapy while two studies with small participant numbers found no significant difference. There may be differences in study protocols between studies with higher and lower patient numbers that could influence patient outcomes. Finally, the two studies that used the VHAI found that there was no significant difference between a high, or a moderate, polyunsaturated polymeric formula and CS therapy, but that a high monounsaturated formula was significantly less effective ( $P < 0.001$ ) than CS therapy at inducing disease remission.

### Newly diagnosed CD

There is some evidence to suggest that EEN therapy is more effective in newly diagnosed CD patients compared with patients who have existing CD. Differences in treatment response rates according to time since diagnosis are not limited to EEN therapy. Response and remission rates achieved with biologic therapy are greater in children than adults<sup>[32]</sup> which may, in part, be due to the duration of disease prior to initiation of the treatment. Similarly, adults with a shorter duration of CD are more likely to respond and achieve remission with biologic

therapy<sup>[32]</sup>. Also the use of immune-modulators early in the disease course in adults and children has been shown to reduce the probability of long term CS and intestinal surgeries<sup>[33]</sup>.

Two adult studies have compared EEN with CS therapy in treatment-naïve patients<sup>[22,26]</sup>. In both studies, 80% of those treated with EEN achieved disease remission after 4-6 wk of an elemental diet (comparable to remission rates in those treated with CS). Other adult studies comparing EEN with CS have not differentiated between patients with newly diagnosed CD and existing CD in their analyses. One study mentioned that both of the newly diagnosed CD patients responded to EEN treatment<sup>[20]</sup>, but the numbers enrolled in the study were too small to show if there was a statistically significant difference in response to treatment between the two groups. A study of 22 patients treated with EEN found that EEN therapy was as effective in newly diagnosed patients as those with existing disease<sup>[21]</sup>, although 40% of patients did not complete the course of EEN. The authors do not indicate how many of those that completed EEN treatment had existing or newly diagnosed disease. The two larger multi-centre European trials did not differentiate between those that had and not had received previous CD treatment<sup>[28,29]</sup>.

Paediatric research suggests that EEN is more effective in treating newly diagnosed CD than existing CD<sup>[9]</sup>. Day *et al.*<sup>[9]</sup> showed that, of 15 newly diagnosed CD patients, 12 (80%) entered remission after eight weeks of EEN, whereas only seven of the 12 (58%) children with long-standing disease entered remission ( $P > 0.05$  by fisher's exact test). In other paediatric studies with newly diagnosed CD patients disease remission was achieved in 79%-93% of those that completed EEN treatment and 70%-79% on an intention to treat basis<sup>[7,34]</sup>.

### Duration of CD

Longer duration of CD is associated with more complications including tissue scarring, fistulae, abscess, strictures, perianal disease and bowel resections<sup>[35]</sup>. EEN therapy has been shown to induce disease remission by reducing mucosal inflammation<sup>[36-38]</sup>. Complications of CD are often non-inflammatory in nature; therefore, EEN may be less effective in treating these patients. Interestingly, a case series of three children with perianal disease at diagnosis found that EEN (used in combination with surgery and antibiotics) was effective at inducing disease remission and assisted in the healing of perianal disease<sup>[39]</sup>. EEN was used as a maintenance therapy in all three children without return of perianal disease. A clinical trial has not been conducted to further investigate the potential role of EEN in the management of perianal CD.

Overall, studies in adult patients of EEN compared with CS therapy have not excluded patients with complicated disease. Usual exclusions included imminent surgery, intestinal perforation, ileus, abscesses, massive bleeding, short bowel syndrome with ileostomy and, in

some cases, previous surgery. The presence of other complications of existing CD such as scarring, perianal disease or previous bowel surgery is not detailed in the adult literature. It is impossible to ascertain whether those who did not respond to EEN therapy had more or less complications than those who did respond. Furthermore, the studies had only small numbers of patients within each disease sub-group and were unable to conduct in-depth statistical analysis of these sub-groups.

### Adherence

Non-adherence with EEN treatment was a limiting factor in the success of EEN therapy in many studies. A number of reasons for non-adherence of adult CD patients with EEN therapy have been postulated including poor taste of the formula, lack of support and poor motivation to complete the treatment.

Un-palatability of the EN formula was the most common reason for non-adherence in the studies performed to date. Many early studies that compared EEN with CS treatment used elemental formulas. The difference between polymeric and elemental formulas is that the protein fraction in polymeric formula is in its whole form rather than as individual amino acids or peptides in semi-elemental formulas and elemental formulas tend to have a low total fat content. Polymeric formula has been shown to be as effective as elemental at inducing disease remission<sup>[40,41]</sup>. Elemental formulas have a distinctive smell and flavour mainly due to the presence of amino acids, which have a bitter flavour. Bitterness is negatively correlated with palatability, whereas sweetness and sourness are positively correlated with palatability<sup>[42]</sup>. Fat content may also affect the palatability of the formula<sup>[43]</sup>. The elemental formulas used in the studies were low fat (1%-3% TE) compared with semi-elemental (9%-33% TE) and polymeric (32%-35% TE) formulas. Hence polymeric formulas are thought to be more palatable. However, there is limited research comparing the palatability of the two formula types. A retrospective study of children who received elemental formula from 1992-2001 and children who received polymeric formula from 2000-2004 found that adherence to treatment did not differ between the two groups but that those receiving polymeric formula were less likely to need a nasogastric tubes (NGT) inserted to deliver the feed<sup>[44]</sup>.

The mode of delivery of the formula may also play a role in patient compliance. Many studies with high adherence rates administered elemental formulas *via* NG or nasoduodenal tubes rather than orally. More recent paediatric studies have encouraged oral intake of polymeric formula and use of NGT only if needed<sup>[7,9,34]</sup>. For free living (non-hospitalised) patients, taking the formula orally may be more socially acceptable. Elemental and polymeric formulas have been shown to be as equally effective at inducing remission of disease in children<sup>[40]</sup> and adults<sup>[18]</sup>.

Studies that used elemental formulas given exclusively *via* NG or nasoduodenal tubes had low rates of non-

adherence (0%-13%)<sup>[26,28]</sup>. Whereas studies that reported high rates of non-adherence (33%-41%) used elemental or semi-elemental formulas given orally and if a patient did not tolerate EEN orally a NGT was placed.<sup>[21,25,29]</sup>

However, three of the six studies using elemental or semi-elemental diet orally reported higher adherence rates<sup>[19,22,24]</sup>. Two of these studies<sup>[19,24]</sup> only used EEN for 2 wk and patients were given a peptide based semi-elemental formula (Peptamen) orally rather than an amino acid-based elemental formula. Of the 19 patients using EEN in these two trials, only 1 patient was non-adherent with the treatment. The third study, by O'Moráin *et al.*<sup>[22]</sup> was one of the first to compare EEN to CS treatment. Patients were asked to take the elemental formula orally for four weeks and if they could not tolerate it a NGT was placed. Of the 11 patients in the EEN group, two (18%) could not tolerate the formula orally or *via* a NGT.

Of the three adult studies that used polymeric formula, two administered it *via* NG or nasoduodenal tubes with 100% adherence<sup>[20,23]</sup>. The third study used a polymeric powder (a high oleic and high linoleic acid formulation) given orally or *via* NGT if not tolerated orally<sup>[27]</sup>. Non-adherence with the treatment was 17%-25%. No published adult studies have used a ready-to-drink polymeric formula given orally. There are, however, various studies with children that have shown that polymeric formulas are palatable orally. Borrelli *et al.*<sup>[7]</sup> studied 19 children with CD who drank an isocaloric polymeric formula (Modulen) as their sole source of nutrition for 10 wk. Thirteen children took the formula orally; four required overnight feeding *via* a NGT, in addition to taking it orally during the day, to meet their nutritional requirements and two children could not manage to take the required volume of formula orally or *via* a NGT. Of the 17 children that successfully completed the 10 wk intervention 15 (88%) achieved disease remission. Day *et al.*<sup>[9]</sup> studied 27 children with CD who were prescribed EEN with isocaloric polymeric formula (Modulen or Osmolite) for up to 8 wk. Nineteen children managed the required volume of formula orally, five needed to take some of the formula *via* a NGT and three could not tolerate the required volume orally or *via* a NGT. Of the 24 children who completed at least 8 wk of EEN, 19 entered remission (79%).

Both of these paediatric studies used an isocaloric polymeric formula. It appears that the major reason for non-adherence in these cohorts was difficulty tolerating the volume required for nutritional requirements rather than un-palatability. It is not clear whether the volume required to meet an adult's nutritional requirements (*e.g.*, 1600-2400 mL of ready-to-drink isocaloric polymeric formula per day) may lead to poor adherence. The use of a concentrated polymeric formula, (*e.g.*, 1.5 kcal/mL formula), may help alleviate this issue.

If adherence with and response to EEN treatment of 90% and 80%, respectively, can be achieved in adults EEN may be a viable treatment option. Ready-to-drink polymeric formula, which may be more palatable orally

than elemental or semi-elemental formulas and more convenient and portable than powdered options, could provide an option for adults with CD wishing to reduce their exposure to CS, induce disease remission and potentially attain the benefits associated with EEN therapy that have been confirmed in children.

### Disease location

Disease location is thought to affect the efficacy of EEN therapy. In particular, colonic disease may be more refractory to treatment than disease with ileal involvement. However, due to the small participant numbers in most adult EEN studies there has been insufficient statistical power for subgroup analyses. A pooled meta-analysis of mainly adult studies from the 1980s and 1990s found that there was insufficient data to perform subgroup analyses by disease location<sup>[18]</sup>.

Some paediatric studies have specifically investigated the impact of disease location on response to EEN therapy. Afzal *et al.*<sup>[13]</sup> studied 65 children aged 8-17 years old with newly diagnosed CD of which 12 had ileal disease, 39 had ileocolonic disease and 14 had isolated colonic disease. They found that disease remission was harder to induce with EEN therapy in patients with colonic disease - remission achieved in 50% compared with 82% in those with ileocolonic disease and 92% in those with ileal CD ( $P = 0.02$ ). They also used colonoscopy to assess mucosal healing after EEN therapy and found that there was no improvement in colonic mucosal inflammation in those with colonic or ileocolonic disease.

Conversely, Buchman *et al.*<sup>[3]</sup> investigated the effect of disease location on remission rates after EEN therapy and found that colonic CD responded just as well as ileocolonic disease. Their study included 114 children (median age 11.6 years), all with recently diagnosed CD. Nineteen patients had colonic disease, four had ileal disease, 29 had ileocolonic disease, 49 had upper gastrointestinal tract disease and 9 had disease that could not be classified using the Vienna classification. Of those with colonic disease 79% went into remission after eight weeks of EEN therapy compared with 86% with ileocolonic disease, 88% with upper gastrointestinal disease and only 25% with ileal disease. It should be noted that there were only 4 patients with ileal disease compared with at least 20 in the other three groups. Further evidence is needed to confirm whether CD location affects the efficacy of EEN.

### Age of patient

Current guidelines suggest that EEN therapy is more appropriate to use in paediatric rather than adult patients<sup>[6,18]</sup>. There are no studies in adults that have assessed whether age affects response to EEN therapy. Although the mean age of adults included in the 11 studies evaluated here was approximately 30 years, the age range varied substantially and was not always published. Of those that did publish the age range of patients it was common to include patients aged 20 up to 50 or 60<sup>[19,22,25]</sup>.

It is unknown if age affects response to EEN therapy or compliance with treatment.

## CONCLUSION

Initial reports demonstrated that EEN was effective in inducing remission in adults with active CD and proposed this intervention as an alternative to CS therapy. However, subsequent larger studies failed to reproduce these results. Since then many studies have been conducted in paediatric populations and numerous benefits over and above achieving disease remission have become apparent. It appears that non-compliance with EEN treatment in early studies adversely affected the efficacy of EEN compared with CS therapy. There is also evidence to support a possible role of EEN with a specific group of adult patients - those newly diagnosed disease and, possibly, those with ileal involvement. Further research with this group is warranted. The use of polymeric formulas provided orally, which has not previously been studied in adult patients, may improve treatment compliance and allow adult patients to reap the many other benefits of EEN that have been shown in children over and above achieving disease remission and improving nutritional status.

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## Diagnosis of IgG4-related sclerosing cholangitis

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**Key words:** IgG4-related sclerosing cholangitis; Primary sclerosing cholangitis; IgG4; Sclerosing cholangitis

**Core tip:** IgG4-related sclerosing cholangitis (IgG4-SC) has become a third distinct clinical entity of sclerosing cholangitis. The diffuse cholangiographic abnormalities observed in IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC), and the presence of segmental stenosis suggests cholangiocarcinoma (CC). IgG4-SC responds well to steroid therapy, whereas PSC is only effectively treated with liver transplantation, and CC requires surgical intervention. IgG4-SC should be carefully diagnosed based on a combination of characteristic clinical, serological, morphological, and histopathological features after cholangiographic classification and targeting of a disease for differential diagnosis.

### Abstract

IgG4-related sclerosing cholangitis (IgG4-SC) is often associated with autoimmune pancreatitis. However, the diffuse cholangiographic abnormalities observed in IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC), and the presence of segmental stenosis suggests cholangiocarcinoma (CC). IgG4-SC responds well to steroid therapy, whereas PSC is only effectively treated with liver transplantation and CC requires surgical intervention. Since IgG4-SC was first described, it has become a third distinct clinical entity of sclerosing cholangitis. The aim of this review was to introduce the diagnostic methods for IgG4-SC. IgG4-SC should be carefully diagnosed based on a combination of characteristic clinical, serological, morphological, and histopathological features after cholangiographic classification and targeting of a disease for differential diagnosis. When intrapancreatic stenosis is detected, pancreatic cancer or CC should be ruled out. If multiple intrahepatic stenoses are evident, PSC should be distinguished on the basis of cholangiographic findings and liver biopsy with IgG4 immunostaining. Associated inflammatory bowel disease is suggestive of PSC. If stenosis is demonstrated in the hepatic hilar region, CC should be discriminated by ultrasonography, intraductal ultrasonography, bile duct biopsy, and a higher cutoff serum IgG4 level of 182 mg/dL.

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### INTRODUCTION

IgG4-related sclerosing cholangitis (IgG4-SC) is a characteristic type of sclerosing cholangitis, with an unknown pathogenic mechanism. Patients with IgG4-SC display increased serum IgG4 levels<sup>[1]</sup> and dense infiltration of IgG4-positive plasma cells with extensive fibrosis in the bile duct wall<sup>[2]</sup>. Circular and symmetrical thickening of the bile duct wall is observed in the areas without stenosis that appear normal on cholangiography, as well as in the stenotic areas<sup>[3]</sup>. IgG4-SC has been recently recognized as an IgG4-related disease. IgG4-SC is frequently associated with autoimmune pancreatitis (AIP). IgG4-related dacryoadenitis/sialadenitis and IgG4-related retroperitoneal

fibrosis are also occasionally observed in IgG4-SC<sup>[4-7]</sup>. However, some IgG4-SC cases do not involve other organs<sup>[8]</sup>. IgG4-SC is most common in elderly men. Obstructive jaundice is frequently observed in IgG4-SC. The clinical and radiological features of IgG4-SC are resolved by steroid therapy, although its long-term prognosis is not clear<sup>[9-12]</sup>. The diffuse cholangiographic abnormalities observed in IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC), and the presence of segmental stenosis suggests cholangiocarcinoma (CC). IgG4-SC responds well to steroid therapy, whereas PSC is effectively treated only with liver transplantation, and CC requires surgical intervention. It is also necessary to rule out secondary sclerosing cholangitis (SSC) caused by diseases with an obvious pathogenesis.

Precise diagnosis is needed before choosing appropriate treatments. Therefore, this paper provides a review of the clinical and pathological characteristics of IgG4-SC, focusing on its differential diagnosis from other biliary diseases such as PSC and CC.

## CLASSIFICATION OF SC

SC has been classified into two categories: PSC and SSC. IgG4-SC has sometimes been described as an isolated biliary tract lesion, even in the absence of pancreatic involvement, and has thus been established as a distinct clinical entity. Therefore, we propose that SC should now be classified into three categories: PSC, IgG4-SC, and SSC. We have identified three reasons why IgG4-SC should be considered independent of other forms of SSC. First, steroid therapy is highly effective for IgG4-SC, which is in contrast to the other types of SC. Second, in comparison with the other forms, IgG4-SC is the most frequently encountered in daily clinical practice. Third, the characteristics of IgG4-SC need to be fully discriminated from those of the other three intractable diseases, that is, pancreatic cancer (PCa), PSC, and CC.

With regard to the diagnosis of SC, SSC should be initially ruled out. Thereafter, IgG4-SC should be suspected, the serum IgG4 level measured, and further exploration for pancreatic involvement or other IgG4-related systemic disease, conducted. Finally, compatibility with the PSC criteria should be ascertained.

### PSC

The following diagnostic criteria for PSC, which were proposed by the Mayo Clinic, have been widely used<sup>[13]</sup>: typical cholangiographic abnormalities involving the intrabiliary and extrabiliary trees, compatible clinical and biochemical findings, and exclusion of other causes of SSC. Liver biopsy had been used in the past to help confirm diagnosis, but its diagnostic specificity and sensitivity have become controversial. Nevertheless, liver biopsy is useful in the diagnosis of small duct PSC, for patients with suspected PSC but normal cholangiographic findings, and for the exclusion of other cholestatic diseases.

Characteristic inflammatory bowel diseases (IBDs) are

frequently observed in PSC patients. Standard ursodeoxycholic acid doses lead to improvements in biochemical abnormalities but not in histological findings, cholangiographic appearance, or patient survival. Liver transplantation is considered effective for end-stage liver disease because of PSC and is associated with improved patient survival. PSC usually leads to cirrhosis, with a mean survival time of 12-17 years.

### IgG4-SC

Recently, IgG4-SC has attracted much attention with the emergence of clinical characteristics that distinguish it as a new clinical entity. The diffuse cholangiographic abnormalities observed in association with AIP may resemble those observed in PSC, and the segmental stenosis suggest CC. IgG4-SC responds well to steroid therapy compared with the other two types of SC.

We have previously reported on the differences between IgG4-SC and PSC<sup>[9]</sup>. The age at clinical onset is significantly older for patients with IgG4-SC. Among the chief complaints in IgG4-SC, obstructive jaundice, reflecting marked concentric stenosis of the large bile duct, is most frequently observed. However, in Japan, patients with PSC who present without symptoms after liver injury are identified by physical examination<sup>[14]</sup>.

An elevated serum IgG4 level is a characteristic feature of IgG4-SC<sup>[15]</sup>. In patients with IgG4-SC, the pancreas is the most common organ involved other than the liver. Patients with IgG4-SC have multiorgan involvement, including sclerosing sialadenitis, retroperitoneal fibrosis, and mediastinal lymphadenopathy<sup>[4-7]</sup>.

### SSC

SSC is a chronic cholestatic biliary disease that can develop after a diverse range of insults to the biliary tree. SSC is considered to develop as a consequence of known injuries or secondary to pathological processes of the biliary tree. The etiology of SSC can usually be identified, although the exact pathogenesis often remains speculative. The most frequently described causes of SSC are long-standing biliary obstruction, surgical trauma to the bile duct, and ischemic injury to the biliary tree in liver allografts.

The different types of SSC have been described in the diagnostic criteria established by the Mayo Clinic<sup>[13]</sup>. Two reviews of SSC cases have been published<sup>[16,17]</sup>. IgG4-SC was previously classified into SSC. We classified the etiology of SSC based on three review articles<sup>[13,16,17]</sup> (Table 1). There are few studies comparing patients with SSC and PSC. A 10-year retrospective review (1992-2002) by the Mayo Clinic identified 31 patients with SSC<sup>[18]</sup>. The documented etiologies in their series included surgical trauma from cholecystectomy, intraductal stones, recurrent pancreatitis, and abdominal injury. Nine of their patients with SSC ultimately required liver transplantation, and four died. In their series, when compared with matched controls with PSC, the patient transplant-free survival was significantly shorter.



**Table 1 Etiology of secondary sclerosing cholangitis**

Congenital	Caroli's disease Cystic fibrosis
Chronic obstruction	Choledocholithiasis Biliary strictures (secondary to surgical trauma, chronic pancreatitis) Anastomotic strictures in liver graft Neoplasms (benign, malignant, metastatic)
Infectious	Bacterial cholangitis Recurrent pyogenic cholangitis Parasitic infection (cryptosporidiosis, microsporidiosis) Cytomegalovirus infection
Toxic	Accidental alcohol, formaldehyde, hypertonic saline instillation in the bile ducts
Immunologic	Eosinophilic cholangitis Acquired immunodeficiency
Ischemic	Vascular trauma Post-traumatic sclerosing cholangitis Post-transplantation hepatic artery thrombosis Hepatic allograft rejection (acute, chronic) Intra-arterial, chemotherapy-related injury Transcatheter arterial embolization therapy
Infiltrative disorders	Systemic vasculitis Amyloidosis Radiation injury Sarcoidosis Systemic mastocytosis Hypereosinophilic syndrome Hodgkin's disease

## IgG4-SC DIAGNOSIS

### Cholangiographic classification

IgG4-SC displays various cholangiographic features similar to those of PCa, PSC, and CC. The characteristic features of IgG4-SC can be classified into four types based on the stricture regions revealed by cholangiography and differential diagnosis (Figure 1)<sup>[19,20]</sup>. Type 1 IgG4-SC displays stenosis only in the lower part of the common bile duct and thus should be differentiated from chronic pancreatitis, PCa, and CC. Type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic and extrahepatic bile ducts, should be differentiated from PSC and is further subdivided into two subtypes: type 2a, characterized with narrowing of the intrahepatic bile ducts with prestenotic dilation; and type 2b, characterized by the narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches, which is caused by marked lymphocytic and plasmacyte infiltrations into the peripheral bile ducts. Type 3 IgG4-SC is characterized by stenosis in the hilar hepatic lesions and the lower part of the common bile duct. Type 4 IgG4-SC presents with strictures of the bile duct only in the hilar hepatic lesions. The cholangiographic findings of types 3 and 4 IgG4-SC should be discriminated from those of CC.

### Serum IgG4 level

Serum IgG4 level has been reported to be a useful marker for discriminating AIP from other pancreatic diseases.

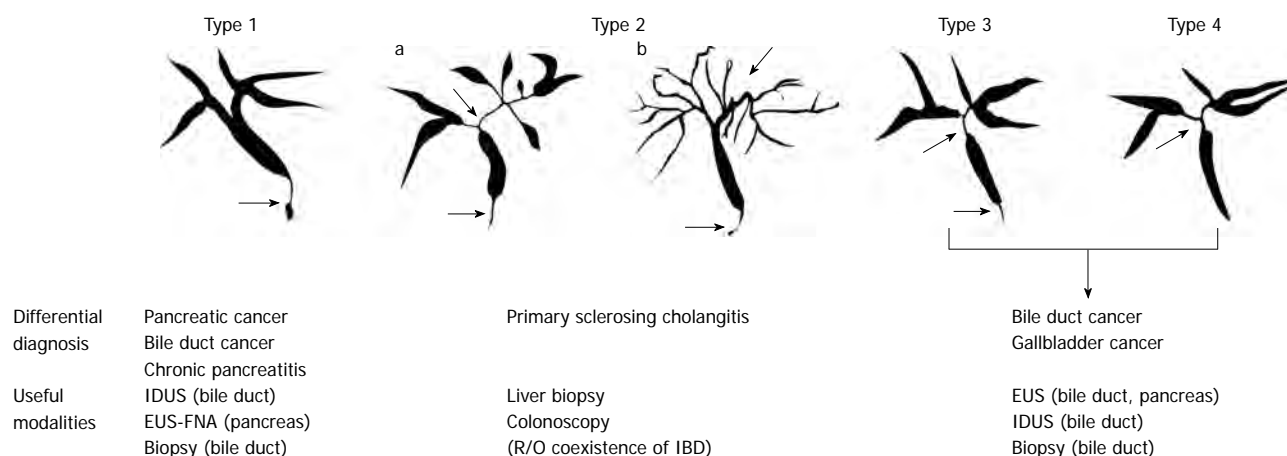
A cutoff IgG4 level of 135 mg/dL is widely used as part of the diagnostic criteria for AIP. However, twice the upper limit of normal is also recommended to discriminate AIP from PCa. In the international consensus diagnostic criteria for AIP, once or twice the upper limit of normal is included in levels 1 and 2 diagnoses, respectively<sup>[21]</sup>.

Only a few reports have been published concerning the cutoff IgG4 level in the diagnosis of IgG4-SC. We published for the first time, the diagnostic criteria for IgG4-SC based on a comparative study<sup>[22]</sup>. The cutoff IgG4 level of 135 mg/dL is useful in discriminating IgG4-SC from PCa and PSC. However, this cutoff level displayed lower specificity in discriminating IgG4-SC from CC. Oseini *et al*<sup>[23]</sup> evaluated the utility of serum IgG4 level in distinguishing IgG4-SC from CC. They reported that among their 126 patients with CC, 17 (13.5%) had elevated (> 140 mg/dL) and four (3.2%) had a > 2-fold increase (> 280 mg/dL) in serum IgG4 levels. PSC was present in 31/126 CC patients, of whom seven (22.6%) had an elevated serum IgG4 level. The authors concluded that some patients with CC, particularly PSC, had elevated serum IgG4 levels and diagnosis using a twofold higher cutoff serum IgG4 level may not reliably distinguish IgG4-SC from CC. However, a cutoff level fourfold higher than the upper limit of normal had 100% specificity for IgG4-SC.

We recently established a cutoff serum IgG4 level to differentiate IgG4-SC from the three other diseases (type 1 IgG4-SC *vs* PCa, type 2 IgG4-SC *vs* PSC, and type 3 IgG4-SC *vs* CC) using serum IgG4 levels measured in nine Japanese high-volume centers<sup>[24]</sup>. The cutoff obtained from receiver operator characteristic (ROC) curves displayed similar sensitivity and specificity to those of 135 mg/dL when all the IgG4-SC cases and controls were compared. However, a new cutoff value was established when IgG4-SC subgroups and controls were compared. A cutoff level of 182 mg/dL can increase the specificity to 96.6% (a 4.7% increase) for distinguishing types 3 and 4 IgG4-SC from CC. A cutoff level of 207 mg/dL might be useful for completely distinguishing types 3 and 4 IgG4-SC from CC.

Alswat *et al*<sup>[25]</sup> demonstrated that serum IgG4 levels could efficiently detect patients with IgG4-SC after excluding SC patients with AIP. However, previously reported IgG4-SC cases without pancreatic involvement displayed no marked increase in serum IgG4 level compared with patients with AIP-associated IgG4-SC. Hamano *et al*<sup>[8]</sup> demonstrated modestly high serum IgG4 levels (119, 122, and 195 mg/dL) in three IgG4-SC cases without an obvious pancreatic mass.

Elevated serum IgG4 level is considered a characteristic feature of IgG4-SC<sup>[1]</sup>. However, Mendes *et al*<sup>[26]</sup> measured the serum IgG4 level in 127 patients with PSC and found that it was elevated in 12 (9%). The patients with elevated IgG4 levels had higher levels of total bilirubin and alkaline phosphatase, higher PSC Mayo risk scores, and lower incidence of IBD. It is important to note that the time to liver transplantation was shorter in



**Figure 1 Cholangiographic classification of IgG4-related sclerosing cholangitis and differential diagnosis.** Stenosis is located only in the lower part of the common bile duct in type 1; stenosis is diffusely distributed in the intra- and extra-hepatic bile ducts in type 2. Type 2 is further subdivided into two. Extended narrowing of the intrahepatic bile ducts with prestenotic dilation is widely distributed in type 2a. Narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches are widely distributed in type 2b; stenosis is detected in both the hilar hepatic lesions and the lower part of the common bile ducts in type 3; strictures of the bile duct are detected only in the hilar hepatic lesions in type 4. IDUS: Intraductal ultrasonography; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; IBD: Inflammatory bowel disease.

the patients with elevated IgG4 levels (1.7 years *vs* 6.5 years,  $P = 0.0009$ ). As only one of the patients in their series had an abnormal pancreatogram, the documented cases appeared to conform to the diagnosis of IgG4-SC. Therefore, clinical trials in which patients with PSC are evaluated for IgG4 and patients presenting elevated levels are treated with corticosteroids should be considered.

### Other organ involvement

The concept of IgG4-related disease has been established internationally<sup>[27]</sup>. IgG4-SC is included in the IgG4-related disease category. Serum IgG4 level elevation and tissue infiltration with IgG4-positive plasma cells are common threads that connect a variety of apparently disparate conditions observed previously in multiple organs. Certain clinical and pathological features help define IgG4-related disease and distinguish it from its potential mimics. IgG4-related disease is a fibroinflammatory condition characterized by a tendency to form tumefactive lesions, a dense lymphoplasmacytic infiltrate rich in IgG4-positive plasma cells, storiform fibrosis, frequent but not invariable elevations in serum IgG4 level, and a swift initial response to glucocorticoids, provided that tissue fibrosis has not supervened.

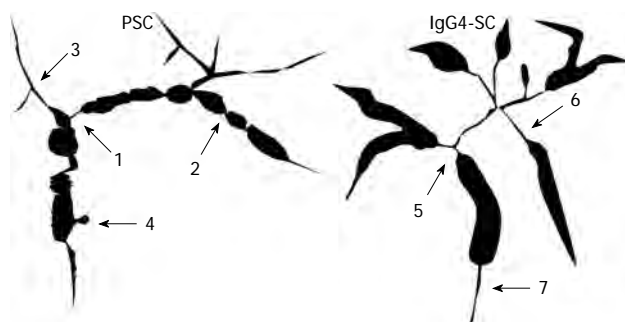
The pancreas was the first organ in which IgG4-related disease was identified, but the disease has now been described in virtually every organ system: the biliary tree, salivary glands, orbital tissues (*e.g.*, lacrimal gland, extraocular muscles, and retrobulbar space), kidneys, lungs, lymph nodes, meninges, aorta, breast, prostate, thyroid gland, pericardium, retroperitoneum, and skin.

Association with AIP is a useful finding in the diagnosis of IgG4-SC. In one study, 59 (95%) of 62 patients with IgG4-SC had associated AIP, with high prevalence<sup>[21]</sup>. Ghazale *et al*<sup>[15]</sup> reported an incidence rate of AIP association of 92% in 53 patients with IgG4-SC, which was a comparatively large sample. However, focal-

type AIP sometimes displays similar imaging findings to those of PCa, making discrimination between the two diseases difficult<sup>[28]</sup>. The sensitivity rates of diagnostic criteria for AIP have been reported to range from 80% to 92%<sup>[29]</sup>. Therefore, useful diagnostic criteria need to be established for IgG4-SC when it is not associated with AIP or when diagnosis of AIP is unclear. IgG4-SC is occasionally associated with other systemic IgG4-related diseases including symmetrical dacryoadenitis/sialadenitis and retroperitoneal fibrosis. These associations are helpful in the accurate diagnosis of IgG4-SC.

### Pathological features

In IgG4-SC, fibroinflammatory involvement is observed mainly in the submucosa of the bile duct wall, whereas the epithelium of the bile duct is intact<sup>[2]</sup>. However, slight injury and/or neutrophil infiltration are occasionally observed in IgG4-SC with associated secondary cholangitis. PSC should be ruled out if inflammation is observed, particularly in the epithelium of the bile duct wall. The characteristic pathological findings of IgG4-SC were first reported as “lymphoplasmacytic sclerosing pancreatitis with cholangitis”<sup>[30]</sup>. Abraham *et al*<sup>[31]</sup> reported frequent fibroinflammatory involvement of the gallbladder and common bile duct in patients with lymphoplasmacytic sclerosing pancreatitis. Zen *et al*<sup>[2]</sup> revealed that the bile duct wall in IgG4-SC had pathological features similar to those of AIP, displaying dense infiltrations of lymphocytes and IgG4-positive plasma cells, with extensive fibrosis and obliterative phlebitis. They classified IgG4-SC into six categories according to the extent of inflammation and association with an inflammatory pseudotumor. IgG4-positive plasma cells are sparse in the affected bile ducts in PSC, and the luminal side of the bile ducts, including lining biliary epithelial cells, is preferentially affected compared with IgG4-SC. In PSC, the fibrosis is denser and older, whereas in IgG4-SC, the entire bile



**Figure 2** Schematic illustration of comparison of cholangiographic (primary sclerosing cholangitis vs IgG4-related sclerosing cholangitis) findings<sup>[28]</sup>. The schematic comparison of cholangiographic findings between IgG4-related sclerosing cholangitis (SC) and primary sclerosing cholangitis (PSC). IgG4-related SC displays segmental and long strictures and stricture of the lower common bile duct, whereas PSC displays band-like strictures (1–2 mm), beaded appearance (short and annular stricture alternating with normal or minimally dilated segments), pruned-tree appearance (diminished arborization of intrahepatic duct and pruning), and diverticulum-like outpouching (outpouchings resembling diverticula, often protruding between adjacent strictures). 1: Band-like stricture; 2: Beaded appearance; 3: Pruned-tree appearance; 4: Diverticulum-like outpouching; 5: Segmental stricture; 6: Long stricture with prestenotic dilation; 7: Stricture of lower common bile duct.

duct wall and periductal tissue are affected. However, a recent study by Zhang *et al.*<sup>[32]</sup> revealed that 23 (23%) of 98 liver explants with PSC had periductal infiltration with abundant IgG4-positive plasma cells [10/high-power field (HPF)] in the hilar area.

### Differential diagnosis of IgG4-SC based on cholangiographic classification

IgG4-SC displays various cholangiographic features similar to those of PCa, PSC, and CC<sup>[9]</sup>. The differential diagnosis based on cholangiographic classification is sufficient in clinical practice because the useful modalities depend on the cholangiographic types (Figure 1)<sup>[20]</sup>.

Type 1 IgG4-SC should be differentiated from chronic pancreatitis, PCa, and CC. The modalities useful for differential diagnosis are intraductal ultrasonography (IDUS)<sup>[3]</sup>, endoscopic ultrasound-guided fine-needle aspiration for the pancreas<sup>[33]</sup>, and cytological examination and/or biopsy of the bile duct<sup>[3,34]</sup>.

Type 2 IgG4-SC should be differentiated from PSC. The modalities useful for differential diagnosis are cholangiography<sup>[35]</sup>, evaluations for associated IBD<sup>[9,12]</sup>, and liver biopsy<sup>[36,37]</sup>. Our discriminant analysis formula for cholangiographic features, including age, was able to discriminate type 2 IgG4-SC from PSC<sup>[35]</sup>. Band-like strictures, a beaded or “pruned tree” appearance, and diverticulum-like outpouching are significantly more frequent in PSC cases. In contrast, segmental strictures, long strictures with prestenotic dilation, and strictures of the lower common bile duct are significantly more common in IgG4-SC. These differences are illustrated in Figure 2. The characteristic cholangiographic features reflect the underlying pathological processes involved in each condition. In PSC, obliterative fibrosis is the main cause of biliary stenosis, creating short strictures. In contrast, in

IgG4-SC, severe lymphoplasmacyte infiltration into bile ducts in the long region is the main cause of biliary stenosis, resulting in long strictures (Figure 3).

In contrast, Kalaitzakis *et al.*<sup>[38]</sup> reported that diagnosing IgG4-SC by cholangiography displayed high specificity but poor sensitivity and may have led to the misdiagnosis of IgG4-SC as PSC or CC.

Associated ulcerative colitis is suggestive of PSC. IBD is present in only 0%–6% of patients with IgG4-SC<sup>[9,12,15]</sup>. IBD is usually not a feature associated with type 1 AIP, unlike the frequent association of IBD with type 2 AIP<sup>[23]</sup>. IBD associated with PSC represents a third phenotype in western countries<sup>[39]</sup>. Backwash ileitis, rectal sparing, and low disease activity appear to be features that characterize IBD when it is associated with PSC<sup>[39,40]</sup>.

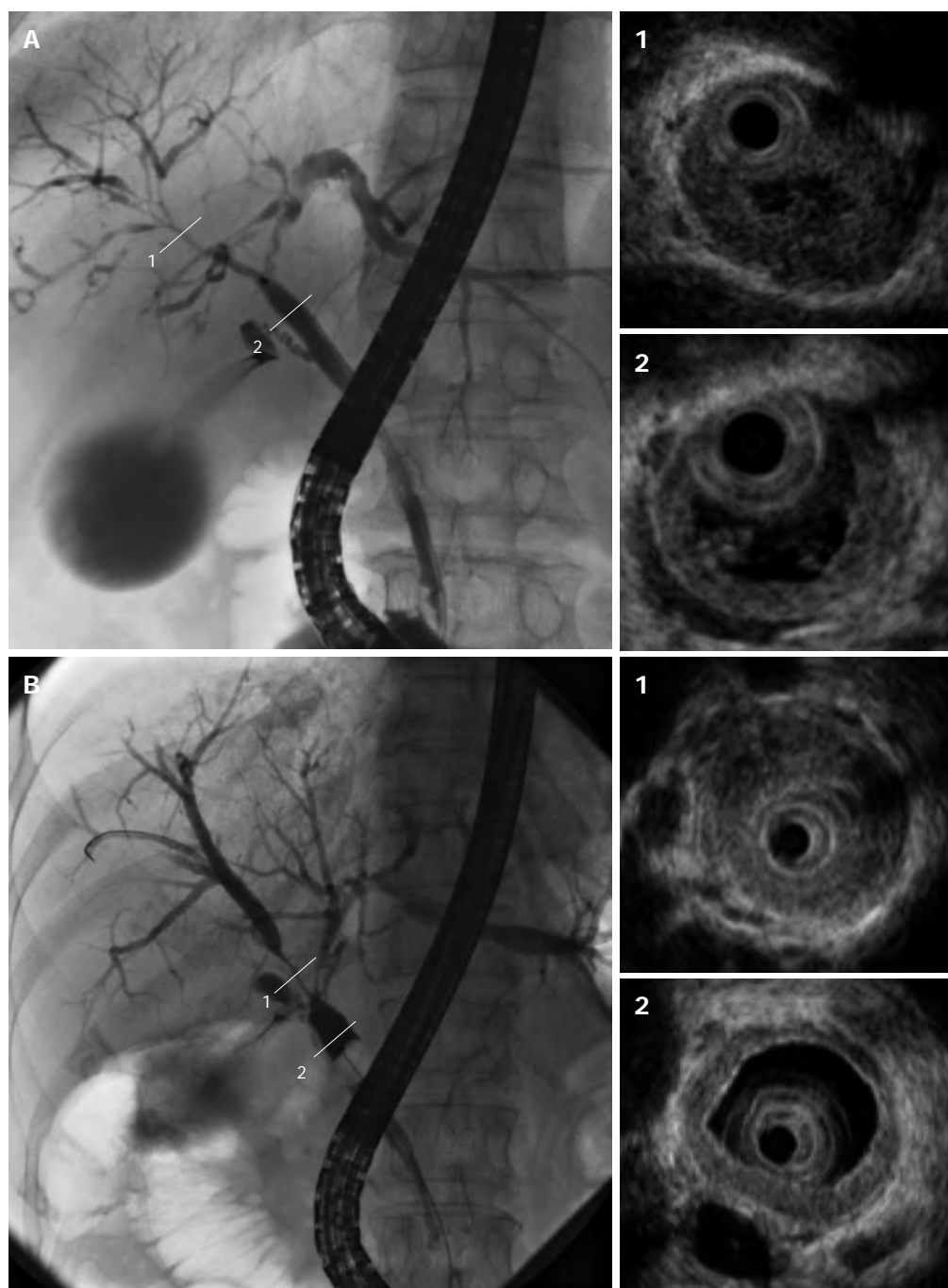
The histological features of IgG4-SC on liver biopsy are distinctive and, in conjunction with IgG4 immunohistochemical staining, help distinguish IgG4-SC from PSC<sup>[36,41]</sup>. We have already reported that liver needle biopsy is especially useful for distinguishing IgG4-SC from PSC in patients with cholangiographically evident intrahepatic biliary strictures<sup>[37]</sup>. Four (57%) of seven patients with type 2 IgG4-SC presented infiltration with  $\geq 10$  IgG4-positive plasma cells per HPF in liver biopsy samples, whereas none of the PSC patients presented this feature.

Types 3 and 4 IgG4-SC need to be discriminated from CC. The modalities useful for the differential diagnosis of types 3 and 4 IgG4-SC are endoscopic procedures<sup>[42]</sup> such as endoscopic ultrasonography, IDUS<sup>[3,43]</sup>, cytological examination, and/or biopsy of the bile duct<sup>[3,34]</sup>. Although we described how type 2 IgG4-SC could be discriminated from PSC on the basis of characteristic cholangiographic features, cholangiography cannot discriminate the segmental stricture of types 3 and 4 IgG4-SC from CC. Therefore, we applied our discriminant analysis formula for cholangiographic features to discriminate between only type 2 IgG4-SC and PSC.

IDUS findings such as circular-symmetrical wall thickening, smooth outer margin, smooth inner margin, and homogeneous internal echo at the stenotic area were useful for the diagnosis of IgG4-SC. The most characteristic IDUS finding in the IgG4-SC cases was thickening of the bile duct wall, which appeared normal on cholangiography<sup>[3]</sup>. Bile duct wall thickening spread continuously from the intrapancreatic bile duct to the upper bile duct in most cases. To differentiate IgG4-SC from CC, 0.8-mm thickness of the bile duct wall that appeared normal on a cholangiogram was the best cutoff as indicated by ROC curves. The sensitivity, specificity, and accuracy were 95%, 90.9%, and 93.5%, respectively, when the cutoff value was 0.8 mm. No CC cases had a bile duct wall thicker than 1 mm. The sensitivity, specificity, and accuracy were 85%, 100%, and 87%, respectively, when the cutoff value was set at 1 mm. We considered a 1-mm thickness as an optimal cutoff wall thickness to completely exclude CC.

Ghazale *et al.*<sup>[15]</sup> reported the usefulness of endoscopic biliary biopsy for diagnosis of IgG4-SC. They





**Figure 3** Cholangiogram displaying stenosis in the intrahepatic ducts (A-1) and hilar hepatic lesions (B-1); intraductal ultrasonography revealing bile duct wall thickening in areas with stenosis (1) and without (2).

reported that 14 (88%) of 16 patients had immunostaining results indicating abundant IgG4-positive cells ( $> 10$  IgG4-positive cells/HPF) in bile duct biopsy specimens. Furthermore, they considered that the absence of malignant cells in the presence of an inflamed mucosa with many IgG4-positive plasma cells provided histological evidence to support the diagnosis of IgG4-SC. However, we were unable to diagnose any case as IgG4-SC on the basis of hematoxylin-eosin and elastin-van Gieson staining alone<sup>[3]</sup>. Abundant IgG4-positive plasma cells were observed in only three (18%) of 17 patients. We were able to diagnose IgG4-SC in only three patients (18%) on

the basis of its characteristic histopathological features. However, it was possible to rule out CC by transpapillary biopsy. In addition, one of 11 CC cases presented with abundant IgG4-positive plasma cells. Zhang *et al.*<sup>[32]</sup> also reported that abundant IgG4-positive plasma cells were evident in seven (18%) of 38 cases of CC. Harada *et al.*<sup>[44]</sup> reported that CC cells could play the role of nonprofessional antigen-presenting cells and Foxp3-positive regulatory cells, inducing IgG4 reactions *via* the production of interleukin-10 indirectly and directly, respectively.

We could rule out CC by transpapillary biopsy. The superficial nature of endoscopic biopsy specimens lim-



its usefulness for demonstrating the characteristic histological features of IgG4-SC. However, Kawakami *et al.*<sup>[34]</sup> reported that the diagnostic rate from ampullary and bile duct biopsies was 52% (15/29 cases) based on the threshold of 10 IgG4-positive plasma cells per HPF, and that bile duct biopsy was valuable for patients with swelling of the pancreatic head. Ampullary biopsy is sometimes useful in the diagnosis of AIP and IgG4-SC<sup>[45,46]</sup>.

Itoi *et al.*<sup>[47]</sup> reported that cholangioscopy was useful in differentiating IgG4-SC from PSC and that monitoring patterns of proliferative vessels on video peroral cholangioscopy may be useful in differentiating IgG4-SC from CC.

### Treatment and prognosis

Although some patients responded to biliary drainage or surgical resection, IgG4-SC displays a good response to steroid therapy, as is the case for pancreatic lesions.

Nishino *et al.*<sup>[11]</sup> reported that bile duct stricture improved to various degrees in all 10 patients treated by steroid therapy but persisted in the lower part of the bile duct in four patients. Hirano *et al.*<sup>[48]</sup> reported that steroid therapy could reduce AIP-related unfavorable events and that multivariate analysis indicated that steroid therapy and obstructive jaundice at onset were significant factors predictive of unfavorable events. Thus, early introduction of steroid therapy is recommended, especially for patients with obstructive jaundice. Ghazale *et al.*<sup>[15]</sup> reported the clinical courses after steroid treatment ( $n = 30$ ), surgical resection ( $n = 18$ ), and conservative management ( $n = 5$ ). Relapses occurred in 53% of cases after steroid withdrawal, whereas 44% relapsed after surgery and were further treated with steroids. The presence of proximal extrahepatic/intrahepatic strictures was predictive of relapse. Steroid therapy normalized liver enzyme levels in 61% of patients, and it was possible to remove biliary stents in 17 of 18 patients. Fifteen patients treated with steroids for relapse after steroid withdrawal responded to the treatment, and seven treated with additional immunomodulatory drugs reportedly remained in steroid-free remission. Topazian *et al.*<sup>[49]</sup> reported that biliary strictures in one patient improved after rituximab therapy and thus the biliary stents were removed. However, the role of immunomodulatory drugs for relapse warrants further study. In one of our series, six of seven cases of IgG4-SC without steroid therapy and IgG levels  $> 2000$  mg/dL were associated with significantly higher incidence of recurrence or progression<sup>[50]</sup>.

Morphological and functional changes in the liver and bile ducts in IgG4-SC during long-term observation have not yet been reported. Our long-term follow-up of IgG4-SC cases without steroid therapy revealed that two patients developed portal obstruction and liver atrophy but no sign of liver cirrhosis or failure<sup>[51]</sup>. Ghazale *et al.*<sup>[15]</sup> reported that four of 53 patients displayed portal hypertension and liver cirrhosis during their clinical courses. It is possible that persistent jaundice without steroid administration could result in liver failure, thus necessitating

orthotopic liver transplantation. However, further study is needed to elucidate the long-term outcome of IgG4-SC.

### Steroid trial

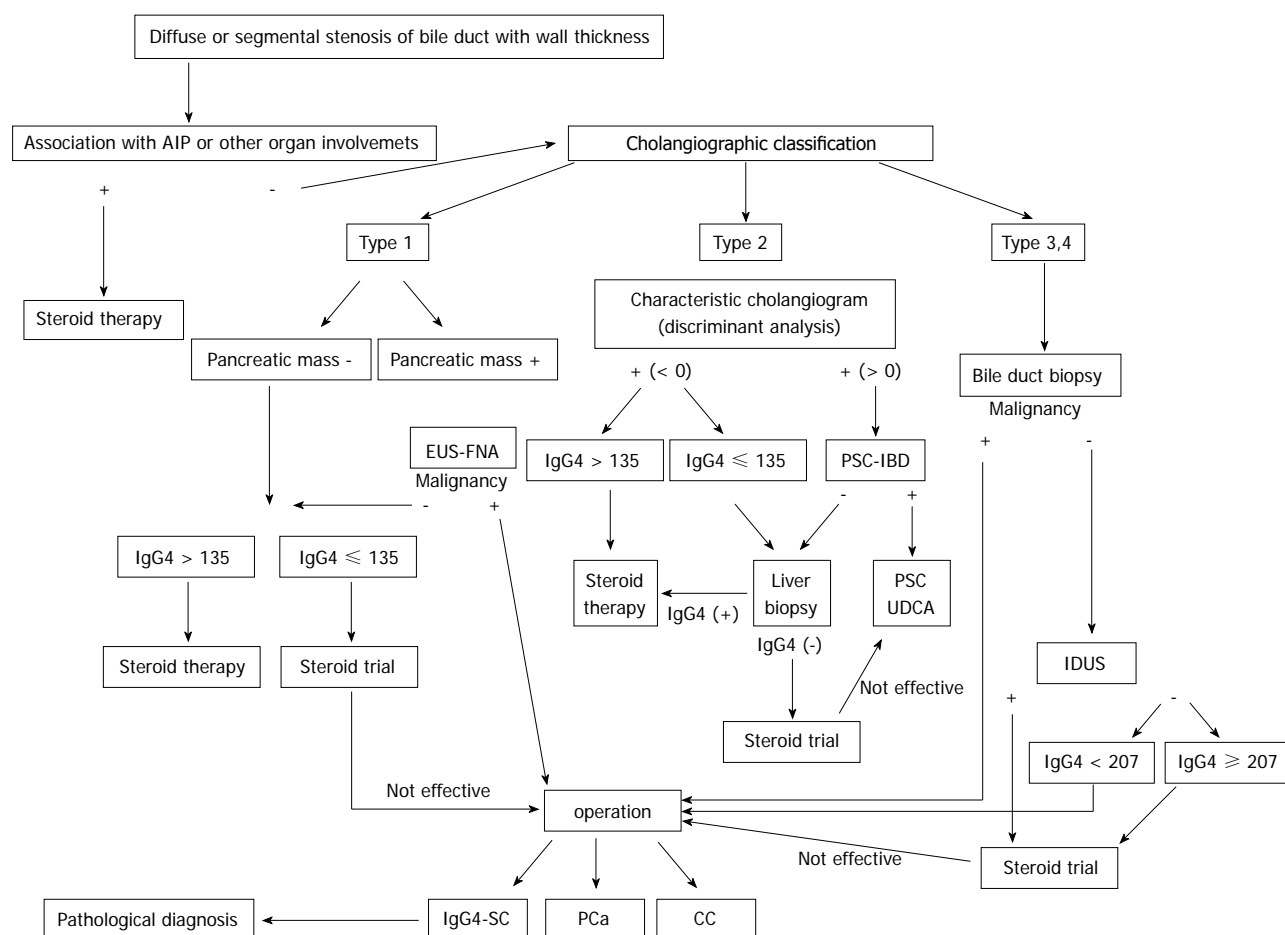
Although, generally, diagnosis should be made before starting therapy, a steroid trial is needed in some cases<sup>[52]</sup>. If a diagnosis cannot be clearly established in type 2 IgG4-SC, then a steroid trial is recommended. If malignancy is not confirmed by bile duct biopsy in types 3 and 4 IgG4-SC and bile duct wall thickening that appears normal on a cholangiogram, a steroid trial is an option. No reports on any steroid trial for IgG4-SC have been published thus far. A short-term steroid trial should be performed carefully only by specialists in pancreatic and biliary diseases. In addition, steroid pulse therapy is also an option<sup>[53]</sup>. Advanced-stage IgG4-SC may sometimes be unresponsive to steroid therapy because cases of AIP and IgG4-SC show a predominantly inflammatory nature at the early stage, followed by relatively less inflammation but marked fibrous scarring later. This should be kept in mind when attempting a steroid trial for IgG4-SC diagnosis<sup>[54]</sup>. The time frame for a steroid trial for IgG4-SC remains unknown. When a cholangiogram is indicative of type 1, 3 or 4 IgG4-SC, IgG4-SC should be discriminated from PCa or CC. It is important not to delay the timing of surgery by performing a long-term steroid trial. If a cholangiogram is indicative of type 2 IgG4-SC, IgG4-SC should be discriminated from PSC. Sufficient time should be devoted to a steroid trial only if an increased risk of biliary infection can be avoided.

### Diagnostic criteria

Only three sets of diagnostic criteria for IgG4-SC have been proposed<sup>[15,20,24]</sup>. AIP should be clinically discriminated only from PCa. However, IgG4-SC should be discriminated from all of the three intractable diseases, that is, PCa, PSC, and CC. Therefore, diagnostic criteria that take into account the differential diagnosis between these three intractable diseases need to be established<sup>[22]</sup>. Our diagnostic criteria provide a concrete diagnostic algorithm for IgG4-SC (Figure 4). Association with AIP and other organ involvements are common useful diagnostic parameters in all three IgG4-SC types. Characteristic cholangiogram, liver biopsy and exclusion of IBD are useful parameters in type 2 IgG4-SC. IDUS findings, exclusion of malignancy by bile duct biopsy and a serum IgG4 cut-off level of 207 mg/dL were useful parameters in type 3 and 4 IgG4-SC. Although, generally, diagnosis should be made before starting therapy, a steroid trial is needed in some cases.

The HISORT criteria for the diagnosis of IgG4-SC<sup>[15]</sup> are based on the characteristic features of IgG4-SC on histological, imaging, and serological examination; other organ involvement; and response to steroid therapy, which parallel the HISORT criteria established for AIP<sup>[55]</sup>.

The Research Committee of IgG4-related Diseases and the Research Committee of Intractable Diseases of Liver and Biliary Tract, in association with the Ministry



**Figure 4** Algorithm for management of IgG4-related sclerosing cholangitis (cited from [22]). CC: Cholangiocarcinoma; PSC: Primary sclerosing cholangitis; IgG4-SC: IgG4-related sclerosing cholangitis; IDUS: Intraductal ultrasonography; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; IBD: Inflammatory bowel disease; UDCA: Ursodeoxycholic acid.

of Health, Labor and Welfare, Japan, and the Japan Biliary Association, proposed clinical diagnostic criteria for IgG4-SC in 2012<sup>[20]</sup>. These diagnostic criteria also include the concept of differential diagnosis based on cholangiographic classification.

## CONCLUSION

Since IgG4-SC was first described, it has become a third distinct clinical entity of SC. IgG4-SC should be carefully diagnosed based on a combination of characteristic clinical, serological, morphological, and histopathological features after cholangiographic classification and targeting of a disease for differential diagnosis.

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## Efficacy of switching to telbivudine plus adefovir in suboptimal responders to lamivudine plus adefovir

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### Abstract

**AIM:** To examine the efficacy of telbivudine (LdT) + adefovir (ADV) *vs* continuation of lamivudine (LAM) + ADV in patients with LAM-resistant chronic hepatitis B (CHB) who show a suboptimal response to LAM + ADV.

**METHODS:** This was a randomized, active-control, open-label, single-center, parallel trial. All eligible patients were enrolled in this study in Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea, between March 2010 and March 2011. Hepatitis Be antigen (HBeAg)-positive CHB patients whose serum hepatitis B virus (HBV) DNA remained detectable despite at least 6 mo of LAM + ADV therapy were included. Enrolled patients were randomized to either switching to LdT (600 mg/d orally) plus ADV (10 mg/d orally) (LdT + ADV group) or to continuation with LAM (100 mg/d orally) plus ADV (10 mg/d orally) (LAM + ADV group), and were followed for 48 wk. One hundred and six patients completed the 48-wk treatment period. Serum HBV DNA, HBeAg status, liver biochemistry and safety were monitored at baseline and week 12, 24, 36 and 48.

**RESULTS:** The duration of prior LAM + ADV treatment was 18.3 (LdT + ADV) and 14.9 mo (LAM + ADV), respectively ( $P = 0.131$ ). No difference was seen in baseline serum HBV DNA between the two groups [3.66 (LdT + ADV) *vs* 3.76 (LAM + ADV)  $\log_{10}$  IU/mL,  $P = 0.729$ ]. At week 48, although there was no significant difference in the mean reduction of serum HBV DNA from baseline between LdT + ADV group and LAM + ADV group ( $-0.81$  *vs*  $-0.47$   $\log_{10}$  IU/mL,  $P = 0.167$ ), more patients in the LdT + ADV group had undetectable HBV DNA levels compared to those in the LAM + ADV group (30.2% *vs* 11.5%,  $P = 0.019$ ). Three patients with LdT + ADV treatment and 2 patients with LAM + ADV treatment achieved HBeAg loss. The patients in both groups tolerated the treatment well without serious adverse

events. The proportion of patients with estimated glomerular filtration rate  $\geq 90$  mL/min per  $1.73\text{ m}^2$  in the LdT + ADV group increased from 49.1% (26/53) at baseline to 58.5% (31/53) at week 48, while that in the LAM + ADV group decreased from 37.7% (20/53) at baseline to 30.2% (16/53) at week 48.

**CONCLUSION:** The switch to LdT + ADV in suboptimal responders to LAM + ADV showed a significantly higher rate of virologic response at week 48. These results suggest that LdT + ADV could be a therapeutic option for patients who are unable to use enofovir disoproxil fumarate for any reason.

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**Key words:** Chronic hepatitis B; Antiviral resistance; Suboptimal response; Telbivudine; Lamivudine

**Core tip:** A suboptimal response is common in patients treated with lamivudine (LAM) + adefovir (ADV) combination therapy and it has also become a new challenge for the management of chronic hepatitis B (CHB) patients. We commenced this study with the effect of telbivudine (LdT) + ADV combination therapy as a rescue therapeutic option in LAM-resistant CHB patients with suboptimal response to LAM + ADV. Our results demonstrated that switching from LAM + ADV to LdT + ADV resulted in superior virologic response, renoprotective effect and similar safety profiles at week 48. These results suggest that LdT + ADV could be a therapeutic option for patients who are unable to use enofovir disoproxil fumarate for any reason.

Park H, Park JY, Kim SU, Kim DY, Han KH, Chon CY, Ahn SH. Efficacy of switching to telbivudine plus adefovir in suboptimal responders to lamivudine plus adefovir. *World J Gastroenterol* 2013; 19(43): 7671-7679 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i43/7671.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7671>

## INTRODUCTION

Worldwide, over 400 million people suffer from chronic hepatitis B (CHB). Patients with CHB have a 15%-40% life-time risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. High hepatitis B virus (HBV) DNA concentration in serum in patients with CHB is known as an independent risk factor for disease progression to cirrhosis and HCC<sup>[2,3]</sup>. Therefore, the treatment goal of antiviral therapy for CHB is to achieve complete suppression of viral replication as rapidly as possible<sup>[4-6]</sup>, because prolonged viremia on therapy can lead to a higher risk of future antiviral drug resistance and therapeutic failure as well as disease progression<sup>[2,7,8]</sup>.

Lamivudine (LAM) has been widely used for treatment

of CHB since its first approval at 2002. However, a major limitation of LAM is the development of LAM-resistant YMDD-motif mutations in the viral DNA polymerase, the prevalence of which increases progressively to about 70% after 4 years of treatment<sup>[9]</sup>. In patients resistant to LAM, add-on combination therapy with LAM and adefovir (ADV) has resulted in lower rates of virologic breakthrough and additional development of genotypic resistance than when switching to ADV or entecavir<sup>[10,11]</sup>. Thus, LAM + ADV combination therapy has been recommended as a rescue therapy in patients with LAM resistant viral strains in many Asian countries, for its considerable effectiveness, lower resistance and affordable price<sup>[5]</sup>.

Unfortunately, a substantial proportion of patients treated with LAM + ADV combination therapy show a suboptimal virologic response<sup>[10-12]</sup>. Because there has been evidence that this suboptimal response to antiviral therapy might have clinical relevance to higher risk of developing resistance to long-term antiviral treatment, suboptimal response to nucleotide analogues (NAs), in addition to drug resistance, has also become a new challenge for the management of CHB patients<sup>[13-15]</sup>. However, there is no standard optimal strategy for the management of suboptimal response to NA therapy at present. Many practice guidelines suggest a combination treatment regimen with tenofovir disoproxil fumarate (TDF) which is a NA with a high barrier to resistance as a highly potent rescue therapeutic option<sup>[4,6]</sup>. TDF, however, remains largely unavailable in Asian countries. Thus, several trials with various combination regimens for these populations have been proceeded<sup>[16,17]</sup>, and the results suggested consistently that combination therapy rather than switching to another drug offers a potentially attractive therapeutic option.

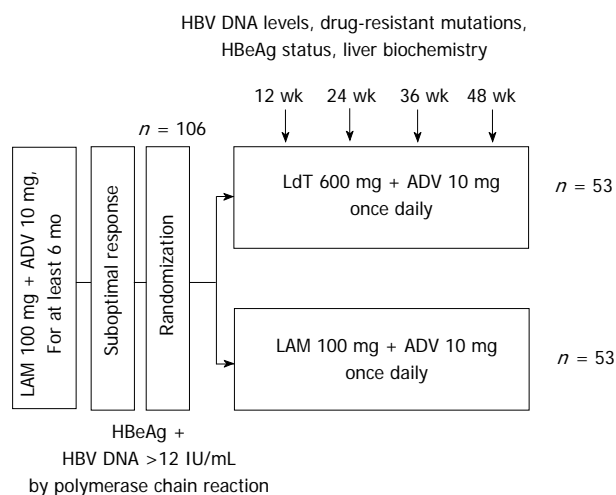
Telbivudine (LdT) is one of the licensed NAs which is structurally related to LAM and highly selective for HBV DNA and inhibits viral DNA synthesis with no effect on human DNA or other viruses<sup>[18]</sup>. The Gestation Linked to Obesity and Environment (GLOBE), the largest trial in CHB, demonstrated that LdT is superior to LAM for all efficacy measures over 2 years of therapy<sup>[19,20]</sup>. Another trial showed a superior viral suppressive effect of LdT even to ADV in treatment naïve CHB patients<sup>[21]</sup>. In addition, LdT + ADV combination treatment showed better outcomes against LAM-resistant HBV than ADV alone<sup>[22]</sup>. Therefore, LdT is a therapeutic option in LAM-resistant hepatitis B patients with suboptimal response to LAM + ADV combination therapy.

In this study, we directly compared the antiviral efficacy of switching to LdT + ADV combination vs LAM + ADV continuation in hepatitis Be antigen (HBeAg) positive LAM-resistant hepatitis B patients who showed suboptimal response to LAM + ADV combination treatment.

## MATERIALS AND METHODS

### Patients

Patients eligible for this study were men and women, aged over 20 years, positive for serum hepatitis B surface antigen (HBsAg) for at least 6 mo, and positive for HBeAg.



**Figure 1** Flow diagram of study participants. LMA: Lamivudine; ADV: Adefovir; LdT: Telbivudine; HBV: High hepatitis B virus; HBeAg: Hepatitis Be antigen.

Inclusion criteria were confirmed mutations in the HBV polymerase gene that confers resistance to LAM (rtM204V/I and/or rtL180M), and serum HBV DNA concentration > 12 IU/mL after combination treatment with LAM (100 mg/d) plus ADV (10 mg/d) for at least 6 mo that was ongoing at the time of randomization. Patients were expected to have well-preserved liver function (Child-Pugh score  $\leq 6$ ) and no history of ascites, variceal bleeding, or encephalopathy.

Patients were excluded if they had previous or current HCC; prior treatment with an antiviral agent other than LAM and/or ADV; coinfection with hepatitis C, hepatitis D, or human immune deficiency virus; concurrent systemic corticosteroids or other immunosuppressive agents; history of alcohol or substance abuse; or other current liver diseases, prior organ transplantation, or a history of malignancy within 3 years.

### Study design

This was a randomized, active-control, open-label, single-center, parallel trial. All eligible patients were enrolled in this study in Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, between March 2010 and March 2011. Patients were randomized to either switching to LdT (600 mg/d orally) plus ADV (10 mg/d orally) (LdT + ADV group) or to continuation with LAM (100 mg/d orally) plus ADV (10 mg/d orally) (LAM + ADV group), and were followed for 48 wk. Randomized patients were evaluated at baseline and week 12, 24, 36 and 48. At each visit, hematology, biochemistry, and prothrombin time/international normalized ratio were assessed. HBV DNA level was measured at baseline and week 12, 24, 36 and 48, using a real-time Polymerase Chain Reaction assay (Abbott Laboratories, Chicago, IL) with a linear dynamic detection range of 12 to  $1 \times 10^9$  IU/mL. Multiplex Restriction fragment mass polymorphism (RFMP) assays of the HBV genome were performed to detect LAM and ADV resistance mutations at baseline and at times as needed<sup>[23]</sup>. Because over 98%

of South Korean patients with CHB have HBV genotype C<sup>[24,25]</sup>, HBV genotype was not determined. HBeAg and anti-HBeAb were assessed at baseline and at week 48, using commercially available enzyme immunoassays (Abbott Laboratories)<sup>[26,27]</sup>. The upper limit of normal (ULN) alanine aminotransferase (ALT) was defined as 40 IU/L. Occurrences of adverse events were assessed at every visit through week 48.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the Guidelines for Good Clinical Practice as well as local regulatory requirements. This study was approved by the Institutional Review Board of Yonsei University of Medical College, and written informed consent was obtained from all patients. This study was registered at ClinicalTrials.gov, number NCT01270165 (<http://www.clinicaltrials.gov/ct2/show/NCT01270165>).

### Study endpoints

The primary endpoint was the proportion of patients in each treatment group who achieved virologic response (serum HBV DNA concentration of < 12 IU/mL) at week 48. Secondary endpoints included mean reduction from baseline in serum HBV DNA concentration at week 48, the proportion of patients with normalized serum ALT levels, HBeAg loss or seroconversion at week 48, and emergence of resistance mutation to drug during study period.

### Statistical analysis

The variables were expressed as mean with SD or ranges, or *n* (%), as appropriate. The  $\chi^2$  or Fisher's exact test and the Mann-Whitney *U* test were used to compare categorical and continuous variables, respectively. Paired related data were analyzed using the Wilcoxon paired test. A two-sided *P* value < 0.05 was considered to indicate statistical significance. All statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Baseline characteristics of patients

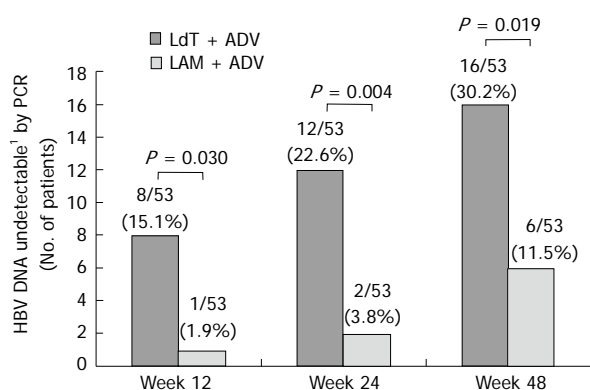
One hundred and ten patients were screened from March 2010 to March 2011, and 106 were randomized (53 in each group). All patients completed 48 wk of treatment after randomization; thus, data from all 106 patients randomized were available for the intention-to-treat analysis (Figure 1).

Overall baseline characteristics of all patients as well as of each group are shown in Table 1. Twenty-four (22.6%) patients had cirrhosis with well-preserved liver function. The mean (SD) serum HBV DNA levels was 3.71 (1.46) log<sub>10</sub> IU/mL. The mean (ranges) duration of LAM + ADV treatment prior to randomization was 17.1 (6-45) mo. At baseline, all patients had LAM resistance mutations, including 27 (25.5%) with rtM204I alone, 1 (0.9%) with rtM204I + rtM204V, 28 (26.4%) with rtM204I + rtL180M, 28 (26.4%) with rtM204V + rtL180M and 22 (20.8%) with rtM204I + rtM204V +

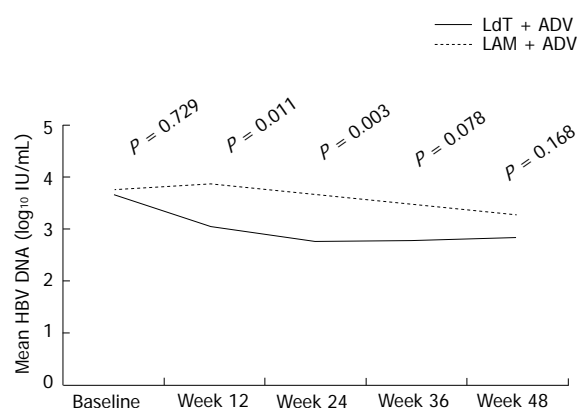
**Table 1** Baseline characteristics of patients *n* (%)

Variables	Total ( <i>n</i> = 106)	LdT + ADV ( <i>n</i> = 53)	LAM + ADV ( <i>n</i> = 53)	<i>P</i> value
Mean age, yr	46.3 (22-76)	49.0 (23-76)	43.7 (22-73)	0.053
Male	79 (74.5)	42 (79.2)	37 (69.8)	0.265
Liver cirrhosis	24 (22.6)	13 (24.5)	11 (20.8)	0.647
Laboratory results				
AST (IU/L)	29 (7-119)	33 (7-119)	28 (10-92)	0.125
ALT (IU/L)	28 (13-125)	26 (15-84)	29 (13-125)	0.098
Total bilirubin (mg/dL)	0.7 (0.3-1.8)	0.7 (0.3-1.8)	0.8 (0.3-1.6)	0.382
Albumin (g/dL)	4.5 (0.7-5.4)	4.4 (3.4-5.4)	4.6 (0.7-5.1)	0.777
Prothrombin time	1.01 (0.91-1.42)	1.00 (0.91-1.42)	1.02 (0.93-1.24)	0.917
Platelet count ( $\times 10^9$ /L)	175 (45-293)	175 (67-290)	174 (45-293)	0.610
AFP (ng/mL)	2.87 (0.86-57.54)	2.61 (1.57-11.66)	2.98 (0.86-57.54)	0.030
Mean prior LAM period, mo (range)	32.2 (8-139)	31.5 (8-77)	33.5 (11-139)	0.695
Mean prior LAM + ADV period, mo (range)	17.1 (6-45)	18.3 (6-45)	14.9 (6-39)	0.131
YMDD mutation	106 (100)	53 (100)	53 (100.0)	-
rtM204I alone	27 (25.5)	13 (24.5)	14 (26.4)	0.643
rtM204I + rtM204V	1 (0.9)	1 (1.9)		
rtM204I + rtL180M	28 (26.4)	15 (28.4)	13 (24.5)	
rtM204V + rtL180M	28 (26.4)	12 (22.6)	16 (30.2)	0.501
rtM204I + rtM204V + rtL180M	22 (20.8)	12 (22.6)	10 (18.9)	0.514
eGFR (mL/min per 1.73 m <sup>2</sup> )	89.1 (56.1-131.6)	89.8 (59.8-131.6)	85.7 (56.1-123.3)	0.437
Serum HBV DNA (log <sub>10</sub> IU/mL)				0.729
Mean (SD)	3.71 (1.46)	3.66 (1.65)	3.76 (1.25)	
Median (range)	3.63 (1.32-8.10)	3.34 (1.32-8.10)	3.78 (1.41-5.94)	

Data expressed as mean (SD), mean (range) or median (range). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LAM: Lamivudine; ADV: Adefovire; HBV: High hepatitis B virus; eGFR: Estimated glomerular filtration rate; LdT: Telbivudine.



**Figure 2** Proportion of patients with undetectable serum hepatitis B virus DNA (< 12 IU/mL) over time. <sup>1</sup>Undetectable < 12 IU/mL. LAM: Lamivudine; ADV: Adefovire; HBV: High hepatitis B virus; LdT: Telbivudine; PCR: Polymerase chain reaction.



**Figure 3** Mean hepatitis B virus DNA levels over time in the two groups. LAM: Lamivudine; ADV: Adefovire; HBV: Hepatitis B virus; LdT: Telbivudine.

rtL180M. There were no genotypic mutations of ADV in all patients at the baseline. Demographic and laboratory characteristics were similar between the two treatment groups, and mean (SD) serum HBV DNA levels in the LdT + ADV group and LAM + ADV group were 3.66 (1.65) log<sub>10</sub> IU/mL and 3.76 (1.25) log<sub>10</sub> IU/mL, respectively ( $P = 0.729$ ). There was no difference in the mean duration of prior LAM treatment as well as that of LAM + ADV treatment prior to randomization between the two groups (prior LAM period, 31.5 mo *vs* 33.5 mo,  $P = 0.695$ ; LAM + ADV period prior to randomization, 18.3 mo *vs* 14.9 mo,  $P = 0.131$ ).

### Virologic response

The efficacy of treatment in the LdT + ADV and LAM

+ ADV groups are summarized and compared in Table 2 and Figures 2-4. During treatment, the number of patients who achieved virologic response (serum HBV DNA level of < 12 IU/mL) gradually increased to 16 (30.2%) patients at week 48 in the LdT + ADV group. In contrast, the number of patients with virologic response in the LAM + ADV group was consistently lower than those in the LdT + ADV group from week 12 to week 48, and only 6 (11.5%) patients in the LAM + ADV group showed virologic response at week 48. The primary efficacy endpoint, the proportion of patients who achieved HBV DNA level of < 12 IU/mL at week 48, differed significantly between the two groups (30.2 % *vs* 11.5 %, respectively,  $P = 0.019$ ) (Figure 2 and Table 2).

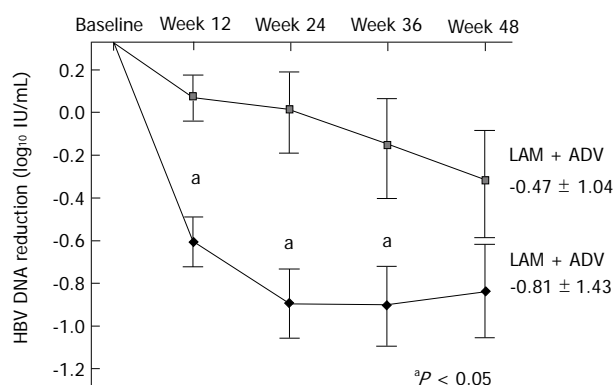
Mean (SD) serum HBV DNA level of the LdT +



**Table 2** Virologic, serologic and biochemical responses during study periods *n* (%)

Variables	Week 12		<i>P</i> value	Week 24		<i>P</i> value	Week 48		<i>P</i> value
	LdT + ADV	LAM + ADV		LdT + ADV	LAM + ADV		LdT + ADV	LAM + ADV	
Serum HBV DNA, mean (SD) (log <sub>10</sub> IU/mL)	3.05 (1.51)	3.84 (1.35)	0.011	2.79 (1.52)	3.65 (1.44)	0.003	2.85 (1.73)	3.29 (1.49)	0.168
Reductions in HBV DNA <sup>1</sup> , mean (SD) (log <sub>10</sub> IU/mL)	-0.68 (0.83)	0.07 (0.60)	< 0.001	-0.88 (1.06)	-0.11 (0.85)	< 0.001	-0.81 (1.43)	-0.47 (1.04)	0.167
HBV DNA undetectable <sup>2</sup>	8 (15.1)	1 (1.9)	0.030	12 (22.6)	2 (3.8)	0.004	16 (30.2)	6 (11.5)	0.019
Virologic nonresponders <sup>3</sup>	-	-	-	33 (62.3)	48 (90.6)	0.001	-	-	-
HBsAg loss	0 (0)	0 (0)	-	0 (0)	0 (0)	-	0 (0)	0 (0)	-
HBeAg negativity,	0 (0)	0 (0)	-	2 (3.8)	0 (0)	-	3 (5.7)	2 (3.8)	0.648
Normal range of ALT <sup>4</sup>	39 (73.6)	38 (71.7)	0.828	37 (69.8)	40 (75.5)	0.513	40 (75.5)	38 (71.7)	0.768
ALT normalization <sup>5</sup>	2/14 (14.3)	6/14 (42.9)	0.209	6/14 (42.9)	5/14 (35.7)	0.704	8/14 (57.1)	3/14 (21.4)	0.053

<sup>1</sup>Reduction of hepatitis B virus (HBV) DNA from baseline; <sup>2</sup>Defined serum HBV DNA of < 12 IU/mL; <sup>3</sup>Defined as a < 1 log<sub>10</sub> IU/mL reduction in serum HBV DNA level from baseline at 24 wk; <sup>4</sup>Upper normal limit of ALT, 40 IU/L; <sup>5</sup>Among patients who have elevated ALT levels at baseline. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LAM: Lamivudine; ADV: Adefovir. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis Be antigen.



**Figure 4** Mean reduction of serum hepatitis B virus DNA levels from baseline. Mean hepatitis B virus (HBV) DNA (log<sub>10</sub> IU/mL) were plotted over time. Error bars indicate the standard deviation (<sup>a</sup>*P* value < 0.05). LAM: Lamivudine; ADV: Adefovir; LdT: Telbivudine.

ADV group was significantly lower than that of the LAM + ADV group at week 12 and week 24 [3.05 (1.51) log<sub>10</sub> IU/mL *vs* 3.84 (1.35) log<sub>10</sub> IU/mL at week 12, *P* = 0.011; 2.79 (1.52) log<sub>10</sub> IU/mL *vs* 3.65 (1.44) log<sub>10</sub> IU/mL at week 24, *P* = 0.003] (Table 2 and Figure 3). However, there was no statistically significant difference in serum HBV DNA levels between the LdT + ADV group and the LAM + ADV group [2.85 (1.73) log<sub>10</sub> IU/mL *vs* 3.29 (1.49) log<sub>10</sub> IU/mL at 48 wk, *P* = 0.168] (Figure 3).

The mean reduction of serum HBV DNA levels from baseline to week 12 or week 24 was significantly greater in the LdT + ADV than in the LAM + ADV group (-0.68 log<sub>10</sub> IU/mL *vs* 0.07 log<sub>10</sub> IU/mL; *P* < 0.001, -0.88 log<sub>10</sub> IU/mL *vs* -0.11 log<sub>10</sub> IU/mL; *P* < 0.001, respectively) (Figure 4 and Table 2). At week 48, however, there was no significant difference in the mean reduction of serum HBV DNA from baseline between the LdT + ADV group and the LAM + ADV group (-0.81 log<sub>10</sub> IU/mL *vs* -0.47 log<sub>10</sub> IU/mL, *P* = 0.167; Table 2 and Figure 4).

The number of patients with virologic nonresponse, defined as < 1 log<sub>10</sub> IU/mL reduction in serum HBV DNA level from baseline at week 24, was significantly lower in the LdT + ADV group than in the LAM + ADV

group [33 (62.3%) *vs* 48 (90.6%), respectively, *P* = 0.001] (Table 2). A total of 8 patients experienced virologic breakthrough ( $\geq 1$  log<sub>10</sub> IU/mL increase in serum HBV DNA from nadir during treatment), 4 patients in the LdT + ADV group and 4 patients in the LAM + ADV group. Most of them (6/8) had poor compliance for taking medication, and there was no new emergence of drug resistance for LAM or ADV in the RFMP examination conducted at the time of virologic breakthrough.

### Biochemical and serologic response

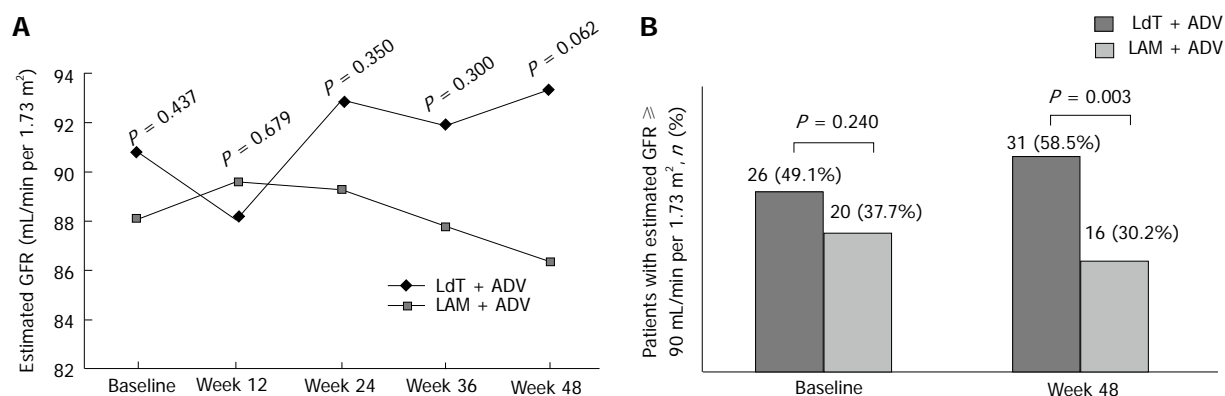
The proportion of patients with normal serum ALT levels at week 48 did not differ significantly between the LdT + ADV group and the LAM + ADV group (75.5% *vs* 71.7%, respectively; *P* = 0.768) (Table 2). Among patients with elevated ALT at baseline, the proportion of patients achieving normalized ALT at week 48 in the LdT + ADV group and LAM + ADV group were 57.1% (8/14) and 21.4% (3/14), respectively, and the difference showed borderline significance between the two groups (*P* = 0.053) (Table 2).

Three patients (5.7%) in the LdT + ADV group and 2 patients (3.8%) in the LAM + ADV group became HBeAg negative at week 48 (*P* = 0.648; Table 2). No patient achieved loss of HBsAg during the treatment period.

### Safety

The majority of patients in the LdT + ADV and LAM + ADV groups tolerated the treatment well without serious adverse events. No patient required dose reduction or discontinuation of treatment due to an adverse event. No patient experienced ALT flare ( $> 10 \times$  ULN), increased serum creatinine kinase level of  $> 150$  U/L, or serum phosphorus level of  $< 1.5$  mg/dL during the treatment period. Neither group reported decompensated cirrhosis or hepatocellular carcinoma from baseline to week 48.

No patient was found to have an elevation of creatinine  $\geq 0.5$  mg/dL. The mean estimated glomerular filtration rate (eGFR) is shown in Figure 5A. Although statistical significance did not exist, eGFR in the LdT + ADV group tended to increase during the treatment pe-



**Figure 5** Estimated glomerular filtration rate over time in the two groups (A), and proportion of patients with estimated glomerular filtration rate  $\geq 90$  mL/min per  $1.73 \text{ m}^2$  at baseline and week 48 (B). LAM: Lamivudine; ADV: Adefovir; HBV: High hepatitis B virus; LdT: Telbivudine. GFR: Glomerular filtration rate

riod, whereas that in the LAM + ADV group tended to decrease. The proportion of patients with eGFR  $\geq 90$  mL/min per  $1.73 \text{ m}^2$  in the LdT + ADV group increased from 49.1% (26/53) at baseline to 58.5% (31/53) at week 48, while that in the LAM + ADV group decreased from 37.7% (20/53) at baseline to 30.2% (16/53) at week 48. The proportion of patients with eGFR  $\geq 90$  mL/min per  $1.73 \text{ m}^2$  was significantly higher in the LdT + ADV group than in the LAM + ADV group at week 48 (58.5% *vs* 30.2%;  $P = 0.003$ ) (Figure 5B). Twenty-six percent (7/27) of the patients with baseline eGFR  $< 90$  mL/min per  $1.73 \text{ m}^2$  shifted to eGFR  $\geq 90$  mL/min per  $1.73 \text{ m}^2$  after 48 wk of LdT + ADV treatment, as compared to 15.2% (5/33) in the LAM + ADV group ( $P = 0.299$ ).

## DISCUSSION

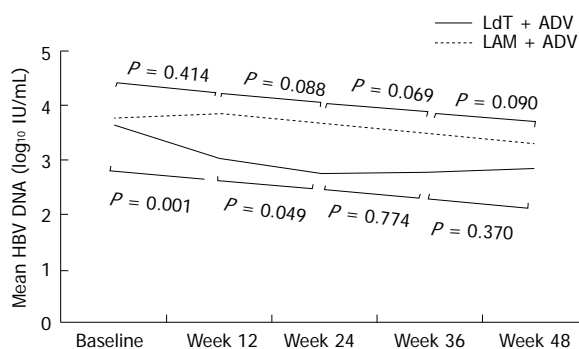
This study is the first study that provides a direct comparison of the antiviral efficacy of LdT + ADV and LAM + ADV in HBeAg-positive LAM resistant CHB patients who have suboptimal response to LAM + ADV. The results of this study show that treatment with LdT + ADV significantly suppressed HBV replication and more patients with LdT + ADV achieved virologic response compared to those with LAM + ADV after 48 wk of treatment. The difference in viral suppressive effect between the two groups was greatest at week 24, which decreased gradually thereafter.

The combination of LAM and ADV has been recommended as a treatment option for patients with LAM resistant CHB<sup>[4-6]</sup>. Because of the unavailability of TDF in many Asian countries, ADV has been used widely as a combination treatment regimen. However, due to the weak antiviral activity of ADV<sup>[28]</sup> and poor susceptibility for drug-resistant viral strains, suboptimal response is particularly common in patients who received LAM + ADV<sup>[29,30]</sup>. Evidence has shown that the persistence of suboptimal response during long-term antiviral treatment is associated with the emergence of multi-drug resistant viral strains, which could result in poorer clinical outcomes<sup>[31,32]</sup>. Thus, management of a suboptimal response to antiviral therapy has recently been of new concern,

and combination with other NAs rather than switching to monotherapy offers a potentially attractive therapeutic option<sup>[33,34]</sup>.

Based on the superior efficacy of LdT over LAM shown in the GLOBE trial<sup>[35]</sup>, a recent study examined switching patients who remained viremic under LAM treatment to LdT and demonstrated that early ( $\leq 24$  wk) switch to LdT improves virologic outcomes in CHB patients with persistent viral replication under LAM treatment<sup>[36]</sup>. In addition, previous two independent short-term studies on patients with poor response to ADV monotherapy demonstrated that a higher proportion of patients in the LdT + ADV group achieved a virological response at week 24 than did patients in the LAM + ADV group<sup>[37,38]</sup>. Based on these prior reports, we conducted this study to investigate the efficacy of switching to LdT + ADV as a substitute therapeutic option for patients who showed a suboptimal response to LAM + ADV combination treatment.

In our study, patients who were switched to LdT + ADV had a superior virologic response at 48 wk compared to those who continued LAM + ADV treatment (30.2% *vs* 11.5%,  $P = 0.019$ ). At 48 wk, the mean serum HBV DNA level was lower and the mean reduction from baseline was greater in the LdT + ADV group than in the LAM + ADV (2.85 log<sub>10</sub> IU/mL *vs* 3.29 log<sub>10</sub> IU/mL and -0.81 log<sub>10</sub> IU/mL *vs* -0.47 log<sub>10</sub> IU/mL, respectively), but the differences between the two groups were not statistically significant ( $P = 0.168$  and  $P = 0.167$ , respectively). As described in Table 2, however, differences of both the mean serum HBV DNA levels and the mean reduction of HBV DNA levels from baseline were significant at 12 wk and 24 wk. These results are ascribable to a different rate of decline in serum HBV DNA levels between the two groups. When we analyzed the rate of decline between adjacent time points in the respective treatment groups, we found that there were no statistically significant declines of serum HBV DNA levels as times go by in the LAM + ADV group (Figure 6). Continuing LAM + ADV with suboptimal response offers little antiviral benefit to patients with LAM-resistant HBV and as much as 90.6% of patients who continued on LAM + ADV re-



**Figure 6** Serial changes of mean serum hepatitis B virus DNA levels. Serum hepatitis B virus DNA levels decreased significantly not only from baseline to 12 wk but also from 12 wk to 24 wk in the LdT + ADV group. LAM: Lamivudine; ADV: Adefovir; HBV: High hepatitis B virus; LdT: Telbivudine.

remained as virologic non-responders (defined as  $< 1 \log_{10}$  IU/mL reduction in baseline serum HBV DNA level at 24 wk) at week 24. In contrast, serum HBV DNA levels decreased significantly not only from baseline to 12 wk but also from 12 wk to 24 wk in the LdT + ADV group ( $P < 0.001$  and  $P = 0.049$ , respectively) (Figure 6). This suggests that the viral suppressive effect emerged in the LdT + ADV group particularly during the early treatment period.

Considering the significantly decreased serum HBV DNA level in the LdT + ADV group during the early treatment period, we speculated the good virologic response in this group may be related to the effect of LdT, because evidence in treatment-naïve patients demonstrates that LdT could significantly increase the rate of virologic response compared to LAM as well as ADV<sup>[19,39,40]</sup>. However, the rate of serum HBV DNA level decline in the LdT + ADV group decreased and became dull at the later part of the study period, which is from week 24 to week 48. It might be correlated to diminished susceptibility to NAs and generally unsatisfying clinical outcomes in a pretreated population with drug-resistant HBV compared to a treatment-naïve population<sup>[41-43]</sup>. Another possible explanation is that the emergence of ADV-resistant HBV strains following suboptimal response to LAM + ADV might attenuate the superior viral suppressive effect of LdT to that of LAM in these study patients. This is supported by a recent study which reported no differences in virologic and biochemical responses in the comparison of two treatments, LdT + ADV and LAM + ADV, in CHB patients with suboptimal response to ADV monotherapy<sup>[44]</sup>.

HBeAg loss is the key goal of antiviral therapy for HBeAg-positive CHB patients, which indicates good prognosis, including lower rates of cirrhosis and slower disease progression<sup>[3,6,45,46]</sup>. In our study, we reported quite low rates of HBeAg loss, with 5.7% in the LdT + ADV group and 3.8% in the LAM + ADV group, suggesting that this pretreated population is particularly refractory to serologic response. It has been reported that HBeAg loss is less common in patients with LAM-resistant mutation

than in those with wild-type HBV, regardless of the adequate rescue therapy<sup>[41-43]</sup>.

Both treatments were well tolerated and showed similar safety profiles. Patients who switched from LAM + ADV to LdT + ADV did not experience any additional spectrum of adverse effects. Interestingly, we found that the patients in the LdT + ADV group showed a favorable effect of improved renal function compared to those in the LAM + ADV group during the treatment period. Although the mechanism has not been clarified, there have been several reports that LdT treatment is associated with renoprotective effects in patients with CHB<sup>[47,48]</sup>. Considering the risk of renal impairment of ADV, the renoprotective effect of LdT could be complementary in patients who were treated with ADV for a long term period.

There are some limitations in this study. First, this prospective study has small sample size and a potential bias. Relatively short follow-up duration was another limitation. Thus, further well controlled studies with sufficient size and longer duration of follow-up are needed.

In conclusion, this trial demonstrated that switching from LAM + ADV to LdT + ADV in LAM-resistant CHB patients with suboptimal response resulted in superior virologic response, renoprotective effect and similar safety profiles at week 48. These results suggest that CHB patients with LAM-resistant HBV and suboptimal response to LAM + ADV treatment should be considered for switching to other combination regimens using more potent drugs. LdT + ADV could be a therapeutic option for patients who are unable to use TDF for any reason. However, a stronger rescue combination therapy should be investigated in this population.

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## COMMENTS

### Background

A substantial proportion of patients treated with lamivudine (LAM) + adefovir (ADV) combination therapy show a suboptimal virologic response. Because there has been evidence that this suboptimal response to antiviral therapy might have clinical relevance to higher risk of developing resistance to long-term antiviral treatment, suboptimal response to nucleotide analogues, in addition to drug resistance, has also become a new challenge for the management of chronic hepatitis B (CHB) patients. However, there is no standard optimal strategy for the management of suboptimal response to nucleotide analogue therapy at present.



## Research frontiers

This study is the first study that provides a direct comparison of the antiviral efficacy of telbivudine (LdT) + ADV and LAM + ADV in hepatitis Be antigen-positive LAM resistant CHB patients who have suboptimal response to LAM + ADV.

## Innovations and breakthroughs

Our results demonstrated that switching from LAM + ADV to LdT + ADV in LAM-resistant CHB patients with suboptimal response resulted in superior virologic response, renoprotective effect and similar safety profiles at week 48.

## Applications

From our study, it was suggested that LdT + ADV could be a therapeutic option for patients who are unable to use tenofovir disoproxil fumarate for any reason.

## Peer review

The authors described a comparison of combination therapy of telbivudine plus adefovir vs lamivudine plus adefovir. This is the first comparison study about these 2 different combination therapies. This information is very important for future antiviral therapy of chronic B hepatitis. Furthermore, the study design is well organized and data is analyzed very well.

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## 8-bromo-7-methoxychrysin inhibits properties of liver cancer stem cells *via* downregulation of $\beta$ -catenin

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### Abstract

**AIM:** To evaluate whether 8-bromo-7-methoxychrysin (BrMC), a synthetic analogue of chrysin, inhibits the properties of cancer stem cells derived from the human liver cancer MHCC97 cell line and to determine the potential mechanisms.

**METHODS:** CD133<sup>+</sup> cells were sorted from the MHCC97 cell line by magnetic activated cell sorting, and amplified in stem cell-conditioned medium to obtain the enriched CD133<sup>+</sup> sphere forming cells (SFCs). The stem cell properties of CD133<sup>+</sup> SFCs were validated by the tumorsphere formation assay *in vitro* and the xenograft nude mouse model *in vivo*, and termed liver cancer stem cells (LCSCs). The effects of BrMC on LCSCs *in vitro* were evaluated by MTT assay, tumorsphere formation assay and transwell chamber assay. The effects of BrMC on LCSCs *in vivo* were determined using

a primary and secondary xenograft model in Balb/c-nu mice. Expressions of the stem cell markers, epithelial-mesenchymal transition (EMT) markers and  $\beta$ -catenin protein were analyzed by western blotting or immunohistochemical analysis.

**RESULTS:** CD133<sup>+</sup> SFCs exhibited stem-like cell properties of tumorsphere formation and tumorigenesis capacity in contrast to the parental MHCC97 cells. We found that BrMC preferentially inhibited proliferation and self-renewal of LCSCs ( $P < 0.05$ ). Furthermore, BrMC significantly suppressed EMT and invasion of LCSCs. Moreover, BrMC could efficaciously eliminate LCSCs *in vivo*. Interestingly, we showed that BrMC decreased the expression of  $\beta$ -catenin in LCSCs. Silencing of  $\beta$ -catenin by small interfering RNA could synergize the inhibition of self-renewal of LCSCs induced by BrMC, while Wnt3a treatment antagonized the inhibitory effects of BrMC.

**CONCLUSION:** BrMC can inhibit the functions and characteristics of LCSCs derived from the liver cancer MHCC97 cell line through downregulation of  $\beta$ -catenin expression.

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**Key words:** Liver cancer; Cancer stem cell; 8-bromo-7-methoxychrysin; Self-renewal;  $\beta$ -catenin

**Core tip:** We successfully obtained liver cancer stem cells (LCSCs) from the liver cancer MHCC97 cell line by employing the combination of magnetic activated cell sorting and tumorsphere culture. We showed for the first time that 8-bromo-7-methoxychrysin (BrMC), a synthetic analogue of chrysin, could preferentially inhibit proliferation and self-renewal, suppress epithelial-mesenchymal transition and invasion of LCSCs, and further eradicate LCSCs *in vivo*. The results of this study support the use of BrMC for liver cancer chemopreven-

tion or chemotherapy.

Quan MF, Xiao LH, Liu ZH, Guo H, Ren KQ, Liu F, Cao JG, Deng XY. 8-bromo-7-methoxychrysin inhibits properties of liver cancer stem cells *via* downregulation of  $\beta$ -catenin. *World J Gastroenterol* 2013; 19(43): 7680-7695 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7680.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7680>

## INTRODUCTION

Human liver cancer is the fifth most common cancer in the world and the third leading cause of cancer-related death<sup>[1,2]</sup>. Although surgery, liver transplantation or chemotherapy offers the possibility of prolonged survival for liver cancer patients, mortality still remains high, largely due to recurrence and drug-resistance<sup>[3,4]</sup>. According to the cancer stem cell hypothesis, this is thought to be due to the survival of a population of chemoresistant cells within the tumor, the cancer stem cells (CSCs) in liver cancer, that are able to regenerate the tumor following chemotherapy<sup>[5]</sup>. However, most currently available therapeutic approaches, including chemotherapy and radiotherapy, lack the ability to effectively kill these CSCs, which may eventually lead to the disease relapse and metastasis<sup>[6,7]</sup>. A number of previous studies have suggested that CD133, originally identified as a hematopoietic stem cell marker, could be used to isolate liver cancer stem cells (LCSCs) from human liver cancer cell lines, xenograft tumors and primary liver cancer specimens<sup>[8-13]</sup>. These CD133<sup>+</sup> liver cancer cells possess many stem cell properties, including extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. Thus, this minor subpopulation of CD133<sup>+</sup> LCSCs may contribute to the high recurrence rate of liver cancer. Therefore, the identification of a compound that can target LCSCs is one of the main steps in improving overall survival of liver cancer patients.

More recently, a number of studies have found that several dietary compounds can directly or indirectly inhibit cancer stem cell self-renewal pathways<sup>[14]</sup>. For example, natural flavonoid, genistein and a synthetic derivative of daidzein, N-t-boc-daidzein, have been reported to possess inhibitory activity against prostate and epithelial ovarian CSCs, respectively<sup>[15,16]</sup>. Chrysin (5,7-dihydroxyflavone), a naturally widely distributed flavonoid, has been shown to possess promising effects on the inhibition of proliferation and induction of apoptosis in a variety of cancer cells<sup>[17]</sup>. 8-bromo-7-methoxychrysin (BrMC) is a synthetic derivative of chrysin, and our previous study demonstrated the effect of BrMC in inhibiting proliferation and induction of apoptosis in colon, gastric and liver cancer cells was stronger than that of chrysin<sup>[18-21]</sup>.

In this study, we investigated the inhibitory effects of BrMC on the characteristics of LCSCs. We showed for the first time that BrMC was able to inhibit cancer

stem cell-like properties of LCSCs and eliminate LCSCs *in vivo*. We also found that BrMC significantly decreased  $\beta$ -catenin expression in LCSCs and knockdown of  $\beta$ -catenin expression could synergize the inhibition of self-renewal of LCSCs induced by BrMC. Together, our results indicated that the downregulation of  $\beta$ -catenin expression appeared to contribute to the inhibitory effects of BrMC on the properties of LCSCs.

## MATERIALS AND METHODS

### Cell culture and reagents

The human liver cancer MHCC97 cell line was purchased from Fuxiang Biotechnology Co., Ltd. (Shanghai, China). MHCC97 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA, United States) in an incubator containing 5% CO<sub>2</sub> at 37 °C. Wnt3a-conditioned medium was prepared as described by Willert *et al.*<sup>[22]</sup>. BrMC was synthesized as described previously<sup>[18]</sup>. MTT was purchased from Sigma (St. Louis, MO, United States). Fetal bovine serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trypsin and DMSO were from Amersco Company (Solon, OH, United States). Antibodies used in this study were as follows: rabbit polyclonal antibodies against ZO-1 (Abcam, Cambridge, MA, United States), mouse monoclonal antibodies against N-cadherin (Upstate Co., Lake Placid, NY, United States), Vimentin (Neo Markers, Fremont, CA, United States), E-cadherin (BD Transduction Labs, Lexington, KY, United States),  $\beta$ -catenin and CD44 (Cell Signaling Technology Inc., Danvers, MA, United States),  $\beta$ -actin (Sigma Chemical Co., St Louis, MO, United States), and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States).

### Cell sorting and flow cytometry

Cell sorting was performed on MHCC97 cells using the cell surface marker CD133<sup>+</sup> with magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cells were trypsinized and washed with PBS, and suspended in Phosphate buffered saline (PBS) containing 0.5% Bovine Serum Albumin (BSA). 100  $\mu$ L Fc receptor (FCR) Blocking Reagent (anti-CD133 antibody) and 100  $\mu$ L CD133-conjugated MicroBeads (AC133, Cell Isolation Kit, Miltenyi Biotec) per 10<sup>8</sup> cells were subsequently added to the sample and incubated in parallel for 30 min on ice. After washing the cells, CD133 positive and negative fractions were each isolated through MACS separation columns. The quality of sorting was controlled by flow cytometry analysis for CD133 expression using PE-conjugated anti-human CD133 antibody and isotype control mouse IgG2b-PE (Biolegend, San Diego, CA, United States). The single cell suspension was cultured in stem cell-conditioned medium (DMEM/F12



medium supplemented with  $1 \times B27$ , 20 ng/mL EGF, 20 ng/mL bFGF, 0.4% BSA, 4  $\mu$ g/mL Insulin, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin; Invitrogen) for the following assays.

### Tumorsphere culture

Single-cell suspensions were suspended at a density of 2000 cells/mL in stem cell-conditioned medium and seeded into ultralow attachment 24-well plates (Corning, NY, United States). When the diameter of the spheroid reached 50  $\mu$ m, suspension cultures were passaged every 6 d. Colonies were counted at 10 different views under a microscope (Nikon, Japan). The volume of the spheroid was estimated using  $V = (4/3) \pi R^3$ . Experiments were repeated 3 times with duplication in each experiment.

### Western blotting analysis

The procedures for preparation of whole cell lysates and western blotting analysis have been previously described<sup>[23]</sup>. Mouse anti-human  $\beta$ -catenin, N-cadherin, vimentin, E-cadherin, ZO-1, CD133, CD44 and  $\beta$ -actin antibodies were used as primary antibodies. Signals were visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, United States).  $\beta$ -actin was used as an internal control. Images were scanned, followed by densitometry analysis with UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, United States).

### MTT assay

CD133<sup>+</sup> sphere-forming cells (SFCs) or parental MHCC97 cells were seeded in a 96-well plate pre-coated with 0.6% agarose at a density of 5000 cells/well as described previously<sup>[24]</sup>. One day after plating, various concentrations of BrMC (0.1, 0.3 1.0, 3.0 or 10.0  $\mu$ mol/L) were added to each well and the culture continued for 48 h. After removal of the medium, cells were incubated with 5 mg/mL of MTT for 4 h. Cells were then extracted with acidic isopropanol and the absorbance at 570 nm ( $A_{570}$ ) was measured by means of an enzyme-labeling instrument (EXL-800 type). The relative cell proliferation inhibition rate = (average  $A_{570}$  of the experimental group/average  $A_{570}$  of the control group)  $\times$  100%.

### Matrigel invasion assay

The invasion ability of tumor cells was examined *in vitro* using a transwell chamber system with 8.0  $\mu$ m pore polycarbonate filter inserts (Corning Coster, Cambridge, MA, United States). The lower side of the filter was coated with 10  $\mu$ L gelatin (1 mg/mL), and the upper side was coated with 10  $\mu$ L of Matrigel. Parental MHCC97 cells or LCSCs ( $2 \times 10^3$ ) were placed in the upper part of the filter. 10% fetal bovine serum was added in the lower part of the chamber as a chemical attractant. The chamber was then incubated at 37 °C for 48 h. Cells that could not invade through the filter were removed with a cotton swab. The cells in the lower part of the chamber were fixed with methanol and stained with crystal violet. The invasiveness of tumor cells was determined by counting

the total number of cells on the lower side of the filter at 100  $\times$  magnification. In the drug-intervention experiment, cells were pretreated with different concentrations of BrMC for 24 h prior to the transwell chamber assay.

### In vivo tumorigenicity experiments

Pathogen-free Balb/c-nu mice aged 5-6 wk were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All animal studies were performed in accordance with the standard protocols approved by the Ethical Committee of Hunan Normal University and the Committee of Experimental Animal Feeding and Management. Mice were randomly divided into 3 groups (4 mice/group) and maintained under standard conditions, according to the standard protocols. Cells were suspended in serum free-DMEM/Matrigel (BD Biosciences, San Jose, CA, United States) mixture (1:1 volume). Each recipient Balb/c-nu mouse was inoculated subcutaneously with various numbers of CD133<sup>+</sup> SFCs ( $2 \times 10^3$ ,  $1 \times 10^4$  and  $1 \times 10^5$  cells) in one flank and parental MHCC97 cells ( $1 \times 10^4$ ,  $1 \times 10^5$  and  $1 \times 10^6$ ) in the other. Tumorigenicity experiments were terminated 2 mo after cell inoculation. Tumor size were measured with a caliper, and the volume was calculated using  $V (\text{mm}^3) = L \times W^2 \times 0.5$ . Harvested tumors were imaged and weighed immediately. Specimens from tumor tissue samples were fixed in 10% neutral buffered formalin, processed in paraffin blocks, and sectioned. The sections were stained with hematoxylin and eosin (HE) and examined for the histopathology.

For BrMC treatment studies,  $5 \times 10^4$  LCSCs per mouse were injected subcutaneously. Two weeks after inoculation, animals were randomly divided into 4 groups. One group underwent daily gastric lavage with refined olive oil as control, and the other 3 groups were treated with 12.5, 25 or 50 mg/kg BrMC. After 20 d of treatment, living cells from the primary tumors were dissociated and injected into 3 groups of mice (4 mice per group). Each mouse was implanted with  $5 \times 10^4$  cells from the control group and from the 50 mg/kg BrMC treated group in each flank. The growth of tumors was monitored, and tumor volumes were measured every 3 d. Animals were humanely sacrificed when the larger of the two tumors reached 500 mm<sup>3</sup>.

### Immunohistochemical examination

For immunohistochemical analysis of CD44 and CD133, tissues of the LCSCs derived-tumors in the nude mouse xenograft model were performed with formalin-fixed, paraffin-embedded sectioning as previously described by Moinfar *et al.*<sup>[25]</sup>. After incubation with 1% non-fat dry milk in PBS (pH 7.4), the sections were then reacted with mouse anti-CD44 monoclonal antibody (1:250, Cell Signaling Technology Inc.) or mouse anti-CD133 monoclonal antibody (1:200, Abzoom, Dallas, TX, United States) for 1 h at room temperature followed by incubation with the secondary biotinylated antibody for 30 min. After washing, sections were subsequently incubated with streptavidin-peroxidase for 30 min. Finally, the results were vi-



sualized after a 15-min incubation with diaminobenzidine.

### RNA interference

A control non-specific small interfering RNA (siRNA) (5'-GACTTCATAAGGCGCATGC-3') and  $\beta$ -catenin siRNA (5'-AGCUGAUUUGAUGGACAGTT-3') were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Transfection of siRNA was carried out with Lipofectamine 2000 (Invitrogen Life Technologies) according to the procedure recommended by the manufacturer. Twenty-four hours after transfection, the cells were treated with DMSO (control) or BrMC at the indicated concentrations for 24 h. The cells were then collected and processed for western blotting and the tumorsphere formation assay.

## RESULTS

### Isolation and characterization of LCSCs derived from MHCC97 cell line

CD133 has previously been classified as a CSC marker in liver cancer. Therefore, we first isolated the CD133<sup>+</sup> subpopulation from MHCC97 cells by MACS. Following sorting, we examined the expression of CD133 by flow cytometry. As shown in Figure 1A, the sorted CD133<sup>+</sup> cells showed a high purity of 57.29%  $\pm$  4.61%, as compared with a purity of 1.02%  $\pm$  0.65% for CD133<sup>-</sup> counterparts and 7.21%  $\pm$  1.34% for non-sorted MHCC97 cells. To establish long-term cultures enriched in stem cells from sorted CD133<sup>+</sup>, we performed the tumorsphere assay by culturing the cells in stem cell-conditioned medium. Within 6 d of culture, we obtained liver cancer spheroids both in CD133<sup>+</sup> cells and parental MHCC97 cells (Figure 1B). As shown in Table 1, the CD133<sup>+</sup> subpopulation exhibited a 2.7- and 2.5-fold enhancement in tumorsphere formation amount and size, respectively, compared with that of parental cells, whereas, CD133<sup>-</sup> counterparts could not grow as spheroids in the nonadherent and serum-free conditions.

To further confirm the stem cell properties and functions of the CD133<sup>+</sup> SFCs, we evaluated their self-renewal capacity and tumorigenic potential. First, we measured the capacity of single cells obtained from these CD133<sup>+</sup> dissociated spheres to form secondary tumorspheres. Within 9 d of culture, we obtained new LCSC spheroids of growing undifferentiated CD133<sup>+</sup> cells (Figure 1C). These suggest an *in vitro* self-renewing capacity of CD133<sup>+</sup> SFCs. In addition, CD133<sup>+</sup> SFCs also expressed an enhanced level of stem cell markers, CD133 and CD44, compared with their parental cells (Figure 1D). Next, we evaluated the tumorigenic potential of CD133<sup>+</sup> SFCs. We investigated the ability of CD133<sup>+</sup> SFCs and parental cells to give rise to tumors in Balb/c-nu mice. As many as  $1 \times 10^6$  parental cells were needed to initiate stable tumor formation 39 d after injection, while, in contrast, as few as  $1 \times 10^4$  CD133<sup>+</sup> SFCs were sufficient to generate visible tumors only 23 d post-injection (Table 2). These data indicate that CD133<sup>+</sup> SFCs, namely LCSCs,

are more tumorigenic than their parental cells *in vivo*. Additionally, HE staining was performed and revealed similar histological characteristics in tumor xenografts derived from CD133<sup>+</sup> SFCs and their parental cells (Figure 1E).

### BrMC inhibits proliferation and self-renewal of LCSCs derived from MHCC97 cell line

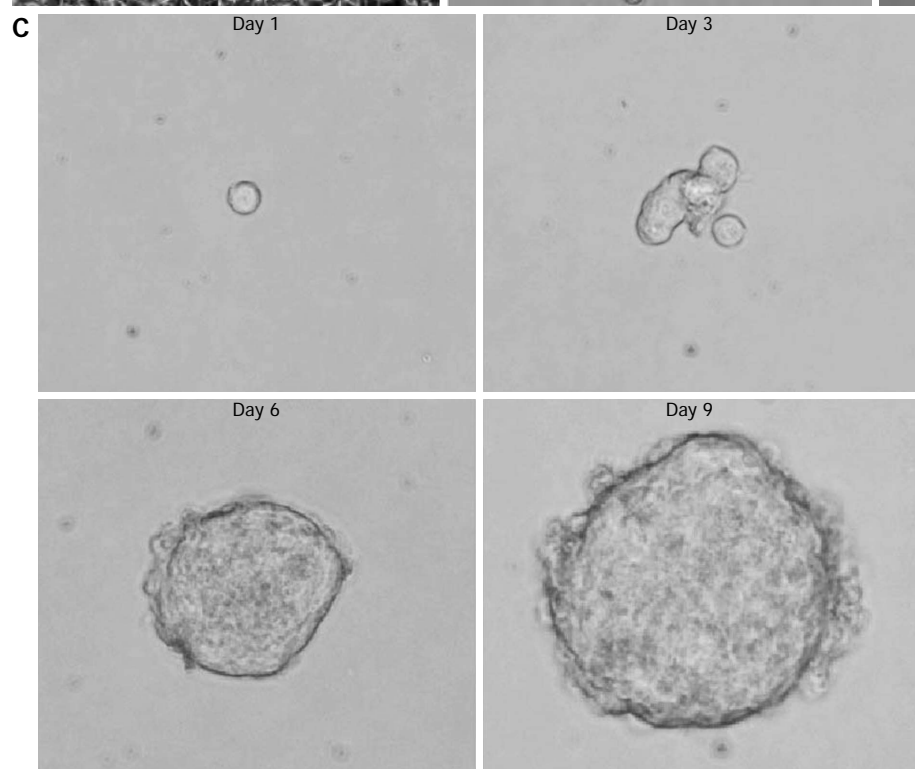
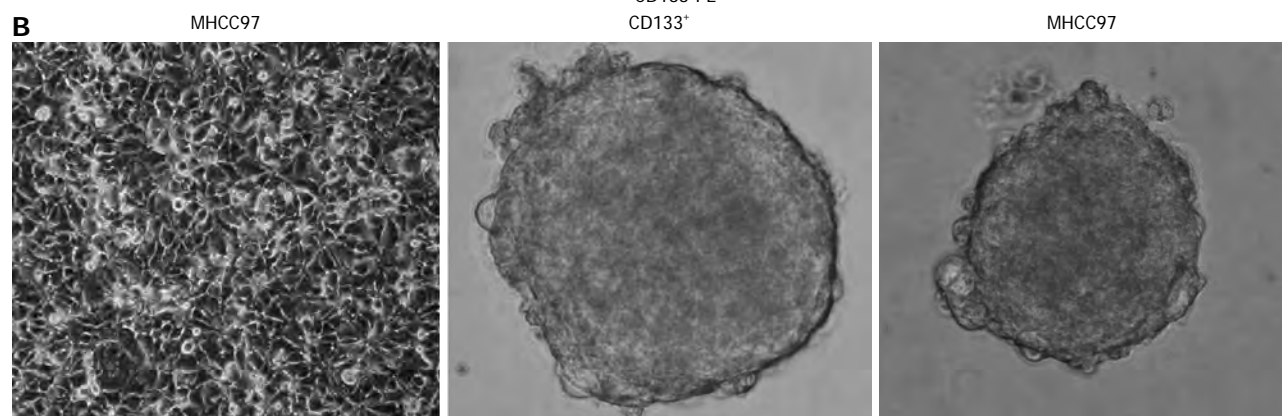
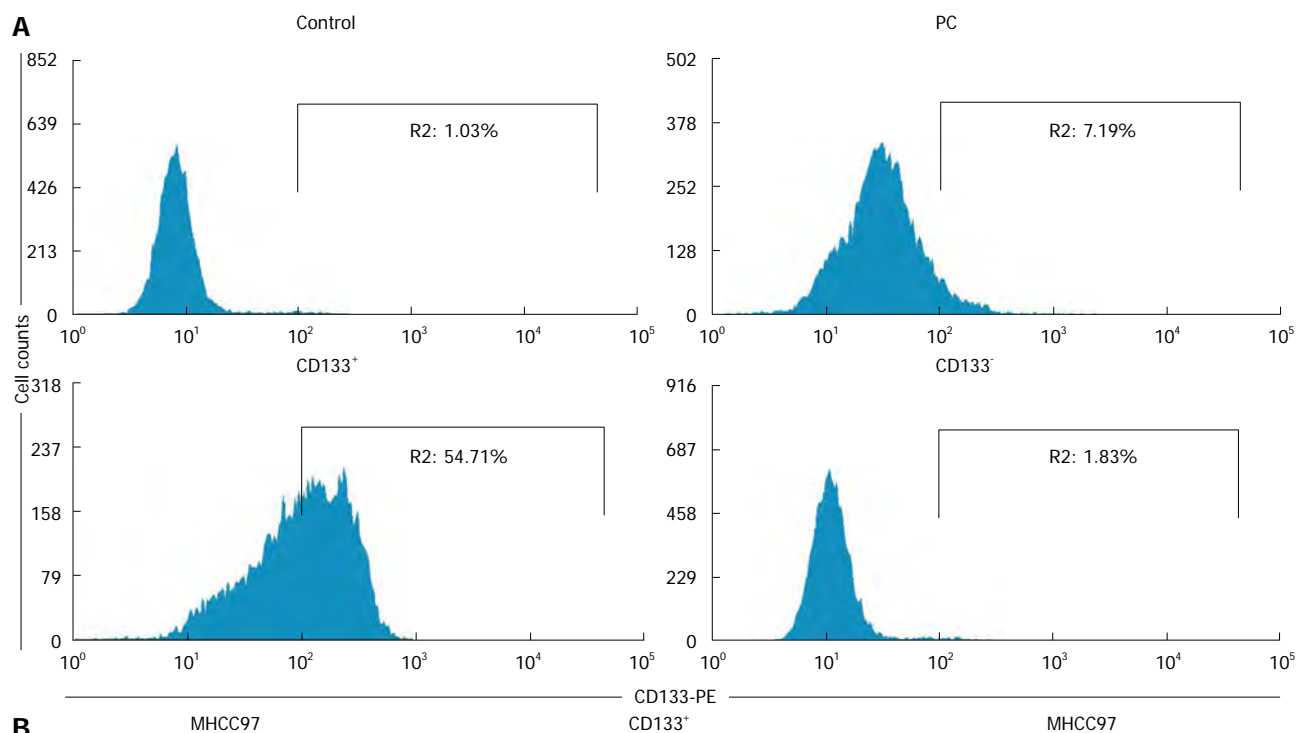
CSCs possess the property of limitless proliferative potential. A number of previous studies have demonstrated that some naturally-occurring polyphenol compounds such as genistein preferentially inhibit proliferation of pancreatic cancer stem cells<sup>[26]</sup>. In this study, we thus evaluated the anti-proliferative effects of BrMC on LCSCs derived from MHCC97 cell line by MTT assay. As shown in Figure 2A, when cells were treated with different concentrations of BrMC for 48 h, BrMC preferentially inhibited proliferation of LCSCs in a dose-dependent manner, with the IC<sub>50</sub> around 0.5  $\mu$ mol/L for LCSCs and 17.9  $\mu$ mol/L for parental MHCC97 cells.

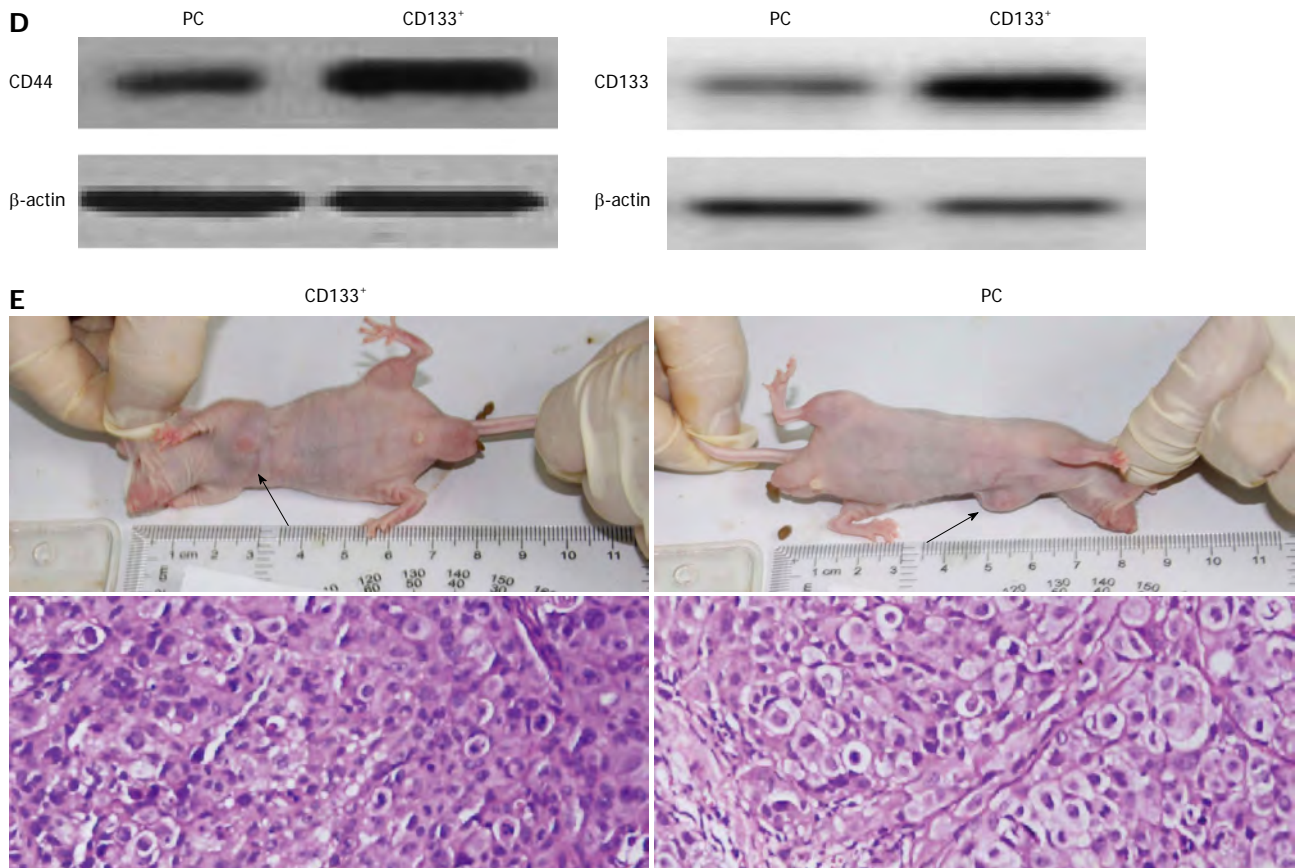
In order to evaluate whether BrMC could suppress the self-renewal of LCSCs derived from the MHCC97 cell line *in vitro*, we treated the primary tumorspheres with varying concentrations of BrMC and then removed the drug and cultured them for another passage to form the secondary spheres. Results showed that BrMC treatment resulted in a decrease both in tumorsphere number and size of LCSCs. Furthermore, a significant decrease in the number and size of the secondary tumorspheres indicated a reduced self-renewal capacity of these LCSCs by BrMC treatment (Figure 2B and C).

### BrMC inhibits Epithelial-mesenchymal transition and invasion of LCSCs derived from MHCC97 cell line

Epithelial-mesenchymal transition (EMT) is an important process during metastasis of LCSCs. Therefore, we sought to examine whether morphological changes existed between LCSCs and parental MHCC97 cells cultured adherently *in vitro*. As observed in Figure 3A, LCSCs exhibited a spindle-like shape, while parental MHCC97 cells displayed a cobble-stone-like phenotype. However, treatment with 0.1  $\mu$ mol/L BrMC suppressed EMT in LCSCs as morphological changes from a spindle-like shape to a cobble-stone-like appearance were displayed. Moreover, similar results were further confirmed by western blotting using specific antibodies against EMT-relative markers. Figure 3B shows that LCSCs expressed higher vimentin and N-cadherin protein levels, which are typically associated with mesenchymal cells, and lower expression of epithelium-associated E-cadherin and ZO-1 proteins. However, BrMC induced the upregulation of epithelial markers E-cadherin and ZO-1 and the downregulation of mesenchymal markers N-cadherin and vimentin after treatment for 24 h of LCSCs derived from MHCC97 cell line.

Since EMT has been identified as being associated with increased cancer cell invasion, we next evaluated the effect of BrMC on cell invasion of LCSCs *in vitro* using a transwell chamber coated with a Matrigel barrier. As





**Figure 1** Isolation and characterization of liver cancer stem cells derived from the MHCC97 cell line. A: Flow cytometry analysis of CD133 expression following sorting. CD133<sup>+</sup> cells from MHCC97 cells formed liver cancer spheroids in stem cell-conditioned medium (200 × magnification); B: Anchorage-dependent growth of MHCC97 cells, tumor spheroid formed by CD133<sup>+</sup> cells, tumor spheroid formed by parental MHCC97 cells; C: Secondary tumorspheres formed by single cells from dissociated primary liver spheroids (400 × magnification); D: Expression of stem cell surface markers CD44 and CD133 in CD133<sup>+</sup> sphere-forming cells (SFCs) and parental cells; E: Hematoxylin-eosin staining revealed similar histological characteristics in tumor xenografts derived from CD133<sup>+</sup> SFCs and their parental cells (100 × magnification).

**Table 1** Tumorsphere formation ability of CD133<sup>+</sup> cells derived from the MHCC97 cell line (mean ± SD, n = 3)

Cell line	Spheroid number/2 × 10 <sup>3</sup> cells		Volume of spheroid (μm <sup>3</sup> )	
	Parental cells	CD133 <sup>+</sup> cells	Parental cells	CD133 <sup>+</sup> cells
MHCC97	61 ± 26	167 ± 31 <sup>a</sup>	153 ± 31	397 ± 45 <sup>a</sup>

<sup>a</sup>P < 0.05 vs parental MHCC97 cells.

shown in Figure 3C and 3D, BrMC significantly reduced the invasiveness capacity of LCSCs in a dose-dependent manner. These results demonstrated that BrMC possesses inhibitory effects on EMT and invasion in LCSCs derived from MHCC97 cell line.

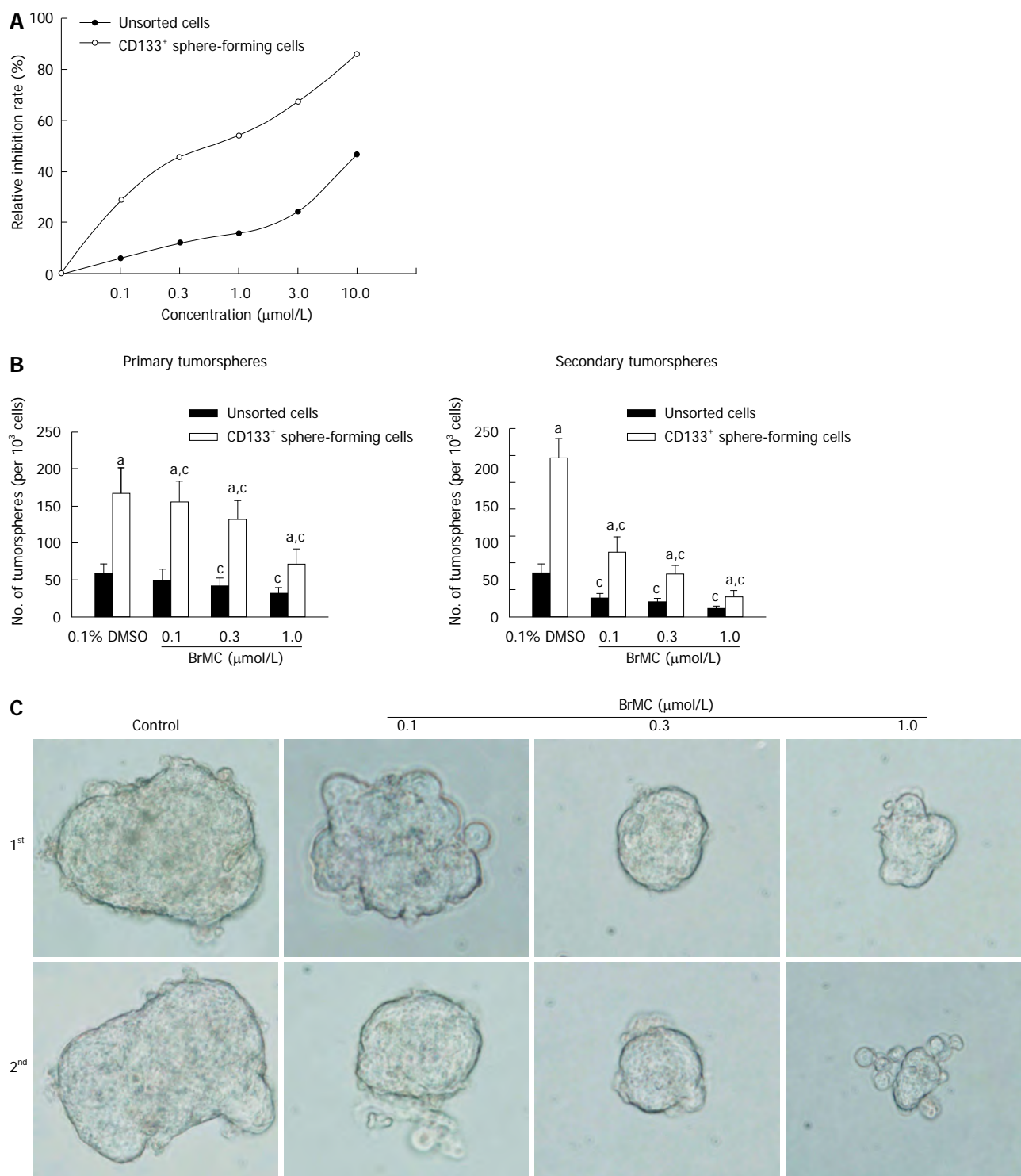
#### BrMC eliminates LCSCs derived from MHCC97 cell line *in vivo*

In order to evaluate whether BrMC could target LCSCs *in vivo*, we utilized the xenograft model of LCSCs from MHCC 97 cells in Balb/c-nu mice. Two weeks after cell inoculation with 5 × 10<sup>4</sup> LCSCs resuspended in Matrigel, animals underwent daily gastric lavage with various concentrations of BrMC. After 20 d of treatment, tumors

in 25 and 50 mg/kg BrMC-treated mice were less than 50% of the size of those in refined olive oil control animals (Figure 4A and B). Immunohistochemical analysis of CD44 and CD133 in LCSC-derived tumors revealed that the LCSC markers CD44 and CD133 were mainly expressed on the cell surface of the cancer cells, and that the tumors derived from CD133<sup>+</sup> SFCs showed significantly higher CD44 and CD133 positive rates than that of tumors derived from parental cells (Figure 4C). Furthermore, BrMC treatment can significantly decrease the CD44 and CD133 expression frequency of the tumors derived from LCSCs (Figure 4D).

To further confirm the results, we investigated the growth of secondary tumors in Balb/c-nu mice inoculated with tumor cells dissociated from primary tumor xenografts. In order to avoid possible variations due to heterogeneity, each recipient mouse was inoculated with 5 × 10<sup>4</sup> cells obtained from 50 mg/kg BrMC-treated tumors and another 5 × 10<sup>4</sup> cells obtained from control tumors in two opposite sides. Interestingly, we found that tumor cells from control animals exhibited rapid tumor re-growth, reaching a final tumor volume of 567–686 mm<sup>3</sup>. However, the tumor cells from 50 mg/kg BrMC-





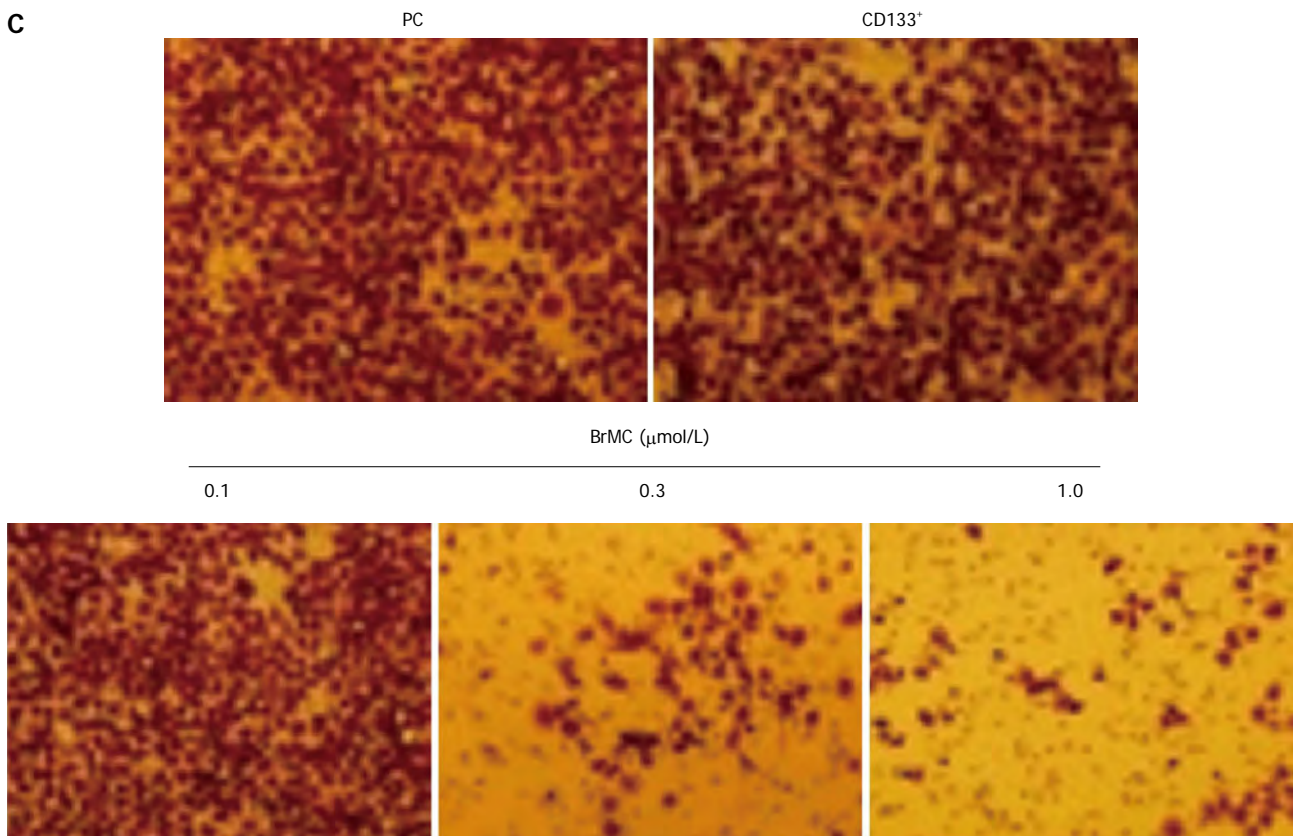
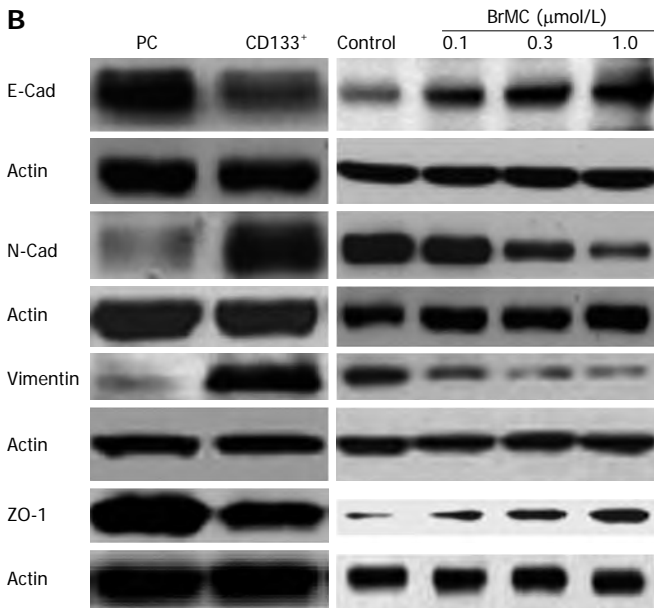
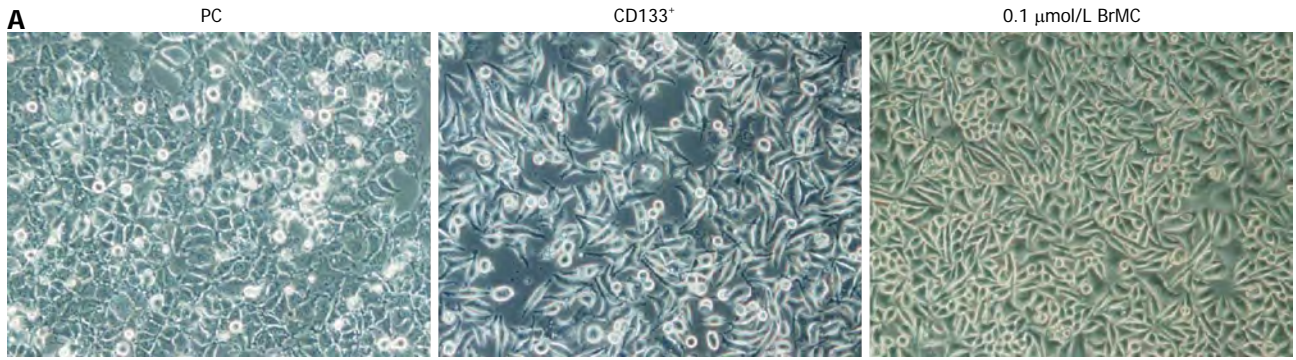
**Figure 2** Effects of 8-bromo-7-methoxychrysin on cell proliferation and self-renewal. 8-bromo-7-methoxychrysin (BrMC) inhibited proliferation (A), self-renewal (B and C) of liver cancer stem cells derived from MHCC97 cell line (mean  $\pm$  SD,  $n = 3$ ). <sup>a</sup> $P < 0.05$  vs unsorted MHCC97 cells treated with corresponding concentrations of BrMC. <sup>c</sup> $P < 0.05$  vs corresponding 0.1% DMSO treated group. Tumor sphere morphology is shown as the phase contrast image (400  $\times$  magnification).

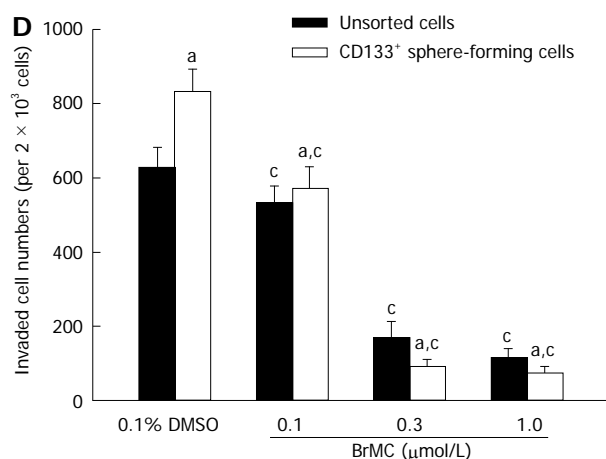
treated mice mostly failed to generate any tumors up to 33 d after inoculation (Table 3). These results suggest that BrMC was able to eliminate LCSCs in primary tumor xenografts, thereby inhibiting tumor regrowth in secondary inoculated mice.

### BrMC inhibits self-renewal in LCSCs through modulation of $\beta$ -catenin expression

To examine whether BrMC could regulate expression of stem cell markers of LCSCs, we determined the expression of CD44 and CD133 following BrMC treatment







**Figure 3** 8-bromo-7-methoxychrysin inhibition of liver cancer stem cells. 8-bromo-7-methoxychrysin (BrMC) inhibited epithelial-mesenchymal transition (EMT, A and B) and invasion (C and D) of liver cancer stem cells derived from the MHCC97 cell line (mean  $\pm$  SD,  $n = 3$ ). Cell morphological changes associated with EMT are shown as the phase contrast image (200  $\times$  magnification). <sup>a</sup> $P < 0.05$  vs unsorted MHCC97 cells treated with corresponding concentrations of BrMC, <sup>c</sup> $P < 0.05$  vs corresponding 0.1% DMSO treated group.

by western blotting analysis. Results showed that BrMC downregulated CD44 and CD133 expression in a dose-dependent manner (Figure 5A). This was in accordance with our previous immunohistochemical analysis in LCSC-derived tumors (Figure 4D).

CD44 has been shown to be a downstream target of the  $\beta$ -catenin signaling pathway<sup>[27]</sup>. Wnt/ $\beta$ -catenin signaling has been implicated in the maintenance of CSCs of liver cancer<sup>[28]</sup>. Therefore, we measured the expression level of stem cell signal molecule  $\beta$ -catenin in LCSCs and parental MHCC97 cells, and examined whether  $\beta$ -catenin was downregulated by BrMC in LCSCs. Western blotting analysis showed that  $\beta$ -catenin was highly expressed in LCSCs compared with that of parental MHCC97 cells. We also found that BrMC (0.1, 0.3, 1.0  $\mu\text{mol/L}$ ) treatment resulted in a significant decrease in  $\beta$ -catenin expression of LCSCs (Figure 5B).

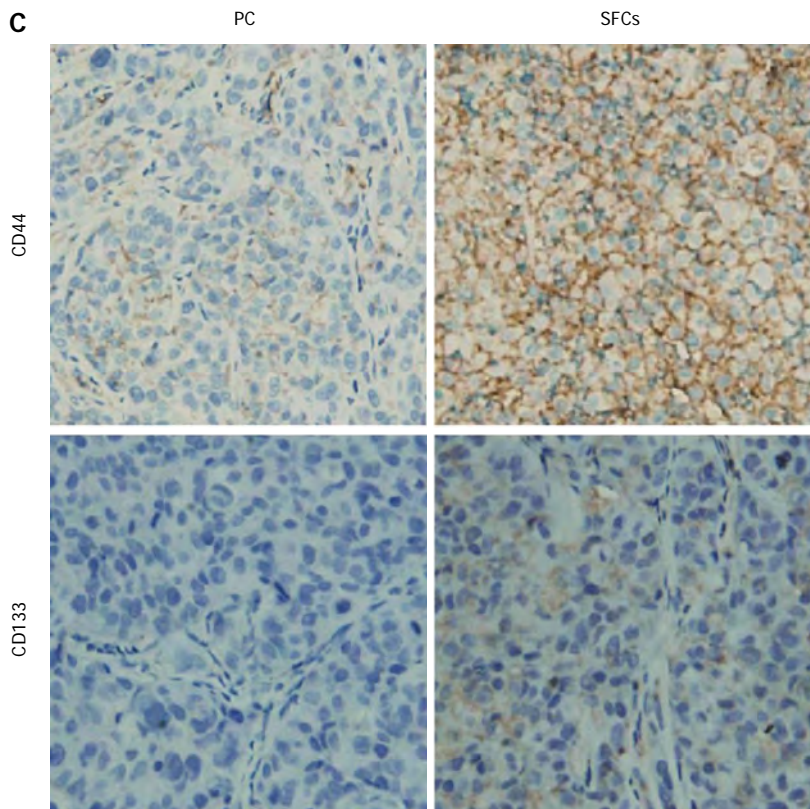
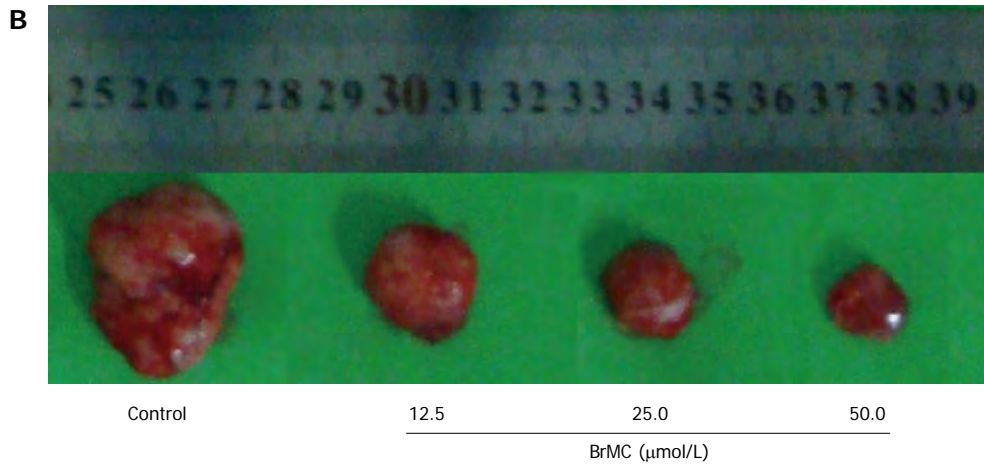
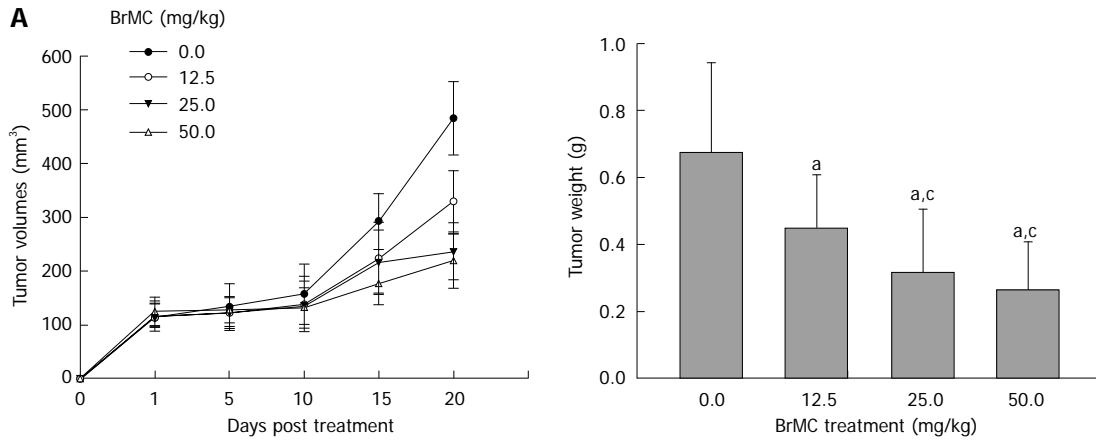
We further determined the role of  $\beta$ -catenin in the maintenance of self-renewal of LCSCs. Silencing of  $\beta$ -catenin by siRNA transfection resulted in less expression of  $\beta$ -catenin protein, as confirmed by Western blotting (Figure 5C). We also found that the downregulation of  $\beta$ -catenin expression significantly decreased the tumorsphere formation ability and inhibited expression of stem cell markers of LCSCs (Figure 5D and 5E). BrMC (0.1  $\mu\text{mol/L}$ ) plus  $\beta$ -catenin siRNA inhibited  $\beta$ -catenin expression to a greater degree compared to either alone (Figure 6A). Moreover,  $\beta$ -catenin siRNA potentiated the BrMC-induced decrease in tumorsphere formation of LCSCs (Figure 6B). We also treated LCSCs with Wnt3a, a ligand known to activate the Wnt/ $\beta$ -catenin pathway. As expected, Wnt3a induced  $\beta$ -catenin stabilization and resulted in a corresponding up-regulation of  $\beta$ -catenin in LCSCs (Figure 6C). This upregulation of  $\beta$ -catenin attenuated BrMC-induced downregulation of  $\beta$ -catenin and stem cell markers and antagonized BrMC-induced inhibition of self-renewal of LCSCs (Figure 6D, 6E and 6F). Taken together, these results provide some molecular

evidence suggesting that the downregulation of  $\beta$ -catenin expression may contribute to the inhibitory effects of BrMC on LCSCs.

## DISCUSSION

Cancer stem cells are defined as a minor population of tumorigenic cells that are capable of continuous self-renewal and differentiation, and undergo unlimited proliferation, giving rise to new tumors<sup>[29,30]</sup>. Therefore, finding compound(s) that are capable of inhibiting or killing the CSCs is extremely important to overcome tumor resistance, reduce relapse, and eventually improve overall survival. Our previous study has shown that BrMC possessed promising inhibitory effects on proliferation and apoptosis of colon, gastric and liver cancer cells. In the current study, we first successfully isolated and identified LCSCs from the liver cancer MHCC97 cell line. Further, we showed for the first time that BrMC could preferentially inhibit proliferation and self-renewal, and suppress EMT and invasion of LCSCs. Moreover, BrMC was able to eradicate LCSCs *in vivo*, as assessed by an *in vivo* tumorigenicity assay using primary and secondary Balb/c-nu mouse models. Secondly, we found that the inhibitory effects of BrMC on stem cell function and properties of LCSCs were mediated by inhibition of  $\beta$ -catenin pathways:  $\beta$ -catenin siRNA transfection and BrMC were synergistic in inhibiting the self-renewal of LCSCs. Conversely, the inhibition of Wnt3a in LCSCs resulted in an opposite effect.

More recently, CD133 has been used as a surface marker of CSCs in various solid tumors, including liver cancer. However, the function of CD133 is not entirely known yet. Thus, the single phenotypic marker CD133 is not sufficient to identify LCSCs. Tumorsphere culture may provide an alternative approach to identify and enrich LCSCs. Under non-adherent serum-free conditions *in vitro*, most tumor cells undergo programmed cell death,





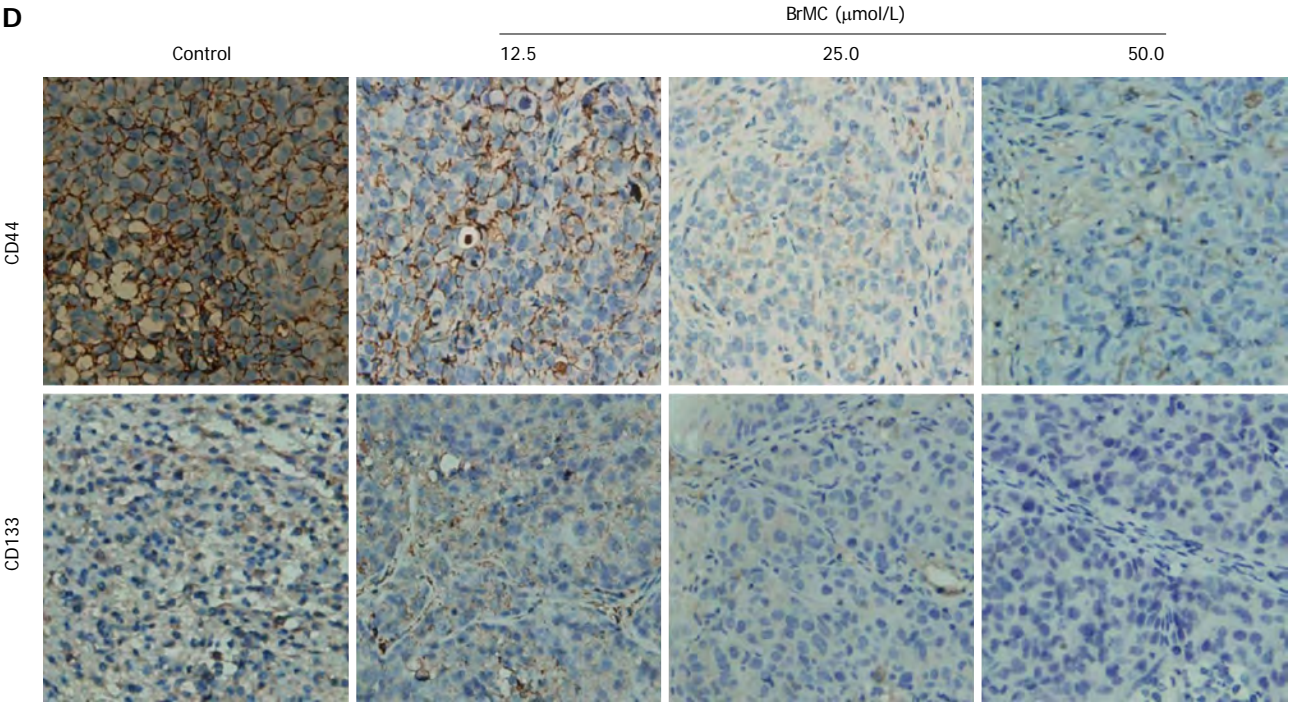


Figure 4 8-bromo-7-methoxychrysin eliminated liver cancer stem cells derived from MHCC97 cell line *in vivo*. Effects of 8-bromo-7-methoxychrysin (BrMC) on growth of primary and secondary tumor xenografts derived from liver cancer stem cells (LCSCs) (A and B, mean  $\pm$  SD,  $n = 12$ ). <sup>a</sup> $P < 0.05$  vs refined olive oil treatment model, <sup>c</sup> $P < 0.05$  vs treatment with 12.5 mg/kg BrMC. Immunohistochemical analysis of CD44 and CD133 in LCSC-derived tumors before and after BrMC treatment (C and D).

Table 2 Tumorigenicity of CD133 <sup>+</sup> sphere forming cell derived from MHCC97 cells in Balb/c-nu mice			
Cell type	No. inoculated cells	Tumor incidence <sup>1</sup>	Latency (d) <sup>2</sup>
Parental cells	1 $\times$ 10 <sup>4</sup>	0/4	-
	1 $\times$ 10 <sup>5</sup>	0/4	-
	1 $\times$ 10 <sup>6</sup>	4/4	39
CD133 <sup>+</sup> SFCs	2 $\times$ 10 <sup>3</sup>	3/4	31
	1 $\times$ 10 <sup>4</sup>	4/4	23
	1 $\times$ 10 <sup>5</sup>	4/4	8

<sup>1</sup>Number of tumors detected/number of injections; <sup>2</sup>Approximate number of days from tumor cell injection to appearance of a tumor. SFC: Sphere forming cells.

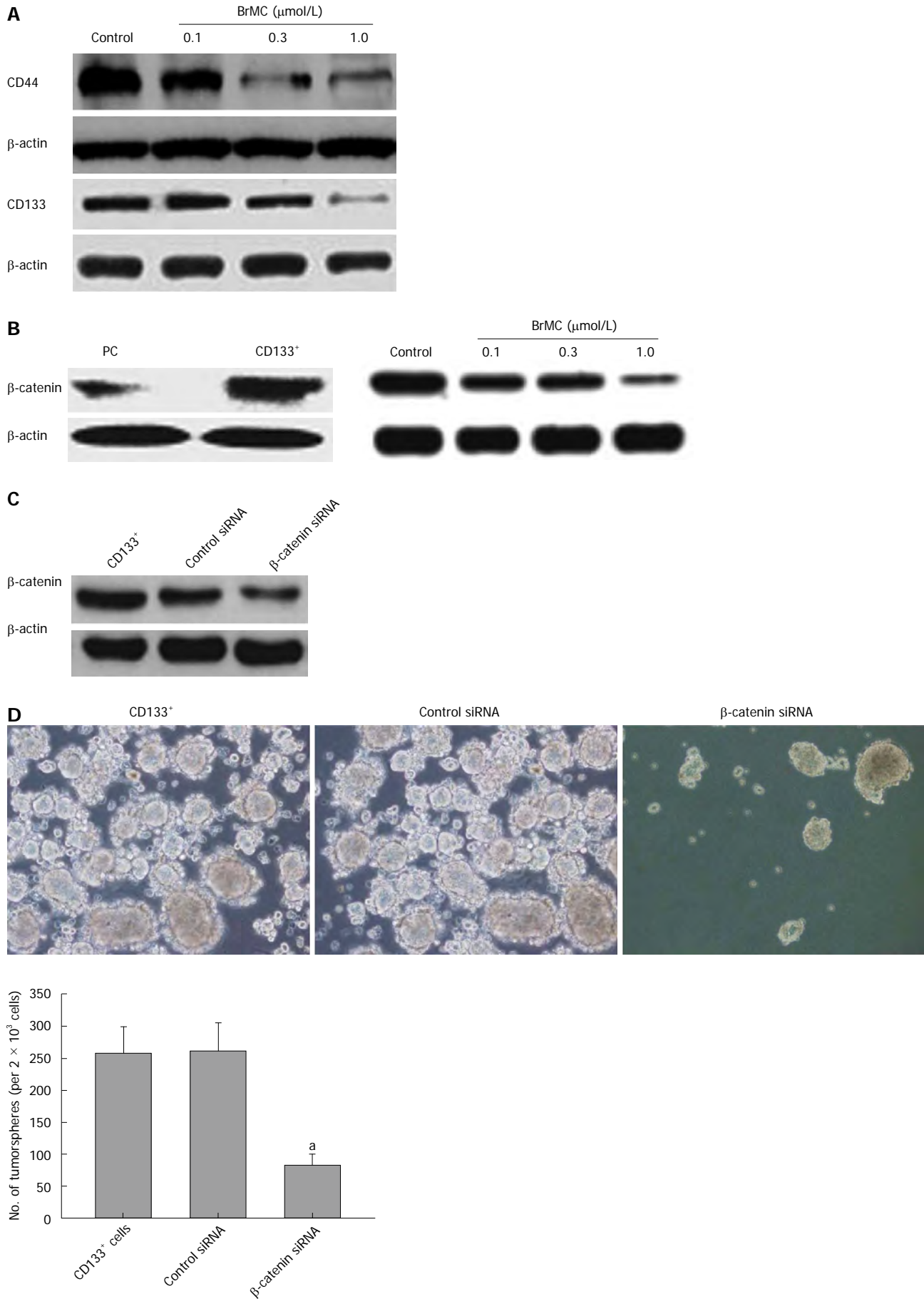
whereas the rare CSCs divide to generate multicellular 3-dimensional spheres<sup>[31,32]</sup>. This assay is a powerful tool to enrich CSCs and further assess the functional properties of the isolated CSCs. By employing a combination of this technique and MACS based on the CD133<sup>+</sup> surface marker, we have successfully obtained the putative LCSCs, namely CD133<sup>+</sup> SFCs, from the MHCC97 cell line. We demonstrated that these CD133<sup>+</sup> SFCs possess stem-like properties, including self-renewal, initiation of tumor growth in mice at very low cell numbers and a higher expression level of stem cell marker compared with their parental cells. These data indicated that the method which we used to isolate and indentify LCSCs from liver cancer cell lines may be faster, more economic and effective, compared with methods based on two or more surface markers.

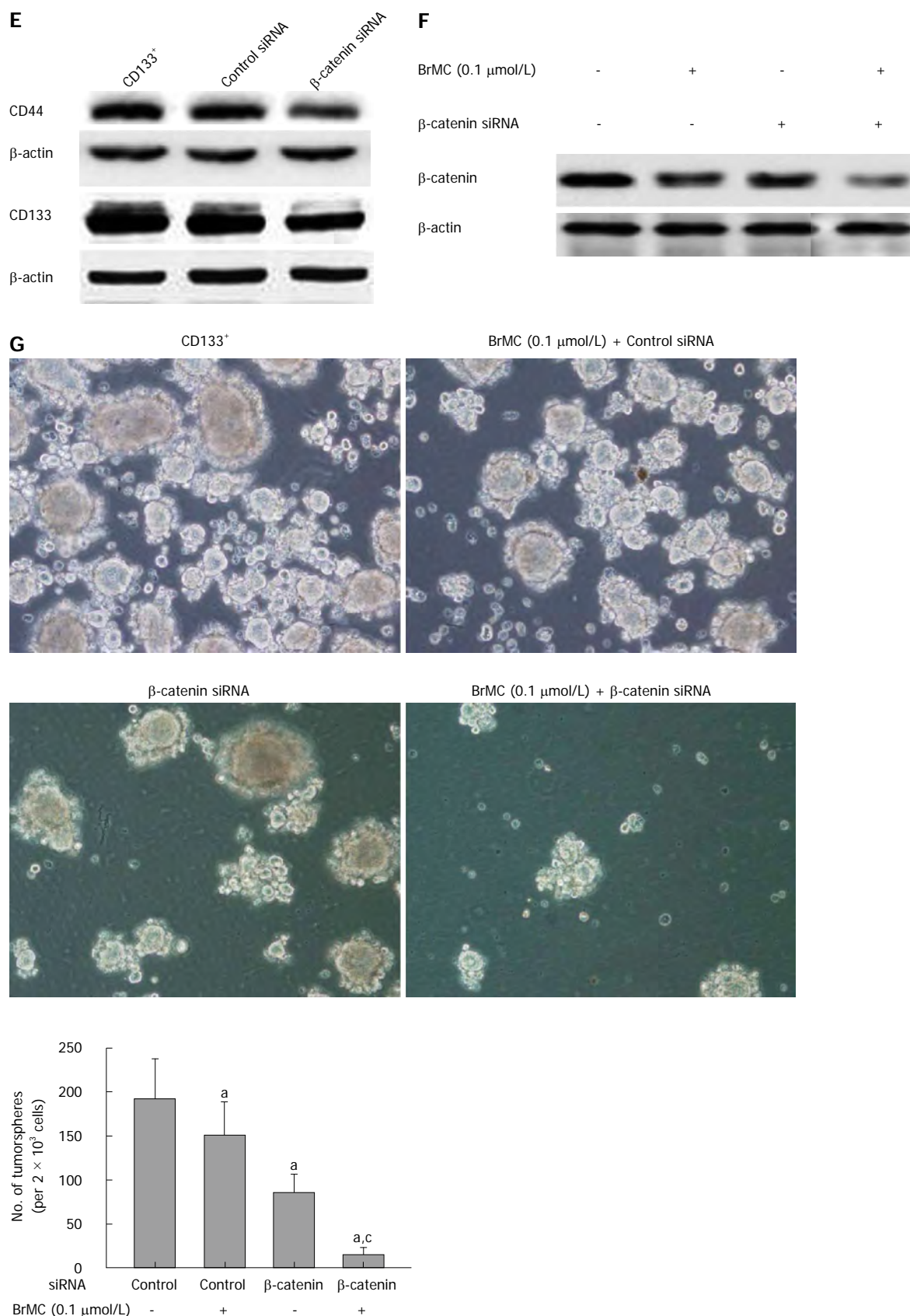
Table 3 Effects of 8-bromo-7-methoxychrysin on growth of secondary tumors in Balb/c-nu mice inoculated with tumor cells obtained from primary xenografts				
Time (d)	Tumor incidence <sup>1</sup>		Volume (mm <sup>3</sup> )	
	Control	BrMC-treated group	Control	BrMC-treated group
1	0/12	0/12	-	-
3	0/12	0/12	-	-
6	4/12	0/12	28 $\pm$ 12	-
12	9/12	0/12	134 $\pm$ 29	-
15	12/12	3/12	321 $\pm$ 63	16 $\pm$ 8
18	12/12	3/12	532 $\pm$ 96	33 $\pm$ 16
21	8/8	2/8	351 $\pm$ 67	23 $\pm$ 13
24	8/8	2/8	593 $\pm$ 131	32 $\pm$ 24
27	4/4	1/4	264 $\pm$ 91	54
30	4/4	1/4	387 $\pm$ 114	67
33	4/4	1/4	567 $\pm$ 126	82

<sup>1</sup>Number of tumors detected/number of injections. BrMC: 8-bromo-7-methoxychrysin

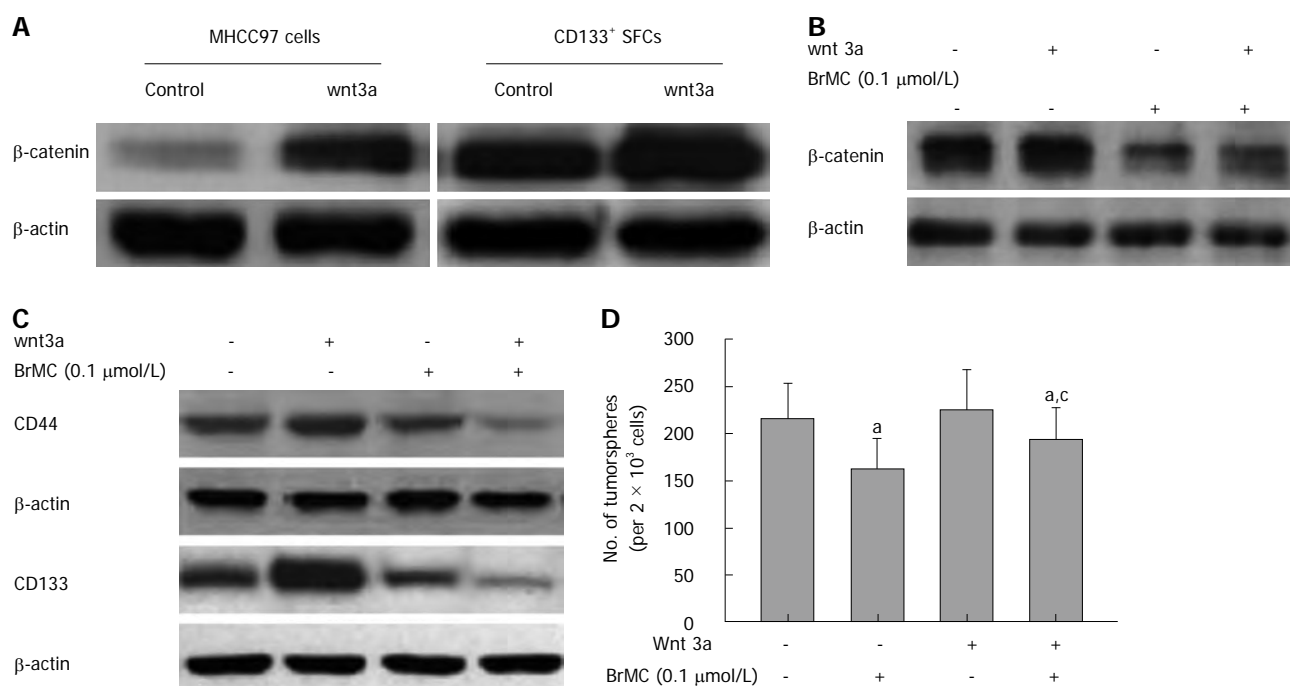
The Wnt/ $\beta$ -catenin pathway is one of the key pathways that modulates stem cell self-renewal<sup>[14]</sup>. For example, overexpression of  $\beta$ -catenin enhanced self-renewal preferentially and mediated radiation resistance of Sca1<sup>+</sup> progenitors in an immortalized mammary gland cell line<sup>[33]</sup>. Hallett *et al.*<sup>[34]</sup> reported that pharmacological inhibitors of Wnt/ $\beta$ -catenin signaling could inhibit the viability and or self-renewal of breast tumor-initiating cells, and target breast tumor-initiating cells in a Her2/Neu mouse model of breast cancer. Consistent with these previous reports, we found that downregulation of  $\beta$ -catenin







**Figure 5**  $\beta$ -catenin siRNA synergized the inhibitory effects of 8-bromo-7-methoxychrysin. 8-bromo-7-methoxychrysin (BrMC) downregulated CD44 and CD133 expression in liver cancer stem cells in a concentration-dependent manner (A).  $\beta$ -catenin was highly expressed in CD133<sup>+</sup> sphere forming cells (SFCs) and was downregulated by BrMC treatment (B).  $\beta$ -catenin siRNA decreased the protein level of  $\beta$ -catenin (C) and stem cell markers (E), and significantly inhibited self-renewal capacity (D) of CD133<sup>+</sup> SFCs (mean  $\pm$  SD,  $n = 3$ ). <sup>a</sup> $P < 0.05$  vs CD133<sup>+</sup> SFCs or control siRNA transfected CD133<sup>+</sup> SFCs. BrMC enhanced  $\beta$ -catenin siRNA induced downregulation of  $\beta$ -catenin expression (F) and inhibition of self-renewal capacity (G) in CD133<sup>+</sup> SFCs (mean  $\pm$  SD,  $n = 3$ ). <sup>a</sup> $P < 0.05$  vs CD133<sup>+</sup> SFCs derived from the MHCC97 cell line. <sup>c</sup> $P < 0.05$  vs 0.1  $\mu$ mol/L BrMC or  $\beta$ -catenin siRNA treated CD133<sup>+</sup> SFCs.



**Figure 6** Wnt3a treatment antagonized the inhibitory effects of 8-bromo-7-methoxychrysin. Wnt3a treatment resulted in an increase in the expression of  $\beta$ -catenin in both liver cancer stem cells (LCSCs) and parental MHCC97 cells (A) and attenuated the effects of 8-bromo-7-methoxychrysin (BrMC) on the expression of  $\beta$ -catenin (B) and stem cell markers (C), and self-renewal capacity (D) of LCSCs derived from the MHCC97 cell line. <sup>a</sup> $P < 0.05$  vs CD133<sup>+</sup> SFCs, <sup>c</sup> $P < 0.05$  vs 0.1 μmol/L BrMC or Wnt 3a alone treated group.

by BrMC resulted in inhibition of CSC function and characteristics of LCSCs, such as significant inhibition of proliferation and self-renewal, suppression of EMT and invasiveness, downregulation of the expression of stem cell markers of LCSCs, and further efficacious promotion of the elimination of LCSCs *in vivo*.

Previous studies have shown that activated Akt was able to phosphorylate Ser9 on GSK3 $\beta$ , which may decrease the activity of GSK3 $\beta$ , thereby leading to stabilization of  $\beta$ -catenin in the cytoplasm<sup>[35]</sup>. Chrysin was reported to induce apoptosis through caspase activation and Akt inactivation in leukemia cells<sup>[36]</sup>. Our previous study also demonstrated that 5,7-dihydroxy-8-nitrochrysin, another synthetic chrysin analogue, could induce activation and nuclear localization of FOXO3a, which was associated with reduced levels of Akt phosphorylation. Therefore, we speculate that the downregulation of  $\beta$ -catenin by BrMC probably occurs *via* reduced levels of Akt and activation of GSK3 $\beta$ , with the consequent degradation of  $\beta$ -catenin. Wnt3a treatment can induce stabilization of  $\beta$ -catenin, with entry into the nucleus and subsequent activation of the  $\beta$ -catenin pathway. Thus, Wnt3a treatment can antagonize the inhibitory effects of BrMC on self-renewal of LCSCs. On the other hand, Su *et al.*<sup>[37]</sup> reported that genistein increases levels of membrane E-cadherin and E-cadherin- $\beta$ -catenin cell adhesion complex, and eventually attenuates  $\beta$ -catenin signaling in mammary epithelial cells. We also found that E-cadherin, an epithelial marker, was upregulated by BrMC in LCSCs. E-cadherin is known to anchor and to sequester  $\beta$ -catenin in the membrane and prevent its

activation. Therefore, we suppose that this inactivation of  $\beta$ -catenin by upregulation of E-cadherin can also contribute to the inhibitory effects of LCSCs by BrMC. Interestingly, the inhibition of  $\beta$ -catenin at the protein level was not optimal, as treatment of  $\beta$ -catenin siRNA can further downregulate  $\beta$ -catenin at the transcription level and synergize the inhibition of self-renewal of LCSCs induced by BrMC.

In conclusion, we have presented supportive evidence for the first time that BrMC, a novel synthetic chrysin analogue, can target LCSCs both *in vitro* and *in vivo*. Furthermore, our study identified the downregulation of  $\beta$ -catenin expression by BrMC as one of the possible mechanisms for its efficacy. These studies support the use of BrMC for liver cancer chemoprevention or chemotherapy. These findings provide a strong rationale for preclinical and subsequent clinical evaluation of BrMC for liver cancer therapy.

## COMMENTS

### Background

Liver cancer is the fifth most common cancer in the world and the third leading cause of cancer-related death. Recent studies indicated that cancer stem cells (CSCs) may be responsible for tumor recurrence and drug-resistance. Therefore, the identification of a compound that can target liver CSCs (LCSCs) is one of the main steps in improving overall survival of liver cancer patients.

### Research frontiers

More recently, a number of studies have found that some dietary compounds can directly or indirectly affect CSC self-renewal pathways. 8-bromo-7-methoxychrysin (BrMC) is a synthetic derivative of chrysin, and their previous study have demonstrated the effect of BrMC on the inhibition of proliferation



and induction of apoptosis in colon, gastric and liver cancer cells was stronger than that of chrysin. However, the inhibitory effects of BrMC on the characteristics of CSCs have not been reported yet.

### Innovations and breakthroughs

The authors firstly showed that BrMC, a novel synthetic chrysin analogue, was able to inhibit cancer stem cell-like properties of LCSCs and eliminate LCSCs *in vivo*. They also found that BrMC significantly decreased  $\beta$ -catenin expression in LCSCs and knockdown of  $\beta$ -catenin expression could synergize the inhibition of self-renewal of LCSCs induced by BrMC. The downregulation of  $\beta$ -catenin expression appears to contribute to the inhibitory effects of BrMC on the properties of LCSCs.

### Applications

The present study provided strong evidences for the first time that BrMC was able to target LCSCs both *in vitro* and *in vivo*. These studies support the use of BrMC for liver cancer chemoprevention or chemotherapy.

### Terminology

Chrysin (5,7-dihydroxyflavone), a naturally wide distributed flavonoid, has been reported to possess anti-cancer activities. BrMC is a novel synthetic chrysin analogue.

### Peer review

This manuscript concludes that 8-bromo-7-methoxychrysin can inhibit the functions and characteristics of liver cancer stem cells derived from liver cancer MHCC97 cell line through downregulation of  $\beta$ -catenin expression. It is a good research with necessary information.

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## Laparoscopy-assisted percutaneous endoscopic gastrostomy enables enteral nutrition even in patients with distorted anatomy

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### Abstract

**AIM:** To analyzed whether laparoscopy-assisted percutaneous endoscopic gastrostomy (PEG) could be a valuable option for patients with complicated anatomy.

**METHODS:** A retrospective analysis of twelve patients (seven females, five males; six children, six young adults; mean age 19.2 years) with cerebral palsy, spastic quadriplegia, severe kyphoscoliosis and interposed organs and who required enteral nutrition (EN) due to starvation was performed. For all patients, standard PEG placement was impossible due to distorted

anatomy. All the patients qualified for the laparoscopy-assisted PEG procedure.

**RESULTS:** In all twelve patients, the laparoscopy-assisted PEG was successful, and EN was introduced four to six hours after the PEG placement. There were no complications in the perioperative period, either technical or metabolic. All the patients were discharged from the hospital and were then effectively fed using bolus methods.

**CONCLUSION:** Laparoscopy-assisted PEG should become the method of choice for gastrostomy tube placement and subsequent EN if PEG placement cannot be performed safely.

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**Key words:** Percutaneous endoscopic gastrostomy; Laparoscopy-assisted percutaneous endoscopic gastrostomy; Severe kyphosis; Malnutrition; Interposed organs

**Core tip:** Enteral nutrition (EN) is a life-saving procedure, preventing complications associated with malnutrition. The best solution for EN is percutaneous endoscopic gastrostomy (PEG). In some cases, however, creating such access is impossible. In those cases, laparoscopy-assisted PEG should become the method of choice for gastrostomy tube placement and subsequent EN if PEG placement cannot be performed safely.

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## INTRODUCTION

Tube enteral feeding is the method of choice when an oral diet is insufficient or impossible for more than seven days, even in patients without apparent malnutrition<sup>[1]</sup>. Tube enteral feeding is also strongly recommended if the latter is present or imminent<sup>[1]</sup>. All these patients should be administered enteral nutrition (EN), and when the oral diet cannot be continued, tube feeding is the best option. Since its introduction by Gauderer in 1980, percutaneous endoscopic gastrostomy (PEG) has become the method of choice for EN in all age groups<sup>[2]</sup>. The primary goal of EN is to improve the patient's well-being by preventing or reversing malnutrition and avoiding its consequences. EN is particularly important in pediatric patients because they need to not only survive but also grow. Neurological disorders place children at a very high risk of malnutrition; hence, this group of patients benefits very quickly from EN. Unfortunately, neurological disorders are very often accompanied by serious distortions in body anatomy.

Problems, such as severe kyphoscoliosis, interposed organs or other forms of distorted anatomy, may prevent effective and safe PEG placement due to an inability to guarantee the three principles of safe PEG placement: endoscopic gastric distension, endoscopically visible focal finger invagination and transillumination<sup>[3,4]</sup>. In such cases, surgical gastrostomy, which is an invasive procedure, is often the only option for EN. However, less invasive measures would be preferable over surgical gastronomy. Our study aimed to determine whether laparoscopy-assisted PEG placement is a useful option for EN in patients with distorted anatomy and who are unable to undergo PEG placement.

## MATERIALS AND METHODS

This study was a retrospective analysis of twelve patients (seven females, five males; mean age 19.2 years), who were treated at the pediatric surgery center in Bialystok, Poznan and Skawina, Poland. Six patients were children, and the remaining six were adults. In all patients, malnutrition requiring nutritional support was diagnosed (body mass index < 14 m<sup>2</sup>/kg), and tube feeding was recognized as the method of choice for EN. In all four patients, PEG placement was impossible due to spastic quadriplegia, severe kyphoscoliosis and interposed organs. Informed consent was collected from all patients. The patients and procedure characteristics are presented in Table 1.

### Technique

PEG tube insertion was performed under general an-

esthesia. A single dose of intravenous antibiotics was given. The patient was positioned in the supine position. After sterilizing the skin on the anterior abdominal wall, a 5-mm port was inserted under the umbilicus using Hasson's open technique<sup>[5]</sup>. Pneumoperitoneum was established *via* a trocar, using carbon dioxide. The intra-abdominal pressure in our patients was 10 mmHg. The peritoneal cavity and the abdominal and gastric walls were inspected for suitable sites for the gastrostomy. After insertion of the gastroscope into the stomach and air insufflation, the gastrostomy site was selected under laparoscopic and endoscopic guidance. The skin was incised above the gastrostomy site with a length of 0.5 cm. A trocar with a needle was pushed through this point into the stomach under complete laparoscopic and endoscopic visualization. The PEG was made using the 'pull' technique: a thread was inserted through the trocar after removing the needle and was then snared. The endoscope was withdrawn by the snare holding the thread. A suitably sized PEG tube was then connected to the thread, and the thread was pulled from the skin incision, pulling the tube into patient's mouth through the esophagus. The PEG tube was retained in the stomach with an internal bolster. An external bolster was placed loosely on the skin<sup>[2,3]</sup>. All the procedures were uneventful and without any intraoperative complications.

## RESULTS

The laparoscopy-assisted PEG insertion was successful in all twelve patients. The mean length of the procedure was 16.5 min. A FloCare PEG tube (Nutricia Ltd., Poland) with a diameter of 14 Cherrier was used as the gastric catheter.

No postoperative complications were observed, and EN was commenced four to six hours after the PEG placement using the bolus method (5 mL × 100 mL). The mean length of the hospital stay was 1.5 d. All the patients were discharged from the hospital and then effectively fed using bolus methods at long-term facilities. The follow-up at twelve months did not reveal any complications. The nutritional status of the patients improved significantly, with the mean BMI reaching 17.5 m<sup>2</sup>/kg (Table 1).

## DISCUSSION

Tube feeding is the method of choice when EN is recommended<sup>[1]</sup>. In most cases, PEG tube insertion is a safe procedure and does not lead to complications<sup>[3,6]</sup>. Fatal outcomes have been reported due to comorbidities when the PEG was inserted in the setting of severe disease<sup>[3,7]</sup>. However, the safe placement of a PEG tube requires a permeable esophagus and transillumination of the stomach through the abdominal wall. Impaired coagulation, severe ascites, peritonitis and local esophageal and general gastrointestinal obstructions are considered to be absolute contraindications<sup>[3,4,8]</sup>. Severe kyphoscoliosis with interposed organs and distorted anatomy are

**Table 1** Patients' characteristics, the reasons for L-percutaneous endoscopic gastrostomy, the length of the procedures, the nutritional statuses before and after L-percutaneous endoscopic gastrostomy

No.	Gender	Age (yr)	Reason of LAPEG	BMI before the procedure/ albumin concentration (g/dL)	BMI after 12 mo follow up	Time from insertion of the gastroscope to the peg placement (total operation length)/min	Time form insertion laparoscope to removal of the laparoscope/min
1	F	7	Tay-Sachs disease	11.9/2.9	14.5	22	16
2	M	5	drug-resistant epilepsy	13.9/3.9	15.2	17	13
3	M	15	cerebral palsy	13.2/4.5	15.2	15	9
4	F	3	Patau syndrome	13.8/5.1	16.0	21	12
5	M	17	cerebral palsy	12.6/3.3	18.4	24	14
6	M	11	cerebral palsy	13.2/3.7	17.1	22	9
7	F	22	Wilson's disease	12.0/28	13.5	21	17
8	F	31.5	Gaucher's disease	14.5/29.5	14.5	18	12
9	M	26.2	cerebral palsy	15.5/27.0	15.2	16	8
10	F	33.2	SLA	14.0/25.0	17.0	12	11
11	F	34	SLA	11.0/2.3	14.5	14	11
12	F	24.7	cerebral palsy	11.5/2.9	15.0	13	7

BMI: Body mass index; LAPEG: LA-percutaneous endoscopic gastrostomy; SLA: Amyotrophic later sclerosis; M: Male; F: Female.



**Figure 1** A 17-year-old patient with severe kyphoscoliosis (138 degrees on Cobb's scale) with interposed organs.

considered to be relative contraindications<sup>[8-10]</sup> (Figure 1). In patients with severe kyphoscoliosis, the PEG tube cannot be placed in the usual locations due to organ displacement. In our patients, the contraindications for PEG included the translocation of the stomach into the left lower quadrant of the abdomen (3 patients) or into the middle part of the abdomen (1 patient) (Figure 2). Therefore, in patients with severe kyphoscoliosis, patients suffering from hepatomegaly, splenomegaly, obesity or an intra-abdominal or peritoneal tumor, patients with previous abdominal surgery, especially involving the stomach, and patients with advanced esophageal cancer, when transillumination of the stomach is not achieved, there is an indication for laparoscopic, fluoroscopic or sonographic guidance during the PEG tube placement.

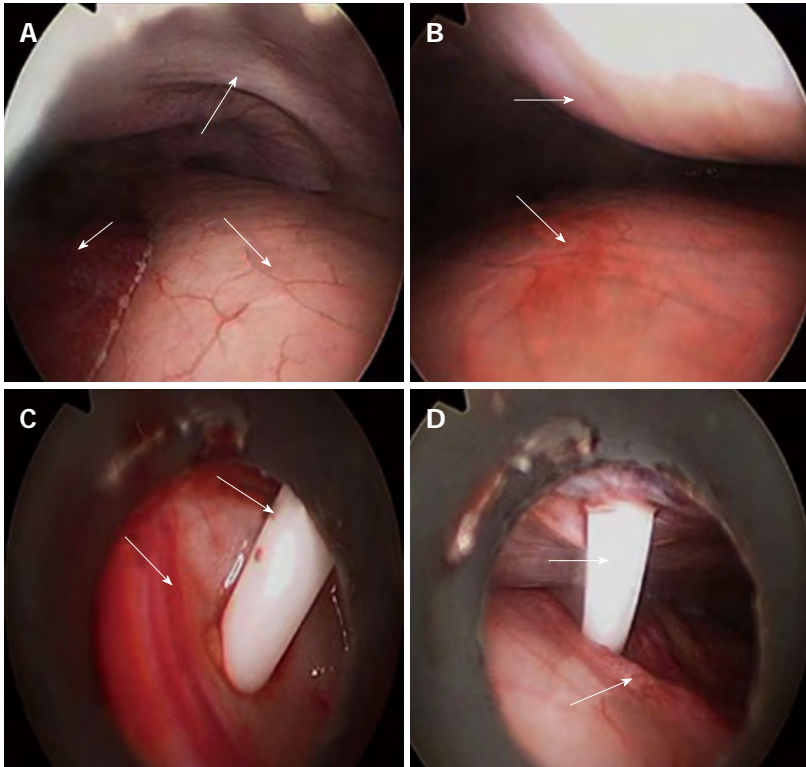
The technique for the laparoscopic-assisted PEG procedure was described for the first time by Raaf *et al*<sup>[11]</sup> in 1993. In 1995, Stringel *et al*<sup>[12]</sup> reported a laparoscopic-assisted PEG procedure in 2 children in whom attempts at a simple PEG had failed. There are also other alternative methods for PEG placement when transillumination is not possible. Radiologic techniques have been used successfully in these patients since 1981<sup>[13,14]</sup>. The gastric tubes

can be placed under ultrasound or fluoroscopic guidance<sup>[15-17]</sup>. Recently, the placement of the gastric tubes has used Computed tomography (CT) fluoroscopy guidance, even in combination with simultaneous gastroscopy<sup>[18]</sup>.

Historically, gastroesophageal reflux was also considered to be a contraindication, as concluded by Yaseen *et al*<sup>[19]</sup>. Recent trials have shown that gastroesophageal reflux might actually improve after PEG placement because the PEG creates a type of anterior pseudo-gastropexy<sup>[20,21]</sup>.

Major complications related to PEG include the following: colonic perforation, esophageal tear, small bowel injury, hepatic or splenic injury, tube migration with or without intestinal obstruction, gastrointestinal bleeding and site or generalized infection. These complications have been reported with variable incidences (5%-17%) in published series<sup>[3,4,7,22]</sup>. The most severe complication, with an incidence of 0.0008%-0.04%, is esophageal perforation<sup>[4,23]</sup>. Predisposing factors include anatomic anomalies in up to 50% of cases<sup>[3]</sup>. Patients with displacement of the transverse colon over the anterior gastric wall are predisposed to colonic injury during the PEG procedure<sup>[3,23,24]</sup>. Colonic injury usually presents with peritonitis, and surgery is then required. Additionally, interposition of the splenic flexure between the anterior abdominal and gastric walls may result in gastrocolic-cutaneous fistulae after PEG placement without direct visual monitoring. The PEG tube is placed through the large bowel into the stomach. Such patients may be almost completely asymptomatic except for transient fever and ileus. The management involves PEG tube removal and spontaneous closure of the fistula<sup>[4,25]</sup>. Small bowel injuries are rather rare because the greater omentum separates the small bowel from the upper abdomen. Small bowel volvulus around the PEG usually presents with a small bowel obstruction caused by a gap between the gastric and abdominal walls<sup>[4]</sup>. Hepatic and splenic PEG-related injuries are also rare. In hemodynamically stable patients, a CT scan can confirm the diagnosis. Hemodynamically unstable patients require emergent surgical exploration, placement of hemostatic sutures in the





**Figure 2** Laparoscopy-assisted percutaneous endoscopic gastrostomy placement: An overview of the procedure. A: General view. The arrows indicate the following (respectively): liver, stomach and abdominal wall; B: Marking the puncture site. The arrows indicate the stomach and abdominal walls, and the pressed wall shows the puncture site; C: Introduction of the needle into the stomach under visual control. The arrows show the gastric wall and the trocar for percutaneous endoscopic gastrostomy (PEG) insertion; D: PEG was performed. The arrows indicate the gastric vessels and the trocar.

*liver or a splenectomy*. Severe hemorrhage is a rare complication of PEG (0.02%-0.06%) and is usually due to anticoagulation, antiplatelet therapy or an anatomic anomaly, as observed in our patients<sup>[3,23]</sup>. We did not notice any major complications from the PEG tube insertion in our patients. In our opinion, only continuous laparoscopic monitoring can ensure that the omentum, colon and small bowel will not be interposed between the stomach and the anterior abdominal wall during gastrostomy fixation in extremely malnourished children with severe kyphoscoliosis.

Minor complications, such as superficial skin infections, superficial granulation tissue formation and tube leak, are common and may occur in up to 50% of patients<sup>[3,4,9,26]</sup>. Minor skin infections usually respond to enteral antibiotics, and granulation tissue usually responds to local cautery with silver nitrate swabs. We did not notice any skin infections, tube leaks or granulation tissue formation in our patients. The patients' families and caregivers were taught how to flush the tube after feeding to avoid tube obstruction.

With respect to the laparoscopy, we use Hasson's open method and an optical access trocar to achieve pneumoperitoneum<sup>[5]</sup>. In our opinion, this method decreases the risk of injuries compared with the blind insertion of the Veress needle; however, Hasson's method may cause air leaks and prolong the operative time. In our cases, the mean duration of the operation time from the insertion of the laparoscope to the removal of the laparoscope

did not differ significantly from that of the classic PEG procedure. We did not have continuous air leaks, and the operating time was not prolonged much because of the laparoscopy. Therefore, we have continued to use this technique as the preferred method for inducing pneumoperitoneum in children. Under laparoscopic observation, the stomach can be punctured in the correct location, avoiding the colon or the liver on its way into the gastric lumen. In our study, the laparoscopic-assisted PEG procedures were performed without difficulty. There was no need to maneuver or relocate the interposed organs during the laparoscopy. This procedure, for which the technical aspects are presented in Figure 2, allowed for successful EN. The subsequent EN helped our patients recover from starvation and decreased their malnutrition-related complication ratio.

In conclusion, the laparoscopy-assisted PEG procedure is a valuable method for gastrostomy tube placement in patients in whom an upper endoscopy is possible but PEG cannot be performed safely. In our opinion, laparoscopy-assisted PEG should become the method of choice for children and adults with distorted anatomy.

## COMMENTS

### Background

Enteral nutrition (EN) is a life-saving procedure that prevents the complications associated with malnutrition. Percutaneous endoscopic gastrostomy (PEG) is the method of choice for this type of intervention because the procedure en-

ables tube feeding.

### Research frontiers

PEG placement is not always possible due to technical difficulties, such as interposed organs or kyphosis. This study aimed to analyze whether laparoscopy-assisted PEG could be a valuable option in those patients in whom the standard procedure is impossible due to distorted anatomy.

### Innovations and breakthroughs

For all the patients, the laparoscopy-assisted PEG tube placement was successful, and the EN was started four to six hours after its placement, proving that this technique is safe and effective.

### Applications

Laparoscopy-assisted PEG should become the method of choice for gastrostomy tube placement and subsequent EN if PEG placement cannot be performed safely.

### Peer review

This is an interesting article of case series of laparoscopy-assisted PEG.

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## Is early limited surgery associated with a more benign disease course in Crohn's disease?

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need for surgery in patients with Crohn's disease (CD).

**METHODS:** Data of 506 patients with incident CD were analyzed (age at diagnosis:  $31.5 \pm 13.8$  years). Both hospital and outpatient records were collected prospectively with a complete clinical follow-up and comprehensively reviewed in the population-based Veszprem province database, which includes incident CD patients diagnosed between January 1, 1977 and December 31, 2008. Follow-up data were collected until December 31, 2009. All patients included had at least 1 year of follow-up available. Patients with indeterminate colitis at diagnosis were excluded from the analysis.

**RESULTS:** Overall, 73 patients (14.4%) required resective surgery within 1 year of diagnosis. Steroid exposure and need for biological therapy were lower in patients with early limited surgery ( $P < 0.001$  and  $P = 0.09$ ). In addition, surgery rates during follow-up in patients with and without early surgery differed significantly after matching on propensity scores ( $P < 0.001$ , HR = 0.23). The need for reoperation was also lower in patients with early limited resective surgery ( $P = 0.038$ , HR = 0.42) in a Kaplan-Meier and multivariate Cox regression ( $P = 0.04$ ) analysis. However, this advantage was not observed after matching on propensity scores ( $P_{\text{Logrank}} = 0.656$ ,  $P_{\text{Breslow}} = 0.498$ ).

**CONCLUSION:** Long-term surgery rates and overall exposure to steroids and biological agents were lower in patients with early limited resective surgery, but reoperation rates did not differ.

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**Key words:** Crohn's disease; Early surgery; Disease course; Disease behavior; Treatment strategy

**Core tip:** An alternative approach may be early limited

### Abstract

**AIM:** To analyze the difference in disease course and



resective surgery in a well-selected group of patients with Crohn's disease. In this population-based study, we found that overall exposure to steroids and biological agents was lower in patients with early limited resective surgery; observed surgery rates were also lower, yet reoperation rates did not differ in the two groups after matching on propensity scores.

Golovics PA, Lakatos L, Nagy A, Pandur T, Szita I, Balogh M, Molnar C, Komaromi E, Lovasz BD, Mandel M, Veres G, Kiss LS, Vegh Z, Lakatos PL. Is early limited surgery associated with a more benign disease course in Crohn's disease? *World J Gastroenterol* 2013; 19(43): 7701-7710 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7701.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7701>

## INTRODUCTION

Crohn's disease (CD) has a variable course, but the majority of patients eventually develop penetrating or stricture complications. In addition, several environmental risk factors (diet, smoking, measles, or appendectomy) may contribute to its etiology and course. A significant adverse outcome is the need for surgery. Nevertheless, surgery is not curative in CD. Surgical resection is typically performed for emergency indications (*e.g.*, obstructive symptoms and hemorrhage) or for failure to respond to medical therapy.

Some years ago, a review article reported that the probability of first resective surgery ranged from 38% to 96% in the first 15 years after diagnosis<sup>[1]</sup>. The overall clinical relapse and reoperation rates after initial resective surgery are 50%-60% and 28%-45%, respectively, during the following 15 years. Surgical resection rates over time vary widely among published studies, ranging between 25 and 61% in the first 5 years. Until recently, there was little evidence that disease outcomes for CD had changed over recent decades. Recently, Peyrin-Biroulet *et al.*<sup>[2]</sup> published a systematic review of the natural history of CD in population-based cohorts. According to the authors' conclusions, the impact of changing treatment paradigms with the increased use of immunosuppressants and biological agents on the natural history of the disease was poorly understood. Available data did not suggest a significant change in outcome of CD, with approximately half of patients requiring surgery within 10 years of diagnosis. The risk of postoperative clinical recurrence within 10 years was 44%-55%.

A recent meta-analysis from International Organization for the Study of Inflammatory Bowel Diseases (IOIBD) Epidemiology Task Force reported that the risk of surgery in CD in prebiologic population-based cohorts has been decreasing during the past decade<sup>[3]</sup>. One of the most striking changes was reported by Jess *et al.*<sup>[4]</sup> in a Danish study. The rate of early surgery (within 1 year of diagnosis) has fallen from 35% (1962-1987) to 12% (2003-2004). During this time, there was a significant

change in patient management; namely, increased and earlier use of immunosuppressants and the introduction of biological therapies. The effect of azathioprine (AZA) on disease prognosis was until recently controversial. Two recent population-based reports confirmed that early AZA use is associated with reduced need for surgery according to a Cox regression analysis and propensity score matching in two population-based cohorts from Wales and Hungary<sup>[5,6]</sup>. Furthermore, in a study from France, an association was reported between the duration of anti-tumor necrosis factor (TNF) and AZA therapy and risk for surgery<sup>[7]</sup>. In contrast, in a previous referral center study from France, the need for intestinal surgery did not decrease despite the increased use of immunosuppressants<sup>[8]</sup>. Of note, in this study, AZA therapy was started only after surgery in the majority of patients. Earlier AZA use is only one of the complex changes in patient management. Other changes have also occurred, including a trend toward tight patient monitoring. Moreover, whether the risk of surgery is affected by the more widespread use of biological agents has yet to be demonstrated by population-based studies.

An alternative approach to the predominant strategy of initially using conservative therapy: using limited resective surgery in a selected group of patients as a primary therapeutic option, may prove advantageous. In a study by Aratari *et al.*<sup>[9]</sup>, early surgery at diagnosis in 207 CD patients with ileocecal disease was associated with a more benign postoperative disease course, in comparison to patients receiving delayed surgery. Nevertheless, reoperation rates were not reduced. Thus, in a subgroup of CD patients, early surgery may represent a valid alternative to medical therapy; particularly in patients with limited, isolated, stenotic ileocecal disease.

Therefore, our aim was to analyze the disease course, drug exposure and need for surgery and reoperation in patients with and without early (within 1 year of diagnosis) limited resective surgery in a population-based cohort from Eastern Europe with a complete clinical follow-up.

## MATERIALS AND METHODS

### Patients

A well-characterized Hungarian cohort of 506 patients with incident CD (male/female: 251/255; age at diagnosis:  $31.5 \pm 13.8$  years) diagnosed between January 1, 1977 and December 31, 2008 were included. Follow-up data were collected until December 31, 2009. All patients included had at least 1 year follow-up available. Patients with indeterminate colitis at diagnosis were excluded from the analysis. The clinical data of CD patients are summarized in Table 1.

### Methods

Clinical data were collected every year from the seven general hospitals (departments of internal medicine, surgery, and pediatrics) and gastroenterology outpatient units. The majority of patients [76% of ulcerative colitis (UC) and



94% of CD patients] were monitored at the Csolnok F. Province Hospital in Veszprem, where data were also registered. Disease behavior was updated yearly. A more detailed description of the data collection method, case assessment, the geographical and socioeconomic background of the province, and the results of surgical and medical management, as well as a detailed description of the Veszprem Province inflammatory bowel disease (IBD) Group, was published in previous epidemiological studies<sup>[10-12]</sup>.

The disease phenotype (age at onset, duration, location, and behavior) was determined according to the Montreal Classification<sup>[13]</sup>. The presence of perianal disease and behavior change during follow-up were also registered. Medical therapy was recorded in detail (as defined by the European Crohn's and Colitis Organisation Consensus Report<sup>[14]</sup>). The need for surgery or reoperation and smoking habits were investigated by review of medical files and by questionnaire.

The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics and by the Csolnok F. Province Hospital Institutional Committee of Science and Research Ethics.

### Treatment policy

The majority of the patients received maintenance therapy with sulfasalazine or a 5-aminosalicylic acid derivative (mesalazine or olsalazine), if tolerated, especially until the mid-1990s. AZA or 6-mercaptopurine (6-MP) was used in selected cases as maintenance therapy for steroid-dependent and -refractory patients or for patients with fistulizing disease. AZA and 6-MP were typically used following resective surgery until the late 1980s, and later on a more widespread basis beginning in the mid-1990s. Short-term oral corticosteroid treatment was used for clinical exacerbations, usually prednisone 40-60 mg/d, tapered and discontinued over the course of 2-3 mo. Beginning in the mid-1990s, methotrexate was used as second-line immunosuppressive therapy in limited cases. Since the late 1990s, infliximab has been used for both induction and maintenance therapy in selected cases.

Surgical resection was performed for emergency indications (*e.g.*, obstructive symptoms and hemorrhage) and for failure to respond to medical therapy. Surgical techniques have also changed during the follow-up period of this study; laparoscopic surgery became available and more widely used from the late 1990s. Limited resections were more widely used from the mid-1990s than in the past, and there is very limited use of defunctioning ileostomy ( $n = 4$ ) formation in routine management of CD. For the majority of patients in the present study, one of the most experienced IBD surgeons in Hungary performed the operations, while laparoscopic surgery and stricturoplasty were performed only in a minority of cases. The definition of early limited surgery was resection of the terminal ileum or ileocecum within 1 year of diagnosis.

Due to Hungarian health authority regulations, a follow-up visit is obligatory for IBD patients at a specialized

gastroenterology center every 6 mo. Otherwise, under the regulations of the Hungarian National Health Insurance (OEP) system, the right to subsidized therapy is forfeited. Consequently, the relationship between IBD patients and specialists is a close one.

### Propensity score model

The authors used two propensity score models to control further for possible confounders, to quantify the probability of surgery and reoperation in patients who had early limited resective surgery versus those who did not.

In the first propensity model, covariates included in the propensity score were selected according to a two-step process. We first constructed an outcome model identifying independent predictors of early limited resective surgery. Subsequently, we included in the model additional predictors known to be associated with surgical outcome (*e.g.*, age at onset and smoking) irrespective of *P* value. We used multivariate logistic regression to estimate propensity scores of early limited resection for each individual. Goodness-of-fit was evaluated by the Hosmer-Lemeshow test, the *P* values of which were not significant. Using the predicted propensity scores from our model, we attempted to match all early limited surgery to identical CD patients without early limited surgery by 5-to-1 greedy matching<sup>[15]</sup>. Additional analysis in patients with ileum-only disease was also performed.

In the second propensity model, we aimed to analyze the probability of reoperation in patients with and without early surgery in a group of CD patients with a history of at least one resective operation. Using the above two-step process and predicted propensity scores from our model, we attempted to match all early surgery CD patients to identical CD patients with a non-early surgical resection through 5-to-1 greedy matching<sup>[15]</sup>.

### Ethical permission

The study protocol was approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics and the Csolnok F. Province Hospital Institutional Committee of Science and Research Ethics.

### Statistical analysis

Variables were tested for normality by the Shapiro-Wilk *W* test. Wilcoxon rank sum test,  $\chi^2$  test, and  $\chi^2$  test with Yates correction and logistic regression were used to test differences in disease phenotype between subgroups of UC and CD patients for dichotomous variables. Kaplan-Meier survival curves were plotted for analysis with log rank and Breslow tests to determine probability of surgical resection. Additionally, Cox regression analysis using the enter method was used to assess the association between categorical clinical variables and time to AZA use and surgical requirements. Variables with  $P < 0.2$  in univariate analysis were included in the multivariate testing. To control further for possible confounders, we developed a propensity score models (see below) for quantifying the probability of reoperation in patients with

**Table 1** Clinical characteristics of patients with Crohn's disease

Characteristics	<i>n</i> = 506
Male/female	251/255
Age at presentation (yr)	31.5 ± 13.8
Median follow-up (yr)	11.4 ± 7.8
Familial IBD	12.9%
Location ( <i>n</i> )	
L1	166
L2	182
L3	155
L4 only	3
Behavior ( <i>n</i> )	
B1	288
At diagnosis B2	100
B3	118
Perianal disease	25.5%
Total steroid exposure/dependent-refractory	68.6%/11.2%
Total azathioprine exposure	45.8%
Total biological exposure	9.1%
Smoking habits ( <i>n</i> )	
No	224
At diagnosis Ex	38
Yes	244

L1: Ileal; L2: Colonic; L3: Ileocolonic; L4: Upper Gastrointestinal; B1: Inflammatory; B2: Stenosing; B3: Penetrating. IBD: Inflammatory bowel disease.

and without early limited resective surgery<sup>[15]</sup>. Matching on propensity scores is one technique commonly used to control for measured confounders in observational studies.  $P < 0.05$  was considered significant. Results for continuous variables were expressed as median (lower and upper quartile) unless otherwise stated. For the statistical analysis, SPSS version 20.0 was used (SPSS, Chicago, IL, United States).

## RESULTS

### Patient phenotype

Five hundred and six residents in Veszprem Province were diagnosed with CD in the 32-year period. Follow-up information was collected up to December 31, 2009, equaling 5758 patient-years of follow-up. The clinical characteristics and disease course according to the year of diagnosis is shown in Table 1. There were significant differences in disease phenotype, drug exposure, and smoking habits in the patient groups diagnosed in the early period and thereafter. Overall, exposure to AZA, systemic steroids and biological agents (after 1998 only) was 45.8%, 68.6% and 9.5%, respectively. Total AZA exposure increased in the subsequent cohorts despite shorter follow-up.

### Prevalence, predictors of surgery and early limited surgery and drug exposure

A total of 204 (40.7%) patients had at least one resective operation (5 patients with resective surgery due to malignant disease were excluded from analysis). The most common surgical procedures were ileocecal resection ( $n$

**Table 2** Predictors of early limited surgery and drug exposures

	Early surgery	No early surgery	<i>P</i> value	OR (95%CI)
Age at onset (A1)	28.4%	19.0%	0.060	1.69 (0.97-2.96)
Ileal location	59.5%	28.1%	< 0.001	
Colonic location	13.5%	39.9%	< 0.001	
Complicated behavior at diagnosis	85.1%	35.9%	< 0.001	10.2 (5.2-20.1)
Overall steroid exposure	52.7%	71.3%	0.001	0.45 (0.27-0.74)
Steroid-dependent course	2.6%	12.3%	0.070	0.19 (0.03-1.40)
Overall biological exposure	4.1%	10.0%	0.090	0.38 (0.12-1.26)
Overall azathioprine exposure	45.9%	45.8%	> 0.050	

= 93), right hemicolectomy ( $n = 59$ ), segmental colonic resection ( $n = 19$ ), and subtotal colectomy/left hemicolectomy ( $n = 11$  and 8, respectively).

A further 36 (7.1%) patients had other surgical procedures (abscess drainage or fistulectomy). Forty-two (8.4%) patients had two resective operations and 17 (3.4%) had three or more operations for CD during follow-up. Ileocecal resection was the most common procedure overall.

The probability of first intestinal surgery due to non-malignant disease after 1, 5 and 10 years was 14.6%, 30.1%, and 51.6%, respectively, in a Kaplan-Meier analysis. The cumulative probability of resective surgery rate decreased in patients diagnosed in the last decade [Group 1 (1977-1998), Group 2 (1999-2008);  $P_{\text{Logrank}} = 0.022$ , and  $P_{\text{Breslow}} = 0.07$ ].

Overall, 73 patients (14.4%) required resective surgery within 1 year of diagnosis. Ten patients were excluded from further analysis from the early surgery group in whom extensive index surgery was performed. The prevalence of early limited resective surgery did not differ significantly in the three cohorts [Cohort A (diagnosed 1977-1989): 11.3%; Cohort B (1990-1998): 12.8%; and Cohort C (1999-2008): 13.4%]. Predictors of early limited resective surgery were ileal location ( $P < 0.001$ ), colonic location ( $P < 0.001$ ), complicated behavior at diagnosis ( $P < 0.001$ ), and age of onset ( $P = 0.06$ , Table 2).

Overall steroid exposure was significantly lower ( $P = 0.001$ ) in patients with early limited resection despite similar follow-up (median: 12 years). The same trend was observed for steroid dependency ( $P = 0.07$ ) and overall biological agent exposure ( $P = 0.09$ ). In contrast, overall AZA exposure was similar between the two groups. Of note, AZA exposure before surgery was 0% *vs* 28.8% ( $P < 0.001$ ), while TNF-antagonist exposure before surgery was 0% *vs* 4.9% ( $P = 0.05$ ) in patients with and without early limited surgery. In logistic regression analysis, disease location ( $P < 0.001$ ) and disease behavior ( $P < 0.001$ ) were associated with the need for early resective surgery (Table 3).

**Table 3** Factors associated with the need for early limited surgery in logistic regression analysis

	<i>P</i> value	OR	95%CI
Age at diagnosis			
A1	0.172	1.62	0.81-3.25
A2		Reference	
Disease location	< 0.001		
L1	< 0.001	7.88	2.92-21.3
L3	0.035	3.21	1.09-9.48
L2		Reference	
Disease behavior at diagnosis	< 0.001		
B2	< 0.001	4.91	2.15-11.2
B3	< 0.001	7.66	3.52-16.7
B1		Reference	
Smoking			
Yes	0.487	1.24	0.68-2.27
No		Reference	

L1: Ileal; L2: Colonic; L3: Ileocolonic; B1: Inflammatory; B2: Stenosing; B3: Penetrating.

**Table 4** Characteristics of patients with and without early resective surgery after matching on propensity scores

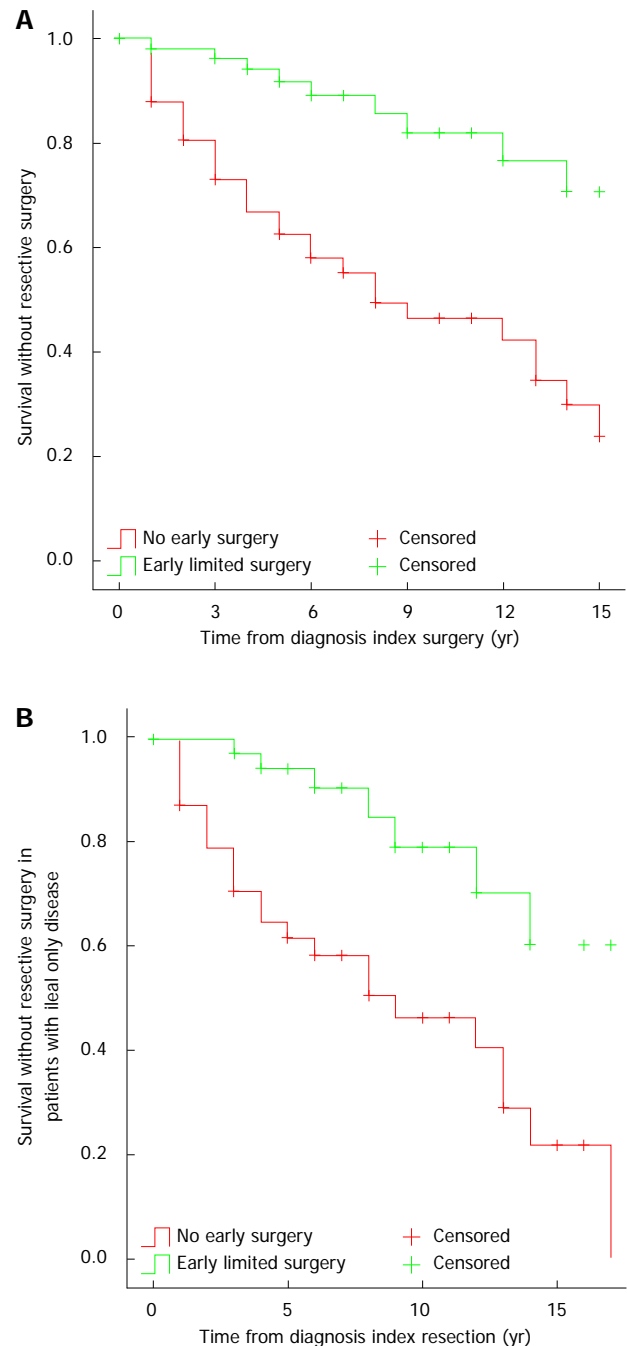
	CD patient with early surgery ( <i>n</i> = 58)	CD patient without early surgery ( <i>n</i> = 58) <sup>1</sup>
Gender (male/female)	32/26	34/24
Age at onset below 40 yr	12	11
Decade of diagnosis		
1977-1989	8	9
1990-1999	22	23
2000-2008	28	26
Disease location		
L1	38	39
L3 <sup>2</sup>	20	19
Disease behavior at diagnosis		
B1	10	10
B2	20	19
B3	28	29
Smoking (yes/no)	36/22	35/23

<sup>1</sup>Early resective surgery: within the year of diagnosis; <sup>2</sup>Ileocecal. CD: Crohn's disease; L1: Ileal; L2: Colonic; L3: Ileocolonic; B1: Inflammatory; B2: Stenosing; B3: Penetrating.

### Risk of operation in patients with and without early limited surgery

In a propensity score model, we compared surgery rates between patients with and without early resective surgery. In the early limited surgery model, propensity scores ranged from 0.02 to 0.56 (median: 0.29) in patients with early limited resection (*n* = 63), and from 0.02-0.55 (median: 0.14) in patients without early limited surgery (*n* = 428). Goodness-of-fit was evaluated by the Hosmer-Lemeshow test, and *P* values were non-significant (*P* = 0.653).

Using a 5-to-1 greedy matching algorithm, we were able to match 58 patients from the early resective surgery group to patients with comparable phenotype without early limited resections (Table 4). The observed first resective surgery rate was 12.1%, 33.2%, and 53.6% after 2, 5



**Figure 1** Need for surgery in Crohn's disease patients with and without an early limited surgery after matching on propensity scores. A: All Crohn's disease (CD) patients (*n* = 58), *P*<sub>Logrank</sub> < 0.001, HR = 0.23, 95%CI: 0.11-0.48; B: CD patients with ileal only disease location (*n* = 38). *P*<sub>Logrank</sub> < 0.001, HR = 0.25, 95%CI: 0.11-0.58.

and 10 years of disease duration, respectively, in the latter group. In contrast, the reoperation rate in the former group was 1.8%, 5.8%, and 17.9% after 2, 5 and 10 years (*P*<sub>Logrank</sub> < 0.001, HR = 0.23, 95%CI: 0.11-0.48; Figure 1A).

If the analysis was restricted to patients with ileum-only location (*n* = 38), the observed first resective surgery rate was 21%, 35.3%, and 59.4% in patients without early surgery after 2, 5 and 10 years of disease duration, respectively. In contrast, the observed reoperation rate in the other group was 0%, 5.8%, and 20.8% after 2, 5 and

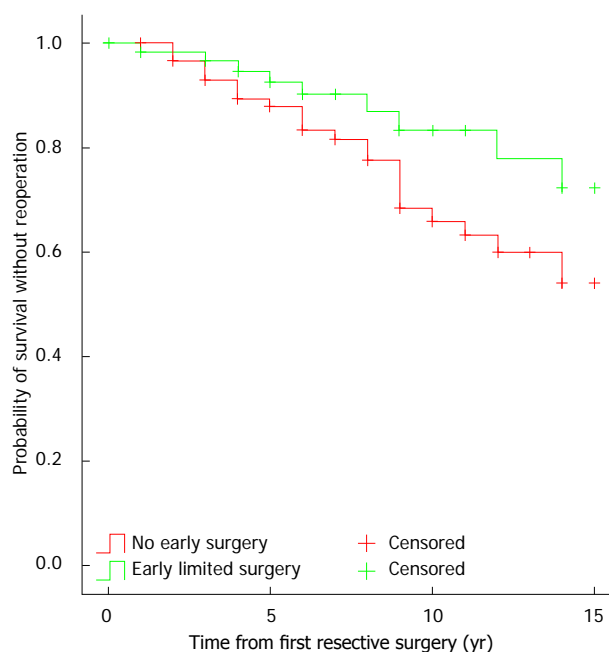


Figure 2 Need for reoperation in patients with and without early resective surgery ( $P_{\text{Logrank}} = 0.038$ ).

10 years ( $P_{\text{Logrank}} < 0.001$ , HR = 0.25, 95%CI: 0.11-0.58) (Figure 1B).

### Reoperation rates and predictors for reoperation

The need for reoperation in patients with early limited resection at 5 years was 7.5%, at 10 years it was 16.5%, while for those without early resective surgery at 5 years it was 12.9%, and 36.3% at 10 years in a Kaplan-Meier analysis ( $P_{\text{Logrank}} = 0.038$ , Figure 2). In a Cox regression analysis, early limited surgery ( $P = 0.04$ ) was the only factor independently associated with the need for reoperation (Table 5).

### Reoperation rates after matching on propensity scores

In addition, we developed a propensity score model to assess further the need for reoperation in patients with and without early resective surgery. After identifying predictors, multivariate logistic regression was used to estimate propensity scores of early limited resection for each individual. Goodness-of-fit was evaluated by the Hosmer-Lemeshow test and  $P$  values were non-significant ( $P = 0.812$ ). In the early limited surgery model, propensity scores ranged from 0.10 to 0.83 (median: 0.45) in patients with early limited resection ( $n = 63$ ), and from 0.02 to 0.69 (median: 0.30) in patients with non-early surgery ( $n = 126$ ). Using a 5-to-1 greedy matching algorithm, we were able to match 54 out of 63 (85.7%) patients with early limited surgery to patients with non-early surgery. As expected, the prevalence of factors included in the propensity score model was well balanced across surgical groups (data not shown).

The observed reoperation rates did not differ between the two groups (early surgery: 1.9%, 5.9%, and 17.7%; *vs* non-early surgery: 2%, 6.7%, and 25.1%, after 1, 5 and 10

Table 5 Factors associated with the need for reoperation in Cox regression analysis

	<i>P</i> value	HR	95%CI
Early surgery <sup>1</sup>			
Yes	0.04	0.42	0.19-0.95
No		Reference	
Age at diagnosis			
A1	0.75	-	-
A2		Reference	
Disease location		0.95	
L2	0.77	-	-
L3	0.95	-	-
Disease behavior at diagnosis		Reference	
B2	0.30	-	-
B3	0.65	-	-
B1		Reference	
Smoking			
Yes	0.29	-	-
No		Reference	

<sup>1</sup>Early resective surgery: Within the year of diagnosis.

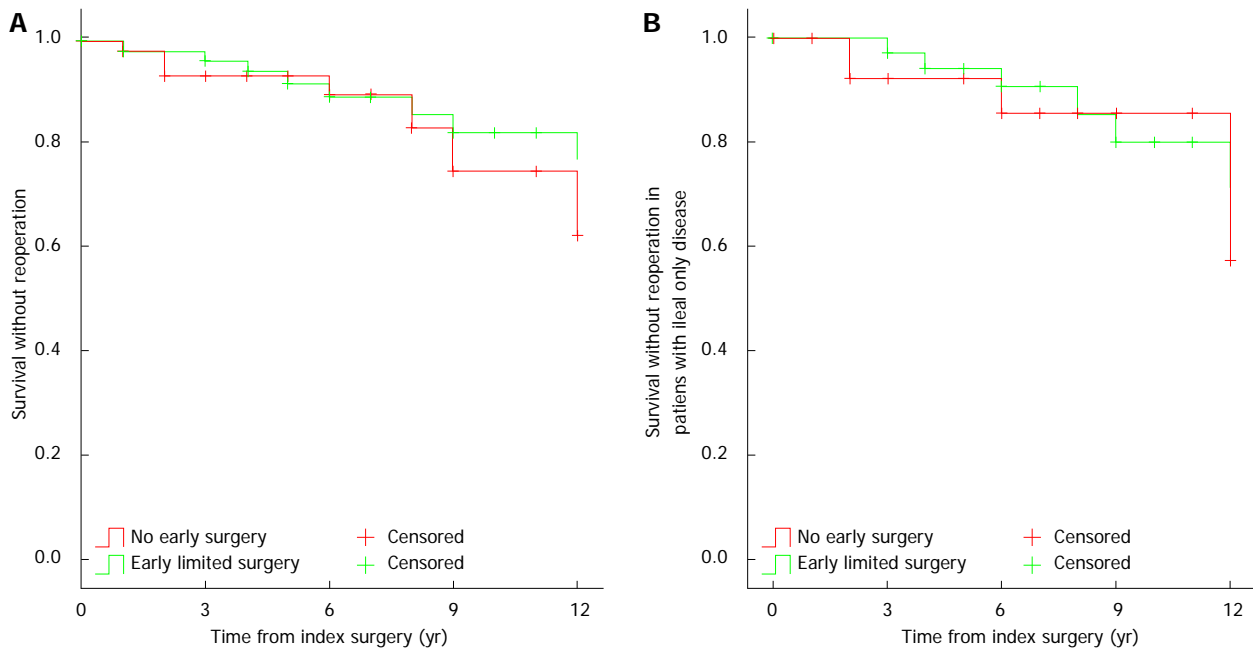
years, respectively,  $P_{\text{Logrank}} > 0.05$ , Figure 3A). Similar results were found if the analysis was restricted to patients with disease limited to the ileum only ( $n = 33$ , early surgery: 0%, 5.8%, and 20%, *vs* non-early surgery: 0%, 7.7%, and 14.3%, after 1, 5 and 10 years, respectively,  $P_{\text{Logrank}} > 0.05$ , Figure 3B).

## DISCUSSION

In the present study, we studied the benefits of early limited resective surgery in patients in a population-based Veszprem Province database. Results from this population-based inception cohort have shown that surgery rates and overall exposure to steroids and biological agents were lower in patients with early limited resective surgery. In contrast, although patients with early limited resective surgery needed less reoperation, by Kaplan-Meier analysis and multivariate Cox regression analysis, in the final propensity-score-matched model, this advantage was lost, and the probability of reoperation was similar in patients with early limited resection and non-early surgery. To the best of our knowledge, this is the first time that the association between early limited resective surgery and reoperation were studied using a propensity score model.

The rates of resective surgery vary significantly according to previous studies with a range from 25% to 61% in the first 5 years. An earlier review article by Walters *et al*<sup>[1]</sup> reported that the probability of first resective surgery was as high as 38%-96% within the first 15 years of diagnosis. The overall recurrence and reoperation rates after first resective surgery is 50%-60% and 28%-45%, respectively, during the subsequent 15 years. More recently, in the IBSEN Study, the cumulative probability of surgery was 13.6%, 27.0% and 37.9%, at 1, 5 and 10 years after diagnosis, respectively, while the risk of reoperation was also lower (9%)<sup>[16]</sup>.





**Figure 3** Need for reoperation in Crohn's disease patients with and without early resective surgery after matching on propensity scores. A: All Crohn's disease (CD) patients ( $n = 54$ ).  $P_{\text{Logrank}} > 0.05$ ; B: CD patients with ileal only disease location ( $n = 33$ ).  $P_{\text{Logrank}} > 0.05$ .

Similarly, in a recent meta-analysis from the IOIBD Epidemiology Task Force, the authors reported that the probability of surgery in CD decreased gradually between 1955 and 2003, even before the advent of the biological era<sup>[3]</sup>. An association with increased and earlier use of immunosuppressants was also suggested in studies from Wales<sup>[6]</sup> and Hungary<sup>[5]</sup>. Ramadas *et al.*<sup>[6]</sup> found that the rates of intestinal surgery decreased during the study period from 59% to 25% within 5 years of diagnosis. There was also a significant reduction in patients having any surgical procedure, from 60% to 35%. Likewise, in a previous referral center study from Hungary, early monotherapy with AZA or combination AZA/biological therapy was associated with a reduced risk for surgery. In earlier published studies from this cohort, the probability of surgical resection was 9.8%, 18.5% and 21.3% after 1, 3 and 5 years, respectively, in patients diagnosed between 2002 and 2006, and a recent decrease in surgical rates (in patients diagnosed after 1998) was observed<sup>[12]</sup>. These changes were associated with increased and earlier use of immunosuppressants<sup>[5]</sup>. Notwithstanding, the change in the use of immunosuppressants could be regarded as a marker of the complex changes in patient management rather than an exclusive factor itself.

Additional predictors of surgery include ileal or colonic disease location, complicated disease behavior, and age at onset - as reported previously in Sweden<sup>[17]</sup> and more recently from the IBSEN cohort<sup>[16]</sup>. Similarly, ileal (HR = 2.35) or ileocolonic (HR = 1.79) location compared to isolated colonic disease, as well as stricturing (HR = 4.33) or penetrating (HR = 3.44) disease at diagnosis, but not perianal disease, were independently associated with time to first surgery in a population-based cohort study from our research group<sup>[18]</sup>. Interestingly, we ob-

served a similar phenotype pattern associated with early limited resection (ileal location,  $P < 0.001$ ; complicated behavior,  $P < 0.001$ ; and age at onset,  $P = 0.06$ ).

However, the risk of surgery, as well as the disease course in patients with primary ileocecal CD, is somewhat different. In an earlier Swedish study, the risk of resective surgery in patients with primary ileocecal CD was 61%, 77% and 83%, after 1, 5 and 10 years of diagnosis, respectively, in 907 patients<sup>[19]</sup>. Relapse rates were 28% and 36% within 5 and 10 years of the first resection, respectively. In an Italian study, clinical and surgical recurrence rates after 5 years were 30.6% and 49.4%, and after 10 years they were 3.6% and 28%, respectively<sup>[20]</sup>. In addition, early surgery (within 3 years of diagnosis) was associated with a longer postoperative course free from clinical recurrence compared with late surgery, but not with reoperation. In the present study, despite higher rates of ileal and complicated disease in patients with early limited surgery, the overall need of steroids (OR = 0.45,  $P < 0.001$ ) during follow-up was lower, and there was a similar tendency for steroid-dependent disease course ( $P = 0.07$ ) and need for biological agents ( $P = 0.09$ ). In contrast, the overall use of AZA was similar in both groups, which may represent an active therapeutic decision by the treating physician rather than a marker of negative disease outcome. Of note, median follow-up was similar for both groups (12 years). We defined early surgery as that performed within 1 year of diagnosis, to avoid a potential bias due to early medical therapy. Of note, a positive effect of early aggressive medical therapy was already observed in patients in whom AZA was started within 18 mo of diagnosis, in a previous study in this cohort<sup>[5]</sup>. In addition, since reoperation rates may be lower in patients with extensive initial surgery due to the anatomical situa-

tion, we excluded these patients from the final analysis.

Comparable data were reported by Aratari *et al*<sup>[9]</sup>. In that study, early limited surgery at diagnosis was associated with less clinical recurrence - defined as need for steroids and lesions documented by endoscopy or radiology ( $P = 0.01$ ), and less need for immunosuppressants ( $P = 0.05$ ), but reoperation rates were not significantly different. Immunosuppressants were started in only 16.2% of patients with early or late surgery, confirming that the medical approach reported by the authors was much more conservative compared to the present study. In addition, the need for immunosuppressants was interpreted as a negative outcome. In another Italian study, the disease course of CD patients diagnosed during emergency surgery was compared to patients without emergency surgery<sup>[21]</sup>. The authors reported that the disease course was more benign in patients requiring surgery at diagnosis, by both univariate and multivariate analysis. Similar to the present study, surgery rates were significantly lower in patients operated on at diagnosis (in the present study with early limited surgery) compared to patients without surgery at diagnosis (in the present study without early limited surgery after matching on propensity scores). Observed surgery rates in the Italian study were 14% and 30% in the surgery-at-diagnosis group and 30% and 44% in the non-early surgery group, respectively. Of note, this design mimics clinical trials comparing two treatment algorithms: (1) early limited resective surgery; and (2) medical therapy but no early surgery. Observed surgery and drug exposure rates were clearly different. However, a different interpretation of our results may be that 40%-45% of patients could avoid surgery despite a similar patient phenotype within 10 years of diagnosis, while in the other group, all patients started with a limited operation. In addition, in the non-early surgery group, only 25.6% of patients received early AZA therapy. Thus, their therapeutic strategy was far from optimized. Moreover, reoperation rates were also analyzed in the present study, which would be very difficult in a clinical trial due to the need for long term follow-up. These were lower in patients with early limited resection versus those without early limited resection by Kaplan-Meier ( $P_{\text{Logrank}} = 0.038$ ) and Cox regression ( $P = 0.04$ ) analysis but the difference was lost after matching on propensity scores. Cost-benefit was not analyzed but with the increasing exposure to biological agents in certain CD populations<sup>[22]</sup>, these studies are urgently awaited.

The authors are aware of the possible limitations of the present study. One such potential limitation is the partially retrospective nature of the study, which may have led to bias in data interpretation. However, data were collected prospectively since 1985 and intestinal resection can be considered an unbiased and solid criterion, even retrospectively, because the indications for surgical intervention are well established. Another possible criticism may be the definition of early surgery. In this subgroup, patients presented partly with a short history of subacute or even acute symptoms. In these patients,

the timing of surgery was determined by the clinical presentation and not by the physician's strategic decision. However, this was also the case for a proportion of non-early surgical patients. Patient management has also changed significantly with regard to surgery techniques (laparoscopy) and imaging (availability of computed tomography and magnetic resonance imaging) that could have potentially affected therapeutic decision making, including indication for surgery. However, in the present study, one leading surgeon performed the majority of the operations and laparoscopic surgery and stricturoplasty was performed only in a minority of the cases. Similarly, there was only limited use of defunctioning ileostomy formation in routine management of CD during the follow-up period. Finally, postoperative management has significantly changed during the follow-up period, including routine endoscopy evaluation and prophylactic therapy; although in our analysis, we matched the groups for decade of diagnosis. In addition, there is accumulating evidence that anti-TNF therapies may reduce the number of operations<sup>[7,23]</sup>, although this was not a universal finding<sup>[24,25]</sup>. Exposure to biological agents was limited in the present study, and most of these patients received induction-only or intermittent infliximab therapy. In contrast, the strengths of this study include the long-term, comprehensive, validated data capture, and the use of both propensity score matching and multiple Cox regression analysis to overcome the limitations present in any partly retrospective study, thereby enabling unbiased analysis. Moreover, a follow-up of several years is needed to assess the reoperation rates, especially in patients without early limited resection, which is almost impossible in a clinical trial.

In conclusion, early limited resective surgery was associated with a lower risk for surgery and lower overall exposure to steroids and biological agents in this population-based cohort but it was not preventive for reoperations after matching on propensity scores. In addition, the similar exposure to immunosuppressants in the two groups may be interpreted as an active medical decision rather than a negative disease outcome. Conversely, resective surgery could be avoided in 40%-45% of CD patients with a similar disease phenotype without an early limited surgery within 10 years of diagnosis.

## COMMENTS

### Background

The optimal initial therapy in patients with limited, isolated, stenotic ileocecal Crohn's disease (CD) is debated. In some cases, early surgery may represent a valid alternative to medical therapy.

### Research frontiers

There are only limited data available on the disease course, including drug exposures, operation and reoperation rates in patients with and without an early limited resective surgery. In addition, data are lacking from population-based cohorts.

### Innovations and breakthroughs

The present long-term, population-based study in a well-characterized cohort of patients with CD has found that the overall exposure to steroids and biologicals was lower in patients with early limited resective surgery, observed surgery

rates were also lower, yet reoperation rates were not different in the two groups after matching on propensity scores

### Applications

Understanding the disease course in this subgroup of patients with CD may lead to more optimized patient management and follow-up.

### Terminology

Disease phenotype is categorized according to the Montreal classification and includes age at onset location and. Early limited surgery was defined as a resective surgery within the year of diagnosis and affecting only the terminal ileum and cecum.

### Peer review

This is good research. To add impact, adding in other centres in Europe should be considered in future.

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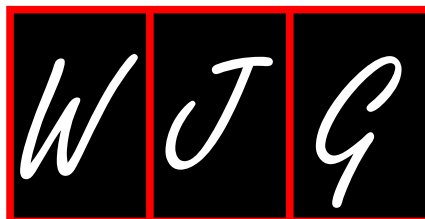
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## Dendritic cell co-stimulatory and co-inhibitory markers in chronic HCV: An Egyptian study

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### Abstract

**AIM:** To assess co-stimulatory and co-inhibitory markers of dendritic cells (DCs) in hepatitis C virus (HCV) infected subjects with and without uremia.

**METHODS:** Three subject groups were included in the study: group 1 involved 50 control subjects, group 2 involved 50 patients with chronic HCV infection and group 3 involved 50 HCV uremic subjects undergoing hemodialysis. CD83, CD86 and CD40 as co-stimulatory markers and PD-L1 as a co-inhibitory marker were assessed in peripheral blood mononuclear cells by real-time polymerase chain reaction. Interleukin-10 (IL-10) and hyaluronic acid (HA) levels were also assessed. All

findings were correlated with disease activity, viral load and fibrogenesis.

**RESULTS:** There was a significant decrease in co-stimulatory markers; CD83, CD86 and CD40 in groups 2 and 3 *vs* the control group. Co-stimulatory markers were significantly higher in group 3 *vs* group 2. There was a significant elevation in PD-L1 in both HCV groups *vs* the control group. PD-L1 was significantly lower in group 3 *vs* group 2. There was a significant elevation in IL-10 and HA levels in groups 2 and 3, where IL-10 was higher in group 3 and HA was lower in group 3 *vs* group 2. HA level was significantly correlated with disease activity and fibrosis grade in group 2. IL-10 was significantly correlated with fibrosis grade in group 2. There were significant negative correlations between co-stimulatory markers and viral load in groups 2 and 3, except CD83 in dialysis patients. There was a significant positive correlation between PD-L1 and viral load in both HCV groups.

**CONCLUSION:** A significant decrease in DC co-stimulatory markers and a significant increase in a DC co-inhibitory marker were observed in HCV subjects and to a lesser extent in dialysis patients.

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**Key words:** Hepatitis C virus; Uremia; Hemodialysis; Dendritic cells; CD83; CD86, CD40; PD-L1; Interleukin-10; Hyaluronic acid

**Core tip:** An assessment of the gene expression of co-stimulatory and a co-inhibitory marker (CD83, CD86, CD40, PD-L1) was conducted in patients with hepatitis C virus (HCV) infection and their correlations with viral load, hepatitis activity score and fibrosis grade were determined. There was a significant decrease in dendritic cell (DC) co-stimulatory markers in HCV infected

subjects, where HCV uremic subjects exhibited a lower degree of reduced co-stimulatory markers. There was a significant increase in the DC co-inhibitory marker in HCV infected subjects, where HCV uremic subjects exhibited a lower degree of increased co-inhibitory marker. All DC markers were significantly correlated with HCV viral load, hepatitis activity index and fibrosis score.

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## INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, with an estimated global prevalence of 3% occurring in about 180 million carriers and approximately 4 million people are newly infected annually<sup>[1]</sup>. It is estimated that up to 70% of individuals exposed to HCV develop viral persistence<sup>[2,3]</sup>. On average, over half a million people in Egypt are infected by HCV annually, far more than any other country in the world, according to a new study published in 2010<sup>[4]</sup>.

A high prevalence of HCV has been reported among hemodialysis (HD) patients worldwide. The prevalence of HCV infection among HD patients is significantly higher than healthy blood donors and the general population<sup>[5]</sup>. HD patients may be at risk for HCV due to the involvement of multiple routes of infection, especially poor blood screening of transfused blood, low standard of dialysis procedures and the need to apply infection control practices.

Patients who spontaneously clear HCV infection have strong and broad T cell responses, while patients with chronic HCV have weak and functionally impaired responses characterized by poor proliferation, impaired cytotoxicity and reduced cytokine secretion after antigen exposure<sup>[6,7]</sup>. Dendritic cells (DCs) are efficient and potent antigen presenters and activators of antigen-specific T cells and adaptive immunity<sup>[8]</sup>. Defective DC activation of T cells may underlie poor T cell responsiveness in HCV infection, and may, in part, determine the response to therapy<sup>[9,10]</sup>.

Human peripheral blood DCs are currently categorized into two major subsets: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs are effective antigen presenters to T cells and secrete interleukin 12, while pDCs are the most potent secretors of antiviral type-I interferons such as interferon  $\alpha$  (IFN- $\alpha$ )<sup>[11]</sup>. DCs migrate to sites of inflammation, sample antigens, and integrate generic microbial danger signals *via* innate immune receptors, named pathogen recognition receptors (PRRs)

that recognize pathogen-associated molecular patterns<sup>[12]</sup>. Signals from PRRs combine with signals from inflammatory cytokines to activate DCs, causing up-regulation of co-stimulatory molecules such as CD40 and CD86. DCs then migrate to lymphoid tissue where they activate antigen-specific CD4 and CD8 T cells by presenting antigens on major histocompatibility complex (MHC) class I and II molecules<sup>[13,14]</sup>.

Reports of global immune dysfunction in HCV infection are controversial; some authors have found faulty responses to general PRRs stimulation including decreased IFN $\alpha$  and IL12 secretion, reduced CD86 expression, decreased HLA-DR (MHC class II) and impaired stimulation of T cells in mixed lymphocyte reaction compared with normal controls<sup>[13]</sup>. Specific HCV proteins such as core and E2 can cause DC dysfunction in tissue culture models<sup>[14]</sup>. Other authors, including those using direct *ex vivo* human samples or a chimpanzee model of HCV have found no defects<sup>[15,16]</sup>. It has been consistently shown in HCV infection that pDC and mDC numbers are reduced in the peripheral compartment compared with normal controls, whereas reports have described increased numbers of DCs in the livers of HCV patients, suggesting hepatic DC sequestration<sup>[17-20]</sup>. The unresolved controversies listed above highlight the need for further study of DCs in HCV infection.

With regard to HD patients with HCV, some researchers reported altered monocyte-derived DC function in patients on HD<sup>[21]</sup>. However, reports on the natural history of hepatitis C in HD patients vary. Several studies stated that HCV disease activity in HD patients is mild, and is not progressive, perhaps due to immunological abnormalities in these patients<sup>[22]</sup>.

The present study was conducted to assess DC response to HCV infection *via* assessment of the gene expression of co-stimulatory markers (CD83, CD86, and CD40) and a co-inhibitory marker (PD-L1) in pDCs and mDCs, and to study the correlations between DC functions and viral load, hepatitis activity score and fibrosis grade.

## MATERIALS AND METHODS

The present study was conducted in the Hepatic Virology Center, Kasr Al-Ainy, Faculty of Medicine, Cairo University. The study involved Group I which included 50 healthy subjects of both genders aged 18-40 years representing the control group and 100 adult age- and sex-matched patients with HCV-related chronic liver disease (CLD).

The patients selected had to comply with the following inclusion criteria: HCV antibody-positive serum and HCV RNA-positive serum by reverse transcription polymerase chain reaction (RT/PCR) for more than 6 months. All patients had to comply with the following exclusion criteria: coinfection with HBV and HCV, hepatocellular carcinoma, severe psychiatric disease, serious co-morbid conditions, HIV-positive patients defined as

having a positive reaction to anti-HIV-1/2 (EIA), auto-immune hepatitis (positive reaction to antinuclear, anti-smooth muscle, anti-mitochondrial and anti-liver-kidney microsomal antibodies), schistosomiasis mansoni (patients with no previous history and negative stool examination), no previous history of regular use of hepatotoxic drugs or alcohol abuse (> 40 g of alcohol/d).

HCV patients were categorized into two groups: Group 2 included 50 HCV subjects with related CLD who were candidates for interferon therapy, and Group 3 included 50 HCV uremic subjects undergoing HD.

Patients were subjected to full clinical examination and abdominal ultrasonography. The following parameters were assessed in all subjects: serum levels of IL-10 and hyaluronic acid (HA) to assess fibrosis, as these parameters have been shown to be accurate in predicting significant fibrosis, severe fibrosis, and cirrhosis with area under characteristic curves (AUCs) of 0.73, 0.77 and 0.97, respectively. Moreover, accurate HA level cut-offs were defined for predicting significant fibrosis, severe fibrosis, and cirrhosis<sup>[23]</sup>. In addition, HA was an accurate noninvasive marker in predicting significant fibrosis in patients with hepatitis C on HD.

Quantitative gene expression of CD83, CD86, CD40 and PD-L1 in peripheral blood mononuclear cells was assessed by real-time PCR<sup>[24-27]</sup>. Histopathological examination of liver biopsy was performed using the Metavir scoring system for grading inflammation and staging fibrosis<sup>[28]</sup>.

Whole blood samples were collected from all subjects. Serum was separated for assessment of HA and IL-10 levels by ELISA kits supplied by Corgenix Inc. (Westminster, CO, United States) according to the manufacturer's recommendations.

The peripheral blood mononuclear cell layer (buffy coat) was isolated using Histopaque-1077 (Sigma, St. Louis, MO, United States) and centrifuged at 400 *g* for 30 min. Total RNA was isolated from the buffy coat using RNeasy purification reagent (Qiagen, Valencia, CA, United States). cDNA was generated from 5 µg of total RNA extracted with 1 µL (20 pmol) antisense primer and 0.8 µL superscript AMV reverse transcriptase for 60 min at 37 °C. The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 7700 sequence detector system (Applied Biosystems, Foster City, CA, United States). PCR primers were designed with Primer-BLAST Software<sup>[29]</sup>, (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) for RNA sequences from GenBank (Table 1). All primer sets had a calculated annealing temperature of 60 °C. Quantitative RT-PCR was performed in duplicate in a 25 µL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems, United States), 900 nmol/L of each primer and 2-3 µL of cDNA. Amplification conditions were 2 min at 50 °C, 10 min at 95 °C and 40 cycles of denaturation for 15 s and annealing/extension at 60 °C for 10 min. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems

**Table 1 Real-time polymerase chain reaction primers of CD83, CD86, CD40 and PD-L1**

Homo sapiens CD40	5'-CCA AAA CGG GCC CTG CTC CA-3' 5'-GAG CCT GGC CCC CTC CAA CA-3' Gene Bank accession number NM_000074.2
Homo sapiens CD86	5'-TAG GAG GTA CGG GGA GCT CGC AA-3' 5'-TTG GCA TGG CAG GTC TGC AGT C-3' Gene Bank accession number 006889.3
Homo sapiens CD83	5'-CGA CGC CGG AGG TGA AGG TG-3' 5'-TCC GGG TCC TGC AGA GTG CA-3' Gene Bank accession number 001040280.1
Homo sapiens PDL1	5'-ACA GAG GGC CCG GCT GTT GA-3' 5'-CTT CGG CCT TGG GGT AGC CC-3' Gene Bank accession number AY254342.1
Homo sapiens GAPDH	5'-GAAGGTGAAGGTCGGAGTCA-3' 5'-GAAGATGGTATGGGATTTTC-3' Gene Bank accession number NC_000019.9

PDL1: Programmed death ligand 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

(Foster City, CA, United States). Relative gene expression of CD83, CD86, CD40 and PD-L1 mRNA was calculated using the comparative Ct method as previously described. All values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and reported as fold change over background levels detected in the control group.

### Statistical analysis

The SPSS program version 16.0.1 (SPSS Inc., Chicago, IL, United States) was used. Numerical data were expressed as mean ± SD. For comparisons between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired *t* test for two groups. Comparisons were considered statistically significant if *P* < 0.05. Spearman correlations were assessed between certain studied parameters.

## RESULTS

The results showed that there was a significant decrease in all co-stimulatory markers; CD83, CD86 and CD40 in group 2 (HCV subjects) and in group 3 (HCV subjects on HD) in comparison to control subjects. Co-stimulatory markers were significantly higher in group 3 in comparison to group 2. With regard to PD-L1, there was a significant increase in groups 2 and 3 in comparison to control subjects. PD-L1 was significantly lower in group 3 as compared to group 2 (Figure 1). This was reflected in viral load, where significant negative correlations were observed between all co-stimulatory markers and viral load in groups 2 and 3, except CD83 in dialysis patients (Figure 2)

The findings in the present study showed a significant elevation in IL-10 and in HA levels in groups 2 and 3, where IL-10 was more significantly elevated in group 3 and HA was significantly lower in group 3 in comparison to group 2 (Figure 3).

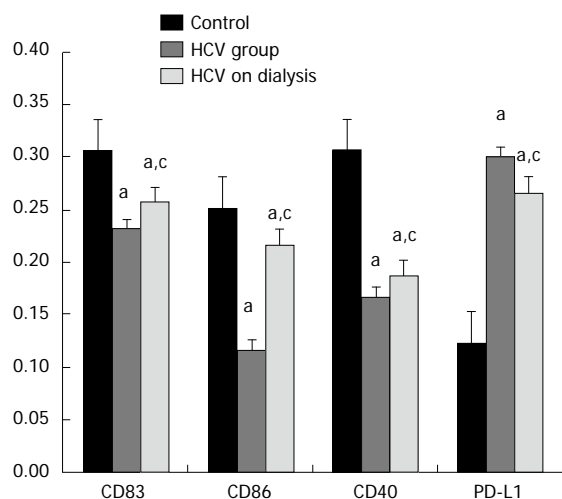


Figure 1 Relative quantitative gene expression of CD83, CD86, CD40 and PD-L1 in all study subjects expressed as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  in both hepatitis C virus (HCV) patient groups vs control group; <sup>c</sup> $P < 0.05$  in the HCV patient group vs HCV uremic patients on hemodialysis.

In group 2, the results showed that there was a significant correlation between HA and hepatitis activity score as well as grade of fibrosis;  $P < 0.001$ . No correlation between IL-10 levels and hepatitis activity score was observed, whereas, there was a significant positive correlation between IL-10 and fibrosis grade,  $P < 0.001$  (Figure 4). Biopsy samples were not taken from group 3 patients (HCV on dialysis).

The results showed that there were significant negative correlations between all co-stimulatory markers and viral load in groups 2 and 3, except CD83 in dialysis patients. There was a significant positive correlation between PD-L1 and viral load in both HCV groups (Figure 2).

Of the 50 non-uremic patients who were candidates for interferon therapy only 4 remained PCR positive for HCV after treatment.

## DISCUSSION

The results of the present study showed that in HCV infected subjects peripheral blood mononuclear cells exhibited lower expression of co-stimulatory markers; CD83, CD86, and CD40 and higher expression of a co-inhibitory marker; PD-L1 in comparison to healthy control subjects. Various studies have assessed DC function in HCV infection. Some have reported that DC function was impaired in HCV infection, which was identified by impaired allostimulatory capacity, decreased DC frequencies, increased mDC IL-10 secretion and decreased IL-12 secretion, as well as decreased pDC IFN- $\alpha$  secretion, while others did not<sup>[15,17,30]</sup>.

Our findings coincide with the results reported by MacDonald *et al.*<sup>[12]</sup>, who stated that monocyte-derived DCs from patients with chronic HCV infection were significantly defective in their capacity to up-regulate the expression of surface molecules involved in antigen presentation (HLA-DR, CD86, CD40) and a classical marker

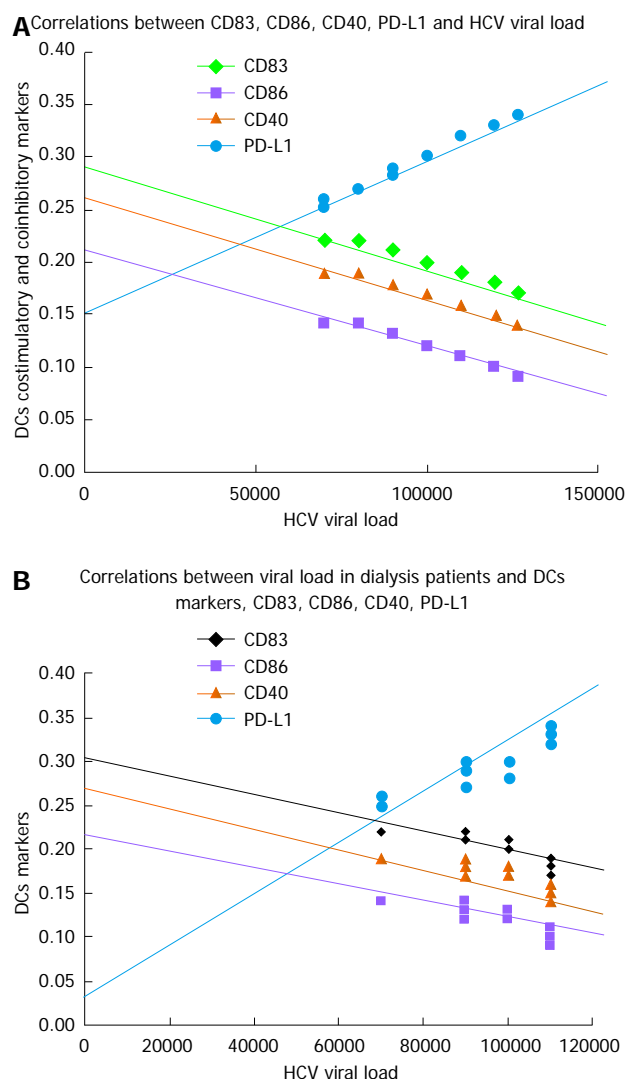
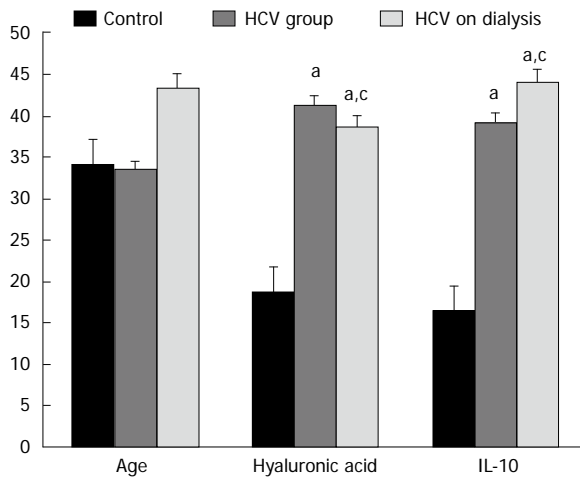


Figure 2 Correlations. A: Between hepatitis C viremia and CD83.  $r = -0.98$ ,  $P < 0.001$ , CD86:  $r = -0.866$ ,  $P < 0.001$ , CD40:  $r = -0.98$ ,  $P < 0.001$ , PD-L1:  $r = 0.889$ ,  $P < 0.001$ ; B: Between hepatitis C viremia in uremic patients on hemodialysis and CD83.  $r = -0.096$ ,  $P > 0.05$  NS, CD86:  $r = -0.588$ ,  $P < 0.05$ , CD40:  $r = -0.946$ ,  $P < 0.001$ , PD-L1:  $r = 0.663$ ,  $P < 0.05$ . HCV: Hepatitis C virus; DC: Dendritic cell

of DC activation (CD83) and at physiological ratios of DCs to T cells, and decreased their ability to present antigen in allogeneic mixed lymphocyte reaction (MLR) assays. More recently, Shen *et al.*<sup>[24]</sup> reported that impaired HCV-specific T cell immunity was associated with the persistence of HCV infection. DC dysfunction was believed to be involved in impaired T cell immunity, but the mechanisms are not understood. The results showed that the expression of both co-stimulatory markers (CD83, CD86, and CD40) and a co-inhibitory marker (PD-L1) was imbalanced in HCV-infected patients compared with healthy controls. The PD-L1/CD86 ratio was increased and positively correlated with PD-L1 expression on DCs in HCV-infected patients. The allostimulatory capacity of DCs was impaired and inversely correlated with PD-L1 expression and the PD-L1/CD86 ratio. These findings agree with our study and suggest that the effect of inhibitory marker PD-L1 overwhelmed the effect of co-



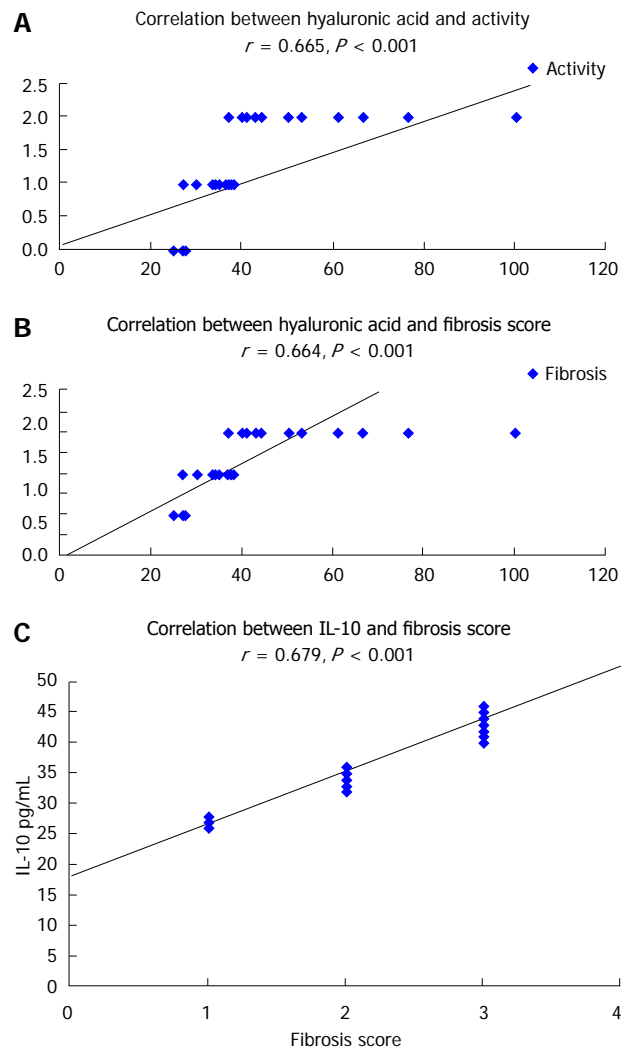


**Figure 3** Serum levels of hyaluronic acid (ng/mL) and interleukin-10 (pg/mL) in all study subjects expressed as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  vs control group, <sup>c</sup> $P < 0.05$  in the hepatitis C virus (HCV) patient group vs HCV uremic patients on hemodialysis. IL-10: Interleukin-10.

stimulatory markers and down-regulated DC-T activation in HCV-infected patients.

HCV may inhibit the immune response of DCs, hindering the adaptive response from T cells<sup>[31]</sup>. It was hypothesized that DC dysfunction may determine the response to PEG-IFN/ribavirin therapy. In the study by Mengshol *et al.*<sup>[32]</sup>, the authors found that pDCs and mDCs were decreased compared to normal controls, consistent with prior studies in HCV and similar to prior studies of patients with HIV<sup>[33]</sup>.

In the present study, the results of HA assessment revealed a significant elevation in HCV infected subjects in comparison to the healthy control group. Moreover, HA levels were positively correlated with hepatitis activity score and grade of fibrosis. These findings can be explained by a recent study conducted on another virus by Spahn *et al.*<sup>[34]</sup>, who stated that ineffective CD8 (+) T cell immunity to adeno-associated virus can result in prolonged liver injury and fibrogenesis. More recently, Jiao *et al.*<sup>[35]</sup> determined whether liver DCs play a role in enhancing regression of liver fibrosis in murine carbon tetrachloride-induced liver injury. They found that conditional DC depletion soon after discontinuation of the liver insult led to delayed regression of fibrosis and reduced clearance of activated hepatic stellate cells, the key fibrogenic cells in the liver. Conversely, DC expansion induced either by Flt3L (fms-like tyrosine kinase-3 ligand) or adoptive transfer of purified DCs accelerated liver fibrosis regression. DC modulation of fibrosis was partially dependent on matrix metalloproteinase (MMP)-9, as MMP-9 inhibition abolished the Flt3L-mediated effect and the ability of transferred DCs to accelerate regression of fibrosis. In contrast, transfer of DCs from MMP-9-deficient mice failed to improve fibrosis regression. Another study conducted by Ryan *et al.*<sup>[36]</sup> proved that in HCV chronically infected subjects, a single nucleotide polymorphism in a c-type lectin expressed by DCs,



**Figure 4** Correlation analysis. A: Between hyaluronic acid levels and hepatitis activity score ( $P < 0.001$ ); B: Between hyaluronic acid levels and fibrosis score ( $P < 0.001$ ); C: Between interleukin-10 (IL-10) levels and fibrosis score ( $P < 0.001$ ).

CD209, was associated with more advanced liver disease and with significantly higher liver fibrosis scores.

With regard to IL-10 levels in our study, the findings revealed significantly elevated IL-10 levels in HCV infected subjects in comparison to healthy controls. Moreover, IL-10 levels were positively correlated with fibrosis grade. These findings agree with results reported by Díaz-Valdés *et al.*<sup>[37]</sup>, who stated that high levels of IL-10 present in chronic HCV infection are associated with the poor antiviral cellular immune responses found in these patients. To overcome the immunosuppressive effect of IL-10 on antigen-presenting cells such as DCs, they developed peptide inhibitors of IL-10 and found that IL-10 inhibiting peptides have important applications in enhancing anti-HCV immune responses by restoring the immunostimulatory capabilities of DCs.

With regard to HD patients, Choi *et al.*<sup>[21]</sup> found that surface expression of major histocompatibility complex class II, CD83, and CD86, and chemokine receptor CCR7 in monocyte derived dendritic cells (moDCs) was

not different between HD patients and healthy controls. Furthermore, moDCs from HD patients produced significantly higher amounts of IL-6, IL-8, IL-1b, and TNF- $\alpha$  when stimulated by cytokine cocktails compared to healthy controls. Abnormalities in cytokine production by moDCs in ESRD patients have also been reported in several previous studies<sup>[38,39]</sup>. Verkade *et al.*<sup>[39]</sup> also demonstrated a marked increase in IL-15 production, a known stimulatory cytokine for DCs, while IL-10 and IL-12p70 levels were not different. In addition, mature moDCs from HD patients showed significantly enhanced allogeneic T cell proliferation compared to healthy controls. These findings could explain our results which showed a significantly lower degree of reduced co-stimulatory markers as well as a significantly lower degree of elevated co-inhibitory marker in HD patients as compared to HCV subjects without uremia.

In conclusion, a significant decrease in DC co-stimulatory markers in HCV infected subjects was observed, where HCV uremic subjects exhibited a lower degree of reduced co-stimulatory markers. There was a significant increase in the DC co-inhibitory marker in HCV infected subjects, where HCV uremic subjects exhibited a lower degree of elevated co-inhibitory marker. All DC markers were significantly correlated with HCV viral load, hepatitis activity index and fibrosis score.

## COMMENTS

### Background

Defective dendritic cell (DC) activation of T cells may underlie poor T cell responsiveness in hepatitis C virus (HCV) infection, and may, in part, determine the response to therapy. It has been consistently shown in HCV infection that plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) numbers are reduced in the peripheral compartment compared with normal controls. Other reports have described increased numbers of DCs in the livers of HCV patients, suggesting hepatic DC sequestration. The unresolved controversies listed above highlight the need for further study of DCs in HCV infection. With regard to hemodialysis (HD) patients with HCV, some researchers have reported altered monocyte-derived dendritic cell function in patients on HD. However, reports on the natural history of hepatitis C in HD patients vary. Several studies stated that HCV disease activity in HD patients is mild, and is not progressive, perhaps due to immunological abnormalities in these patients. The present study was conducted to assess DC response to HCV infection with and without uremia via assessment of the gene expression of co-stimulatory markers (CD83, CD86, and CD40) and a co-inhibitory marker (PD-L1) in pDCs and mDCs, and to study the correlations between DC functions and viral load, hepatitis activity score and fibrosis grade.

### Research frontiers

Researchers have recently explored the mechanisms by which DC function is regulated during HCV infection, leading to impaired antiviral T cell responses and so to persistent viral infection. Recently, DC-based vaccines against HCV have been developed. Several studies describe the current understanding of DC function during HCV infection and explore the prospects of DC-based HCV vaccines. In particular, they describe the biology of DC, the phenotype of DC in HCV-infected patients, the effect of HCV on DC development and function, studies on new DC-based vaccines against HCV infection, and strategies to improve the efficacy of DC-based vaccines.

### Innovations and breakthroughs

A recent study stated that the immature pDC phenotype and sustained pDC and mDC hyperresponsiveness are associated with spontaneous resolution of acute HCV infection. Several investigators found that injection of DCs presenting viral proteins constitutes a promising approach to stimulate T cell immunity

against HCV. They also describe the strategy implemented to enhance antigen loading and immunostimulatory functions of DCs used in the preparation of therapeutic vaccines.

### Applications

Assessment of DC functions in HCV patients may be applicable to anticipate the response to HCV standard of care therapy and to assign patients to specific therapeutic protocols. DC-based immunotherapy may be used in selected HCV cases with poor therapeutic response, high fibrosis index and high hepatitis activity score.

### Terminology

Plasmacytoid DCs (pDCs), myeloid DCs (mDCs), interferon  $\alpha$  (IFN $\alpha$ ), pathogen recognition receptors (PRRs), pathogen-associated molecular patterns (PAMPs), co-stimulatory markers (CD83, CD86, and CD40), a co-inhibitory marker (PD-L1), matrix metalloproteinase (MMP).

### Peer review

Interesting subject with sufficiently large cohorts to detect difference in response to Hepatitis C infection in those with liver disease vs others under dialysis treatment for chronic kidney failure. The key finding that there is "a significant decrease in dendritic cell co-stimulatory markers in HCV infected subjects, where HCV uremic subjects exhibited lower degree of the decrease. There was a significant increase in dendritic cell co-inhibitory marker in HCV infected subjects, where HCV uremic subjects exhibited lower degree of the elevation." If proved in an individual HCV patient may prove important in directing management of HCV infected persons.

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## Characteristics of nonvariceal upper gastrointestinal hemorrhage in patients with chronic kidney disease

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### Abstract

**AIM:** To evaluate the clinical characteristics of non-variceal upper gastrointestinal hemorrhage (NGIH) in patients with chronic kidney disease (CKD).

**METHODS:** From 2003 to 2010, a total of 72 CKD patients (male  $n = 52$ , 72.2%; female  $n = 20$ , 27.8%) who had undergone endoscopic treatments for NGIH were retrospectively identified. Clinical findings, endoscopic features, prognosis, rebleeding risk factors, and mortality-related factors were evaluated. The characteristics of the patients and rebleeding-related data

were recorded for the following variables: gender, age, alcohol use and smoking history, past hemorrhage history, endoscopic findings (the cause, location, and size of the hemorrhage and the hemorrhagic state), therapeutic options for endoscopy, endoscopist experience, clinical outcomes, and mortality.

**RESULTS:** The average size of the hemorrhagic site was  $13.7 \pm 10.2$  mm, and the most common hemorrhagic site in the stomach was the antrum ( $n = 21$ , 43.8%). The most frequent method of hemostasis was combination therapy ( $n = 32$ , 44.4%). The incidence of rebleeding was 37.5% ( $n = 27$ ), and 16.7% ( $n = 12$ ) of patients expired due to hemorrhage. In a multivariate analysis of the risk factors for rebleeding, alcoholism (OR = 11.19,  $P = 0.02$ ), the experience of endoscopists (OR = 0.56,  $P = 0.03$ ), and combination endoscopic therapy (OR = 0.06,  $P = 0.01$ ) compared with monotherapy were significantly related to rebleeding after endoscopic therapy. In a risk analysis of mortality after endoscopic therapy, only rebleeding was related to mortality (OR = 7.1,  $P = 0.02$ ).

**CONCLUSION:** Intensive combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic.

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**Key words:** Chronic kidney diseases; Gastrointestinal hemorrhage; Endoscopy; Peptic ulcer; Alcoholics

**Core tip:** Patients with chronic kidney disease (CKD) have increased hemorrhagic complications, including nonvariceal upper gastrointestinal hemorrhage (NGIH). These individuals also have a higher risk of rebleeding than patients without renal dysfunction. Initial intensive

combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic. Factors of the consumption of alcohol, endoscopic monotherapy, and endoscopists' lack of experience are associated with rebleeding, which is the most important factor for the prediction of mortality in CKD patients with NGIH.

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## INTRODUCTION

The hemostasis rate of nonvariceal upper gastrointestinal hemorrhage (NGIH) is 90%<sup>[1]</sup>. However, rebleeding develops in approximately 20% of patients, and the mortality rate has been reported as ranging from 6%-25%<sup>[1-8]</sup>. In patients with chronic kidney disease (CKD), gastrointestinal hemorrhage is a common complication<sup>[9]</sup>, and hemorrhage from the upper gastrointestinal tract from these patients accounts for 7.8%-12.2% of patients with total upper gastrointestinal hemorrhage<sup>[10]</sup>. Peptic ulcer is the most common cause of upper gastrointestinal hemorrhage in CKD, followed by erosive gastritis, esophagitis, vascular ectasia, and angiodysplasia<sup>[9,12]</sup>. Several reports have suggested that the prevalence of peptic ulcer in patients with CKD is higher than in the general population<sup>[13,14]</sup>. Many studies on the outcome of and risk factors for peptic ulcer bleeding in patients with normal renal function have been reported. However, there are few studies on the outcome of acute hemorrhage due to peptic ulcer and the risk factors for rebleeding in patients with CKD<sup>[10,12]</sup>.

Although the pathogenesis of hemorrhage in patients with CKD is not completely understood, three hypotheses have been proposed to explain the mechanism. First, uremic platelet dysfunction is believed to be the most important factor<sup>[12,15]</sup>. Second, a high rate of platelet dysfunction may be responsible for the increased frequency of rebleeding compared with the frequency in patients without renal dysfunction<sup>[9,16,17]</sup>. Lastly, previous studies on peptic ulcer and upper gastrointestinal hemorrhage in patients with renal dysfunction have suggested that hemorrhage in these patients is associated with acid secretion and mucosal integrity<sup>[11,18-20]</sup>. Moreover, hemodialysis, heparin use, abnormal platelet function, and anemia could be related to NGIH in patients with end-stage renal disease (ESRD), although the evidence is limited. Thus, the objective of this study was to evaluate the clinical characteristics of upper gastrointestinal ulcer hemor-

rhage in CKD patients and to determine the risk factors for rebleeding in patients undergoing endoscopic therapy.

## MATERIALS AND METHODS

### Patients

Between December 2003 and December 2010, a total of 72 CKD patients (M:F = 52:20, mean age:  $63.9 \pm 11.1$ ) who had undergone endoscopic therapy for NGIH were retrospectively evaluated. CKD was defined according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative guidelines for nephrologists, which describe the presence of either kidney damage or decreased renal function (glomerular filtration rate  $< 60$  mL/min per  $1.73 \text{ m}^2$ ) for more than 3 mo. ESRD was defined as chronic kidney failure (glomerular filtration rate  $< 15$  mL/min per  $1.73 \text{ m}^2$ ) treated by dialysis. Patients who were not treated with endoscopic management and who had mallory-weiss syndrome were excluded. The clinical and endoscopic characteristics of patients with rebleeding were reviewed and compared with the characteristics of patients without rebleeding. Data were recorded for the following variables: gender, age, dialysis use, the method of dialysis, alcohol use and smoking history, past hemorrhage history, endoscopic findings (the cause, location, and size of the hemorrhage and the hemorrhagic state), endoscopic therapy, endoscopist experience, clinical outcomes, and mortality. Written informed consent to treatment was obtained from each patient. The study was performed in accordance with the Helsinki Declaration.

The patients' vital signs were checked every 10 min before endoscopy, every 2 h for the 24 h after endoscopy, and every 6 h after follow-up endoscopy. The hemoglobin level was checked more than once per day, and a blood transfusion was performed if the hemoglobin level decreased to below 9 g/dL. Rebleeding was defined as fresh hematemesis, fresh melena with blood pressure  $< 100$  mmHg, a drop in the hemoglobin level of more than 2 g/dL, or endoscopic confirmation of hemorrhage or pathologic lesions necessitating endoscopic management within 7 d after initial therapy. The hemorrhagic state was classified into five groups based on Forrest's classification: active pumping, active oozing, vessel exposure, red clots, and black clots.

### Endoscopic therapy

All of the patients presented to the hospital with NGIH and underwent an endoscopic examination within 24 h. Endoscopic management for peptic ulcer bleeding was performed. Intravenous proton pump inhibitors (PPIs) were prescribed to promote healing of the lesion before and after endoscopic therapy. Levin tube irrigation with more than 3000 cc of normal saline was performed before initial endoscopy. Thirteen experienced gastroenterologists ( $> 6000$  cases of endoscopy) performed therapeutic endoscopic procedures for NGIH during the study period. The therapeutic options used to treat NGIH were

**Table 1** Clinical characteristics of chronic kidney disease patients with peptic ulcer hemorrhage

Variables	Number
Gender (Male/female)	52/20
Age (yr) <sup>1</sup>	63.9 ± 11.1
Alcohol (Yes/no)	25/47
Smoking (Yes/no)	29/43
Past hemorrhage History (Yes/no)	11/61
ESRD (Yes/no)	50/22
Dialysis	
Hemodialysis	45
Peritoneal dialysis	5
Symptoms (Hematemesis/Melena/Syncope/Others)	32/28/2/10
Initial blood pressure	
Systolic (mmHg) <sup>1</sup>	128.7 ± 40.0
Diastolic (mmHg) <sup>1</sup>	75.9 ± 16.5
Initial heart rates <sup>1</sup>	94.0 ± 24.5
Hb (g/dL) <sup>1</sup>	7.4 ± 2.0
Platelet (10 <sup>9</sup> /L) <sup>1</sup>	208 ± 156
INR/PTT (second) <sup>1</sup>	1.14 ± 0.35/47.2 ± 55.6

<sup>1</sup>mean ± SD. ESRD: End-stage renal disease; INR: International normalized ratio; Hb: Haemoglobin.

1:10000 epinephrine injection, fibrin glue (Greenplast, Green Cross, Chung won, South Korea) injection, hemoclipping, electrocoagulation, endoscopic band ligation, and argon plasma coagulation. The patients were scheduled for a follow-up endoscopic examination within 24 h. If their general condition was not suitable for endoscopy, the follow-up examination was delayed. At the follow-up examination, oral intake was initiated, and endoscopic biopsy and *Helicobacter pylori* (*H. pylori*) tests were performed. If active bleeding or vessel exposure was observed during the follow-up endoscopic examination, this event was considered as rebleeding, and second endoscopic hemostasis was attempted. If continuous bleeding developed, not controlled by endoscopic hemostasis, operational or interventional radiologic management was performed. During fasting periods, intravenous pantoprazole sodium as a 40 mg bolus was supplied twice per day. After starting oral intake, a standard dose of oral PPIs was administered every morning for 6–8 wk. If the tests for *H. pylori* were positive, eradication medications (PPI + amoxicillin + clarithromycin) were administered for 7 d. Laboratory tests, abdominal ultrasonography, and routine abdominal X-ray were performed after the procedure to evaluate possible complications, including rebleeding or perforation.

### Statistical analysis

The  $\chi^2$  test and Student's *t* test were used to evaluate baseline characteristics. Categorical variables were analyzed by the  $\chi^2$  test, and continuous variables were assessed by the Student's *t* test. Univariate analysis and multivariate logistic regression were used to detect independent risk factors related to rebleeding during follow-up periods and prognosis. A *P* value < 0.05 was considered as significant for all tests. Analyses were performed using SPSS software, version 18.0 (SPSS Inc., Chicago,

**Table 2** Endoscopic findings, therapy and prognosis of clinical risk factors patients with peptic ulcer hemorrhage

Variables	Numbers
Size of ulcer (mm) <sup>1</sup>	13.7 ± 10.2
Hemorrhage state (Pumping/oozing/vessel/red/black)	6/38/18/5/5
Location 1 (Gastric/Duodenum)	48/24
Location 2 (Antrum/Angle/Body/Cardia)	21/11/15/1
Location 3 (Anterior/Posterior/Lesser/Greater)	11/7/22/8
Endoscopic therapy (Injection/Coagulation/Clip/Combination)	27/8/5/32
Amount of epinephrine (cc) <sup>1</sup>	15.6 ± 12.9
Experience of endoscopists (yr) <sup>1</sup>	3.5 ± 2.7
<i>H. pylori</i> infection (Yes/no)	19/33
Rebleeding (Yes/no)	27/45
Hemorrhage related death (Yes/no)	12/60

<sup>1</sup>mean ± SD. *H. pylori*: *Helicobacter pylori*.

IL, United States).

## RESULTS

### Characteristics of patients

During the 4-year study period, 72 CKD patients with peptic ulcer hemorrhage were identified. The clinical characteristics of these patients are summarized in Table 1. The mean age of the patients with CKD was 63.9 ± 11.1. In total, 61 (84.7%) patients were experiencing their first hemorrhagic episode; 8 (11.1%) patients, their second; and 3 (4.2%) patients, their third. In this study, 50 (69.4%) ESRD patients were detected, of whom 45 (90%) patients were undergoing hemodialysis, and 5 (10%) were undergoing peritoneal dialysis. The initial systolic blood pressure of the patients was 128.7 ± 40.0 mmHg, and the diastolic blood pressure was 75.9 ± 16.5 mmHg. The hemoglobin level of the patients was 7.4 ± 2.0 g/dL, and the platelet count was 208 ± 156 (10<sup>9</sup>/L).

All of these patients were managed by endoscopy for peptic ulcer hemorrhage. The endoscopic findings and treatments of these patients are shown in Table 2. The mean ulcer size (mm) was 13.7 ± 10.2, and the hemorrhagic location the stomach in 48 (66.7%) cases and the duodenum in 24 (33.3%) cases. The most common hemorrhagic site in the stomach was the antrum (43.8%).

The therapeutic method of endoscopy was injection for 27 (37.5%) patients, coagulation for 8 (11.1%) patients, clipping for 5 (6.9%) patients, and combination therapy for 32 (44.4%) patients. The most common combination was epinephrine and glue injection (*n* = 11), followed by epinephrine injection and coagulation (*n* = 10) and epinephrine injection and clipping (*n* = 10). The mean number of years of experience of the endoscopists was 3.5 ± 2.7 years. The total number of patients with rebleeding was 27 (37.5%), and hemorrhage-related death was observed in 12 patients (16.7%, Table 2).

### Univariate analysis of risk factors for rebleeding

The incidence of rebleeding was 37.5% (*n* = 27), and

**Table 3** Univariate analysis for clinical risk factors of rebleeding

Characteristics	Rebleeding ( <i>n</i> = 27)	No Rebleeding ( <i>n</i> = 45)	<i>P</i> value
Gender (Male/female)	18/9	34/11	NS
Age (yr) <sup>1</sup>	62.9 (11.5)	64.6 (11.0)	NS
Heart rate <sup>1</sup>	95 (17)	93 (29)	NS
CKD (not ESRD)/ESRD	10/17	12/33	NS
Previous hemorrhage history (Yes/no)	4/23	7/38	NS
Blood pressure			
Systolic (mmHg) <sup>1</sup>	128 (32)	129 (45)	NS
Diastolic (mmHg) <sup>1</sup>	76 (16)	76 (17)	NS
Lab			
Hb (g/dL) <sup>1</sup>	7.4 (1.9)	7.5 (2.1)	NS
Platelet (10 <sup>9</sup> /L) <sup>1</sup>	207 (174)	208 (145)	NS
INR/PTT	1.2/46.7	1.1/48.0	NS
Alcohol (Yes/no)	15/12	10/35	< 0.01
Smoking (Yes/no)	15/12	14/31	< 0.05

<sup>1</sup>mean ± SD. NS: Not significant; CKD: Chronic kidney disease; ESRD: End-stage renal disease; INR: International normalized ratio; Hb: Haemoglobin.

16.7% (*n* = 12) of patients expired due to bleeding. In the univariate analysis of clinical risk factors for rebleeding, there was no statistically significant difference in gender, age, dialysis method, or previous hemorrhage history between the rebleeding and the no-rebleeding groups (Table 3). Alcohol consumption was noted for 15/27 (55.6%) patients in the rebleeding group and 10/45 (22.2%) patients in the no-rebleeding group (*P* < 0.01). Additionally, smoking was reported by 15/27 (55.6%) patients in the rebleeding group and 14/45 (31.1%) patients in the no-rebleeding group (*P* < 0.01). The univariate analysis of endoscopic risk factors for rebleeding is shown in Table 4. Hemorrhagic states, ulcer sizes, and therapeutic methods were not significantly different between the rebleeding and the no-rebleeding groups. However, the number of years of experience of the endoscopists was 2.8 years for the rebleeding group and 4.0 years for the no-rebleeding group, which was a statistically significant difference (*P* < 0.05). In an analysis according to the endoscopists' status, being a doctor on fellowship was associated with rebleeding compared with being a doctors on the faculty (OR = 2.1, *P* = 0.02).

#### Multivariate analysis of risk factors for rebleeding and mortality

In the multivariate analysis of risk factors for rebleeding, the consumption of alcohol, endoscopic monotherapy, and endoscopists' lack of experience were associated with rebleeding development (Table 5). The alcohol-consuming group had an OR of 11.19 (*P* = 0.02) for rebleeding compared with the non-alcohol-consuming group. Although therapeutic methods were not associated with rebleeding in the univariate analysis, combination endoscopic treatment was associated with less frequent development of rebleeding in the multivariate analysis (OR = 0.06, *P* = 0.01). The experience of endoscopists

**Table 4** Univariate analysis for endoscopic risk factors of rebleeding

Variables	Rebleeding	No rebleeding	<i>P</i> value
Hemorrhage state			NS
Active pumping	1	5	
Active oozing	15	23	
Blood vessel	7	11	
Red or black clot	4	6	
Ulcer size (mm) <sup>1</sup>	14.4 (11.9)	13.2 (9.1)	NS
Location	11/7	21/9	NS
(antrum and angle <i>vs</i> body)			
<i>H. pylori</i> infection (Yes/no)	7/20	17/28	NS
Therapy			NS
Injection	11	16	
APC or electrocoagulation	4	4	
Clip	2	3	
Combination	10	22	
Amount epinephrine <sup>1</sup>	16.9 (15.3)	14.7 (11.3)	NS
Endoscopists' experience <sup>1</sup>	2.8 (1.9)	4.0 (3.0)	< 0.05
Endoscopists' status			< 0.05
Fellowship doctor	20	19	
Faculty doctor	7	26	

<sup>1</sup>mean ± SD. NS: Not significant; *H. pylori*: *Helicobacter pylori*; APC: Argon plasma coagulation

was significantly associated with the development of rebleeding in the multivariate analysis (OR = 0.56, *P* = 0.03). The risk factor associated with prognosis was rebleeding alone (OR = 7.10, *P* = 0.02, Table 6).

## DISCUSSION

Patients with CKD have increased hemorrhagic complications<sup>[10]</sup>. Additionally, there are a higher rebleeding risk and greater mortality in patients on dialysis than in patients without renal dysfunction<sup>[11]</sup>. The current study investigated peptic ulcer hemorrhage in CKD patients and found that these individuals are at high risk of rebleeding. In this study, the rebleeding rate in patients with CKD was 37.5%. This result is higher than for the CKD (14%) or normal renal function (12%) group and similar to rebleeding in ESRD patients (38%) in a previous study<sup>[12]</sup>. Moreover, the result is higher than the rate determined for Korean CKD patients (14.3%) in another study<sup>[21]</sup>. However, the definition of rebleeding differed. In the present study, rebleeding was confined to episodes within 7 d after endoscopic therapy, whereas there was no clear statement about time in previous studies, in which even rebleeding at 30 d after the first hemorrhage was included<sup>[12]</sup>. Taking these results together, CKD patients have a higher risk of rebleeding than patients without renal dysfunction, with approximately more than one third of CKD patients experiencing rebleeding.

NGIH is associated with high mortality. The mortality of NGIH complicating acute renal dysfunction is 68.3% and of NGIH complicating severe liver cirrhosis (LC) is 68.4%<sup>[22,23]</sup>. In the present study, the mortality of CKD patients with NGIH was 16.7%. This result is higher than the 13% value determined in a Taiwanese study and



**Table 5** Multivariate analysis for risk factors of rebleeding

Variables	P value	OR
Age	NS	
Gender	NS	
Smoking	NS	
Hb (g/dL)	NS	
Platelet ( $10^9/L$ )	NS	
INR	NS	
Ulcer size	NS	
Location	NS	
Hemorrhage state	NS	
Amount of epinephrine	NS	
Alcohol (Yes/no <sup>1</sup> )	0.02	11.19
Therapy (Combination therapy/monotherapy <sup>1</sup> )	0.01	0.06
Experiences of endoscopists (yr)	0.03	0.56

<sup>1</sup>Reference category. NS: Not significant; INR: International normalized ratio; Hb: Haemoglobin B.

the 8.6% value reported in another Korean study<sup>[10,21]</sup>. In particular, rebleeding was related to the mortality of the CKD patients (OR = 7.1,  $P = 0.02$ ). According to a study that used nationwide inpatient samples from the United States, mechanical ventilation, severe sepsis, disseminated intravascular coagulation, cancer, age (> 65 years), coagulation defects, and venous thromboembolism were predictors of mortality in patients with ESRD and NGIH<sup>[24]</sup>. However, that study did not include rebleeding in the statistical analysis, although other studies have emphasized the importance of this parameter<sup>[12,21,24]</sup>. Overall, rebleeding is the most important factor for the prediction of mortality in CKD patients with NGIH, and the prevention of rebleeding should be a goal of clinical practice.

Regarding risk factors for rebleeding, the experience of endoscopists was one of the main factors (OR = 0.56,  $P = 0.03$ ). In a retrospective study in Canada, ESRD itself and ulcer with high-risk stigmata were the factors associated with rebleeding<sup>[12]</sup>. However, the experience of endoscopists was not analyzed. According a study of risk factors for rebleeding in NGIH patients, regardless of renal function, lower hemoglobin levels, endoscopist inexperience (< 2 years of experience), and comorbidity with CKD or LC were the associated factors<sup>[25]</sup>. These results indicate that insufficient or inappropriate endoscopic management by inexperienced endoscopists could result in rebleeding in CKD patients with NGIH. Moreover, considering that emergency endoscopic hemostasis procedures are frequently performed at night or on holidays by only one endoscopist, without the help of colleagues, intensive endoscopic treatments are important.

Combined endoscopic management was associated with a reduced risk of rebleeding in this study (*vs* monotherapy, OR = 0.06,  $P = 0.01$ ). Recent studies have suggested that combined endoscopic hemostasis treatments are superior to single treatments<sup>[26,27]</sup>. In a study of the risk factors for rebleeding in NGIH patients, regardless of renal function, combination therapy (injection + thermal therapy) was associated with lower mortality compared with injection therapy alone<sup>[25]</sup>. These results

**Table 6** Multivariate analysis for risk factors of prognosis

Variables	P value	OR
Age	NS	1.07
Gender (Male/female <sup>1</sup> )	NS	1.87
Hemorrhage state	NS	
Active pumping		0.07
Active oozing		0.00
Blood vessel		0.17
Red clot		0.00
Black clot <sup>1</sup>		0.77
Rebleeding (Yes/no <sup>1</sup> )	0.02	7.10

<sup>1</sup>Reference category. NS: Not significant.

are consistent with the findings of our study. Among the endoscopic therapeutic options, injection therapy ( $n = 27$ , 67.5%) was preferred over thermal ablation ( $n = 8$ , 20%), such as argon plasma coagulation or electrocoagulation, when used as single method in this study. This preference was due to concerns about tissue injury or loss in thermal therapy, which could result in rebleeding in patients with a hemorrhagic tendency. Another finding of this study was that being an endoscopic doctor on fellowship was associated with rebleeding compared with being a doctor on the faculty (OR = 2.1,  $P = 0.02$ ). Overall, intensive endoscopic treatment by experts using a combined method can result in better outcomes in CKD patients with NGIH.

Another finding of this study was related to alcohol use. In the multivariate analysis of risk factors for rebleeding, alcohol use was demonstrated to be an important risk factor (OR = 11.19,  $P = 0.02$ ). Additionally, the most common hemorrhagic site was the lesser curvature of the antrum of the stomach. *H. pylori* infection was detected in only 33.3% of patients. The exact mechanism underlying the association of alcohol use and *H. pylori* with NGIH in patients with CKD is not understood. However, endoscopists have to pay more attention when a patient is an alcoholic because of rebleeding risk, which is associated with high mortality.

There are several limitations of this study. First, it was retrospective study, and a small number of patients were evaluated. Second, hospital stay and blood transfusion, which were evaluated in other studies<sup>[12,28]</sup>, were not assessed. Further studies are needed to improve outcomes in CKD patients with NGIH.

In conclusion, initial intensive combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic, as rebleeding after endoscopic treatment is a risk factor for mortality.

## COMMENTS

### Background

Peptic ulcer is the most common cause of upper gastrointestinal hemorrhage in chronic kidney disease (CKD). Additionally, there are a higher rebleeding risk and greater mortality in patients on dialysis than in patients without renal dysfunction. Many studies on the outcome of and risk factors for peptic ulcer

bleeding in patients with normal renal function have been reported. However, there have been few studies on the outcome of acute hemorrhage due to peptic ulcer and on the risk factors for rebleeding in patients with CKD.

### Research frontiers

According to a study that used nationwide inpatient samples from the United States, mechanical ventilation, severe sepsis, disseminated intravascular coagulation, cancer, age (> 65 years), coagulation defects, and venous thromboembolism were predictors of mortality in patients with end-stage renal disease and nonvariceal upper gastrointestinal hemorrhage (NGIH).

### Innovations and breakthroughs

In the previous study on predictors of mortality in patients with ESRD and NGIH, rebleeding was not determined to be a major predictor of mortality. However, only rebleeding was related to mortality (OR = 7.1,  $P = 0.02$ ) in the current study. Moreover, alcoholism (OR = 11.19,  $P = 0.02$ ), the experience of endoscopists (OR = 0.56,  $P = 0.03$ ), and combination endoscopic therapy (OR = 0.06,  $P = 0.01$ ) compared with monotherapy were significantly related to rebleeding after endoscopic therapy in this study.

### Applications

Initial intensive combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic. These factors are associated with rebleeding, which is the most important factor for the prediction of mortality in CKD patients with NGIH.

### Peer review

This is a well done study. It is an important topic. The manuscript is interesting, well done and well written.

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## Events associated with apoptotic effect of *p*-Coumaric acid in HCT-15 colon cancer cells

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### Abstract

**AIM:** To investigate the events associated with the apoptotic effect of *p*-Coumaric acid, one of the phenolic components of honey, in human colorectal carcinoma (HCT-15) cells.

**METHODS:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide assay was performed to determine the antiproliferative effect of *p*-Coumaric acid against colon cancer cells. Colony forming assay was conducted to quantify the colony inhibition in HCT 15 and HT 29 colon cancer cells after *p*-Coumaric acid treatment. Propidium iodide staining of the HCT 15 cells using flow cytometry was done to study the changes in the cell cycle of treated cells. Identifica-

tion of apoptosis was done using scanning electron microscope and photomicrograph evaluation of HCT 15 cells after exposing to *p*-Coumaric acid. Levels of reactive oxygen species (ROS) of HCT 15 cells exposed to *p*-Coumaric acid was evaluated using 2', 7'-dichlorofluorescein-diacetate. Mitochondrial membrane potential of HCT-15 was assessed using rhodamine-123 with the help of flow cytometry. Lipid layer breaks associated with *p*-Coumaric acid treatment was quantified using the dye merocyanine 540. Apoptosis was confirmed and quantified using flow cytometric analysis of HCT 15 cells subjected to *p*-Coumaric acid treatment after staining with YO-PRO-1.

**RESULTS:** Antiproliferative test showed *p*-Coumaric acid has an inhibitory effect on HCT 15 and HT 29 cells with an IC<sub>50</sub> (concentration for 50% inhibition) value of 1400 and 1600  $\mu\text{mol/L}$  respectively. Colony forming assay revealed the time-dependent inhibition of HCT 15 and HT 29 cells subjected to *p*-Coumaric acid treatment. Propidium iodide staining of treated HCT 15 cells showed increasing accumulation of apoptotic cells ( $37.45 \pm 1.98$  vs  $1.07 \pm 1.01$ ) at sub-G<sub>1</sub> phase of the cell cycle after *p*-Coumaric acid treatment. HCT-15 cells observed with photomicrograph and scanning electron microscope showed the signs of apoptosis like blebbing and shrinkage after *p*-Coumaric acid exposure. Evaluation of the lipid layer showed increasing lipid layer breaks was associated with the growth inhibition of *p*-Coumaric acid. A fall in mitochondrial membrane potential and increasing ROS generation was observed in the *p*-Coumaric acid treated cells. Further apoptosis evaluated by YO-PRO-1 staining also showed the time-dependent increase of apoptotic cells after treatment.

**CONCLUSION:** These results depicted that *p*-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway.

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**Key words:** Honey; Apoptosis; Rhodamine-123; Sub-G1; Merocyanine; *p*-Coumaric acid; Reactive oxygen species

**Core tip:** This article describes apoptotic effect of *p*-Coumaric acid, one of the phenolic components of honey, against colon cancer cells. *p*-Coumaric acid treatment resulted in the inhibition of proliferation and colony forming ability of human colorectal carcinoma (HCT-15) and HT 29 cells. Major events associated with growth-inhibition are increasing reactive oxygen species generation, increasing lipid layer breaks and a fall in Mitochondrial membrane potential. Further, membrane blebbing and shrinkage of *p*-Coumaric acid exposed HCT 15 cells insinuated apoptosis. Hence our results depicted that *p*-Coumaric acid is a prospective candidate for chemoprevention of colon cancer.

Jaganathan SK, Supriyanto E, Mandal M. Events associated with apoptotic effect of *p*-Coumaric acid in HCT-15 colon cancer cells. *World J Gastroenterol* 2013; 19(43): 7726-7734 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7726.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7726>

## INTRODUCTION

Phenolic compounds are present in various dietary agents. Consumption of such agents has been linked to improve various disease conditions like cancer, diabetes and cardiac disorders. Diet is believed to be much influential in explaining the susceptibility to cancer. Most interestingly, colon cancer is more vulnerable to diet because these epithelial cells are chronically exposed to these dietary agents<sup>[1,2]</sup>. Since, cancer of colon is among the most common malignancy among the Western and Asian nations, research communities explore various new dietary agents rich in phenolic compounds to purge this malignancy.

In our laboratory, experiments in studying the preventive effect of honey against colon cancer had been constantly done. Previous results depicted honey could inhibit the colon cancer cell proliferation. Antiproliferative effect was found to vary with the phenolic content present in the honey<sup>[3-5]</sup>. Since honey containing higher phenolic content was found to induce apoptosis significantly, the scope of this research was extended to study the apoptosis induced by one of the phenolic components of honey, *p*-Coumaric acid, against the colon cancer cells.

*p*-Coumaric acid is the abundant isomer of cinnamic acid and also widely found in edible plants such as peanuts, tomatoes, carrots etc. *p*-Coumaric acid is reported to have antitumor and anti-mutagenic activities<sup>[6,7]</sup>. In a study, *p*-Coumaric acid along with the combination of hydrocaffeic acid found to reduce the UV-B oxidation damage in human conjunctival cells *in vitro* and in cornea and sclera of rabbits *in vivo*<sup>[8]</sup>. In one of the latest studies, the ability of *p*-Coumaric acid to protect rat's heart against doxorubicin (DOX)-induced oxidative stress

was investigated. It showed that *p*-Coumaric acid could reduce the DOX-induced high serum levels of lactic dehydrogenase and creatine phosphokinase<sup>[9]</sup>. In one of the most recent studies, effect of *p*-Coumaric acid against the colonic epithelial cells (Caco-2) was studied. *p*-Coumaric acid at a concentration of 1500  $\mu$ mol/L was found to inhibit the proliferation of Caco-2 cells by 43%-75% after 24-72 h of treatment<sup>[10]</sup>. However, literature available does not depict the mechanism of *p*-Coumaric acid induced apoptosis in colon cancer cells.

Apoptosis is the major form of cell death accompanied by morphological changes like membrane blebbing and shrinkage of cells. Further, events like nuclear and chromatin condensation, DNA fragmentation and segregation of apoptotic bodies were the characteristic features of apoptosis. Reactive oxygen species (ROS) is involved in various biochemical functions like cell proliferation and apoptosis. Recent studies reported ROS mediated apoptosis is accompanied with the loss of mitochondrial membrane potential<sup>[11,12]</sup>.

This current study, deals with the growth inhibitory effect of *p*-Coumaric acid in colon cancer cells. Further, an attempt has been made to explore the ROS and mitochondrial dependent mechanism in the apoptosis induced by the *p*-Coumaric acid.

## MATERIALS AND METHODS

### Reagents

DMEM, RPMI-1640, fetal bovine serum (FBS), *L*-glutamine, sodium pyruvate, nonessential amino acids, vitamin solution, penicillin and streptomycin were obtained from Life Technologies, Inc., Grand Island, NY, United States. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT), propidium iodide, mercury orange, rhodamine-123, RNase and *p*-Coumaric acid were purchased from Sigma-Aldrich, United States. Merocyanine 540 and YO-PRO-1 were obtained from Invitrogen Inc, United States.

### Cell culture

Colon carcinoma cell line HT 29 and human colorectal carcinoma (HCT-15) (Organ: Colon, Disease: Colorectal adenocarcinoma; Organism: Human; procured from National Centre for Cell science, Pune, India) was grown in DMEM and RPMI medium respectively, supplemented with 10% FBS, *L*-glutamine, penicillin, sodium pyruvate, nonessential amino acids and vitamin solution. Adherent monolayer cultures of HCT 15 were maintained in T-25 flasks and incubated at 37 °C in 5% carbon dioxide (CO<sub>2</sub>). The cultures were free of mycoplasma and maintained no longer than 12 wk after recovery from frozen stocks.

### Cell proliferation assay

Thiazolyl blue tetrazolium bromide (MTT) assay was carried out as follows: Cells were trypsinized, counted and 1000 cells were seeded per well in 96-well plate. The following day, 100  $\mu$ L of medium containing the desired

concentration of *p*-Coumaric acid was added to the appropriate wells. The cells were then kept at 37 °C in 5% CO<sub>2</sub> for the desired length of time. Control used in these experiments was untreated cells kept for 48 h. For all the experiments performed below, control cells remained untreated and kept for the same duration as the longest time-point of the respective experiment. At this point, 100 µL of (5 mg/mL) MTT reagent was added to each well, and the plate was placed at 37 °C in the incubator for 2 h. 200 µL of dimethyl sulfoxide was added to each well, after aspirating the supernatant. Colored formazan product was assayed spectrophotometrically at 570 nm using enzyme-linked immunosorbent assay plate reader<sup>[12]</sup>.

### Colony forming assay

HCT 15 and HT 29 cells were treated with *p*-Coumaric acid at a concentration of 1400 and 1600 µmol/L respectively for definite time periods (12, 24 and 48 h) and collected by trypsinization. The cells were counted and seeded again in triplicate on a 6-well tissue culture plate with 3000 cells/well. The cells were cultured for 15 d with growth media replaced after every two days. The cells were stained with 0.5% crystal violet (in methanol) and colonies were counted<sup>[12]</sup>.

### Cell cycle analysis

After the appropriate treatment with *p*-Coumaric acid, HCT 15 cells were washed with phosphate-buffered saline, then resuspended in 50 µg/mL propidium iodide containing 0.1% sodium citrate with 0.1% Triton X-100 for 20 min at 4 °C. Cells were then analyzed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems), and the sub-G<sub>1</sub> fraction was used as a measure of the apoptotic cells. Control used in the experiments was untreated cells kept for 48 h. Analysis was performed in linear amplification mode in case of cell cycle analysis. Remaining experiments of flow cytometry were performed in logarithmic amplification mode unless otherwise stated<sup>[13]</sup>.

### Estimation of ROS generation

Dichlorofluorescein-diacetate (DCFH-DA) was cleaved by the intracellular nonspecific esterase to form DCFH. DCFH are oxidized by ROS to form the fluorescent compound DCF. *p*-Coumaric acid treated cells (1400 µmol/L) were harvested using trypsin/EDTA and resuspended in PBS. Working solution (20 µmol/L) of DCFH-DA was directly added cells and then it was incubated at 37 °C for 15 min. Cells were washed and resuspended in PBS and kept on ice immediately before analyzing by flow cytometry<sup>[12]</sup>. This fluorescent intensity of DCF was measured and correlated with the ROS generated in the cells.

### Determination of mitochondrial membrane potential

HCT 15 colon cancer cells were treated with *p*-Coumaric acid (1400 µmol/L) for different time points. After-

wards, cells were harvested and resuspended in 1 mL of rhodamine-123 (5 µg/mL) for 1 h at 37 °C. The intensity of fluorescence from rhodamine-123 was measured by flow cytometry<sup>[12]</sup>.

### Detection of membrane lipid organization

Colon cancer cells (HCT 15) were treated with *p*-Coumaric acid (1400 µmol/L) for different time points. Cells were harvested and re-suspended in 1 mL of merocyanine 540 (10 µg/mL) for 15 min at 37 °C. The intensity of fluorescence was measured by flow cytometry<sup>[13]</sup>.

### YO-PRO-1 staining

YO-PRO-1 permits analysis of apoptotic cells without interfering cell viability. After treatment with *p*-Coumaric acid (1400 µmol/L), the cell pellets were mixed in 1 µmol/L YO-PRO-1 for 20 min at room temperature. After incubation intensity was measured using flow cytometry<sup>[13]</sup>.

### Scanning electron microscope and photomicrograph images

Fixed amount of HCT 15 cells were seeded in a sterilized glass slide and incubated for 24 h. *p*-Coumaric acid at a concentration of 1400 µmol/L was added for 48 h time interval. After incubation, cells were harvested by using trypsin/EDTA and centrifuged for 5 min at room temperature. Then the supernatant was decanted and pellet was dried. Pellet was treated with 2.5% glutaraldehyde in distilled water for 45 min in hybrid oven shaker at 37 °C. Cells were washed thrice with PBS for 5 min and then dehydrated by ethyl alcohol of different concentration (30%, 50%, 70%, 95% and 100%) for 5-10 min. Fixing of cells was done with hexamethyl disilazane and the sample was taken for scanning electron microscope analysis. Photomicrograph images of HCT 15 and HT 29 cells were acquired using microscope.

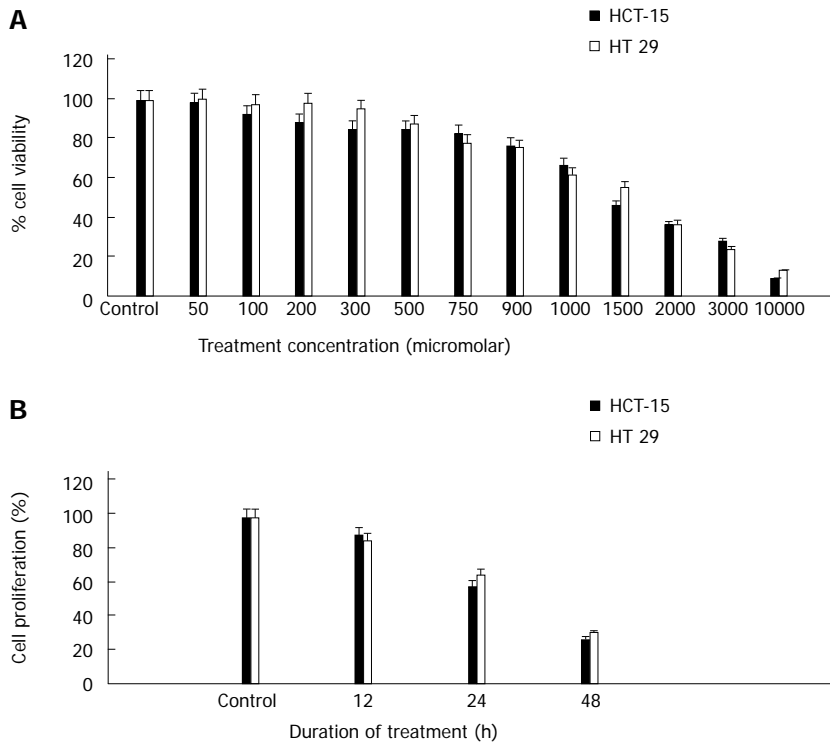
### Statistical analyses

All values are expressed as the mean ± SE. Figures were plotted using Graphpad Prism software. All experiments were performed three times independently (biological triplicates). One-way ANOVA was performed to find statistical significance.

## RESULTS

### MTT assay

MTT assay of treated cells was performed after 48 h of treatment. Colon cancer cells (HCT 15 and HT 29) growth was inhibited in a dose-dependent manner. Both HCT-15 and HT-29 cell growth were inhibited significantly with an IC<sub>50</sub> of around 1400 µmol/L and 1600 µmol/L respectively (Figure 1A). HCT 15 cells were found more sensitive to *p*-Coumaric acid, however at higher concentrations both cell lines were found to be equally affected. Statistical analysis showed that *p*-Coumaric acid treatment results in significant inhibition (*P*



**Figure 1** Antiproliferative effect, colony inhibitory of *p*-Coumaric acid against colon cancer cells. A: Both human colorectal carcinoma (HCT-15) and HT-29 cells grown in 96-well plate were treated with various concentration of *p*-Coumaric acid (0-10000  $\mu\text{mol/L}$ ) diluted in the media for 48 h. The mean of the percentage cell viability (% of control) along with their standard error is indicated; B: After various incubation periods of *p*-Coumaric acid treatment, colonies formed were stained with 0.5% crystal violet and counted, and percentage of survival was calculated by normalizing the values. Data reported as the mean  $\pm$  SE from three different observations. Mean differences are significant at 12, 24 and 48 h.

$< 0.05$ ) compared with untreated control cells at 200  $\mu\text{mol/L}$  and 500  $\mu\text{mol/L}$  for HCT 15 and HT 29 cells respectively (Figure 1A).

#### Colony forming assay

*p*-Coumaric acid treated HCT 15 cells showed a maximum of 94, 67, 32 colonies after 12, 24 and 48 h of treatment. Untreated HCT 15 cells produced a maximum of 105 colonies. Similar experiment with HT29 cells displayed a maximum of 131, 101, 51 colonies after 12, 24 and 48 h treatment whereas the control HT 29 cells produced 154. A time-dependent inhibition of colony formation was clearly evident from this experiment (Figure 1B). There was a significant reduction ( $P < 0.05$ ) in the number of colonies formed under the various time intervals examined (both HCT 15 and HT 29 cells) when compared with corresponding untreated cells (Figure 1B).

#### Cell cycle analysis

Cell populations were tabulated among the sub- $G_1$ ,  $G_0/G_1$ , S and  $G_2/M$  phases of the cell cycle. It showed an increasing sub- $G_1$  arrest from 1.00% (control) to 37.45% after 48 h (Table 1). Statistical analysis of the sub- $G_1$  column indicated significant increase ( $P < 0.05$ ) of cells in the sub- $G_1$  phase insinuating apoptosis increases with the time-dependency.

#### ROS generation

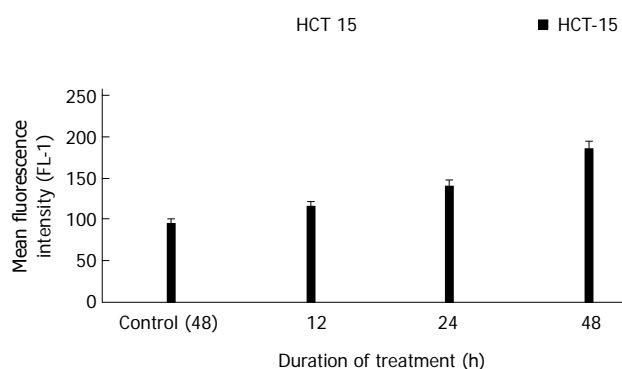
ROS levels were increased significantly after treatment. The increasing mean fluorescent intensity was found to be 116, 141, and 185 during 12, 24 and 48 h respectively. Untreated control cells showed an intensity of 96 after 48 h. ROS intensity after 48 h treatment was almost double the intensity of the control cells. Moreover, the differences in the ROS levels at various h examined were significant, compared to control with a  $P$  value of less than 0.05 (Figure 2).

#### Mitochondrial membrane potential

The decreasing mean fluorescent intensity was found to be 147, 91 during 6 and 12 h of treatment respectively. Untreated control cells showed an average intensity of 229 after 12 h. From the results, it was observed that *p*-Coumaric acid treatment reduced the potential by 2.5 fold after 12 h. There was also statistically significant reduction ( $P < 0.05$ ) of potential at the estimated intervals compared to untreated cells (Figure 3A).

#### Lipid layer breaks

Untreated cells displayed a mean intensity of 33 after 6 h. Treated cells showed 37 and 50 after 3 and 6 h respectively (Figure 3B). It is evident from the above results that treated cells displayed an increase in the lipid layer breaks.



**Figure 2** *p*-Coumaric acid induced reactive oxygen species generation. Human colorectal carcinoma (HCT-15) cells were cultured in the presence or absence of *p*-Coumaric acid for the specified time points. Dichlorofluorescein-diacetate fluorescence intensity was detected by using flow cytometry. Data is representative of three independent experiments and the mean differences are significant at 12, 24 and 48 h.

**Table 1** Cell cycle distribution of human colorectal carcinoma-15 cells after *p*-Coumaric acid treatment

Time in h	Sub G <sub>1</sub> <sup>1</sup>	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
Control	1.07 ± 1.01	42.82 ± 1.92	8.03 ± 1.23	40.07 ± 2.85
12 h	5.98 ± 1.17	23.06 ± 3.15	10.29 ± 4.01	46.67 ± 1.89
24 h	16.46 ± 2.03	23.92 ± 1.74	9.91 ± 3.29	39.03 ± 1.58
48 h	37.45 ± 1.98	12.79 ± 4.45	4.9 ± 3.82	17.12 ± 4.65

<sup>1</sup>Mean differences are significant at  $P < 0.05$ . Data represents mean  $\pm$  SD.

### Photomicrograph and scanning electron microscope images

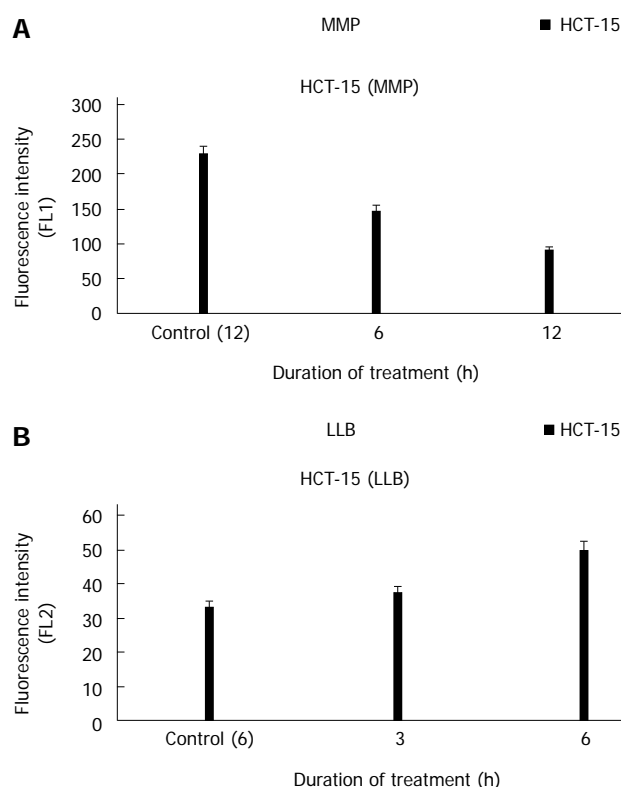
Scanning electron microscope (SEM) images of *p*-Coumaric acid treated cells (48 h) showed typical signs of apoptosis like membrane blebbing and shrinkage as indicated by arrow marks. Normal cells were found almost spherical without marked shrinkage (Figure 4A). This was further corroborated with the photomicrograph images (Figure 4B).

### Yo-Pro-1 staining

The percentage of cells distributed in M2 population signifying apoptosis increased depending upon the duration of treatment. It was found to be 20, 33 after 24 and 48 h of *p*-Coumaric acid treatment. M2 phase population of untreated control cells was found to be 8% after 48 h (Figure 5).

## DISCUSSION

Diet consumption and cancer have been linked by various studies<sup>[14,15]</sup>. They postulated that consistent pattern of consumption of diets which are rich in vegetables and fruits may reduce the risk of cancer. Phenolic compounds, one of the classes of non-nutritive phytochemicals, are widely distributed in our foods and suggested to have preventive effect against various disease conditions like cancer, diabetes and several cardiac disorders<sup>[16,17]</sup>.

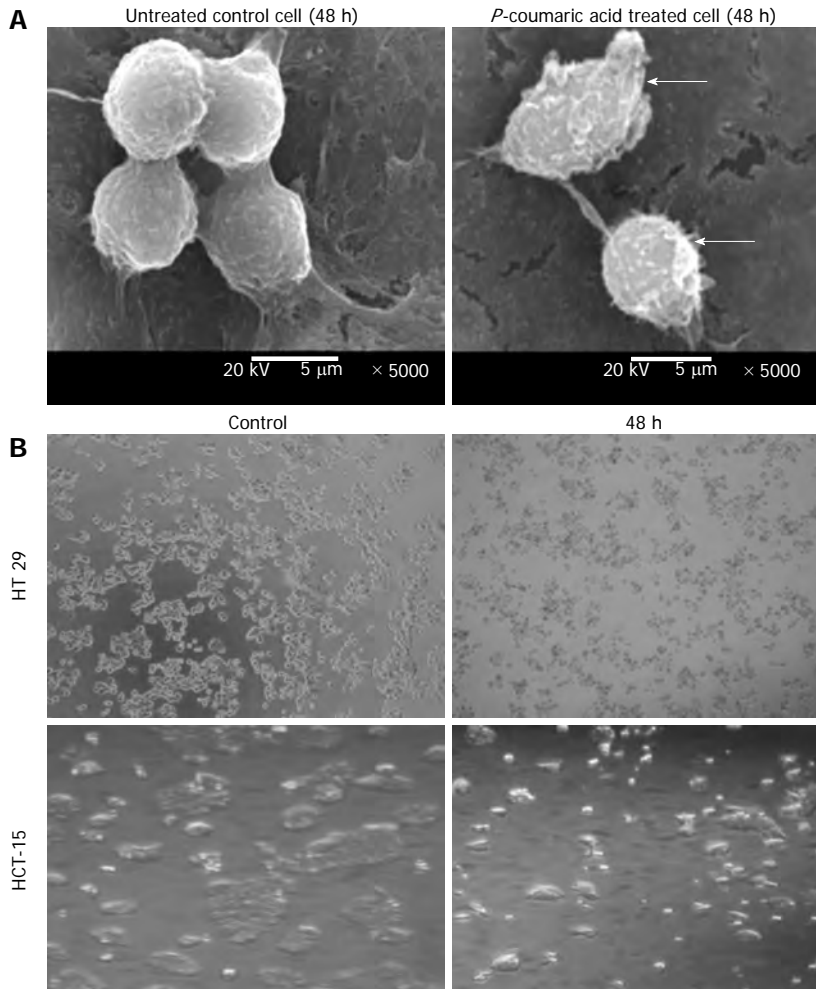


**Figure 3** Events associated with growth-inhibitory effect of *p*-Coumaric acid. A: Human colorectal carcinoma (HCT-15) cells were treated with *p*-Coumaric acid for specified time-periods and then mitochondrial membrane potential were determined using rhodamine-123 by flow cytometry. Mean differences are significant at 6 and 12 h compared with untreated control cells ( $P < 0.05$  vs untreated control cells); B: HCT 15 cells were treated with *p*-Coumaric acid and evaluated using merocyanine 540 to quantify the lipid layer breaks (LLBs). Data is representative of three independent experiments and mean differences are significant at 3 and 6 h compared with untreated control cells ( $P < 0.05$  vs untreated control cells). MMP: Mitochondrial membrane potential.

From our laboratory, it was showed that honey rich in phenolic content was able to induce apoptosis significantly in colon cancer cells. Hence, in this research the effect of *p*-Coumaric acid, one of the phenolic constituents of honey, induced apoptosis in colon cancer cells was studied.

*p*-Coumaric acid inhibited the proliferation of colon cancer cells. Both HCT-15 and HT-29 cell growth were inhibited significantly with an  $IC_{50}$  of around 1400  $\mu$ mol/L and 1600  $\mu$ mol/L respectively. This was similar to the previously published report on the antiproliferative effect of *p*-Coumaric acid against Caco-2 cells<sup>[10]</sup>. Bioavailability of phenolic constituents is a major factor when we would like to examine the effect of *p*-coumaric acid in *in vivo*. In one of the researches, it was showed that bioavailability of coumaric acid after consumption of 200 g plum is in the range of 28-230 mg/serving<sup>[18]</sup>. In a colonic volume of 200 mL, this would yield a concentration in the range of 850 to 7000  $\mu$ mol/L. Hence, it is believed that estimated  $IC_{50}$  values against these colon cancer cells are achievable internally. Human diet is complex and the supply of coumaric acid from different diets has to be evaluated simultaneously to have an idea





**Figure 4** Morphological assessment of *p*-Coumaric acid treated cells. A: Human colorectal carcinoma (HCT-15) cells were treated with *p*-Coumaric acid for 48 h and the cells were observed under scanning electron microscope. Treated cells showed membrane blebbing and shrinkage compared to untreated normal control cells; B: HT 29 and HCT 15 cells were subjected to *p*-Coumaric acid treatment for 48 h and observed under light microscopy. Treated cells displayed apoptotic features like blebbing and shrinkage compared to untreated normal control cells.

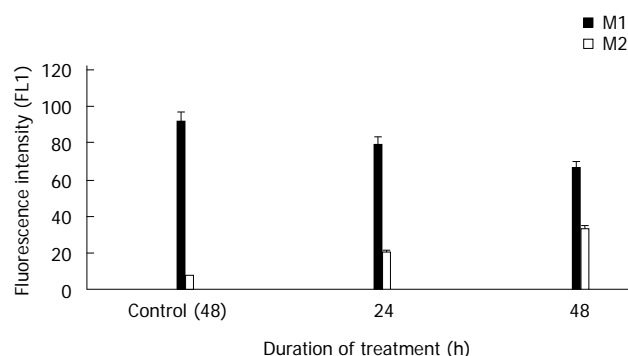
about its bioavailability. To add further, bioavailability varies among the individuals and this makes estimation of intakes and prediction of physiological range of phenolics in body fluids is a mammoth task. The biggest drawback is that bioavailability of *p*-Coumaric acid will be in pulses depending upon the food intake whereas in cell culture environments it is constant<sup>[10]</sup>.

*p*-Coumaric acid significantly inhibited the colony formation *in vitro*. This is indispensable, since most of the chemotherapeutic drugs were shown to inhibit the colony formation<sup>[12]</sup>. The effect of *p*-coumaric acid against intestinal epithelial cells (IEC) isolated from the mouse was evaluated. It was found that *p*-Coumaric acid was not toxic to these cells. Even at a higher concentration of 5.1 mmol/L nearly 80% cells were viable (results not shown). Sparing nature of *p*-Coumaric acid against mouse IEC was interesting and would warrant further study with normal human colonic cells.

Mitochondrial malfunction is one of the key events occurring at the initial stages of apoptosis. Studies reported a fall in the mitochondrial membrane potential

during apoptosis induced by various chemotherapeutic drugs. Mitochondrial membrane potential of *p*-Coumaric acid treated cells using rhodamine-123 showed decreasing intensity, confirming the mitochondrial malfunction. ROS is involved in various biochemical functions like cell proliferation and apoptosis. Generally, ROS stress is oncogenic and it is found to increase the metabolic activity. It also stimulates further ROS generation through mitochondrial respiratory chain and maintains the cancer phenotype. On the other hand, high dose of ROS for prolonged duration could induce cellular damage and apoptosis<sup>[19,20]</sup>. Hence by utilizing time and dose-dependent ROS generation, we can trigger cell death by using exogenous ROS-generating agents. Our experiment involving DCFDH-DA staining indicated increasing ROS generation in the *p*-Coumaric acid treated cells. Hence, *p*-Coumaric acid may be considered as a potential exogenous candidate (generating ROS) to induce apoptosis in colon cancer cells.

The most notable property of phenolic phytochemicals is that they have antioxidant activity. This is due to



**Figure 5 Apoptosis evaluation using Yo-Pro-1 dye by flow cytometry.** Human colorectal carcinoma (HCT-15) cells were treated with *p*-Coumaric acid for specified time points. The distribution of cell population changed according to the exposure time as indicated by M1 and M2. Percentage of M2 population depicting apoptosis increased on the basis of the duration of treatment. Data is representative of three independent experiments and the differences in the values of M2 were significant at 24 and 48 h ( $P < 0.05$  vs untreated control cells) compared to untreated control cells.

the ability of phenolic hydroxyl groups which can provide hydrogen atoms in scavenging the ROS. Hence it is suggested that phenolic phytochemicals could scavenge the ROS molecules and inhibit the mitogen activated protein kinase (MAPK) signaling and blocking the nuclear factor kappaB and activator protein 1 activation which eventually lead to inhibit cancer cell proliferation. Although antioxidant properties of phenolic phytochemicals were explained for its mechanism of inhibiting cancer cells, they also show pro-oxidant activity under certain experimental conditions<sup>[21]</sup>. ROS generation was observed in the cell culture media containing EGCG, quercetin and gallic acid in both time and concentration-dependent manner<sup>[22]</sup>. In our case, *p*-Coumaric acid was also found to increase ROS generation in a time-dependent manner. Hence, treating the cancer cells with *p*-Coumaric acid can produce significant ROS resulting stressful or cytotoxic effects. Excess of ROS generation by phenolic phytochemicals induces apoptosis through MAPK activation. Simultaneously, increased p53 activation was mediated by Ras/MAPK kinase/MAPK pathway as observed in the apoptosis of EGCG and resveratrol<sup>[23,24]</sup>. Hence, we hypothesize that the increased ROS generation may result in the activation of p53 in the *p*-Coumaric acid treated cells. This may in-turn would have caused the up-regulation of Bax and down-regulation of Bcl2 which are the down-stream targets of p53 resulting in apoptosis.

Apoptosis, a distinguished form of cell death, is characterized by membrane blebbing and DNA fragmentation. Electron Microscopy is used as a golden standard in detecting apoptosis<sup>[25-27]</sup>. In our case, both scanning electron microscope and photomicrograph images of *p*-Coumaric acid treated cells showed typical membrane blebbing and shrinkage portraying apoptosis. Sub-G<sub>1</sub> arrest of cell cycle is considered as a sign of apoptosis<sup>[28-30]</sup>. *p*-Coumaric acid treatment showed increasing accumulation of cells in the sub-G<sub>1</sub> phase confirming

apoptosis. This was similar to the most anticancer drugs which induced apoptosis by arresting the cells at sub-G<sub>1</sub> phase<sup>[31-33]</sup>. At an early stage of apoptosis, there will be considerable damage to plasma membrane and the lipid layer will be disorganized. Nowadays in addition to the nuclear and morphological assessment, lipid layer perturbations in plasma membrane can also insinuate apoptosis. Merocyanine staining of treated cells for lipid layer organization showed increasing fluorescence intensity depicting apoptosis. This observation was similar to eugenol induced apoptosis shown recently<sup>[13]</sup>.

In conclusion, *p*-Coumaric acid exerted antiproliferative activity against colon cancer cells like HT 29 and HCT 15. Both the cell lines growth was repressed significantly by inducing apoptosis. Apoptosis induced by *p*-Coumaric acid involved various physical and biochemical changes. To enumerate, cells showed membrane blebbing and shrinkage as depicted by SEM and photomicrograph images. Earlier lipid layer breaks were associated with the *p*-Coumaric acid induced apoptosis. Cell cycle progression was arrested at sub-G<sub>1</sub> phase by *p*-Coumaric acid treatment. Mitochondrial membrane potential of treated cells also showed a decrease after *p*-Coumaric acid treatment. Moreover, there was increase in the ROS generation and lipid layer breaks after treatment. These results insinuate that *p*-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway. However, further experiments in preclinical and clinical settings will promote this as a likely candidate for chemoprevention of colon cancer.

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## COMMENTS

### Background

Consumption of phenolic components has been linked to improve various disease conditions like cancer, diabetes and cardiac disorders. Diet is believed to be much influential in explaining the susceptibility to cancer. Most interestingly, colon cancer is more vulnerable to diet because these epithelial cells are chronically exposed to these dietary agents. Honey has been reported to possess protective effect in many inflammatory diseases and oxidative stress-related injuries. Recent works from the laboratory showed phenolic components of honey were attributed with inherent potential to inhibit colon cancer cells. In this article *p*-Coumaric acid, one of the phenolic components of honey, has been examined for its growth inhibitory effects.

### Research frontiers

Chemotherapy utilizes antineoplastic or dietary agents for treating colon cancer. However, there is still a continuous search for novel agents with improved efficiency. To their knowledge *p*-Coumaric acid, one of the phenolic components of honey, have never been examined for its inhibitory mechanism against colon

cancer.

### Innovations and breakthroughs

Events associated with the inhibitory nature of *p*-Coumaric acid are clearly highlighted in this manuscript. Authors have shown that *p*-Coumaric acid inhibited the colon cancer cells in dose-dependent manner. Further it was deciphered that *p*-Coumaric acid induced apoptosis is accompanied with increasing reactive oxygen species (ROS) levels, a fall in the mitochondrial membrane potential and increased lipid layer breaks. Hence authors concluded that *p*-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway.

### Applications

*p*-Coumaric acid induced apoptosis in colon cancer cells through ROS-mitochondrial pathway. Hence, further experiments in preclinical and clinical settings will promote *p*-Coumaric acid as a plausible candidate for chemoprevention of colon cancer.

### Peer review

This work describes the events associated with the growth-inhibitory effect of *p*-Coumaric acid in colon cancer cells. Since *p*-Coumaric acid is one of the phenolic components of honey, the study has a clear interest in the field of chemoprevention of colon cancer. The results of this study are interesting and demonstrate that *p*-Coumaric acid has antiproliferative activity against colon cancer cells inducing apoptosis and causing physical and biochemical changes.

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## Screening of *SLC25A13* mutation in the Thai population

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### Abstract

**AIM:** To determine the prevalence of *SLC25A13* mutations in the Thai population.

**METHODS:** A total of 1537 subjects representing the Thai population were screened for a novel pathologic allele p.Met1? (c.2T > C) and six previously known common *SLC25A13* mutations: [I] (c.851\_854delGTAT), [II] (g.IVS11 + 1G > A), [III] (c.1638\_1660dup), [IV] (p.S225X), [V] (IVS13 + 1G > A), and [XIX] (g.IVS16ins3kb) using a newly developed TaqMan and established HybProbe assay, respectively. Sanger sequencing was employed for specimens showing an aberrant peak to confirm the targeted mutation as well as the unknown aberrant peaks detected. Frequencies of the mutations identified were compared in each region. Carrier frequency and disease prevalence of citrin deficiency caused by *SLC25A13* mutations were estimated.

**RESULTS:** p.Met1? was identified in the heterozygous state in 85 individuals, giving a carrier frequency of 1/18, which suggests possible selective advantage of this variant. The question of p.Met1? homozygote lethality remains unanswered which may serve as an explanation as to why this homozygote has yet to be identified in patients/controls even with high allele frequency. The p.Met1? mutation has rarely been studied in populations other than Thai and Chinese; therefore, may have been overlooked. Development of the TaqMan assay in the present study would allow a simple, rapid, and cost-effective method for mass screening. Heterozygous mutations: [XIX] and [I] were identified in 17 individuals, giving a carrier rate of 1/90 and a calculated homozygote rate of 1/33000. Two novel variants, g.IVS11 + 17C > G and c.1311C > T, of unknown clinical significance were identified at low frequency.

**CONCLUSION:** This study highlighted the current underestimation of citrin deficiency and suggests the possible selective advantage of the p.Met1? allele.

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**Key words:** Aspartate-glutamate carrier; Isoform 2; Citrin deficiency; Type II citrullinemia; Neonatal intrahepatic cholestasis caused by citrin deficiency; *SLC25A13*

**Core tip:** Citrin deficiency is underestimated in various populations and the high prevalence of some *SLC25A13* variants possibly contribute to uncharacterized predisposition/protection of certain disorders.

Wongkittichote P, Sukasem C, Kikuchi A, Aekplakorn W, Jensen LT, Kure S, Wattanasirichaigoon D. Screening of *SLC25A13* mutation in the Thai population. *World J Gastroenterol* 2013; 19(43): 7735-7742 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7735.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7735>

## INTRODUCTION

Citrin deficiency (CD) is a genetic disorder inherited in an autosomal recessive pattern<sup>[1]</sup>. It is caused by mutations in the *SLC25A13* gene encoding the citrin protein, a mitochondrial aspartate-glutamate carrier, isoform 2 (AGC2) and is expressed mainly in the liver<sup>[2-5]</sup>. The major function of AGC2 is to export mitochondrial aspartate in exchange for cytosolic glutamate<sup>[5]</sup> and is involved in several metabolic pathways with major contributions to the urea cycle and malate-aspartate shuttle<sup>[1,6,7]</sup>.

There are three distinct age-dependent clinical phenotypes of CD: the mild phenotype of neonatal intrahepatic cholestasis caused by citrin deficiency [Neonatal Intrahepatic Cholestasis caused by Citrin Deficiency (NICCD), Online Mendelian Inheritance in Man (OMIM): 605814], asymptomatic period or failure to thrive and dyslipidemia in young children, and a fatal phenotype of type II citrullinemia [Adult Onset Type II Citrullinemia (CTLN2), OMIM: 603471] in older children and adults (11-80 years)<sup>[1,8,9]</sup>. Misdiagnosed or mistreated CTLN2 patients usually have poor outcomes that often result in death due to hyperammonemic encephalopathy<sup>[8]</sup>.

CD is a panethnic disorder, but is relatively more common among East Asian populations. The carrier prevalence among Japanese and Chinese populations has been reported to be approximately 1/65<sup>[10-16]</sup>. Despite its high prevalence, clinical features and metabolic profiles are diverse among patients, thus making correct diagnosis difficult<sup>[1,17,18]</sup>. A definitive diagnosis of CD typically requires DNA sequencing analysis of the *SLC25A13* gene, which is time consuming and costly. Alternative methods such as polymerase chain reaction restriction fragment length polymorphisms, GeneScan and SNaPshot have

been adapted to detect mutations in the *SLC25A13* gene<sup>[4,13,14,16,18-20]</sup>. Recently, Kikuchi *et al.*<sup>[21]</sup>, reported a new method for identifying common mutations using a real-time polymerase chain reaction (PCR)-based technique combined with melting curve analysis using HybProbes. This method appears to be a rapid and economical approach that is also suitable for the use with a high-throughput platform<sup>[21]</sup>.

Over 30 *SLC25A13* mutations have been described<sup>[13,14,19]</sup> and our group has previously shown that mutation [I] was the most common (8/10) mutated allele identified among NICCD Thai infants with the remaining identified as mutations [III] (c.1638\_1660dup) and [XIX] (g.IVS16ins3kb)<sup>[16]</sup>. In addition, the heterozygous state of a novel p.Met1? (c.2T > C) variant was identified in an infant with idiopathic cholestasis and in 3 out of 100 healthy controls<sup>[16]</sup>. This variant was also observed in Chinese control individuals with equivalent carrier prevalence<sup>[20]</sup>. The pathogenic property of p.Met1? has been confirmed in a yeast model of CD and this mutant exhibits loss of citrin function<sup>[22]</sup>. Here, we conducted a detailed investigation to capture the true prevalence of CD in the Thai population given the small number of Thai patients with CD that have been characterized at the molecular level and with a high prevalence of p.Met1? carriers in an earlier study.

## MATERIALS AND METHODS

### Subjects

Eligible DNA samples were anonymous specimens previously stored and obtained through the Thai 4<sup>th</sup> National Health Examination Survey, during August 2008 and March 2009 by the National Health Examination Survey Office, Health System Research Institute. Sample size was calculated based on the estimated carrier prevalence of *SLC25A13* mutations, 1/110, which was derived from a previous patient-based study<sup>[16]</sup>. Using the Power and Sample Size Calculations Program (version 3.0.43), for alpha error of 10% and power of 90%, the target sample size needed to achieve statistical significance was 669. Even in a worst case scenario (carrier rate of 1/200) with the same alpha error and power of testing, the minimum sample size required was 1337. To offset sample loss due to insufficient DNA quantity or the degradation of DNA extract quality, 15%-20% additional specimens were added to the sample size. A computer-based simple randomization was used to randomly select samples from each of the five regions in Thailand including; Bangkok, Central, Northeastern, Northern and Southern (Figure 1). The number of samples from each region was in accordance to the population distribution reported by the National Statistical Office ([www.nso.go.th/](http://www.nso.go.th/)). This ensured an avoidance of ascertainment bias of the study population since there may or may not have been the possibility of ethnic differences of the *SLC25A13* variants among subpopulations in Thailand. Moreover, Thais from the Southern region of the country are more ethnically re-



Figure 1 Map of Thailand and geographic distribution.

lated to Malay descendents and those from the North-eastern region are more ethnically related to descendants of Laos and Cambodia. In total, 1569 specimens were received for the study following the approval of Ramathibodi Hospital institutional review board.

#### Detection of the *p.Met1?* allele by TaqMan assay

Real-time PCR using hydrolysis probes that were specific to each allele were employed to detect the *p.Met1?* mutation. Probes and primers were designed according to the *SLC25A13* gene sequence (GenBank accession no. NM\_014251). Primer sequences were 5' GTCAGT-GGGTCCCGCAGTC 3' and 5' GCACCCCATTTT-GCTCCG 3' as a forward and reverse primer, respectively. The probe for detecting the wild-type allele was tagged with 6-FAM at the 5' end, whereas the mutant allele contained VIC. Sequences of the wild-type and mutant probes were 6-FAM 5'AACCGGGGCGAATCATG-GCG 3' minor groove binder (MGB) and VIC 5'AACCGGGGCGAATCACGGCG 3' MGB, respectively.

Each real-time PCR reaction contained 0.9  $\mu\text{mol/L}$  of each primer, 0.25  $\mu\text{mol/L}$  of each probe, 20 ng of genomic DNA and 5  $\mu\text{L}$  of TaqMan<sup>®</sup> Genotyping Master Mix (Applied Biosystem). The thermal profile started with an initial denaturation at 95 °C for 10 min followed by 55 amplification cycles at 30 s, and denaturation at 95 °C. Finally, annealing and extension for 90 s at 62 °C. The ViiA<sup>™</sup> 7 System (Applied Biosystem) was used for detection. Positive samples were confirmed using PCR-

*EagI* restriction digest as previously established<sup>[16]</sup>.

#### Detection of six common mutations by HybProbe assay

Six common mutations accounting for 91%-100% of the mutant alleles previously reported in Japanese, Chinese, and Thai patients were selected for screening in the present study<sup>[14,16,21]</sup>. These mutations were: [I] (c.851\_854delGTAT), [II] (g.IVS11+1G>A), [III] (c.1638\_1660dup), [IV] (p.S225X), [V] (IVS13 + 1G > A) and [XIX] (g.IVS16ins3kb). HybProbe assays were performed using probes and primers for *SLC25A13* previously validated<sup>[21]</sup>. Real-time PCR was performed using a LightCycler 480 (Roche Applied Science). A 10- $\mu\text{L}$  real-time PCR reaction contained 0.5  $\mu\text{mol/L}$  of each forward and reverse primer (except 0.1  $\mu\text{mol/L}$  of reverse primer in the probe-primer set B), 0.2  $\mu\text{mol/L}$  of each donor and acceptor probe, 20-40 ng of genomic DNA and 5  $\mu\text{L}$  of LightCycler 480 probe Master (Roche Applied Science) or Premix Ex Taq<sup>™</sup> (Perfect Real Time) (Takara Bio Company). All positive samples were confirmed using PCR-*Hpy*CH4IV restriction digest for mutation [I]<sup>[16]</sup>, long-range PCR for mutation [XIX]<sup>[14]</sup>, and/or direct sequencing (Research Center, Ramathibodi Hospital). Long range PCR conditions were as follows: 94 °C for 5 min; 35 cycles of 98 °C for 20 s, 60 °C for 30 s, 68 °C for 15 min; 72 °C for 20 min and 15 °C for  $\infty$ . It should be noted that conventional nomenclature of mutations of the *SLC25A13* gene has been widely used; therefore, to ensure that the readers would easily grasp the mutation type concept, conventional nomenclature was used alongside standard nomenclature.

#### Statistical analysis

Fisher's Exact test was used to determine the statistical difference for the frequencies of the mutant alleles identified in different geographic regions. A *P* value of < 0.05 was considered statistically significant. Analysis of variance to compare sex and age distribution among regions was performed using the SPSS program (version 16.0, SPSS Inc., Chicago, IL, United States).

## RESULTS

Complete DNA specimens from 1537 individuals were included in the analysis, 758 males (mean age  $47 \pm 21$  years) and 779 females (mean age  $49 \pm 21$  years), with an overall mean age of  $48 \pm 21$  years. Sex and age distribution were not significantly different in the five different regions, although the mean age of subjects from the Northeastern region was slightly higher than those from the other areas of the country.

#### High prevalence of *p.Met1?* allele in the Thai population

The *p.Met1?* allele was found in 85 individuals giving a 1/18 carrier frequency. This mutation was evenly distributed throughout geographic regions (Table 1). One of these individuals was compound heterozygous with mutation [I]; however, since the specimens in our analy-



**Table 1** Number of individuals with *SLC25A13* mutations and carrier prevalence in the Thai population according to geographical distribution

Region	Mutation [ I ] (c.851_854delGTAT)	Mutation XIX (g.IVS16ins3kb)	p.Met1?	Total carriers (p.Met1? not included)	Total carriers (p.Met1? included)
Bangkok (n = 142)	2 (1/71) <sup>1</sup>	2 (1/71)	5 (1/28) <sup>1</sup>	4 (1/36)	8 (1/18) <sup>1</sup>
Central (n = 387)	0	3 (1/129)	17 (1/23)	3 (1/129)	20 (1/19)
Northeastern (n = 498)	4 (1/125)	0	27 (1/18)	4 (1/125)	31 (1/16)
Northern (n = 291)	1 (1/291)	3 (1/97)	21 (1/14)	4 (1/74)	25 (1/12)
Southern (n = 219)	1 (1/219)	1 (1/219)	15 (1/15)	2 (1/110)	17 (1/13)
Total (n = 1537)	8 (1/192)	9 (1/171)	85 (1/18)	17 (1/90)	101 (1/15)
P value <sup>2</sup>	0.18	0.067	0.366	NA	0.413

<sup>1</sup>One individual was found with compound heterozygote of Mutation [ I ] (c.851\_854 delGTAT) and p.Met1?; <sup>2</sup>Statistical analysis using Fischer's Exact test giving P value for comparison of the carrier prevalence identified across the regions. NA: Not available.

sis were anonymous it was impossible to determine the phenotype of this person.

### Carrier frequency of six other *SLC25A13* mutations

A total of 17 individuals were heterozygotes for mutant alleles. Excluding the p.Met1? variant, 8 mutation [ I ] and 9 mutation [XIX] (Table 1) were observed, giving a 1/90 carrier rate. Frequencies of mutations [ I ] and [XIX] identified in each region were not statistically different ( $P = 0.180$  and  $0.067$ , respectively). The four other mutations were not found. Melting point analysis revealed four specimens with heterozygous peaks that were distinct from mutation [ II ] (IVS11 + 1G > A) (Figure 2A). Sequencing of these specimens revealed a novel single nucleotide polymorphism (SNP), IVS11 + 17C > G. This SNP was located in the anchor-probe binding sequence. Prediction by NNSplice<sup>[23]</sup> ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)) indicated no change in the splice-site score.

Screening of mutation [V] revealed one specimen with an abnormal melting peak (Figure 2B) where direct sequencing revealed an SNP, c.1311C > T, at the binding site of the reporter probe. This novel variant was located at the last base of a codon and at the last base of exon 13, likely resulting in a synonymous change, c.C437C. The calculated donor splice score remained unchanged (0.97) suggesting that this variant is likely to be a rare polymorphism.

## DISCUSSION

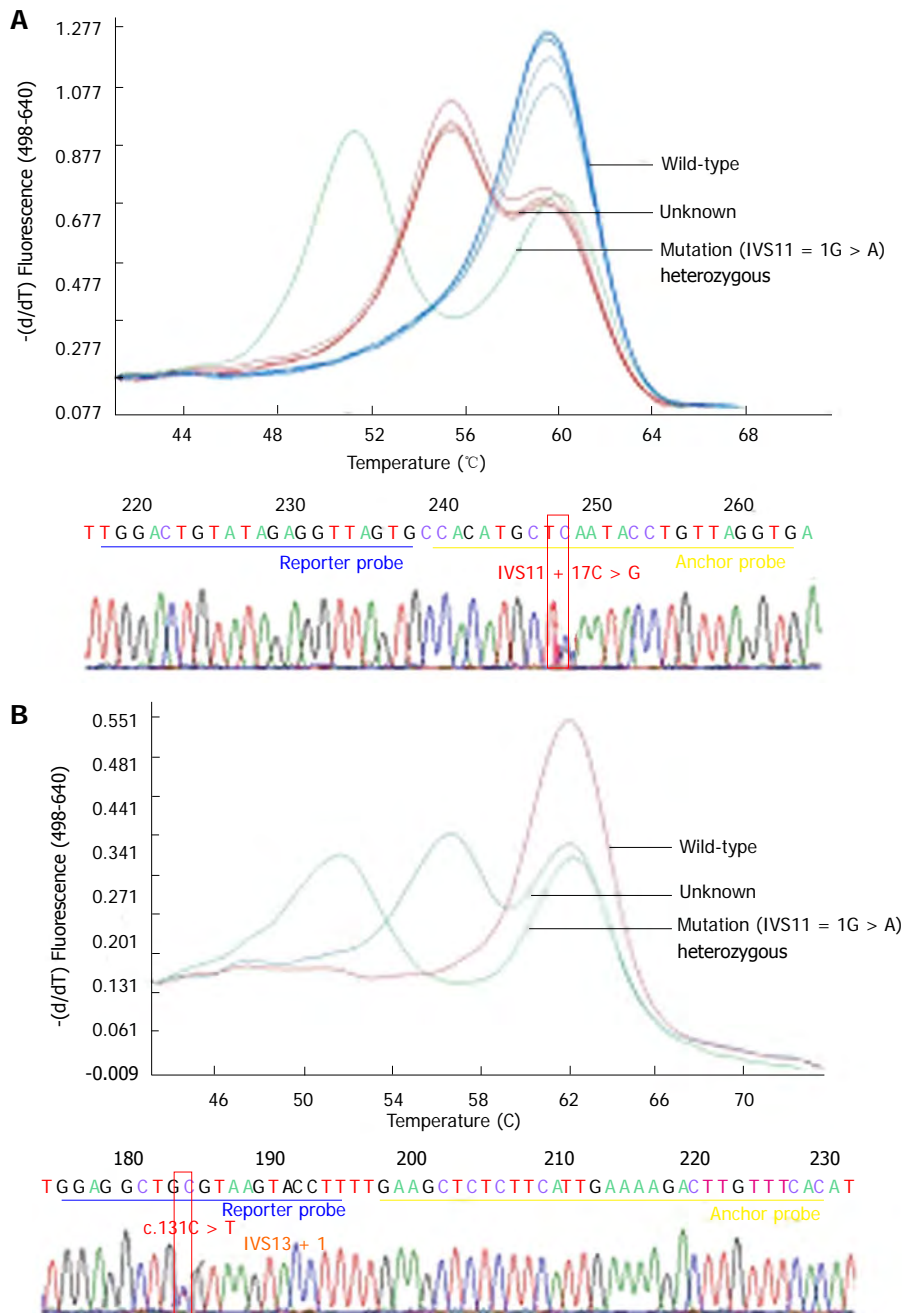
We have demonstrated a carrier rate of at least 1 in 90, and a homozygote/compound homozygote rate of 1 in 33000 for the known and previously identified *SLC25A13* mutations, excluding the p.Met1? variant among the general Thai population. This number is different, although similar, to the estimated carrier prevalence of 1/110 and homozygotes/compound homozygote rate of 1/48000 that was obtained from the only other available patient-based study<sup>[16]</sup>. Our findings are also consistent with the prevalence of *SLC25A13* homozygotes/compound heterozygotes of 1/17000 in the Japanese population and the disease frequency of 1/17000-34000 for NICCD, and 1/100000-230000 for CTLN2<sup>[8,24,25]</sup>.

No studies of the p.Met1? variant had been carried out prior to the recent description in affected and unaffected populations of China and Thailand<sup>[8,14-16,20]</sup>. This mutant allele has a high carrier frequency of 1/18 and a predicted homozygote rate of 1/1300. Based on our analysis, approximately 50000 individuals homozygous for p.Met1? are predicted to be present in the Thai population. Proven deleterious effects of the p.Met1? variant in a yeast model<sup>[22]</sup> and identification of a compound heterozygote between (paternally inherited) p.Met1? and the (maternally inherited) *SLC25A13* pathologic allele (r.16\_212dup) in a Chinese NICCD patient<sup>[20]</sup> supports the pathogenicity of the p.Met1? allele. When taking the p.Met1? allele into account with the other six previously described common mutations identified in the present study, a very high carrier rate, 1 in 15, and homozygote rate, 1 in 900, is predicted for *SLC25A13* mutations.

Patient- and population-based analyses of the prevalence of the p.Met1? variant in other ethnic backgrounds would help reveal its clinical relevance in CD and other disorder (s), if any. Based on the yeast model, the p.Met1? variant is expected to cause citrin protein production loss. It is also possible that in the p.Met1? variant, an alternative translation initiation site may produce a truncated citrin protein lacking 34 amino acid residues. However, the loss of the 34 N-terminal citrin residues causes the mislocalization and impaired function of the aberrant protein<sup>[22]</sup>, indicating that even if this truncated protein was produced, it would not contribute to normal citrin activity. Currently, we have not identified a p.Met1? homozygotic individual and it is possible that p.Met1? may lead to embryonic lethality, similar to homozygotes of the Southeast Asian Ovalocytosis mutant allele in the anion-exchanger 1<sup>[26,27]</sup>. Several questions regarding the p.Met1? mutation still remain unanswered. Despite its deleterious nature in the yeast model, high prevalence in Thai and Chinese populations from tropical areas, resistance to infectious diseases, and unexplainable unidentified homozygotes in patients even with a high allele frequency, its clinical pathology in humans, ethnic and regional prevalence, selective advantage against infectious diseases, and its lethality warrant further study.

There are two noteworthy limitations of the current study: subject age and the investigation of other





**Figure 2** Novel variants in the anchor probe binding sites, detected using the HybProbe assay. A: Upper panel shows unknown heterozygous melting peaks distinguishable from mutation [II] (IVS11 + 1G > A) the heterozygous positive control; lower panel is a sequenogram, confirming a variant IVS11 + 17C > G; B: Upper panel shows an aberrant heterozygous melting peak deviated from mutation [V] (IVS13 + 1G > A) heterozygote; lower panel indicates a sequenogram, confirming a novel variant c.1311C > T.

mutations. Given that 48 million people in Thailand are represented in our study, based on the inclusion age of 15 or older, over 1455 individuals may possibly have CD considering the confirmed disease causing mutations (excluding p.Met1?). The carrier rate derived from the present study may be underestimated due to the age of the population and mutations screened. Subjects involved in the study were living adults with an average age of 48 years. Some homozygotes/compound heterozygotes might not have survived prior to the start of the survey due to severe phenotypes, and thus would not have been included in our analysis. However, the age of the subjects may not have such a significant effect on the results of this study since the severe phenotype is relatively rare. Another limitation of the present study was that only six mutations were screened, thus the contribution of other

mutations was not included in our analysis. The other two less common mutations among the Chinese and Japanese population: [VI] (c.1799\_1800insA) and [VIII] (c.1801G > T)<sup>[21]</sup> were not selected for screening due to budget limitations.

CD is relatively common in East Asian populations<sup>[13,14]</sup>. Data from this study suggest that it is also common in Thais. While our analysis did not reveal any significant changes in mutation distribution in the five regions of Thailand, it is possible that an increase in sample size from each region may be necessary to reveal any other variations. CD has also been reported in Vietnamese and Malaysians<sup>[14,15,28]</sup>. Additional population studies in Southeast Asian populations will shed more light on the geographical distribution of this disease.

When considering each individual confirmed disease

**Table 2** Frequency of *SLC25A13* mutations in population studies

Mutation		Number of carriers (allele frequency)				
		Japanese <sup>[21]</sup>	Japanese <sup>[13,14]</sup>	Korean <sup>[13]</sup>	Chinese <sup>[13]</sup>	Thai (present study)
I	851del4	4 (0.48)	4 (0.15)	11 (0.22)	45 (0.54)	8 (0.26)
II	IVS11 + 1G > A	3 (0.36)	9 (0.33)	8 (0.16)	0 (0.00)	0 (0.00)
III	1638ins23	0 (0.00)	1 (0.04)	1 (0.02)	3 (0.04)	0 (0.00)
IV	S225X	0 (0.00)	5 (0.18)	0 (0.00)	0 (0.00)	0 (0.00)
V	IVS13 + 1G > A	2 (0.24)	1 (0.04)	0 (0.00)	0 (0.00)	0 (0.00)
VII	R605X	0 (0.00)	0 (0.00)	2 (0.04)	0 (0.00)	NA
VIII	E601X	1 (0.12)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
X	IVS6 + 5G > A	0 (0.00)	0 (0.00)	0 (0.00)	15 (0.18)	NA
XI	R184X	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.01)	NA
XIX	IVS16ins3kb	0 (0.00)	1 (0.04)	NA	NA	9 (0.29)
	p.Met1?	NA	NA	NA	NA	85 (2.77)
	<i>n</i>	420	1372	2455	4169	1537
p.Met1? not included						
	Number of carriers	10	20	22	64	17
	Carrier rate	1/42	1/65	1/112	1/65	1/90
	Homozygote rate	1/7100	1/17000	1/50000	1/17000	1/33000
p.Met1? included						
	Number of carriers		NA			101
	Carrier rate					1/15
	Homozygote rate					1/900

NA: Not available.

causing mutations, [XIX] and [I] were the two leading mutations identified in the general Thai population with equivalent frequency, 9/1537 and 8/1537, respectively (Table 2), whereas patient-based studies indicate a higher frequency of mutation [I] (8/10 mutated alleles)<sup>[16]</sup>. The discrepancy between the frequencies of mutant alleles identified from population-based studies and those obtained from patient-based analyses is also evident in other studies<sup>[13,21]</sup>.

Of the known disease causing mutations, [I] and [XIX] are most common in Thai and Chinese populations, whereas mutations [II] and [I] variants are most common among Japanese populations and patients (Table 2)<sup>[13,14,21]</sup>. In further exploration of this difference in ethnic mutation, when comparing between Chinese and Japanese, there is a possibility that the Thai ethnic background is more closely related to that of the Chinese. This may be linked to the ancient migration of certain Chinese ethnic groups to Thailand<sup>[29]</sup>.

The discovery of g.IVS11 + 17C > G and c.1311C > T variants which are located on the anchor/reporter probe binding sites raised the possibility that an SNP located in the same area may affect the dissociation of probes from the target DNA and interfere with detection of the target SNP. Therefore, direct sequencing should be performed on positive subjects in order to confirm the presence of the target SNP. Moreover, the presence of double SNPs in *αs* could possibly obscure detection of the target SNP; however, this possibility remains to be demonstrated. Bioinformatic analysis of the g.IVS11 + 17C > G and c.1311C > T variants showed no change in splice score or amino acid sequence, suggesting a benign nature of these SNPs. However, the possibility of either being a pathogenic allele cannot be completely excluded

due to its surprisingly low prevalence, 4 and 1 in 1537, respectively.

Overall, this study revealed that CD is not uncommon in the Thai population and there is a high frequency of the p.Met1? allele. Once the optimization for TaqMan/HybProbe analysis for each mutation is complete, it will serve as a rapid, efficient, robust, convenient, and cost-saving method for large scale analysis that will enable general population and newborn screening across the country. This has already been demonstrated by the successful establishment of the TaqMan assay for the p.Met1? mutation. Procedures utilized in the present study should prove valuable in examining the distribution and frequencies of *SLC25A13* mutations including p.Met1? among Southeast Asian populations.

Further investigations are required to establish the clinical relevance of p.Met1? both in patients and controls. Demonstration of the molecular pathogenic mechanism of p.Met1? in human/mammalian models, although it is predicted to be a loss of function mutation<sup>[22]</sup>, will also aid in further understanding. The unusually high frequency of the p.Met1? mutation suggests it may have a role in the predisposition and/or protection of disorder (s), perhaps similar to the selection of red cell polymorphisms in areas endemic for malarial infection<sup>[30-32]</sup>. Our further work will examine the possible connection between the p.Met1? mutation and protection against infectious tropical diseases.

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## COMMENTS

### Background

Citrin deficiency (CD) leads to three distinct phenotypes; two of which are non-fatal. These phenotypes include: Neonatal intrahepatic cholestasis caused by citrin deficiency, failure-to-thrive and dyslipidemia. The third, a fatal phenotype of type II citrullinemia, has marked elevation of ammonia level mimicking a primary disorder of the urea cycle.

### Research frontiers

Treatment for CD is distinct from other urea cycle disorders. Epidemiological data are needed to predict disease prevalence and aid in creating awareness for diagnosis among physicians. In this study, the authors screened 1537 subjects from the general population for a novel pathologic allele, p.Met1?, and six common mutations using newly developed Taqman and established HybProbe assays. They demonstrated a carrier frequency of 1/18 for p.Met1? allele, and the carrier rate of 1/90 for mutations: [XIX] and [I], and calculated homozygote rate of 1/33000 for the two latter mutations.

### Innovations and breakthroughs

The question of p.Met1? homozygote lethality remains unanswered which may serve as an explanation as to why this homozygote has yet to be identified in patients/controls even with high allele frequency. The p.Met1? mutation has been rarely studied in populations other than the Thai and Chinese and therefore, may have been overlooked. The high carrier rate of p.Met1? suggests the possible selective advantage of this particular allele.

### Applications

The unusually high frequency of the p.Met1? mutation suggests its possible role in the predisposition and/or protection of disorder(s), which is perhaps similar to that of the selection of red cell polymorphisms in endemic areas for malarial infection. The established TaqMan assay would allow for a simple, rapid, and cost-effective method for p.Met1? mass screening.

### Terminology

CD is caused by a mutation in the *SLC25A13* gene encoding for an aspartate-glutamate carrier isoform 2 (AGC2) and is expressed mainly in the liver. The major function of AGC2 is to export mitochondrial aspartate in exchange for cytosolic glutamate and it is involved in several metabolic pathways with major contributions to the urea cycle and malate-aspartate shuttle.

### Peer review

The article covers a topic area of increasing interest. It contains novel information and data affirms on available literature. 1537 subjects from general population were screened for a novel pathologic allele p.Met1? and six common mutations using newly developed Taqman and established HybProbe assays. The study highlighted the current underestimation of citrin deficiency and suggested the possible selective advantage of the p.Met1? allele.

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## Single-incision vs three-incision laparoscopic cholecystectomy for complicated and uncomplicated acute cholecystitis

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### Abstract

**AIM:** To compare the clinical outcome of single-incision laparoscopic cholecystectomy (SILC) and three-incision laparoscopic cholecystectomy (3ILC) for acute cholecystitis.

**METHODS:** From July 2009 to September 2012, 136 patients underwent SILC or 3ILC for acute cholecystitis at a tertiary referral hospital. One experienced surgeon performed every procedure using 5 or 10 mm 30-degree laparoscopes, straight instruments, and conventional ports. Five patients with perforated gallbladder and diffuse peritonitis and 23 patients with mild acute cholecystitis were excluded. The remaining 108 patients were divided into complicated and uncomplicated groups according to pathologic findings. Patient demog-

raphy, clinical data, operative results and complications were recorded and analyzed.

**RESULTS:** Fifty patients with gangrenous cholecystitis, gallbladder empyema, or hydrops were classified as the complicated group, and 58 patients with acute cholecystitis were classified as the uncomplicated group. Twenty-three (46.0%) of the patients in the complicated group ( $n = 50$ ) and 39 (67.2%) of the patients in the uncomplicated group ( $n = 58$ ) underwent SILC; all others underwent 3ILC. The postoperative length of hospital stay (PLOS) was significantly shorter in the SILC subgroups than the 3ILC subgroups ( $3.5 \pm 1.1$  d vs  $4.6 \pm 1.3$  d,  $P < 0.01$  in the complicated group;  $2.9 \pm 1.1$  d vs  $3.7 \pm 1.4$  d,  $P < 0.05$  in the uncomplicated group). The maximum body temperature recorded at day 1 and at day 2 following the procedure was lower in the SILC subgroups, but the difference reached statistical significance only in the uncomplicated group ( $37.41 \pm 0.56$  °C vs  $37.80 \pm 0.72$  °C,  $P < 0.05$  on postoperative day 1;  $37.10 \pm 0.43$  °C vs  $37.57 \pm 0.54$  °C,  $P < 0.01$  on postoperative day 2). The operative time, estimated blood loss, postoperative narcotic use, total length of hospital stay, conversion rates, and complication rates were similar in both SILC and 3ILC subgroups. The complicated group had longer operative time ( $122.2 \pm 35.0$  min vs  $106.6 \pm 43.6$  min,  $P < 0.05$ ), longer PLOS ( $4.1 \pm 1.3$  d vs  $3.2 \pm 1.2$  d,  $P < 0.001$ ), and higher conversion rates ( $36.0\%$  vs  $19.0\%$ ,  $P < 0.05$ ) compared with the uncomplicated group.

**CONCLUSION:** SILC is safe and efficacious for patients with acute cholecystitis. The main benefit is a faster recovery than that achieved with 3ILC.

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**Key words:** Single-incision laparoscopic cholecystectomy; Single-incision laparoscopic surgery; Laparoen-

doscopic single site surgery; Cholecystectomy; Acute cholecystitis; Complicated cholecystitis; Gangrenous cholecystitis

**Core tip:** single-incision laparoscopic cholecystectomy (SILC) is an alternative treatment for uncomplicated benign gallbladder diseases, but its role in acute cholecystitis remains unclear. This comparative analysis of SILC with three-incision laparoscopic cholecystectomy for treating acute cholecystitis represents the largest series to date and proportion of gangrenous cholecystitis patients (30.6%). The well-known drawbacks of SILC - longer operative time and higher cost - were alleviated by the larger paraumbilical incisions facilitating extraction of inflamed gallbladders and reliance on conventional instruments only. The low procedure conversion rate observed for SILC indicated its safety and efficacy for treating acute cholecystitis. SILC providing a faster recovery time was the main benefit to these patients.

Chuang SH, Chen PH, Chang CM, Lin CS. Single-incision vs three-incision laparoscopic cholecystectomy for complicated and uncomplicated acute cholecystitis. *World J Gastroenterol* 2013; 19(43): 7743-7750 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7743.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7743>

## INTRODUCTION

Single-incision laparoscopic cholecystectomy (SILC) is a novel technique comparable to traditional multi-incision laparoscopic cholecystectomy (LC) for uncomplicated benign gallbladder diseases in respect of safety and efficacy<sup>[1-3]</sup>. In addition to well-established cosmetic advantage; decreased post-operative pain and faster recovery are potential benefits<sup>[4-6]</sup>. However, higher complication rates in SILC have been reported<sup>[7-9]</sup>. Therefore, application of this technique in cases of acute cholecystitis should be done with caution<sup>[10]</sup>. Published SILC studies contain a small number of patients with acute cholecystitis<sup>[11-15]</sup>, while reports comparing SILC and traditional LC for acute cholecystitis are very rare<sup>[11]</sup>.

SILC was developed as a step-by-step evolution of three-incision laparoscopic cholecystectomy (3ILC) and two-incision laparoscopic cholecystectomy (2ILC) in March 2010<sup>[16]</sup>. Importantly, only conventional instruments were used. Initially, this procedure was only adopted in patients with simple benign gallbladder disease. Since May 2011, however, SILC has been offered as an optional procedure for acute cholecystitis by our clinical practice. This study compares the clinical outcomes following SILC and 3ILC for acute cholecystitis over a period of 39 mo.

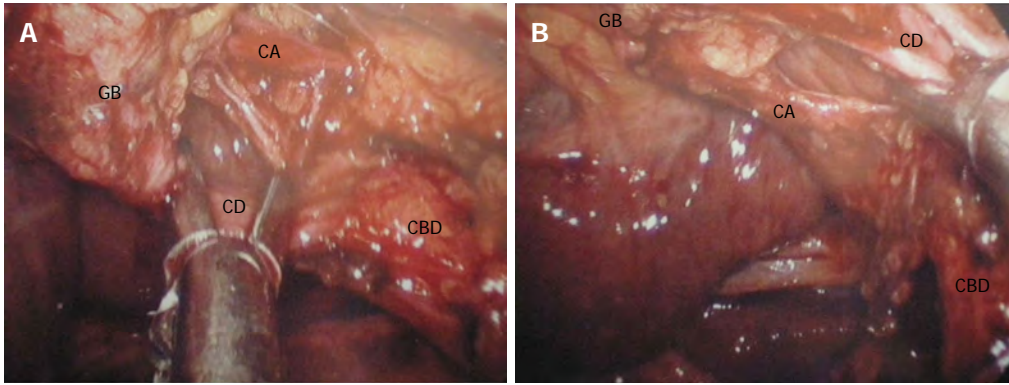
## MATERIALS AND METHODS

From July 2009 to September 2012, 136 consecutive patients with acute cholecystitis underwent cholecystectomy by a single surgeon at a tertiary referral hospital in Hsin-Chu city, Taiwan. Five patients had perforated gallbladder and diffuse peritonitis and were excluded from the analysis. The role of laparoscopic operation in patients with perforated gallbladders and diffuse peritonitis remains controversial<sup>[17-19]</sup>. Twenty-three patients with "mild acute cholecystitis" were also excluded. The clinical course of this disease is similar to that of a biliary colic. To eliminate the bias related to disease severity, the enrolled 108 patients were divided into complicated and uncomplicated groups, according to operative and pathologic findings. Gangrenous cholecystitis, gallbladder empyema or hydrops were defined as complicated cholecystitis, while all other findings were defined as uncomplicated cholecystitis.

Patient demography, clinical data, operative results and complications were recorded. A modified APACHE II was used as the preoperative prognostic score, namely, low risk: < 5 points, intermediate risk: 6-9 points, and high risk: ≥ 10 points<sup>[20]</sup>. The operative time was defined as the interval from initial skin incision to skin closure. Postoperative narcotic use was recorded as the intramuscular pethidine dose (mg) per kilogram of patient body weight (*i.e.*, 1 mg/kg). The postoperative length of hospital stay (PLOS) was defined as the duration between the day of surgery and the day of discharge in the same hospitalization. The total length of hospital stay referred to the total hospitalization duration including readmission for late-onset complications. The maximum body temperature (BT; °C) of each day was recorded from postoperative day 1 to day 4 for patients who were still hospitalized. Any procedure that failed to be fulfilled as scheduled was regarded as converted. The complications were recorded according to the five-grade Clavien-Dindo classification system<sup>[21]</sup>.

### Surgical technique

The details of the surgical techniques have been described previously<sup>[16]</sup>. In SILC, two 5 mm straight instruments and a 5 mm 30-degree rigid laparoscope were inserted into the abdominal cavity *via* three 5 mm ports in a vertical line at a 2 cm paraumbilical incision on the left side. An optional 2 mm right subcostal incision was made for the passage of a transcystic duct catheter to perform an intraoperative cholangiography (IOC). An assistant controlled the retraction grasper in the middle port. The operator controlled the working instrument in the upper port with the right hand and the laparoscope in the lower port with the left hand (self-camera technique). At the end of the procedure, the lower 5 mm port was upgraded to a 10 mm reusable port for specimen extraction into a retrieval bag. All the fascial defects and the skin incision



**Figure 1** Critical view of safety during a single-incision laparoscopic cholecystectomy for acute cholecystitis. Anterior (A) and posterior (B) views are shown. GB: Gallbladder; CA: Cystic artery; CD: Cystic duct; CBD: Common bile duct.

were sutured. In 3ILC, a 10 mm 30-degree rigid laparoscope was inserted *via* a 10 mm reusable port at a 1 cm infraumbilical incision. The 5 mm working instrument and retraction grasper were inserted *via* two separate 5 mm ports at the epigastrium and right flank respectively.

When a severely inflamed gallbladder or dense pericholecystic fibrosis was encountered at an early stage of the procedure, the threshold for using additional port sites was low. A suction irrigation device was used for decompression of a severely distended gallbladder and meticulous dissection in an unclear operative field. Every effort was made to obtain the critical view of safety (Figure 1), following the recommendations by Strasberg *et al.*<sup>[22]</sup>. If the anatomy of the Calot's triangle was obscure, dissection would be started from the gallbladder dome (retrograde cholecystectomy). In difficult situations, the gallbladder neck or posterior wall was not disturbed (subtotal cholecystectomy)<sup>[23,24]</sup>. The cystic duct stump or gallbladder neck was secured with intracorporeal suturing if the diameter was too big or the tissue was too fragile to be clipped. In infrequent cases, the liver bed was packed with gauze temporarily if the monopolar electrocautery had failed to achieve hemostasis. Wound extension to fit a firm and swollen gallbladder was usually carried out at the infraumbilical incision in 3ILC, but it was largely unnecessary in SILC. When a subhepatic drain was placed, it was always removed within 48 h after the operation if there was no bile leakage. After discharge, all the patients attended follow-up periods of more than 1 mo.

### Statistical analysis

Data were analyzed using Pearson's  $\chi^2$  test and Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

The complicated group (gangrenous cholecystitis, gallbladder empyema or hydrops) consisted of 50 patients, and the uncomplicated group (acute cholecystitis) consisted of 58 patients. Twenty-three (46.0%) of the patients in the complicated group and 39 (67.2%) of the

patients in the uncomplicated group underwent SILC; the remaining patients all underwent 3ILC. The demographic characteristics, clinical data, and pathologic findings showed no statistically significant differences between the SILC and 3ILC subgroups in either the complicated or uncomplicated groups (Table 1). Patients with gangrenous cholecystitis constituted a major portion of the complicated group (65.2% in the SILC subgroup and 66.7% in the 3ILC subgroup). Preoperative endoscopic retrograde cholangiopancreatography (ERCP) was performed on 13 patients during the same hospitalization to address suspicious concomitant choledocholithiasis (Table 2). Nine of the patients showed a positive result and subsequently underwent immediate therapeutic endoscopic sphincterotomy (EST) for stone clearance. Eleven patients had suspicious concomitant choledocholithiasis without preoperative ERCP and subsequently underwent IOC; the results for all were negative. In cases of positive IOC, common bile duct exploration was performed under laparoscopy.

PLOS and postoperative BT were the only two parameters that displayed a statistical difference between the two subgroups (Tables 2 and 3). The SILC subgroup had a shorter PLOS than the 3ILC subgroup in the complicated and uncomplicated groups ( $P < 0.01$  and  $< 0.05$ , respectively). The SILC subgroups had a lower maximum BT than the 3ILC subgroups on the postoperative day 1 and day 2 (Figure 2), but the difference reached statistical significance only in the uncomplicated group ( $P < 0.05$  for postoperative day 1 and  $P < 0.01$  for postoperative day 2). Additional port sites were needed to fulfill the operations in eighteen patients of the complicated group and eleven patients of the uncomplicated group. Converted to an open cholecystectomy (OC) was not necessary in any case. The conversion rates in the SILC and 3ILC subgroups were similar (34.8% *vs* 37.0% in the complicated group; 17.9% *vs* 21.1% in the uncomplicated group).

Fourteen complications occurred in 11 patients (Table 4). The differences in complication rates between the SILC and 3ILC subgroups were statistically insignificant. Four patients experienced mild pulmonary effusion and/or atelectasis, a grade I complication that resolved spon-

**Table 1 Patient characteristics and pathology *n* (%)**

	Complicated acute cholecystitis		<i>P</i> value	Uncomplicated acute cholecystitis		<i>P</i> value
	SILC group ( <i>n</i> = 23)	3ILC group ( <i>n</i> = 27)		SILC group ( <i>n</i> = 39)	3ILC group ( <i>n</i> = 19)	
Age (yr)	51.2 ± 15.3	58.0 ± 17.3	0.147	49.1 ± 13.9	54.6 ± 14.5	0.167
Sex (male/female)	7/16	10/17	0.623	24/15	9/10	0.306
Body mass index (kg/m <sup>2</sup> )	25.24 ± 3.36	26.36 ± 3.59	0.272	25.01 ± 2.67	26.99 ± 4.35	0.081
Modified APACHE II score (points)			0.318			0.595
0-5, low risk	21 (91.3)	22 (81.5)		36 (92.3)	17 (89.5)	
6-9, intermediate risk	2 (8.7)	5 (18.5)		2 (5.1)	2 (10.5)	
10-11, high risk	0	0		1 (2.6)	0	
Previous abdominal surgery	7	3	0.089	6	5	0.319
Previous biliary symptoms	12	10	0.283	30	11	0.135
Duration of acute symptoms > 72 h	14	14	0.522	34	13	0.087
Pathology			0.985			
Gangrene	15 (65.2)	18 (66.7)		-	-	
Empyema	6 (26.1)	7 (25.9)		-	-	
Hydrops	2 (8.7)	2 (7.4)		-	-	
Acute inflammation	-	-		39 (100)	19 (100)	

SILC: Single-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy.

**Table 2 Operative modifications and results *n* (%)**

	Complicated acute cholecystitis		<i>P</i> value	Uncomplicated acute cholecystitis		<i>P</i> value
	SILC group ( <i>n</i> = 23)	3ILC group ( <i>n</i> = 27)		SILC group ( <i>n</i> = 39)	3ILC group ( <i>n</i> = 19)	
Preoperative ERCP	0	3	0.099	0	1	0.148
Preoperative ERCP and EST	2	0	0.118	3	4	0.143
Intraoperative cholangiography	1	0	0.274	5	5	0.202
Operative time (min)	119.8 ± 38.8	124.3 ± 32.1	0.660	100.9 ± 42.1	118.4 ± 45.5	0.154
Estimated blood loss (mL)	43.2 ± 29.8	31.0 ± 26.6	0.156	24.2 ± 31.5	29.5 ± 29.9	0.548
Pethidine dose (mg/kg)	0.624 ± 0.505	0.535 ± 0.740	0.632	0.618 ± 0.485	0.549 ± 0.427	0.601
Postoperative length of hospital stay (d)	3.5 ± 1.1	4.6 ± 1.3	< 0.010	2.9 ± 1.1	3.7 ± 1.4	< 0.050
Total length of hospital stay (d)	6.0 ± 3.6	5.8 ± 3.1	0.814	4.1 ± 1.9	6.4 ± 4.7	0.053
Conversion						
Overall	8 (34.8)	10 (37.0)	0.869	7 (17.9)	4 (21.1)	0.777
2ILC	4	-		3	-	
3ILC	2	-		1	-	
4ILC (standard LC)	2	10		3	4	
OC	0	0		0	0	

SILC: Single-incision laparoscopic cholecystectomy; 2ILC: Two-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy; 4ILC: Four-incision laparoscopic cholecystectomy; OC: Open cholecystectomy; ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy.

**Table 3 Postoperative body temperature**

Maximum body temperature (°C)	Complicated acute cholecystitis		<i>P</i> value	Uncomplicated acute cholecystitis		<i>P</i> value
	SILC group	3ILC group		SILC group	3ILC group	
Post-op day 1	37.73 ± 0.57	38.03 ± 0.66	0.096	37.41 ± 0.56	37.80 ± 0.72	< 0.050
Post-op day 2	37.38 ± 0.59	37.58 ± 0.46	0.180	37.10 ± 0.43	37.57 ± 0.54	< 0.010
Post-op day 3	37.12 ± 0.49	37.30 ± 0.49	0.215	37.04 ± 0.45	37.04 ± 0.56	0.990
Post-op day 4	37.18 ± 0.44	37.19 ± 0.45	0.974	36.88 ± 0.41	36.86 ± 0.33	0.915

Including only patients who were still hospitalized. SILC: Single-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy; post-op: Postoperative.

taneously within a few days<sup>[21]</sup>. Three patients developed grade II complications, including infected nonbilious subhepatic collection (*n* = 1), relapsed cholangitis with bacteremia (*n* = 1), and refractory diarrhea (*n* = 1); all were treated conservatively with intravenous antibiotics and fluid therapy. Three patients had grade IIIa complica-

tions, including infected nonbilious subhepatic collections (*n* = 2) and retained bile duct stones (*n* = 1); the first two patients were managed with percutaneous pigtail drainage and intravenous antibiotics, and the last underwent an ERCP with EST. One patient underwent a laparotomy to remove retained impacted bile duct stones, a grade IIIb



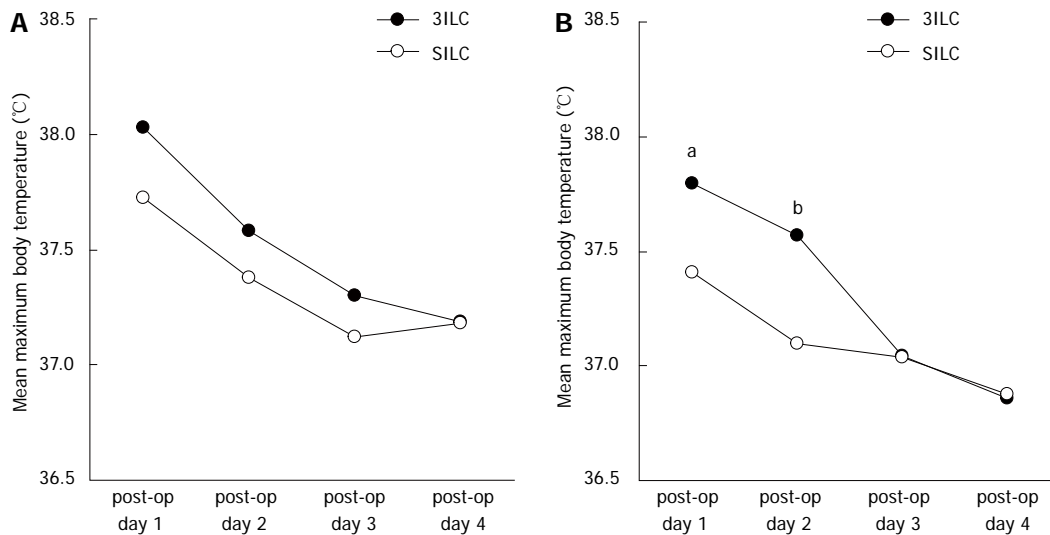


Figure 2 Postoperative mean maximum body temperature for the complicated acute cholecystitis group (A) and the uncomplicated acute cholecystitis group (B). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs SILC. 3ILC: Three-incision laparoscopic cholecystectomy; SILC: Single-incision laparoscopic cholecystectomy; post-op: Postoperative.

Table 4 Complications  $n$  (%)

Complications	Complicated acute cholecystitis		$P$ value	Uncomplicated acute cholecystitis		$P$ value
	SILC group ( $n = 23$ )	3ILC group ( $n = 27$ )		SILC group ( $n = 39$ )	3ILC group ( $n = 19$ )	
Overall <sup>1</sup>	3 (13.0)	3 (11.1)	0.834	3 (7.7)	2 (10.5)	0.718
Grade I	1 (4.3) <sup>1</sup>	1 (3.7) <sup>2</sup>		2 (5.1) <sup>3</sup>	0	
Grade II	1 (4.3) <sup>4</sup>	0		1 (2.6) <sup>5</sup>	1 (5.3) <sup>6</sup>	
Grade IIIa	1 (4.3) <sup>7</sup>	2 (7.4) <sup>8</sup>		0	0	
Grade IIIb	0	0		0	1 (5.3) <sup>9</sup>	
Grade IVa, IVb, V	0	0		0	0	

<sup>1</sup>Pleural effusion and atelectasis; <sup>2</sup>Pleural effusion; <sup>3</sup>One was pleural effusion and atelectasis; the other was pleural effusion; <sup>4</sup>Refractory diarrhea; <sup>5</sup>Infected subhepatic collection; <sup>6</sup>Relapsed cholangitis with bacteremia; <sup>7</sup>Infected subhepatic collection and pleural effusion; <sup>8</sup>One was infected subhepatic collection; the other was retained bile duct stones; <sup>9</sup>Retained bile duct stones. SILC: Single-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy.

Table 5 Overall comparison of complicated and uncomplicated acute cholecystitis groups

	Complicated acute cholecystitis ( $n = 50$ )	Uncomplicated acute cholecystitis ( $n = 58$ )	$P$ value
Age (yr)	54.9 ± 16.6	50.9 ± 14.2	0.184
Sex (male/female)	33/17	33/25	0.333
Modified APACHE II score			0.320
0-5, low risk	43 (86.0)	53 (91.4)	
6-9, intermediate risk	7 (14.0)	4 (6.9)	
10-11, high risk	0	1 (1.7)	
Operative time (min)	122.2 ± 35.0	106.6 ± 43.6	< 0.050
Estimated blood loss (mL)	36.1 ± 28.3	26.0 ± 30.8	0.092
Pethidine dose (mg/kg)	0.577 ± 0.633	0.595 ± 0.464	0.867
Postoperative length of hospital stay (d)	4.1 ± 1.3	3.2 ± 1.2	< 0.001
Total length of hospital stay (d)	5.9 ± 3.3	4.8 ± 3.3	0.098
Conversion	18 (36.0)	11 (19.0)	< 0.050
Complications	6 (12.0)	5 (8.6)	0.563

Data are expressed as absolute numbers (percentage) or mean ± SD.

complication. The seven patients with grade II, IIIa and IIIb complications all needed a secondary hospitalization (range: 5-16 d) and recovered uneventfully.

In summary, the complicated group experienced longer operative times ( $P < 0.05$ ), longer PLOS ( $P < 0.001$ ), and higher conversion rates ( $P < 0.05$ ) (Table 5).

## DISCUSSION

SILC, also known as laparoendoscopic single site cholecystectomy, has increased in popularity worldwide in recent years. While multiple studies have reported this novel technique to be as safe as traditional LC for the

treatment of uncomplicated benign gallbladder disease<sup>[1-3]</sup>, some have demonstrated that SILC is associated with a higher complication rate<sup>[7-9]</sup>. Applying SILC in more complex circumstances, such as acute cholecystitis, becomes an interesting angle in which to study SILC in complex circumstances. To date, the published SILC studies have focused on only a small number of patients with acute cholecystitis and comparative studies have been rare<sup>[11-15]</sup>.

According to the 2010 Society of American Gastrointestinal and Endoscopic Surgeons guideline for the clinical application of laparoscopic biliary tract surgery, the indications, contra-indications and preoperative preparation for SILC are the same as those for multi-port cholecystectomy<sup>[17]</sup>. Both procedures should share the same safety standards with a low conversion threshold. We strictly followed these safety guidelines. Before adopting this technique for acute cholecystitis in May 2011, we had performed over 50 complication-free SILC procedures for simple benign gallbladder disease. Additionally, we are proficient at modified techniques to manage gallbladder complications, such as decompression, meticulous dissection with a suction irrigation device, retrograde cholecystectomy, subtotal cholecystectomy, and intracorporeal suturing the cystic duct stump or gallbladder neck. In SILC, a subhepatic drain always passed through an additional port site. Firm, fragile or severely inflamed gallbladders are usually difficult to retract. Therefore, conventional straight instruments were used in our cases, as the more elastic nature of curved or angulated instruments are not suitable. Because we only used conventional instruments, the procedures could be easily and rapidly converted to multi-incision laparoscopic or open operations for safety concerns.

Pathologic findings often have an effect on operative results of LC<sup>[25-27]</sup>. To eliminate this bias, we divided patients into two groups according to disease severity. The comparison between complicated and uncomplicated groups showed significant differences in operative time ( $P < 0.05$ ), PLOS ( $P < 0.001$ ), and conversion rates ( $P < 0.05$ ) (Table 5). The findings implicated that the two groups were different. The difference in complication rates did not reach statistical significance. This may be due to inadequate patient number and low complication rates.

The finding that the SILC subgroups had a shorter PLOS than the 3ILC subgroups was consistent with our previous study (Table 2)<sup>[16]</sup>. Even small traumatic effects can influence postoperative recovery. In case of acute cholecystitis, we followed a rule that patients who tolerated oral feeding well and had a BT under 37.5 °C for more than 24 h should be discharged. In this study, all the patients resumed oral feeding the morning after the operation, and most of them tolerated it well. Accordingly, postoperative fever became the critical factor leading to longer PLOS. The finding that the SILC subgroups had a lower maximum BT than the 3ILC subgroups on the postoperative day 1 and day 2 explained the shorter PLOS in the SILC subgroups (Table 3, Figure 2). Al-

though the difference in maximum BT reached statistical significance only in the uncomplicated group, we were more concerned about the postoperative fever in patients with complicated acute cholecystitis. We tended to associate the febrile episodes with postoperative infection in these patients. Accordingly, the small difference in maximum postoperative BT between the SILC and 3ILC subgroups in the complicated group influenced the PLOS significantly. The occurrence of postoperative fever was related to the inflammatory response to cholecystitis, atelectasis, and postoperative septic sequelae. Considering the similar pathologic distributions (disease severity) and postoperative complication rates in the SILC and 3ILC subgroups, it is possible that atelectasis may account for the difference in postoperative BT. Upper abdominal incision (upper midline or subcostal incisions) is a well-established risk factor for the development of atelectasis after abdominal surgery<sup>[28]</sup>, and traditional multi-incision LC was associated with impaired postoperative pulmonary function and an incidence of atelectasis up to 30% in several studies<sup>[29-31]</sup>. A lower incidence of febrile episodes following LC correlated with improved postoperative pulmonary function and minimal surgical trauma was observed<sup>[32]</sup>. Thus, 3ILC caused more febrile episodes in the first two postoperative days, and small upper abdominal incisions played a role in impaired postoperative pulmonary function (atelectasis) for patients with acute cholecystitis. We hypothesize that the faster recovery following SILC may be derived not from decreased pain severity, but rather location.

The operative duration and pethidine dose showed no significant difference between the two subgroups (Table 2). It is our opinion that the longer SILC duration in our previous study simply reflected an effect of the learning curve<sup>[16,33,34]</sup>. The typical SILC procedure for simple benign gallbladder disease takes less than one hour. In addition, the 1 cm infraumbilical incision in a 3ILC was too small to fit a swollen gallbladder, and it took some time to enlarge the incision during specimen extraction. The operative duration spent in SILC and 3ILC was comparable in most cases, for both simple and complicated gallbladder disease. Although we failed to reveal the difference in postoperative narcotic use between the two subgroups, it is too early to make a conclusion. In our clinical practice, a steady intramuscular pethidine dose is available, but the patients may feel pain in different degrees. To clarify the issue of postoperative pain related to the procedures, more detailed studies are necessary.

The outcome following a converted LC is worse than that following a successful LC<sup>[35,36]</sup>. A qualified laparoscopic surgeon should never hesitate to convert the procedure in an early stage if patient safety is questionable in difficult situations. The high conversion rate in our study (36% in the complicated group and 19% in the uncomplicated group) reflected our safety concerns (Table 5). Consistent with other studies, no procedure was converted to an OC<sup>[23,24]</sup>. The above-mentioned modified laparoscopic procedures for severe cholecystitis, such as

gallbladder decompression, dissection with a suction irrigation device, retrograde cholecystectomy, subtotal cholecystectomy and intracorporeal suturing, might reduce the open conversion rates tremendously without increasing the complication rates.

In conclusion, SILC with conventional instruments is as safe and efficacious as traditional multi-incision LC for both complicated and uncomplicated acute cholecystitis in experienced hands. The complication rate is low, and the major benefit for patients is faster postoperative recovery. Before applying SILC in difficult gallbladders, a surgeon must be proficient in this novel technique for simple gallbladder disease and the modified laparoscopic techniques for severe cholecystitis. A low threshold for converting the procedure should be maintained for patient safety. Further prospective randomized trials are needed to verify our findings.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Single-incision laparoscopic cholecystectomy (SILC) is a novel technique, with safety and efficacy profiles that are comparable to traditional multi-incision laparoscopic cholecystectomy (LC) for uncomplicated benign gallbladder diseases. For complicated gallbladder diseases, such as acute cholecystitis, the published studies regarding SILC have thus far been conducted with only a small number of patients. Studies comparing SILC and traditional LC for acute cholecystitis are rare, but necessary.

### Research frontiers

Single-incision laparoscopic surgery (SILS; also known as laparoendoscopic single site surgery) is a novel minimally invasive technique, compared with the traditional multi-incision laparoscopic surgery. Besides the obvious cosmetic advantage (producing no visible scar), decreased post-operative pain and faster recovery are the potential benefits of SILS. However, the higher complication rate that accompanies a beginner operator's learning curve must be accounted for when choosing to apply this technique.

### Innovations and breakthroughs

SILC with conventional instruments is as safe and efficacious as traditional multi-incision LC for both complicated and uncomplicated acute cholecystitis when performed by a physician with experienced hands. In particular, the patient benefits are low complication rate and faster postoperative recovery. Before applying SILC in difficult gallbladders, a surgeon must be proficient in this novel technique for simple gallbladder disease and the modified laparoscopic techniques for severe cholecystitis. A low threshold for converting the procedure should be maintained to help ensure patient safety.

### Applications

This study suggests that SILC with conventional instruments can be applied to patients with acute cholecystitis safely and efficaciously, particularly when performed by physicians with experienced hands. Better cosmetic outcome and faster recovery time are major advantages.

### Terminology

Single-incision laparoscopic surgery is a minimally invasive surgical procedure, in which the surgeon operates through a small single entry site - often the navel. As such, this procedure is considered a type of scarless surgery. The SILS

procedure is a good alternative approach (compared to the traditional surgical cholecystectomy procedure) for treating acute cholecystitis, an acute inflammation of the gallbladder characterized by unendurable pain in the right upper abdominal quadrant and is closely correlated with gallbladder stones.

### Peer review

This comprehensive comparative study of SILC and the traditional multi-incision LC treatment approach for acute cholecystitis represents the largest case series investigation of SILC in acute cholecystitis published to date. As well as better cosmetic outcome, SILC was shown to have a faster recovery time and less complications. The postoperative complication of fever remains to be fully understood and may be primarily related to the body's inflammatory response to the cholecystitis rather than the surgical procedure itself. Unfortunately, the well known difference in cost between the two procedures and the longer operating time required by SILC make it difficult to justify further prospective studies.

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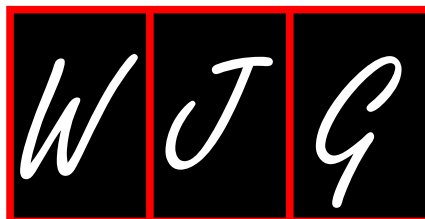
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## Transanal natural orifice specimen extraction for laparoscopic anterior resection in rectal cancer

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### Abstract

**AIM:** To investigate whether transanal natural orifice specimen extraction (NOSE) is a better technique for rectal cancer resection.

**METHODS:** A prospectively designed database of a consecutive series of patients undergoing laparoscopic low anterior resection for rectal cancer with various tumor-node-metastasis classifications from March 2011 to February 2012 at the First Affiliated Hospital of Sun Yat-Sen University was analyzed. Patient selection for transanal specimen extraction and intracorporeal anastomosis was made on the basis of tumor size and distance of rectal lesions from the anal verge. Demographic data, operative parameters, and postoperative outcomes were assessed.

**RESULTS:** None of the patients was converted to laparotomy. Respectively, there were 16 cases in the low anastomosis and five in the ultralow anastomosis groups. Mean age of the patients was 45.4 years, and mean body mass index was 23.1 kg/m<sup>2</sup>. Mean distance of the lower edge of the lesion from the anal verge

was 8.3 cm. Mean operating time was 132 min, and mean intraoperative blood loss was 84 mL. According to the principle of rectal cancer surgery, we performed D2 lymph node dissection in 13 cases and D3 in eight. Mean lymph nodes harvest was 17.8, and the number of positive lymph nodes was 3.4. Median hospital stay was 6.7 d. No serious postoperative complication occurred except for one anastomotic leakage. All patients remained disease free. Mean Wexner score was 3.7 at 11 mo after the operation.

**CONCLUSION:** Transanal NOSE for total laparoscopic low/ultralow anterior resection is feasible, safe and oncologically sound. Further studies with long-term outcomes are needed to explore its potential advantages.

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**Key words:** Transanal specimen extraction; Natural orifice specimen extraction; Laparoscopic anterior resection; Low/ultra-low anastomosis; Total mesorectal excision

**Core tip:** Natural orifice specimen extraction (NOSE) is an emerging technique that has been recently applied to the field of rectal cancer resection. However, which is the better approach for rectal cancer remains controversial. In this paper, we present our surgical technique and short-term outcomes of transanal NOSE in total laparoscopic low/ultralow anterior resection (L-AR) for patients with rectal cancer. Based on our limited experience, transanal NOSE in L-AR for rectal cancer is feasible, safe and oncologically sound.

Han FH, Hua LX, Zhao Z, Wu JH, Zhan WH. Transanal natural orifice specimen extraction for laparoscopic anterior resection in rectal cancer. *World J Gastroenterol* 2013; 19(43): 7751-7757 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7751.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19>

## INTRODUCTION

The incidence of rectal cancer is higher in Asia compared with western countries<sup>[1]</sup>. Technically, resection of low rectal cancer may be the most difficult among all colorectal operations.

At present, traditional colorectal surgery has increasingly given way to laparoscopic anterior resection with total mesorectal excision (L-AR/TME). Evidence-based medicine has established that L-AR/TME is a feasible surgical approach for managing rectal cancer. There were similar results in recent short-term therapeutic effects, local recurrence rate and postoperative survival rate between laparoscopic surgery (LS) and traditional open surgery for radical colon cancer<sup>[2]</sup>. Meanwhile, the mean estimated blood loss, discharge time after operation, and postoperative hospital stay were significantly reduced in the LS<sup>[3]</sup>. However, incision of the abdomen is still necessary in order to remove the specimens in LS, which could cause incision infection and increase the incidence of incisional hernia<sup>[4]</sup>.

Natural orifice specimen extraction (NOSE) may be an effective way to address the challenge. NOSE is feasible and safe technically for radical colorectal cancer surgery by traditional laparoscopic techniques, and for removal of the specimens through a natural orifice<sup>[5,6]</sup>.

Total laparoscopic hemicolectomy has been performed successfully by transvaginal NOSE<sup>[7]</sup>. However, due to its innate limitations, transvaginal NOSE is difficult for radical rectal cancer surgery, especially in low rectal cancer. Here, we introduce a technique used for the laparoscopic radical rectal surgery with TME, and the specimen was removed and anastomosis was accomplished through the anus.

## MATERIALS AND METHODS

This study was approved by the ethics committee of the hospital and written informed consent was obtained from the patients. Twenty-one patients with rectal adenocarcinoma underwent the procedure from March 2011 to February 2012 (Table 1). All patients with preoperative diagnosis of rectal cancer were confirmed by endoscopic colonoscopy, pathology, endosonography, and staged by specialized oncologists at our hospital, and preoperatively managed following the guidelines of the National Comprehensive Cancer Network (NCCN). All operations were performed by a single surgeon who was proficient in various laparoscopic colorectal procedures and laparotomy at our hospital.

These patients with tumor stage T4, tumor covering over half of the circumference of the rectum, metastasis in the liver or lungs on preoperative imaging assessment, or body mass index > 28 were excluded.

Three patients whose tumor-node-metastasis

**Table 1 Patient demographic data**

Patient demographic data	Value
Age (yr)	45.4 ± 3.6
Body mass index	23.1 ± 2.8
Sex (male/female ratio)	12/9
Mean wexner score	3.7 ± 1.6

Wexner Score was obtained in follow-up at 6 mo.

classification of T3 was confirmed by endosonography, magnetic resonance imaging (MRI) and computed tomography (CT) received three cycles of chemotherapy prior to surgery. Radiotherapy followed by resection was conducted based on the national guidelines. The feasibility of the surgery was reappraised at 2 wk after the treatment. All three patients had symptom relief, the tumor was reduced in size, and there were limited side effects of neoadjuvant chemotherapy.

The day before the operation, all patients underwent systemic bowel preparation, and used prophylactic antibiotics.

### Surgical procedure

**Laparoscopic phase.** The patient was positioned in a modified lithotomy position, and the abdomen was then insufflated with 10-12 mmHg CO<sub>2</sub>. Four ports were used in the following procedure. The first port was a 12-mm blunt-tip for the laparoscope, which was placed in the umbilicus using the minilaparotomy technique. The second to fourth ports were 10-mm operating ports in the right lower quadrant, and two 5-mm ports in the right middle abdomen and left lower quadrant, respectively.

Colon mobilization, lymph node dissection, and mesenteric excision were performed laparoscopically in the usual manner. First, the sacral promontory was separated by ultrasonic scalpel (Harmonic ACE; Ethicon Endo-Surgery, OH, United States) from the right side of the rectum. Second, before the tumor was mobilized, the inferior mesenteric artery was ligated at its point of pedicle from the aorta with a large or oversized Hem-o-lock. The I-III branches of artery and vein of sigmoid were cut off while the marginal artery of the proximal colon was preserved. Next, the inferior mesenteric vein was ligated at the corresponding height to the artery. We mobilized the splenic flexure in two patients because there were existing tensions in the anastomosis. Third, the posterior mesorectal fascia was identified and the dissection was extended to the level of the sacral promontory in the avascular plane. The rectum was fully dissociated to the levator ani muscle plane as far as possible along the Denonvillier's fascia. The fragment of the distal rectum that was located 2 cm above the tumor was clamped with a detachable clip.

We preserved the inferior hypogastric nerves as far as possible during the procedure.

**Perineal phase.** The anus was dilated gently until it



**Figure 1** Surgical procedure. A, B: Dilating the anus with a home-made dilator in which the bottom can be folded; C: Exteriorizing the specimen through the anus; D: Placing the anvil shaft into the stump of the proximal colon; E: Placing the anastomosis body into the anus; F: Completion of manual anastomosis procedure; G: Postoperative appearance of the anus; H: Appearance of abdominal wall 3 mo after surgery.

could accommodate four fingers. A home-made anus dilator and fine silk traction sutures were placed into the proximal lip of the exposed mucosal edge at a vertical orientation, in order to expand the anus and expose the rectum (Figure 1A and B). The level of intended transection had to maintain a margin of 2 cm distal from the tumor<sup>[8]</sup>. After irrigating the rectum with 1 L diluted povidone-iodine solution, we sutured two parallel circle-purse-strings in the distal rectal wall with 2-0 prolene lines through the dilated anus. The upper one maintained a minimal margin of 1-1.5 cm distal from the tumor, while the lower was located in the rectal mucosa at 1 cm above the dentate line. Between the two circle-purse-strings, full-thickness rectal circumferential dissection was extended by ultrasonic scalpel. At this point, the peritoneal cavity was extended circumferentially cephalad as far as possible, and then joined the perineal and laparoscopic

dissection planes.

The stump of the proximal rectosigmoid was exteriorized through the dilated anus and opened stump of the distal rectum (Figure 1C). After clamping with purse string forceps, the section of proximal colon, which had to be maintained at a minimal margin of 10 cm above the tumor, was transected under direct vision. After purse-string suturing, an anvil shaft was placed into the stump of the proximal colon, then it was pushed gently back into the peritoneal cavity (Figure 1D). The purse-string suture was tied to the anvil shaft before connecting it to the central shaft of the circular stapler (CDH 29; Ethicon Endo-Surgery, OH, United States). After tightening the lower circle-purse string, the anastomosis was placed into the anus (Figure 1E). Then the anastomat was fired to create an end-to-end coloanal anastomosis in the usual manner<sup>[9]</sup>. An air test was conducted through the anus.



The stitching was reinforced by bioabsorbable suture if necessary. A pelvic drain was inserted.

We performed the procedure successfully in 16 patients. Due to the low position of the stump of the distal rectum, we performed manual anastomosis and protective loop ileostomy in 5 patients (Figure 1F).

The negative margins were confirmed in all patients by intraoperative frozen biopsy. The mesorectal integrity<sup>[10]</sup> and circumferential situation<sup>[11]</sup> of the resected specimens were evaluated by a senior surgeon and qualified pathologist macroscopically and microscopically, in order to ensure that the tumor had been resected completely. The status of the mesorectal specimens was graded into three categories. We differentiated them as complete (intact mesorectum > 5 cm, while defect of mesentery < 5 mm); nearly complete (intact mesorectum > 5 cm, while defect of mesentery > 5 mm); and incomplete (incomplete mesorectum). We defined a positive margin if the circumferential margin from the tumor was < 2 mm under microscopy.

## RESULTS

We successfully performed the procedure in all 21 patients, and none of them was converted to laparotomy (Table 2). There were 16 and five patients in the low or ultralow anastomosis groups, respectively. According to macroscopic specimen assessment of TME, the status was complete for 18 patients, while nearly complete for three patients. In addition, the circumferential resection margin was negative in all patients (Table 3).

The mean maximum tumor diameter was  $4.6 \pm 1.7$  cm. According to the principle of rectal cancer surgery and no-touch isolation technique, we performed D2 lymph node dissection in 13 patients and D3 dissection in eight patients. The postoperative course was unremarkable in most patients, with prompt return of bowel activity and short postoperative stay, except for one patient who was complicated by anastomotic leakage (Table 4). Anastomotic leakage was confirmed by stools leaking from a drain. He was treated with nil by mouth, decompression of the rectum by transanal drainage, and antibiotic infusion until the leak healed spontaneously. He was discharged on the postoperative day 15.

According to the guidelines of NCCN, all patients with T3/T4 or postoperative node-positive tumors underwent postoperative chemotherapy for 6-9 cycles. The follow-up period ranged from 11 to 23 mo. Follow-up examinations were scheduled at 2 wk and 1, 2, 3, 6, 9 and 12 mo, and every 6 mo thereafter until 5 years. All patients underwent CT of the chest, abdomen, and pelvis every 6 mo and colonoscopy at 12 mo, but remained disease free. All five patients who had handsewn coloanal anastomosis with a diverting ileostomy had their ileostomies reversed at 3-6 mo after the operation, based on the diagnosis of free from tumor recurrence and anastomotic stenosis, which were confirmed by endoscopic colonoscopy, barium enema, MRI, and CT. Anal continence was measured with the validated Wexner fecal incontinence

**Table 2** Intraoperative information

Intraoperative information	Value
Mean operation time (min)	132 ± 85
Mean intraoperative blood loss (mL)	84 ± 15
Mean tumor diameter (cm)	4.6 ± 1.7
Distance of lesion from anal verge (cm)	8.3 ± 3.5
Protective ileostomy	5 (23.8)
Defecation time after operation (d)	2.5 ± 1.4

**Table 3** Patient pathological parameters

Items	Number of cases
Pathological diagnoses	
Well differentiated	10
Poorly differentiated	7
Myxoadenocarcinoma	4
Specimen macro-assessment of TME	21 (radical resection)
Circumferential resection margin	21 (Negative)
Postoperative pathology staging (TNM)	
T1-4N0M0	7
T1-2N1M0	5
T3-4N1M0	6
T3-4N2M0	3
Lymph nodes harvest (mean)	17.8 ± 4.6
Metastatic lymph nodes (mean)	3.4 ± 1.8

TME: Total mesorectal excision; TNM: Tumor node metastasis

scoring system (0 = perfect continence, 20 = complete incontinence). The mean Wexner score was 3.7 (range 0-5) at > 11 mo after the operation.

## DISCUSSION

In the past 10 years, L-AR has been performed at our hospital according to the principle of TME for patients with low rectal cancer. Traditional large abdominal incision has been replaced gradually by small abdominal incision. L-AR benefits patients not only in terms of cosmetics and postoperative rehabilitation, but also in reducing surgical interference, maintaining immune function and homeostasis, rapid recovery, and relieving psychological stress after surgery. However, L-AR is still considered imperfect due to the requirement for abdominal incision of 5-7 cm at minimum to remove the specimen completely. There are still some complications, such as abdominal incision infection, postoperative somatic pain, and incisional hernia<sup>[12]</sup>. According to bulk analysis of cases, wound infections occurred in 13.5% of patients after L-AR (2.7% trocar and 10.8% extraction site), and incisional hernias developed in 24.3%, and extraction sites accounted for 85.7% of all wound complications<sup>[13]</sup>.

In order to reduce the impact of L-AR incision and eliminate abdominal incision completely, natural orifice transluminal endoscopic surgery (NOTES) has increased in recent years, which can avoid incisional infection and hernia, and achieve better cosmetic results<sup>[14,15]</sup>.

Recently, transvaginal (posterior fornix incision) has been the main approach of NOTES in most colectomy



**Table 4 Postoperative complications *n* (%)**

Length of hospitalization (d)	6.1 ± 2.7
Postoperative complications	
UTI	2 (9.5)
Anastomotic leakage	1 (4.7)
Anastomotic bleeding	0
Incision infection	0
Intestinal obstruction	0
Impotence	1 (4.7)
Fecal incontinence	0
Anal stenosis	0
Total	4 (18.9)

The data of fecal incontinence, impotence, and anal stenosis was obtained at 1-year follow-up. UTI: Urinary tract infection.

procedures<sup>[16-18]</sup>. However, there are still some negative factors in low/ultralow rectal cancer which hinder the application of transvaginal approach NOTES. First, there are technical shortcomings, such as lack of experience and technical complexity, additional adjacent organ injury, extended operation time, and specialized equipment requirement, which account for the increased cost of the operation. Second, it is sometimes difficult to remove a larger tumor specimen through the posterior vaginal fornix incision. Third, there are many technical difficulties in achieving sphincter preservation for low/ultralow rectal cancer by the transvaginal approach. Finally, the transvaginal approach is obviously limited to female patients, which is a major hindrance for widespread use of the technique in clinical practice.

As a result, more surgeons have been trying to find new approaches for NOTES in low rectal cancer. With regard to the applicability of NOTES in colorectal surgery, the transanal access route of NOTES is intuitively the optimal one. First, rather than creating an opening through an otherwise healthy organ to perform the rectal anterior resection, enterotomy is carried out on the diseased organ itself. Second, the enterotomy is ultimately closed by incorporating it into a standard colorectal anastomosis, which is the requirement of surgery regardless of whether it is achieved *via* NOTES or standard surgery. Finally, transanal NOTES could have substantial benefits over standard transabdominal approaches<sup>[19]</sup>.

At present, transanal access NOTES in radical colorectal cancer surgery has been completed successfully in animal models, but few surgeons have put it into clinical practice due to potential technical difficulties, such as intra-abdominal intestinal fecal contamination, or increased possibility of infection through the colon lumen. All of these factors may affect the safety of the procedure. For example, clinical reports have confirmed that common complications included wound infection (56.7%), septicemia (31.7%), and enterocutaneous fistula (16.7%) in patients who sustained penetrating colon injuries<sup>[20]</sup>. However, with the improvement in anatomical techniques and equipment, transanal NOTES has been performed for resection of the rectum in pig models *in vivo* or fresh cadavers<sup>[21]</sup>, as well as laparoscopy-assisted

transanal NOTES for left-sided colorectal resection<sup>[22]</sup> and sigmoidectomy<sup>[23]</sup>. Unfortunately, these techniques require expensive equipment, which limits the clinical application of NOTES, especially in developing countries.

As a development of NOTES, transanal NOSE is an emerging technique that has been recently applied to the field of rectal excision. Darzi *et al*<sup>[24]</sup> have described a technique of total laparoscopic left-sided colonic resection and transanal specimen delivery. Franklin *et al*<sup>[25]</sup> have reported that laparoscopic colectomy in patients with stage III colorectal cancer is oncologically adequate. Fukunaga *et al*<sup>[26]</sup> have performed radical rectal cancer surgery with removal of the specimens through the anus, thus avoiding abdominal incision. Transanal specimen extraction can also resolve the problems found in obese patients with short or hypertrophic mesentery, or deep abdominal wall, which have been challenges for transabdominal specimen removal<sup>[27]</sup>. It has been confirmed that transanal NOSE is technically feasible. It may be a bridge between NOTES and the conventional laparoscopic approach for radical colorectal cancer surgery.

Our current experience showed that transanal NOSE, combined with TME and L-AR techniques for rectal cancer, could be adapted for radical tumor resection and minimally invasive surgery. Its technical feasibility and oncological principles have been demonstrated by many surgeons<sup>[28]</sup>. The rectal stump is a “necessary” trauma. We can accurately determine the distal cutting edge of the rectum through the full use the rectal stump. Combination of traditional laparoscopic techniques and removal of specimens through a natural orifice can minimize surgical injury<sup>[29]</sup>. Traditional laparoscopic surgical techniques provide a large operating space, mature technology and broad vision, which allows one to dissect accurately the mesorectal, pelvic visceral and parietal fascia. We can ensure that the inferior mesenteric artery is ligated at the root, in order to block the tumor blood supply and venous drainage, and minimize the chance of metastasis. Care is required to avoid any injury to the mesenteric arcades so as to guarantee an adequate blood supply to the descending colon. The operation was carried out following the “holy plane”, which is placed between the pelvic visceral fascia and rectal fascia propria, and then to the anterior Denonvillier’s fascia. The mesorectum should be completely mobilized while the pelvic autonomic nerve is preserved.

After the anus was fully dilated, we used a home-made anal dilator and fine silk traction sutures to evert the anus and expose the rectum, then placed a protective bag into the anus. In the premise of protecting blood supply of the residual colon, the pre-cut specimen was fully freed in the peritoneal cavity, then gently pulled out through the anus.

We paid attention to protecting the functions of anal sphincter while performing a standard radical resection of rectal cancer. Even if the specimen is a relatively large one, for example, the hypertrophic mesorectum, it can be removed smoothly from the fully dilated anus routinely

without tearing the rectum or damaging the anal sphincter. The anus and rectum can be returned to their normal diameter after the operation (Figure 1H).

When the stump of the proximal rectum was exteriorized through the dilated anus and opened stump of the distal rectum, we transected the proximal colorectum under direct vision<sup>[30]</sup>. After intracorporeal purse-string sutures with 2/0 prolene, we used an anastomat to create an end-to-end coloanal anastomosis in the usual manner. Although some studies have found that the J-pouch is superior to end-to-end reconstruction for low rectal cancer<sup>[9,31]</sup>, the latter resulted in acceptable anal function at 6 mo follow-up in our study, due to the careful protection of the anal sphincter, with no tension and a good blood supply in the anastomotic stoma.

In order to prevent peritoneal seeding and trocar-site metastasis, we implemented the general rules for laparoscopic surgery, such as the no-touch technique, appropriate resection margins, early bagging of the resected specimen, and wound protection into our laparoscopic colorectal procedures. Compared to the traditional laparoscopic techniques, our technique had good cosmetic results and reduced the chance of metastasis in the abdominal wall, without increasing complications<sup>[32]</sup>.

Laurent *et al.*<sup>[33]</sup> reported that the conversion rate of laparoscopic radical resection for low rectal cancer was 15.5%. The conversion rate was higher due to the difficulties experienced in fixing colorectal, separating in the pelvic, unexpected intraoperative bleeding, and failure of the closure device, or anastomosis. However, such difficulties did not jeopardize our treatment due to the elasticity and compliance of the tissue while we used mature laparoscopic techniques to remove the specimens through the anus. With full use of the natural orifice of the anus and rectum, total laparoscopic rectal resection is feasible and safe. Such a technique decreases the abdominal surgery complications, and maintains the operation time and the cost of surgery to those of standard L-AR. It also provides significant improvement of the traditional laparoscopic techniques.

However, the present surgical indications are limited to patients with early cancer. Mesorectal invasion and tumor diameter > 6 cm are not included here due to the lack of a large randomized controlled study for this procedure. The operation field is narrowed and the vision is not clear through the anal approach in some conditions, such as a narrow pelvis or large tumor. Although there are reports of microsurgical resection through the anus<sup>[34]</sup>, there is no specialized surgical instrument to complete the procedures for anus dilation, specimen removal, and distal suturing. There is urgency to develop better-adapted tools such as a modified flexible transanal endoscopic platform, longer and more flexible dissecting instruments, staplers and hemostatic devices to permit safe completion of these procedures without any transabdominal assistance. This technique requires further regulation and improvement.

In our limited experience, transanal specimen extrac-

tion in total laparoscopic low/ultralow anterior resection is feasible, safe, and oncologically sound for selected cases. The majority of patients have an acceptable functional outcome. Further studies with long-term outcomes are needed to explore the potential advantages of this technique.

## COMMENTS

### Background

The incidence of rectal cancer is higher in Asia compared with western countries. Technically, the resection of low rectal cancer may be one of the most difficult among all colorectal surgery procedures.

### Research frontiers

At present, traditional colorectal surgery has increasingly given way to laparoscopic anterior resection with total mesorectal excision (L-AR/TME). Evidence-based medicine has established that L-AR/TME is a feasible surgical approach for managing rectal cancer. There have been similar results recently for short-term therapeutic effects, local recurrence rate, and postoperative survival rate between laparoscopic surgery and traditional open surgery for radical colon cancer.

### Innovations and breakthroughs

This study showed that transanal specimen extraction by total laparoscopic low/ultralow anterior resection is feasible, safe, and oncologically sound for selected cases. The majority of patients had an acceptable functional outcome.

### Applications

There is an urgency to develop better adapted tools such as a modified flexible transanal endoscopic platform, longer and more flexible dissecting instruments, and staplers and hemostatic devices to permit safe completion of these procedures without any transabdominal assistance. This technique needs further standardization and improvement.

### Peer review

This study was interesting and highly innovative in terms of colorectal surgical technique, especially for a surgical rather than gastroenterological audience.

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## MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2

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### Abstract

**AIM:** To investigate the function of microRNA-143 (miR-143) in gastric cancer and explore the target genes of miR-143.

**METHODS:** A quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to evaluate miR-143 expression in gastric cancer cell lines. After transfecting gastric cancer cells with miR-143-5p and miR-143-3p precursors, Alamar blue and apoptosis assays were used to measure the respective proliferation and apoptosis rates. Cyclooxygenase-2 (COX-2) expression was determined by real-time RT-PCR and Western blot assays after miR-143 transfection. Reporter plasmids were constructed, and a luciferase reporter assay was used to identify the miR-143 binding site on COX-2.

**RESULTS:** Both miR-143-5p and miR-143-3p were sig-

nificantly downregulated in multiple gastric cancer cell lines. Forced miR-143-5p and miR-143-3p expression in gastric cancer cells produced a profound cytotoxic effect. MiR-145-5p transfection into gastric cancer cells resulted in a greater growth inhibitory effect ( $61.23\% \pm 3.16\%$  vs  $46.58\% \pm 4.28\%$ ,  $P < 0.05$  in the MKN-1 cell line) and a higher apoptosis rate ( $28.74\% \pm 1.93\%$  vs  $22.13\% \pm 3.31\%$ ,  $P < 0.05$  in the MKN-1 cell line) than miR-143-3p transfection. Further analysis indicated that COX-2 expression was potently suppressed by miR-143-5p but not by miR-143-3p. The activity of a luciferase reporter construct that contained the 3'-untranslated region (UTR) of COX-2 was downregulated by miR-143-5p ( $43.6\% \pm 4.86\%$ ,  $P < 0.01$ ) but not by miR-143-3p. A mutation in the miR-145-5p binding site completely ablated the regulatory effect on luciferase activity, which suggests that there is a direct miR-145-5p binding site in the 3'-UTR of COX-2.

**CONCLUSION:** Both miR-143-5p and miR-143-3p function as anti-oncomirs in gastric cancer. However, miR-143-5p alone directly targets COX-2, and it exhibits a stronger tumor suppressive effect than miR-143-3p.

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**Key words:** Gastric cancer; MicroRNA-143; Anti-oncomir; Cyclooxygenase-2; Apoptosis

**Core tip:** MicroRNA-143 (miR-143) has been reported to be a tumor suppressor. However, the functions of miR-143-5p and miR-143-3p have never been compared. In this study, we found that both miR-143-5p and miR-143-3p function as tumor suppressors in gastric cancer; however, miR-143-5p alone directly targets cyclooxygenase-2, and it exhibits a stronger tumor suppressive effect than miR-143-3p.

Wu XL, Cheng B, Li PY, Huang HJ, Zhao Q, Dan ZL, Tian DA,



Zhang P. MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2. *World J Gastroenterol* 2013; 19(43): 7758-7765 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7758.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7758>

## INTRODUCTION

MicroRNAs (miRNAs) are short (19-24 nt) non-coding RNAs that control target mRNA translation and stability by binding to regulatory sites that are mostly located in the 3'-untranslated region (UTR) of transcripts<sup>[1]</sup>. Numerous miRNAs have been shown to display tumor suppressor activity, while others reportedly act as oncogenes<sup>[2]</sup>. The expression levels of these RNAs are altered in many human tumors, resulting in distinct miRNA networks in various tumor types<sup>[3]</sup>. Some targets of these miRNAs have been identified, but many of the critical cancer proteins and pathways that they regulate remain unknown.

MiR-143 is considered a pivotal regulator of gene expression because it directly targets multiple mRNAs that code proteins involved in cell proliferation, differentiation, survival and apoptosis, including cyclooxygenase-2 (COX-2)<sup>[4]</sup>, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)<sup>[5,6]</sup>, B-cell lymphoma 2 (Bcl-2)<sup>[7,8]</sup>, plasminogen activator inhibitor-1 (PAI-1)<sup>[9]</sup>, myosin VI (MYO6)<sup>[10]</sup>, matrix metalloproteinase 13 (MMP-13)<sup>[11]</sup>, DNA (cytosine-5)-methyltransferase 3A (DNMT3A)<sup>[12]</sup> and E twenty-six-like transcription factor 1 (ELK1)<sup>[13]</sup>. The relevance of miR-143 as a putative cancer biomarker is increasing, because this miRNA is downregulated in various human tumors and can suppress tumor growth in cancers of the urogenital system<sup>[7,9,10]</sup>, digestive system<sup>[14-16]</sup>, respiratory system<sup>[17,18]</sup> and nervous system<sup>[19]</sup>, as well as some sarcomas<sup>[11,20]</sup> and B-cell malignancies<sup>[21]</sup>. MiR-143 expression has also been reported to be downregulated in human gastric cancer tissues and cell lines<sup>[22]</sup>. The expression of miR-143 is significantly decreased in stage IV gastric cancer, compared to stages I and II cancers<sup>[23]</sup>. However, the role of miR-143 in gastric cancer and the underlying mechanisms require further investigation.

Among the target genes regulated by miR-143, COX-2 is particularly important. COX, also known as prostaglandin (PG) H2 synthase, is the rate-limiting enzyme in the conversion of arachidonic acid into PGs. COX-2 expression in cells and animal models is associated with tumor cell growth and metastasis, enhanced cellular adhesion and apoptosis inhibition<sup>[24]</sup>. Pharmacologic inhibitors of COX-2 can decrease the growth of certain human tumors<sup>[25,26]</sup> and prevent tumorigenesis in animal models<sup>[27]</sup>. A pathological study showed increased COX-2 expression levels in gastric cancer<sup>[28,29]</sup>. Reduced COX-2 expression in gastric cancer cells led to markedly decreased proliferation and metastatic capability, demonstrating that COX-2 activity is necessary for gastric cancer cell proliferation and metastasis<sup>[30]</sup>. All of the above evidence indicates that COX-2 plays an important role

in gastric cancer. In this study, we investigated the roles of miR-143 and COX-2 in gastric cancer and found that both miR-143-5p and miR-143-3p function as tumor suppressors in gastric cancer; however, miR-143-5p alone directly targeted COX-2 and exhibited a stronger tumor suppressive effect than miR-143-3p.

## MATERIALS AND METHODS

### Cell culture

The human gastric cancer cell lines MKN-1, MKN-7, AGS, SGC-7901 and BGC-823 and the normal gastric epithelium cell line GES-1 were grown in RPMI 1640 medium supplemented with 10% FBS (Hyclone). The cell cultures were incubated in room air at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Reverse transcription and real-time polymerase chain reaction to quantify mature miR-143

Total RNA was extracted with TRIzol (Invitrogen). For mature miRNA expression analysis, cDNA was synthesized with the Taqman MiRNA Reverse Transcription kit (Applied Biosystems) and 100 ng of total RNA (100 ng/μL), along with 1 μL of miR-143-5p (Applied Biosystems) or miR-143-3p (Applied Biosystems) specific primers that were supplied with the miRNA Taqman MicroRNA Assay, according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) analyses were performed in triplicate on a 7900HT Real-Time PCR System (Applied Biosystems), and the data were normalized to RNU6B (Applied Biosystems) for each reaction. The thermal cycling profile used was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Quantification was performed according to the standard  $\Delta\Delta CT$  method.

### Transfection of the miR-143 precursor

Cells were seeded 24 h prior to transfection into 24-well or 6-well plates or 6 cm dishes. Hsa-miR-143-5p (Applied Biosystems), hsa-miR-143-3p (Applied Biosystems) or a miRNA mimic control (Applied Biosystems) was transfected with Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nmol/L. The sequences of the mature miR-143-5p and miR-143-3p used in this study were GGUGCAGUGCUGCAUCUCUGGU and UGAGAUAGAAGCACUGUAGCUC, respectively. The cells were harvested at 24 h (for RNA extraction), 48 h (for protein extraction) or 72 h (for apoptosis assays).

### Cell viability assays

An Alamar blue assay was used to measure cell proliferation. This assay is based on the quantitative metabolic conversion of blue, non-fluorescent resazurin to pink, fluorescent resorufin by living cells. After 72 h of incubation, an Alamar blue (Invitrogen) stock solution was aseptically added to the wells to equal to 10% of the total incubation volume. The resazurin reduction in the cultures was determined after a 2-6 h incubation with

Alamar blue by measuring the absorbances at 530-nm and 590-nm wavelengths on a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments).

### Apoptosis assay

Following maintenance in culture, the cells were harvested and stained with phycoerythrin-conjugated Annexin V according to the manufacturer's instructions (BD Biosciences). The cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences). The cells were considered viable if double negative, early apoptotic if positive for Annexin V alone and necrotic or late apoptotic if double positive.

### MiRNA target prediction

RNA22 (<http://cbcsrv.watson.ibm.com/rna22.html>) was used to identify miRNA-target sites in the 3'-UTR of COX-2 mRNA and the corresponding RNA/RNA complexes and folding energies.

### Western blot

Cells were lysed with Radio Immunoprecipitation Assay buffer (Sigma-Aldrich), and the total protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad). Proteins (40 µg) were separated by 10% SDS/PAGE and electrotransferred onto nitrocellulose membranes. The membranes were then incubated overnight with a COX-2 (Cell Signaling) or poly (ADP-ribose) polymerase (PARP) primary antibody (Cell Signaling) at 4 °C, and subsequently incubated with an HRP-conjugated anti-rabbit secondary antibody (Bio-Rad) for 1 h at room temperature. Protein bands were detected with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

### Reverse transcription and real-time PCR to quantify COX-2 mRNA

Total RNA was extracted with TRIzol (Invitrogen). DNase I (Amplification Grade, Invitrogen) and the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) (Invitrogen) were used for cDNA preparation. Primers and probes were ordered from IDT Inc. The following primers and probes were used: COX-2: Primer-F: 5'-CAAATCCTTGCTGTTCCACCCAT-3', Primer-R: 5'-GTGCACTGTGTTTGGAGTGGGTTT-3', Probe: 5'-AAGTGC GATTGTACCCGGACAGGATT-3', β-GUS: Primer-F: 5'-CTCATTTTGAATTTTGCCGATT-3', Primer-R: 5'-CCGAGTGAAGATCCCCCTTTT-TA-3', Probe: 5'-TGAACAGTCACCGACGAGAGT-GCTGG-3'. The reactions were incubated in a 96-well plate at 95 °C for 12 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 45 s. All reactions were performed in triplicate.

### Luciferase reporter assay

The human COX-2 3'-UTR was amplified and cloned into the XbaI site of the pGL3-control vector (Promega, United States), downstream of the luciferase gene, to

generate the plasmid pGL3-WT-COX2-3'-UTR. pGL3-MUT-5p-COX2-3'-UTR was generated from pGL3-WT-COX2-3'-UTR by deleting the "ACTGTAC" binding site for miR-143-5p. For the luciferase reporter assay, cells were cotransfected with the luciferase reporter vectors and miR-143-5p, miR-143-3p or a miRNA mimic control, using Lipofectamine 2000. A β-actin promoter Renilla luciferase reporter was used for normalization. After 48 h, luciferase activity was analyzed by the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's protocols.

### Statistical analysis

Experimental results were assessed for significance with one-tailed unpaired *t* tests. A *P* value less than 0.05 was considered significant.

## RESULTS

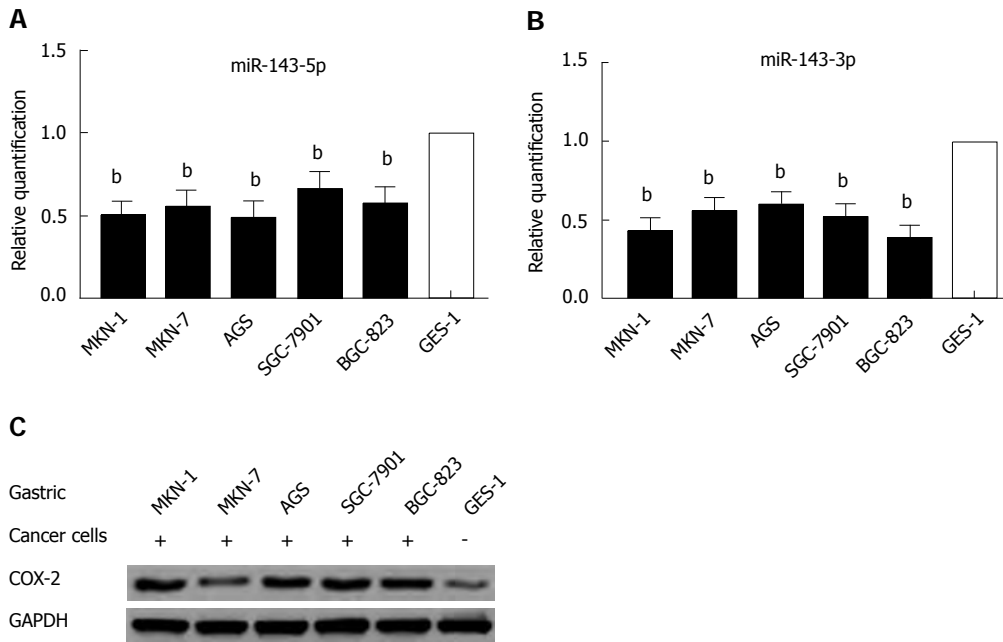
### MiR-143-5p and miR-143-3p expression is reduced in gastric cancer cells

Using real-time PCR to quantify mature miR-143-5p and miR-143-3p in five human gastric cancer cell lines and a normal gastric epithelium cell line, we found that both the miR-143-5p and miR-143-3p expression levels were markedly reduced in gastric cancer cells (*P* < 0.01; Figure 1A and B). Western blot analysis indicated that COX-2 protein expression was increased in the five human gastric cancer cell lines (Figure 1C); this expression was inversely correlated with the miR-143 levels.

### MiR-143 decreases viability and increases apoptosis in gastric cancer cells

The reduced miR-143 expression in gastric cancer suggests that this miRNA could have anti-proliferative effects. To test this hypothesis, we evaluated the effects of transient transfection with miR-143-5p and miR-143-3p in MKN-1 and BGC-823 gastric cancer cell lines. The Alamar Blue assay, a redox assay similar to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, showed significant decreases in gastric cancer cell viability following the transfection of either miR-143-5p or miR-143-3p. The decrease in viability after miR-143-5p transfection was greater than that after miR-143-3p transfection (*P* < 0.05; Figure 2A). Additionally, cell counts were significantly decreased after transfection with either miR-143-5p or miR-143-3p (*P* < 0.05; Figure 2B). Consistent with the results of the Alamar Blue assay, miR-143-5p showed a stronger inhibitory effect than miR-143-3p.

An apoptosis assay after transfection with miR-143-5p or miR-143-3p indicated a marked increase in cell apoptosis. Cells transfected with miR-143-5p had a significantly higher apoptosis rate than those transfected with miR-143-3p (*P* < 0.05; Figure 2C and D). Furthermore, both miR-143-5p and miR-143-3p transfection were accompanied by increased levels of cleaved PARP, a product of apoptosis (Figure 2E).



**Figure 1** MicroRNA-143 expression is downregulated in gastric cancer cell lines. **A:** Quantitative real-time polymerase chain reaction analysis was performed in five gastric cell lines and a normal gastric epithelium cell line (GES-1). Mature microRNA-143-5p (miR-143-5p) expression levels were significantly downregulated in gastric cancer cells, compared to normal gastric epithelium cells ( $^bP < 0.01$ ). The mean value from the GES-1 cell line was normalized to 1; **B:** Mature miR-143-3p expression levels were significantly downregulated in gastric cancer cells, compared to normal gastric epithelium cells ( $^bP < 0.01$ ); **C:** Western blot analysis showed that cyclooxygenase-2 protein expression in the five human gastric cancer cell lines inversely correlated with the miR-143 levels. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; COX-2: Cyclooxygenase-2.

### MiR-143 directly inhibits COX-2 expression via its 3'-UTR

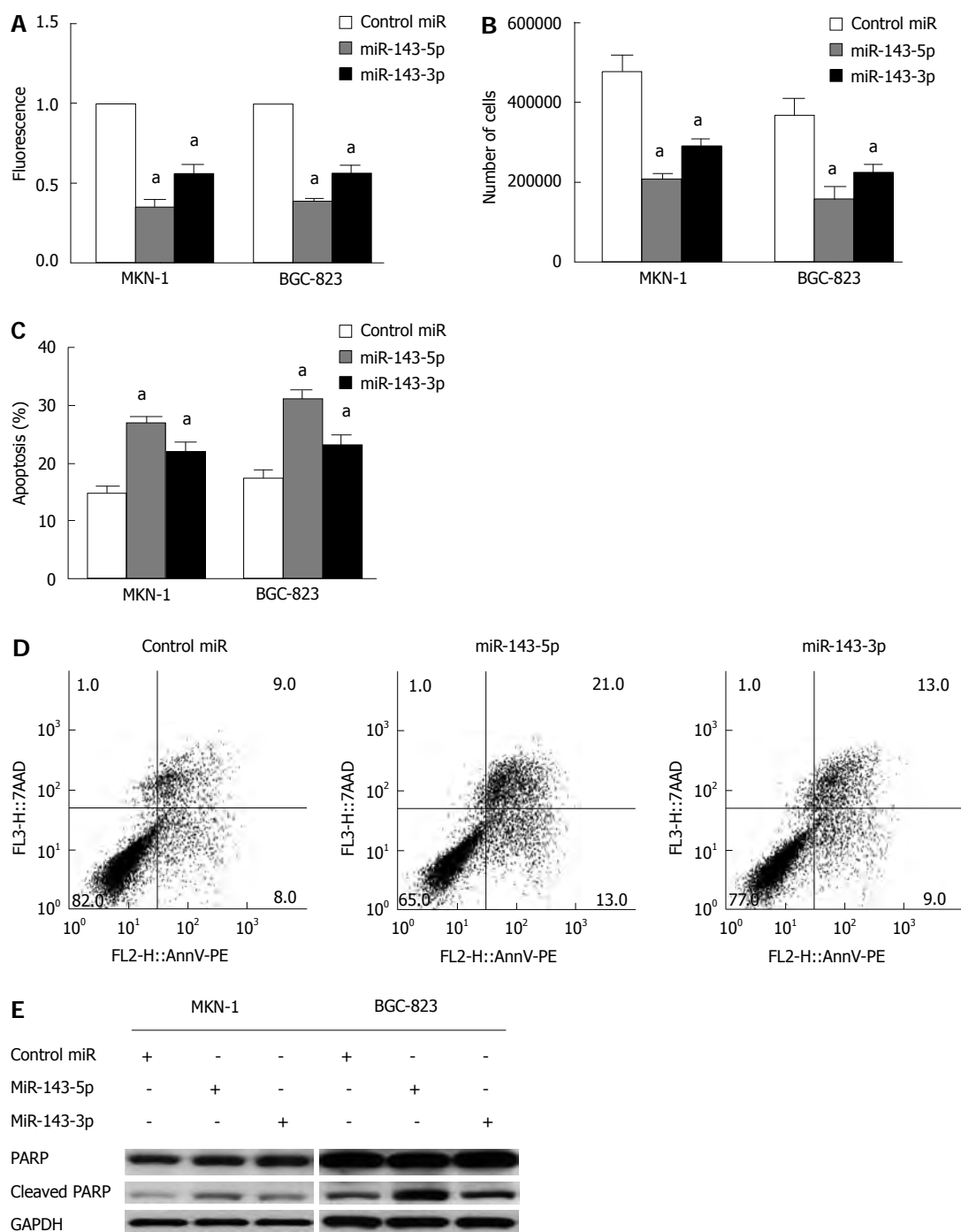
A bioinformatics analysis, conducted with RNA22, indicated that the 3'-UTR of the human COX-2 mRNA (NM\_000963) harbors a putative miR-143-5p binding site (nucleotides 3515-3536) (Figure 3A) but has no miR-143-3p binding site. To assess the inhibitory effects of miR-143 on COX-2, MKN-1 and BGC-823 cells were transfected with miR-143-5p, miR-143-3p or a control. Western blot analysis revealed a profound decrease in the COX-2 protein level after transfection with miR-143-5p but not miR-143-3p (Figure 3B). Consistent with transcriptional inhibition by miRNA, we noted decreased COX-2 mRNA levels after miR-143-5p transfection ( $P < 0.05$ ; Figure 3C).

To confirm the direct action of miR-143 on the COX-2 3'-UTR, transient transfection experiments were performed with the 3'-UTR of COX-2, containing mutated or non-mutated putative miR-143-5p matching sites, downstream of the luciferase open reading frame. Transfection with miR-143-5p inhibited the normalized activity of the COX-2 3'-UTR reporter by 57% ( $P < 0.01$ ), whereas miR-143-3p had no effect on this reporter activity (Figure 3D). In contrast, co-transfection of the mutant reporter plasmid with either miR-143-5p or miR-143-3p had no effect on luciferase activity in the transfected cells (Figure 3E). These results demonstrated that only miR-143-5p bound to the seed sequence present in the 3'-UTR of human COX-2 mRNA to inhibit COX-2 expression.

### DISCUSSION

In the present study, the expression levels of both miR-143-5p and miR-143-3p were found to be downregulated in gastric cancer. This finding is consistent with reports from other research groups<sup>[22,23]</sup>. In a recent report, the authors used real-time RT-PCR and chip assays to analyze 70 paired samples of gastric cancers and benign tissues<sup>[23]</sup>. The authors found that miR-143 was among the most strongly downregulated miRNAs in gastric cancers, compared to benign tissues. The miR-143 expression level was associated with gastric cancer progression and was more significantly reduced in stage IV cancers, compared with stage I and II cancers. Consistent with our observations, a study by Takagi *et al.*<sup>[22]</sup> also indicated that miR-143 was downregulated in gastric cancer cell lines and that transfection with miR-143-3p inhibited the gastric cancer cell viability by targeting ERK5 and AKT. However, the function of miR-143-5p has never been investigated in gastric cancer.

In the miRNA biogenesis pathway, long primary transcripts (pre-miRNAs) that have been transcribed from the genome are processed by the cellular RNase enzyme III Drosha into 60-110-nt structures called precursor miRNAs (pre-miRNA)<sup>[30]</sup>. Pre-miRNAs are cleaved by the RNase III enzyme Dicer-1 to produce short, imperfect, double-stranded miRNA duplexes that are subsequently unwound by helicases to create mature miRNAs. In some cases, two mature miRNAs can be excised from

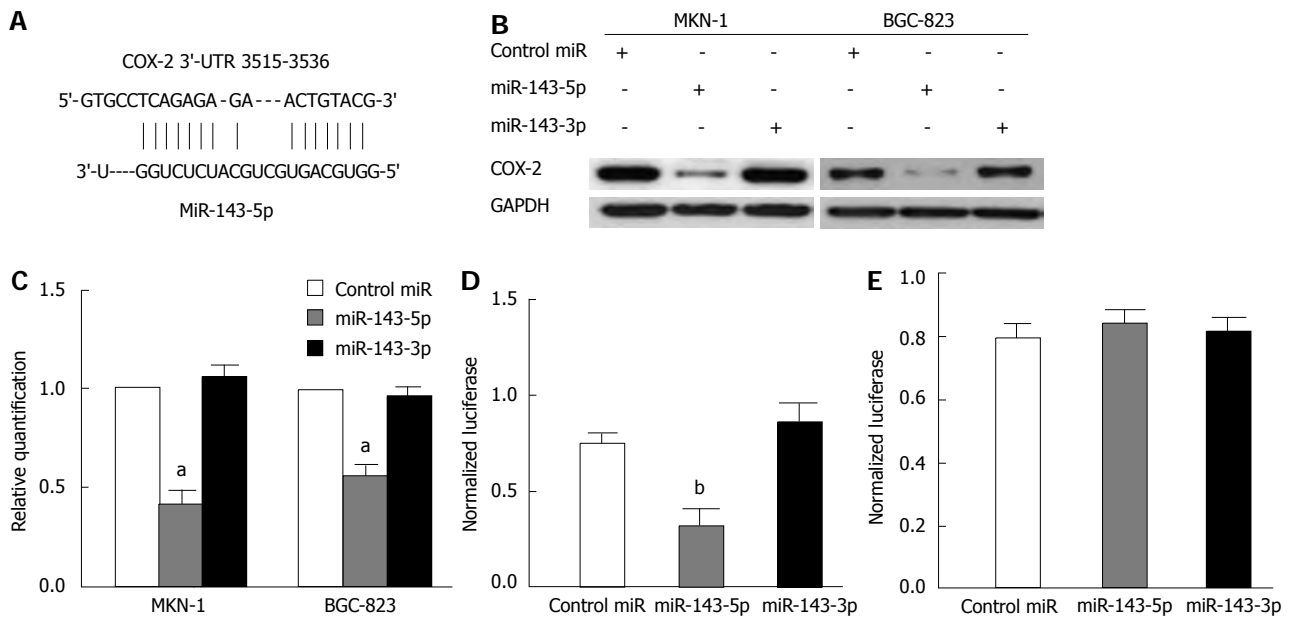


**Figure 2** Transfection with microRNA-143 inhibits gastric cancer cell viability and induces apoptosis. **A:** An Alamar Blue assay was performed 3 d after transfection with microRNA-143-5p (miR-143-5p) or miR-143-3p to measure the viability of MKN-1 and BGC-823 gastric cancer cells. The results showed significant decreases in cell viability following transfection with either miR-143-5p or miR-143-3p ( $P < 0.05$ ); this decrease was greater after miR-143-5p transfection than after miR-143-3p ( $P < 0.05$ ); **B:** Cell counts performed 3 d after transfection into MKN-1 and BGC-823 gastric cancer cells showed decreased cell numbers after transfection with either miR-143-5p or miR-143-3p ( $P < 0.05$ ). Consistent with the results of the Alamar Blue assay, the decrease in cell number after miR-143-5p transfection was greater than that after miR-143-3p transfection ( $P < 0.05$ ); **C and D:** An Annexin V/PE cell apoptosis assay revealed increased apoptosis in gastric cancer cells after transfection with either miR-143-5p or miR-143-3p ( $P < 0.05$ ). Cells transfected with miR-143-5p had a significantly higher apoptosis rate than those transfected with miR-143-3p ( $P < 0.05$ ); **E:** Western blots of PARP protein in MKN-1 and BGC-823 cell lines at 3 d after transfection with miR-143-5p or miR-143-3p. GAPDH was used as a control. The results showed increased expression of cleaved PARP after miR-143-5p and miR-143-3p transfection. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. PARP: Poly (ADP-ribose) polymerase.

the same stem-loop pre-miRNA<sup>[31]</sup>. These “5p” and “3p” miRNAs, although generated from a single primary transcript, have different sequences and therefore target different mRNAs. In humans, two different mature miRNA

sequences are excised from opposite arms of the stem-loop pre-miR-143 to generate two different miRNAs, hsa-miR-143-5p and has-miR-143-3p. Despite nearly a decade of studies on the roles of miRNA in cancers, the





**Figure 3** MicroRNA-143-5p directly inhibits cyclooxygenase-2 expression. **A:** Sites of miR-143-5p seed matches in the cyclooxygenase-2 (COX-2) 3'-untranslated region (3'-UTR) (nucleotides 3515-3536); **B:** Western blot of COX-2 protein in the MKN-1 and BGC-823 gastric cancer cell lines at 3 d after transfection with miR-143-5p, miR-143-3p or microRNA (miRNA) mimic control. GAPDH was used as a control. The results showed a profound decrease in COX-2 protein expression after transfection with miR-143-5p but not miR-143-3p; **C:** Real-time reverse transcription-polymerase chain reaction to determine COX-2 mRNA expression was performed 2 d after transfection with miR-143-5p, miR-143-3p or a control in MKN-1 and BGC-823. The mean expression in the control group was normalized to 1. Consistent with transcriptional inhibition by miRNA, the COX-2 mRNA level was reduced after miR-143-5p transfection ( $^*P < 0.05$ ); **D:** Normalized activity of the wild-type COX-2 3'-UTR luciferase reporter in BGC-823 cells, 2 d after transfection with miR-143-5p, miR-143-3p or a control. The luciferase activity was significantly decreased by miR-143-5p ( $^*P < 0.01$ ) but not miR-143-3p; **E:** Normalized activity of the mutant-type COX-2 3'-UTR luciferase reporter in BGC-823 cells, 2 d after transfection with miR-143-5p, miR-143-3p or a control. The results showed that cotransfection of the mutant reporter plasmid with miR-143-5p or miR-143-3p had no effect on luciferase activity in the transfected cells. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

comparative roles of strand-specific mature miRNAs that originate from the same stem-loop precursor (5p and 3p) have yet to be fully studied. Previously, a number of studies demonstrated that ectopic miR-143 expression inhibited cancer cells in various types of human tumors<sup>[4,6-8,16,20,22]</sup>. However, the role of miR-143 in gastric cancer has not been fully investigated, and differences between miR-143-5p and miR-143-3p have never been reported.

Our functional analysis of miR-143 expression in gastric cancer cell lines indicated tumor suppressor functions for both miR-143-5p and miR-143-3p. Our data revealed that the restoration of both miR-143-5p and miR-143-3p expression suppressed cell proliferation and promoted apoptosis in gastric cancer cells. The tumor-suppressive effect of miR-143-5p was stronger than that of miR-143-3p.

Further analysis demonstrated that only miR-143-5p directly binds to COX-2 mRNA. The luciferase reporter assay revealed that COX-2 contains a binding site for miR-143-5p but no binding site for miR-143-3p. A recent report also demonstrated different functions of miR-28-5p and miR-28-3p; specifically, miR-28-5p altered the expression of CCND1 and HOXB3, whereas miR-28-3p bound to NM23-H1 in colorectal cancer<sup>[32]</sup>. Our study provides further evidence that strand-specific "5p" and "3p" miRNAs could have different targets. This could explain why miR-143-5p is a stronger tumor suppressor in gastric cancer and suggests the existence of other

potential targets of miR-143-3p. For example, other studies have revealed multiple targets for miR-143, such as KRAS<sup>[5,6]</sup>, Bcl-2<sup>[7,8]</sup>, PAI-1<sup>[9]</sup>, MMP-13<sup>[11]</sup>, DNMT3A<sup>[12]</sup>, ELK1<sup>[13]</sup> and MYO6<sup>[10]</sup>. Further studies are needed to determine other targets of miR-143-5p and miR-143-3p in gastric cancer.

In conclusion, both miR-143-5p and miR-143-3p are downregulated in gastric cancer and function as anti-oncomirs. MiR-143-5p is more strongly tumor suppressive than miR-143-3p. Our data also indicate that COX-2 is a direct target of miR-143-5p but not of miR-143-3p. Further studies are needed to define the detailed mechanisms and identify more miR-143 targets.

## COMMENTS

### Background

MicroRNA-143 (miR-143) is considered a pivotal regulator of gene expression and directly targets multiple mRNAs that code for proteins involved in cell proliferation, differentiation, survival and apoptosis. It is downregulated in various human tumors and suppresses tumor growth in cancers of the urogenital system, digestive system, respiratory system and nervous system, as well as some sarcomas and B-cell malignancies. MiR-143 expression has also been reported to be downregulated in human gastric cancer tissues and cell lines, but the mechanism by which miR-143 regulates cancer cells is not fully clear.

### Research frontiers

MiR-143 has been reported to be a tumor suppressor. However, the role of miR-143 in gastric cancer has not been fully investigated. The functional differences between miR-143-5p and miR-143-3p with regard to cancer have never been reported. In this study, the authors compared the tumor suppressive func-

tions of miR-143-5p and miR-143-3p and explored the associated underlying mechanism.

### Innovations and breakthroughs

This functional analysis of miR-143 expression in gastric cancer cell lines indicated that both miR-143-5p and miR-143-3p act as tumor suppressors. The restoration of either miR-143-5p or miR-143-3p suppressed cell proliferation and promoted apoptosis in gastric cancer cells. The tumor-suppressive effect of miR-143-5p was stronger than that of miR-143-3p. Further analysis demonstrated that only miR-143-5p directly bound to the cyclooxygenase-2 (COX-2) mRNA. The luciferase reporter assay revealed that COX-2 contained a binding site for miR-143-5p but not for miR-143-3p. Western blot showed a profound decrease in COX-2 protein expression after transfection with miR-143-5p but not with miR-143-3p. The data in this article indicate that COX-2 is a direct target of miR-143-5p but not of miR-143-3p.

### Applications

Both miR-143-5p and miR-143-3p function as anti-oncomirs and are downregulated in gastric cancers. MiR-143-5p is more strongly tumor suppressive than miR-143-3p and could be a potential gastric cancer therapeutic target.

### Terminology

MiR-143 is considered a pivotal regulator of gene expression because it directly targets multiple mRNAs that code for proteins involved in cell proliferation, differentiation, survival and apoptosis. It is downregulated in various human tumors and suppresses tumor growth in cancers from the urogenital, digestive, respiratory and nervous system, as well as B-cell malignancies.

### Peer review

The authors examined the effects and mechanisms of miR-143 subtypes on gastric cancer cell lines. They examined the effects of these oncomirs on growth, apoptosis and COX-2 activity. For the most part, the paper is straightforward and well written. The experiments are well described and the results are clearly presented.

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## PU.1-silenced dendritic cells prolong allograft survival in rats receiving intestinal transplantation

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### Abstract

**AIM:** To investigate the function of PU.1-silenced semi-mature dendritic cells (DCs) and the possibility of utilizing cell immunity in rat intestinal transplantation.

**METHODS:** DCs were isolated from the bone marrow of F344 rats and cultured using the adherent method. The *PU.1* gene was knocked down in DCs using small interfering RNAs (siRNAs) for 24 h, and the cells were then incubated with lipopolysaccharide for 48 h. The PU.1 siRNA that had the highest silencing efficiency was screened using reverse transcription-polymerase chain reaction and Western blot for further study. The tolerance capacity was analyzed and compared between PU.1-silenced DCs (siRNA PU.1 group), negative control-silenced DCs (siRNA NC group) and immature DCs (control group) both *in vitro* and *in vivo*.

**RESULTS:** Blocking expression of the *PU.1* gene *in vitro* led to a reduction in DC maturation and an increased tolerance capability. PU.1-silenced DCs expressed moderate levels of major histocompatibility complex (MHC)-II and low levels of co-stimulatory molecules, and produced more interleukin (IL)-10, but less IL-12. Compared with the negative control group, surface molecules cluster of differentiation 80 (CD80), CD86 and MHC-II in the siRNA PU.1 group were  $27.0\% \pm 5.6\%$ ,  $23.6\% \pm 4.8\%$  and  $36.8\% \pm 6.8\%$ , respectively, and showed a significantly lower trend ( $P < 0.05$ ). *In vivo* treatment of recipients with PU.1-silenced DCs injected before intestinal transplantation (siRNA PU.1 group), significantly prolonged allograft survival and resulted in better tissue histopathology compared with the siRNA NC group and control group. Mean survival time after transplantation was  $14.3 \pm 3.3$  d in the siRNA PU.1 group ( $P < 0.05$ ).

**CONCLUSION:** PU.1-silenced semi-mature DCs induced partial immune tolerance both *in vitro* and *in vivo*, which could be used as a new strategy to promote transplantation tolerance.

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**Key words:** Dendritic cell; PU.1; Tolerance; Intestinal transplantation; Immune tolerance

**Core tip:** The inhibition of dendritic cells (DCs) maturation can promote their tolerogenicity in transplantation. PU.1 is a newly discovered transcription factor which is required for the regulation of dendritic cell maturation in all DCs subsets. We silenced the *PU.1* gene using siRNA and showed, for the first time, that PU.1-silenced DCs had immune tolerance. This may be a new strategy to prevent graft rejection following intestinal transplantation.

Xu XW, Ding BW, Zhu CR, Ji W, Li JS. PU.1-silenced den-



dratic cells prolong allograft survival in rats receiving intestinal transplantation. *World J Gastroenterol* 2013; 19(43): 7766-7771 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7766.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7766>

## INTRODUCTION

Dendritic cells (DCs) are key antigen-presenting cells, which play an important role in regulating adaptive immune responses. Studies have shown that whether immune responses are induced or suppressed greatly depends on the degree of DC maturation and specific subsets<sup>[1,2]</sup>. Immature DCs, which express low levels of major histocompatibility complex (MHC-II) and co-stimulatory molecules, such as cluster of differentiation 80 (CD80), CD86 and CD40, have a lower ability to capture antigens for presentation to specific T cells<sup>[3,4]</sup>. Therefore, various approaches have been explored to inhibit the maturation of DCs and to promote their tolerogenicity<sup>[5]</sup>.

MicroRNA-155 has emerged as an important regulator in the immune system<sup>[6,7]</sup>. MicroRNA (mRNA)-155 knockout mice showed aberrant immune functions, such as defective B and T cell immunity, abnormal function of antigen-presenting cells, and a failure in the production of high-affinity Immunoglobulin G (IgG)<sub>1</sub> antibodies<sup>[8,9]</sup>. These phenotypes are related to the impaired ability of mRNA-155 to target the E-twenty six transcription factor PU.1, which was first discovered to have multiple roles in hematopoiesis. PU.1 is an essential regulator of both cDC and pDC lineages<sup>[10,11]</sup>, and can regulate numerous genes within the myeloid and lymphoid lineages<sup>[12]</sup>. Recent studies have shown that PU.1 can partially direct the important cytokine receptor Flt3 and play a critical role in DC development and function. Therefore, PU.1 is a major and critical regulator of DC maturation.

In this study, we silenced PU.1 expression in rat bone marrow DCs (BM cells) using small interference RNA (siRNA) molecules and stimulated the cells with lipopolysaccharide (LPS) to obtain semi-mature DCs. These semi-mature DCs were then used to determine whether they could induce tolerance and have an effect on intestinal transplantation in rats.

## MATERIALS AND METHODS

### Animals

F344 and Wistar rats (weighing 180-220 g) were purchased from the Vital River Corporation (Beijing, China) and kept under specific-pathogen-free conditions. Animal experiments and maintenance were approved and regulated by the Ethics Committee of Jinling Hospital (Nanjing, China).

### *In vitro* generation of bone marrow-derived immature DCs

BM cells of F344 rats were used for DC generation fol-

lowing the method described by Lutz *et al.*<sup>[13]</sup> and Yang *et al.*<sup>[14]</sup>. Briefly, the femur and tibia were mechanically obtained, and the marrow cells were flushed out using phosphate-buffered saline (PBS). The obtained single cell suspensions were centrifuged, treated with 0.15 mol/L NH<sub>4</sub>Cl for 5 min and washed twice. The harvested BM cells were cultured in six-well plates (density,  $4 \times 10^6$ /mL) in RPMI1640 with 5 ng/mL recombinant rat granulocyte-macrophage colony-stimulating factor and 5 ng/mL interleukin (IL)-4 (Peprotech, NJ, United States). Non-adherent granulocytes were removed after 48 h of culture. From day 3, half of the medium was replaced with fresh medium every other day. On day 7, non-adherent and loosely adherent cells were harvested and identified as immature DCs, which were ready for transfection, and the supernatants were used for cytokine detection.

### Treatment of DCs

For *in vitro* studies, siRNAs targeting the *PU.1* gene were synthesized by Jima Corporation (Shanghai, China)<sup>[10,15]</sup>. The siRNAs were transiently transfected into the cells using Lipofectamine 2000 (Invitrogen, United States) for 24 h according to the manufacturer's instructions. The sequences of a PU.1-specific siRNA were: sense, 5'-AGCGAUCACUAUUGGGAUUTT-3'; and antisense, 5'-AAUCCCAAUAGUGAUCGCUTT-3'. The sequences of a negative control siRNA were: sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Transfected DCs were cultured in the presence of 10 µg/mL LPS (Sigma-Aldrich, United States) for a further 48 h. Cells and supernatants were harvested for later use, and the cells were designated as PU.1-silenced-LPS DCs (siRNA PU.1 group), negative control-silenced-LPS DCs (siRNA NC group) or immature DCs (control group).

### Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen, United States). RNA (1 pg) was reverse transcribed using an oligo-(dT) primer and reverse transcriptase (Invitrogen). All the measurements were performed in triplicate for each sample and normalized to the β-actin gene. The primer sequences for PU.1 were: forward, 5'-GAGTTTGAGAACTTCCCTGAG-3'; and reverse, 5'-TGGTAGGTCATCTTCTTGCGG-3'. Primer sequences for β-actin were: forward, 5'-ATGGATGACGATATCGCT-3'; and reverse, 5'-ATGAGGTAGTCTGTCAGGT-3'<sup>[15]</sup>.

### Western blot

Cells were homogenized in RIPA lysis buffer and used for Western blot assays. Briefly, equal amounts of protein extracts were boiled in sodium dodecyl sulfate (SDS)-sample buffer for 5 min before being electrophoretically resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% fat-free dried milk and bovine serum albumin dissolved in Tris Buffered Saline with

Tween-20 for 1 h at room temperature and incubated overnight at 4 °C with antibodies raised against PU.1 (Santa Cruz, United States) according to the manufacturer's instructions. Binding of these primary antibodies was visualized with goat anti-rabbit secondary antibodies (1:2000 dilution; Santa Cruz, Texas, United States). Finally, the membranes were washed and an emitter-coupled logic signal detection kit was used (Amersham, IL, United States) for signal detection.

### Flow cytometric analysis

The following antibodies were purchased from eBioscience Corporation (CA, United States): phycoerythrin (PE)-coupled anti-CD86, PE-coupled anti-CD80 and fluorescein isothiocyanate-coupled anti-MHC-II. OX62-Alexa Fluor was obtained from BioLegend (CA, United States). After 7 d of cultivation, the prepared cells mentioned above were stained using the above antibodies at 4 °C for 30 min in PBS containing 0.1% sodium azide. Phenotypic analysis of DCs was performed on a fluorescence activated cell sorter Calibur flow cytometer equipped with Cell Quest (Becton Dickinson, New Jersey, United States).

### Purification of T cells and mixed lymphocyte reaction

T cells ( $2 \times 10^5$ ) purified from rat splenocytes (responder cells) were plated with immature DCs, PU.1-silenced DCs or negative control-silenced DCs (stimulator cells) at varying ratios. Cells were cultured for 3 d and pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine (PerkinElmer, Woodbridge, United States) for the final 18 h. The cells were subsequently harvested onto glass fiber filters, and incorporated radioactivity was quantified using a liquid scintillation counter.

### Detection of IL-12p70 and IL-10

The supernatants from each group as described above were collected and the cytokines IL-12p70 and IL-10 were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R and D Systems, Minneapolis, United States).

### Intestinal transplantation and treatment

Recipient Wistar rats, six in each group, were treated with PU.1-silenced DCs, negative control-silenced-LPS DCs or immature DCs from donor F344 rats ( $1 \times 10^6$  cells), seven days prior to intestinal transplantation *via* tail vein injection. Heterotopic intestinal transplantation was performed using the technique described by Zhang *et al.*<sup>[16]</sup>. The state of intestinal health/rejection was monitored and evaluated daily by examining the color of the graft and secretions from the stoma. Recipient rats that died within three days were regarded as technical failures and excluded from further analysis. The allografts were collected from a location 5 cm from the origin of the jejunum on day 5 after transplantation. Tissues from the three groups were sectioned and subjected to HE staining to evaluate morphologic changes.

### Statistical analysis

Data were reported as mean  $\pm$  SD. One-way analysis of variance was used for data analysis within groups. *P* values less than 0.05 were considered significant.

## RESULTS

### *In vitro* silencing of PU.1 with siRNAs

In order to silence the *PU.1* gene in DCs, we constructed three pre-siRNA vectors targeting PU.1 and one vector carrying a negative control siRNA. We incubated synthetic siRNAs with DCs which were induced with GM-CSF and IL-4 for 7 d to validate the efficiency of gene silencing. The most efficient plasmid to silence PU.1 was selected by assaying the PU.1 mRNA and protein expression. Forty-eight hours after transfection, PU.1 expression in the siRNA PU.1 group was reduced by approximately 85% at the protein level compared with the siRNA NC group (Figure 1A).

### Characteristics of semi-mature DCs and expression of cytokines

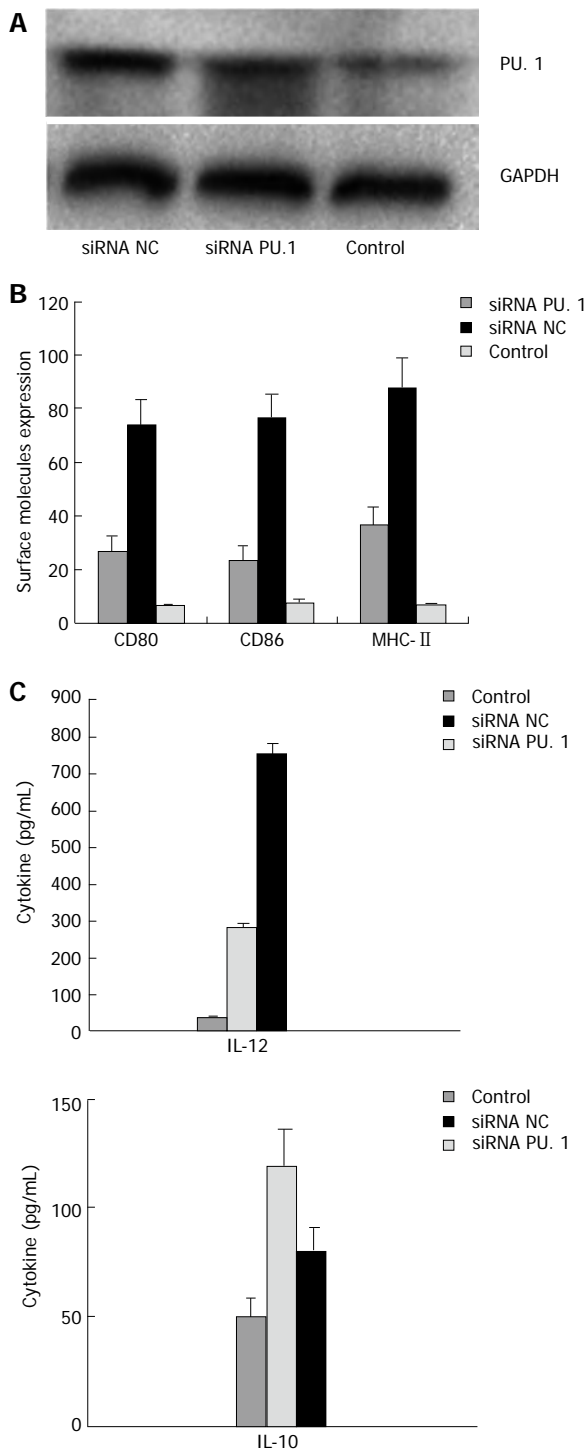
Similar to the characteristics of mature DCs, the DCs in the siRNA NC group also expressed high levels of MHC class II and co-stimulatory molecules. However, in the siRNA PU.1 group, the DCs were semi-mature, with the expression of CD80, CD86 and MHC-II ( $27.0\% \pm 5.6\%$ ,  $23.6\% \pm 4.8\%$  and  $36.8\% \pm 6.8\%$ , respectively) significantly lower than that in the siRNA NC group ( $74.0\% \pm 9.4\%$ ,  $76.5\% \pm 8.7\%$  and  $87.8\% \pm 11.3\%$ , respectively) (Figure 1B,  $P < 0.05$ ). The ability of DCs in the three groups to produce cytokines in cell culture supernatants was also determined, and an opposite trend was noted between IL-12p70 and IL-10 production ( $P < 0.05$ ) (Figure 1C). These data indicate that the PU.1 silencing partially inhibits DC maturation.

### Impaired ability of semi-mature DCs to stimulate T cell proliferation

Purification of T cells and MLR analysis were performed to observe the *in vitro* activity of DCs. The proliferation of Wistar rat splenic T cells in a primary mixed lymphocyte reaction (MLR) in response to stimulation with DCs in the siRNA PU.1 group was significantly reduced compared with that in the siRNA NC group ( $P < 0.05$ , Figure 2), suggesting that PU.1-silenced DCs have an impaired capacity to stimulate T cell proliferation.

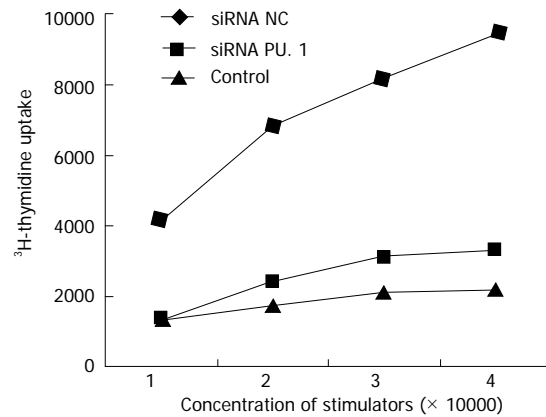
### Treatment with PU.1-silenced DCs prolongs allograft survival

Since the results of the *in vitro* study showed that silencing of PU.1 reduced DC maturation and inhibited allogeneic T cell proliferation, we postulated that knockdown of this key transcription factor might prevent graft rejection. To determine this, we treated Wistar recipients with different groups of DCs 7 d before performing intestinal transplantation. While recipient survival was short in the



**Figure 1 Identification of PU.1-silenced dendritic cells *in vitro*.** A: Analyses of PU.1 gene expression by Western blot; B: Flow cytometry analysis of CD80, CD86 and major histocompatibility complex (MHC)-II in dendritic cells; C: Analyses of cell culture supernatants by enzyme-linked immunosorbent assay. CD: Cluster of differentiation; GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase.

siRNA NC group ( $7.8 \pm 1.5$  d,  $n = 6$ ,  $P < 0.05$ ) and the control group ( $8.0 \pm 2.5$  d,  $n = 6$ ,  $P < 0.05$ ), the infusion of siRNA PU.1 DCs significantly prolonged survival ( $14.3 \pm 3.3$  d). Consistent with our surmise, morphological features of acute rejection were prominent in the siRNA NC group and in the control group. Histological examination



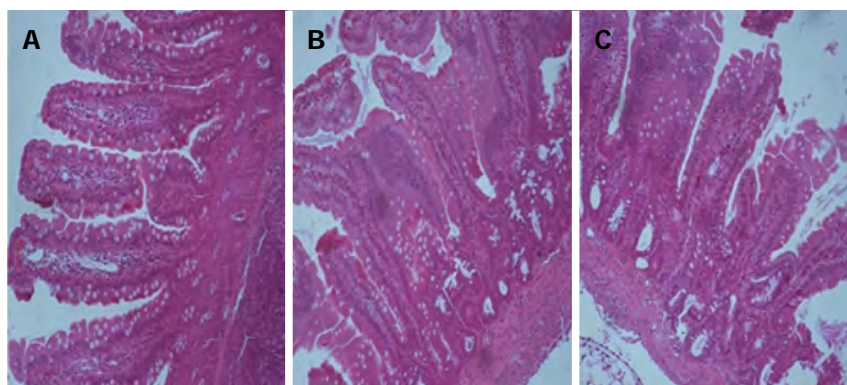
**Figure 2 PU.1-silenced dendritic cells suppress allogeneic T cell proliferation.** Bone marrow dendritic cells (DCs) were cultured and transfected with PU.1 siRNA as described. After that, three groups of DCs were collected and co-cultured with allogeneic T cells in a 96-well plate at various ratios as indicated. [ $^3$ H] was added 48 h after coculture, and its incorporation was measured as an indicator of T cell proliferation ( $P < 0.05$  siRNA PU.1 vs siRNA NC group).

showed different degrees of lymphocyte infiltration and villous edema. In contrast, PU.1-silenced DCs delayed and reduced the immune response and injury, with mild lymphocyte infiltration and reduced inflammation observed in the allograft intestine (Figure 3).

## DISCUSSION

Recently, the role of innate immunity in shaping the adaptive response has been focused in transplantation research, and studies have shown that the infusion of donor immature DCs can prolong graft survival after organ transplantation<sup>[17,18]</sup>, mainly because they are capable of inducing tolerance by inducing T cell anergy or apoptosis<sup>[19,20]</sup>. Immature DCs express low levels of MHC II and co-stimulatory molecules and fail to elicit naïve T cells to modulate the adaptive immune response. However, they are not stable *in vivo* and can easily be stimulated to transform into mature DCs through several signaling pathways<sup>[21]</sup>, which limits their preservation and utilization. Recent studies show that by controlling ambient conditions *in vitro*, semi-mature DCs are obtained from immature DCs following LPS stimulation. These cells are phenotypically stable and hard to differentiate or mature. Yang *et al.*<sup>[14]</sup> found that silencing of MyD88, a proximal component of nuclear factor-kappaB (NF- $\kappa$ B) signaling, affected the maturation of immature DCs by increasing the secretion of IL-10 and decreasing the secretion of IL-12p70. The NF- $\kappa$ B signaling pathway plays a critical role in DC maturation, and IL-10 is regarded as an immunosuppressive cytokine which can downregulate the synthesis of a broad range of inflammatory cytokines and inhibit allogeneic T cell proliferation<sup>[22]</sup>. Therefore, the silencing of key factors involved in DC maturation may lead to a stunted capacity to prime the immune response and better stability<sup>[23,24]</sup>. As PU.1 is highly expressed and plays a critical role in DC maturation, suppression of this gene may result in interruption of DC maturation.





**Figure 3** Histopathology of intestinal allograft from recipient rats. Treatment with PU.1 small interference RNA (siRNA) prevents allograft rejection and is associated with mild lymphocyte infiltration and villous damage. Samples from the three groups were compared (magnification  $\times 40$ , scale bar 20  $\mu\text{m}$ ). A: The siRNA PU.1 group; B: The siRNA NC group; C: The control group.

Unsurprisingly, we found that PU.1-silenced semi-mature DCs<sup>[25]</sup> had a better effect in reducing the inflammatory response than immature DCs in an intestinal allograft model.

It is difficult to perform rat intestinal transplantation due to complex microvascular techniques and high mortality. Many animals died of immune rejection within several days. Although immature DCs express low levels of MHC II and determine tolerogenicity, current evidence for the application of immature DCs in rodent transplantation models is equivocal. In our experiment, rat survival, cytokine production and intestinal histological changes were evaluated to test the immunosuppressive function of semi-mature DCs. We found that acute rejection was significantly alleviated on day 5 compared to the controls, along with prolonged survival time and better condition in these rats. Rats in the siRNA PU.1 group showed slowed neointima formation and reduced inflammation and fibrosis in the allograft intestine. These results can be explained by increased secretion of IL-10 and decreased IL-12p70. The increase in IL-10 may play a crucial role in mediating the functions of semi-mature DCs. The surface expression of co-stimulatory molecules, such as CD86, CD80 and CD40, also showed a reduced trend, which is consistent with the results of T-cell proliferation. Thus, our experiments demonstrated that *PU.1* gene silencing induced partial tolerance in this animal transplantation model.

However, we do not know whether the number of semi-mature DCs fluctuated or changed in recipient rats, and whether these DCs reduced the number and maturation of native DCs at the time of small bowel transplantation. More *in vivo* studies in both donors and recipients are required to identify the mechanism related to better graft survival.

In conclusion, we have provided evidence that silencing of the *PU.1* gene can impair DC maturation, inhibit allogeneic T cell proliferation, and induce immunosuppressive activity. Since PU.1 silencing can prolong intestinal transplant survival in rats, it may be used as a new strategy and viable therapeutic option to prevent graft rejection following intestinal transplantation.

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## COMMENTS

### Background

As key antigen-presenting cells, whether dendritic cells (DCs) induce or suppress immune responses greatly depends on the degree of DC maturation and specific subsets. MicroRNA-155 has emerged as an important regulator in the immune system, and the transcription factor PU.1 is a direct target of miR-155, which has recently been found to play multiple critical roles in DC maturation and function.

### Research frontiers

PU.1 is a major and critical regulator of DC maturation. However, the mechanism of action of PU.1 in DC maturation and tolerogenicity has not been unequivocally addressed. In this study, the authors silenced the *PU.1* gene using siRNA and demonstrated that PU.1 silencing impaired DC maturation and inhibited allogeneic T cell proliferation. PU.1-silenced DCs also prolonged intestinal transplant survival and improved the general state of the graft.

### Innovations and breakthroughs

Recent reports have highlighted the important role of PU.1 in DC maturation and function. This is the first study to report that PU.1-silenced DCs can induce tolerogenicity, which can be applied in rat intestinal transplantation. Furthermore, the authors' *in vitro* studies suggest that inhibiting a key factor in a given signaling pathway is an effective way of inducing DC tolerogenicity.

### Applications

The authors' finding that PU.1-silenced dendritic cells can prolong intestinal transplant survival in rats suggest that PU.1 silencing may be used as a new strategy and viable therapeutic option to prevent graft rejection following intestinal transplantation.

### Terminology

PU.1 is an EPS transcription factor which was first discovered to play multiple roles in hematopoiesis. Recent studies have shown that PU.1 is an essential regulator of both cDC and pDC lineages and can regulate numerous genes within the myeloid and lymphoid lineages. Such a mechanism is thought to be crucial in inducing stable immature DCs. Not surprisingly, the DCs the authors cultivated in the study showed tolerogenicity both *in vivo* and *in vitro*.

### Peer review

The authors examined the surface molecule expression of PU.1-silenced DCs, T cell proliferation and cytokines of cell culture supernatants. The cells were injected to recipient rats to test the immunogenicity *in vivo*. It revealed that they induced immunosuppressive activity; and the increased interleukin-10 expression may play a crucial role in the semi-mature DC functions and induce immunogenicity. The results are interesting and may supplement molecular mechanism of PU.1.

## ACKNOWLEDGMENTS

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## Biliary casts after liver transplantation: Morphology and biochemical analysis

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Author contributions: Yang YL conceived and designed the study; Yang YL, Zhang C, Lin MJ, Shi LJ, Zhang HW, Li JY and Yu Q performed the examinations; Yang YL, Lin MJ, Shi LJ, Zhang HW and Li JY performed the surgery; and Zhang C wrote the manuscript.

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### Abstract

**AIM:** To investigate the pathogenesis of biliary casts after liver transplantation relative to their morphology and biochemical markers.

**METHODS:** The microstructure of biliary casts was assessed using scanning electron microscopy and Hematoxylin and eosin staining assessed their histology. The expression levels of CD3, CD5, CD34, CD68 and CD79a in these biliary casts were evaluated immunohistochemically.

**RESULTS:** Biliary casts differed widely in their microstructure, with some containing blood vessels positive for CD34 and collagen fibers with positive Masson staining. Large numbers of neutrophils and other inflammatory cells were present, but only on the edge of the biliary casts; although the boundaries were clear without crossover. None of the biliary casts contained T-lymphocytes, B-lymphocytes, macrophages and other inflammatory cells.

**CONCLUSION:** The microcostructure of biliary casts

differed. Bacteria and acute rejection are not clearly related to their formation.

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**Key words:** Biliary cast; Biliary cast syndrome; Liver transplantation; Blood vessels; Acute rejection

**Core tip:** This experimental study employed scanning electron microscopy, Hematoxylin and eosin staining and immunohistochemistry to investigate biliary casts following liver transplantation. The results indicated that blood vessels and collagen fibers are present in biliary casts; however, bacteria and acute rejection are not clearly related to their formation, as evidenced by blood vessels positive for CD34 and collagen fibers with positive Masson staining, and no T-lymphocytes, B-lymphocytes, macrophages and other inflammatory cells. Thus, although bile duct injury after liver transplantation is significantly associated with biliary cast formation, their role in acute rejection is unclear.

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Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7772.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7772>

### INTRODUCTION

Despite advances in the management of patients who have undergone cadaveric liver transplantation, 6%-34% patients experience biliary complications<sup>[1]</sup>. Biliary cast syndrome (BCS), first described in 1975<sup>[2]</sup>, occurs less frequently than biliary sludge and stones, with an incidence of 2.5% after orthotopic liver transplantation<sup>[3]</sup>. Multiple intrahepatic biliary strictures, ductal dilatation,

intrahepatic abscesses, and biliary anastomotic leakage characterize BCS. The clinical symptoms of BCS usually include high fever, jaundice and cholestatic liver enzyme elevation, similar to the symptoms observed in some patients with intrahepatic bile duct stones. Surgical management is the treatment of choice, and endoscopic techniques have been successful and safe in the removal of biliary casts<sup>[4-6]</sup>. Morphologically, biliary casts are a similar shape to bile ducts, appearing as a hardened, dark material in the biliary ductal system. Biliary casts can prevent bile drainage, resulting in biliary obstruction and inducing biliary tract infection. Biliary casts can ultimately cause substantial injury to the liver, with some transplant recipients requiring retransplantation. Although the associations between biliary casts and clinical treatment have been assessed recently, less is known about the associations between biliary casts and biochemical markers. We therefore investigated the pathogenesis of biliary casts after liver transplantation relative to their morphology and biochemical markers.

## MATERIALS AND METHODS

### *Isolation of biliary casts*

We evaluated 15 patients with a history of orthotopic liver transplantation, who were treated in our department for jaundice, recurrent cholangitis and high fever. There were 10 males and 5 females, with a mean age of 52.1 years (range, 34-78 years). Of these patients, five underwent deceased donor liver transplantation for hepatitis B-induced cirrhosis and primary liver cancer, one for primary hepatocellular carcinoma and nine for cirrhosis during the decompensated period. Choledochoscopy and duodenoscopy have been used frequently to assess patients with biliary complications after liver transplantation<sup>[7,8]</sup>. Patients with T-tube fistulae can be evaluated by insertion of a cholangioscope directly into the common hepatic duct, whereas patients without T-tube fistulae are evaluated preferably by percutaneous transhepatic cholangioscopy or endoscopic retrograde cholangiopancreatography<sup>[9]</sup>. The distal aspect of the cast was secured using a basket, allowing each cast to be successfully removed as a single piece. All the casts were stored in liquid nitrogen.

### *Scanning electron microscopy*

Following their isolation, biliary casts that were kept at room temperature were rinsed in sterile normal saline solution, fixed with 10% neutral formalin for 12 h at 4 °C, rinsed in 0.1 mol/L phosphate buffer (pH 7.0) and dehydrated through a graded series of ethanol (10 min each at 10%, 30%, 50%, 70% and 90%, and 15 min each three times at 100%). After critical point drying at 30 °C with CO<sub>2</sub> for 6 h, the samples were mounted, coated with 1-μm gold particles and evaluated using a Hitachi S 4800 field emission scanning electron microscope at 2 kV.

### *Histological and immunohistochemical examination*

Biliary casts stored in liquid nitrogen were rinsed in sterile

normal saline solution, fixed with 10% neutral formalin for 12 h at 4 °C, embedded in paraffin, cross-sectioned into 10 mm slices and placed onto glass slides. Some of these histological sections were stained with hematoxylin and eosin (HE) and Masson trichrome, according to standard procedures. The remaining histological sections were deparaffinized, rehydrated, incubated in 3% hydrogen peroxide/absolute methanol for 5 min to block endogenous peroxidase activity and rinsed in distilled water. Nonspecific binding of antibodies was blocked by incubation with 5% normal goat serum for 10 min at room temperature. After washing, the sections were incubated with primary rabbit antibodies against human CD3, CD5, CD34, CD68 and CD79a, overnight at 4 °C. The sections were subsequently incubated with biotinylated secondary antibody for 30 min at 37 °C, with streptavidin biotin complex reagent for 30 min at 37 °C, and with DAB Plus reagent for 10 min, with the sections repeatedly washed with PBS, pH 7.4, between incubations. The sections were counterstained with hematoxylin, mounted and examined by optical microscopy. All antibodies and reagents for immunohistochemistry were purchased from the Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China.

## RESULTS

### *Biliary casts have a variety of morphological structures*

Morphologically, biliary casts have a cordlike, columnar, dendritic shape within the biliary ductal system (Figure 1A). Scanning electron microscopy, however, showed that biliary casts were present in a variety of forms: irregular sheets composed of imbricated accumulations (Figure 1B); honeycombs with porous structures and adherent crystalline substances (Figure 1C); and filamentous structures (Figure 1D).

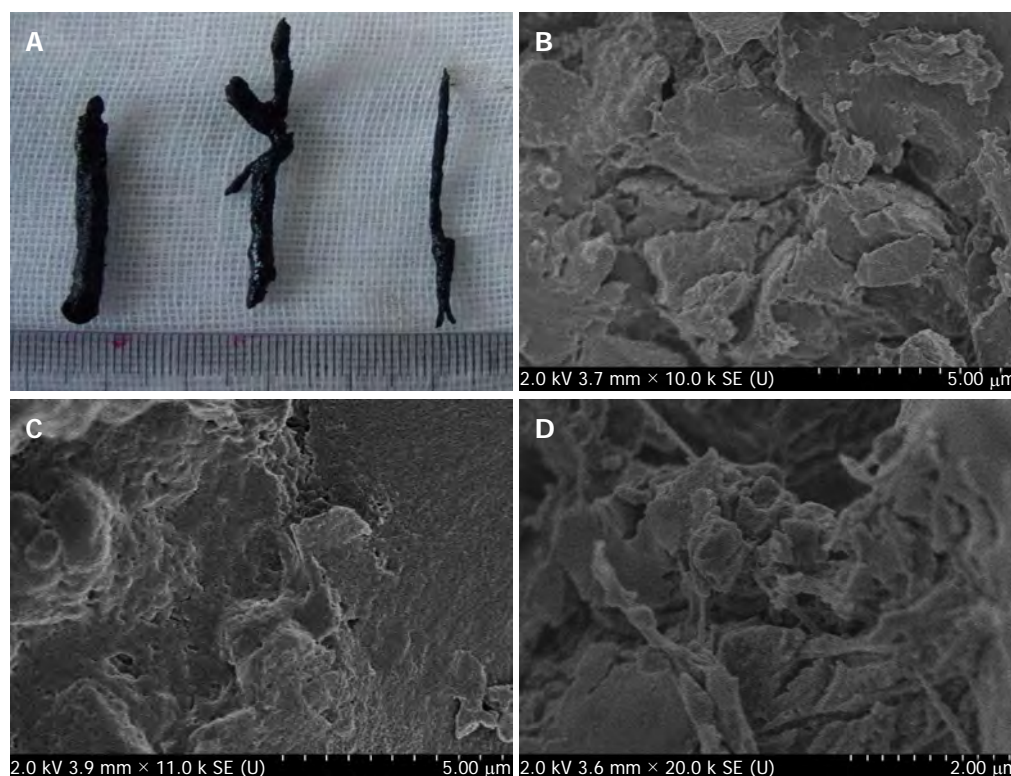
### *Biliary casts contain blood vessels and collagen fibers*

HE staining revealed large numbers of lacunae containing bilirubin, tubiform (Figure 2A) and filamentous structures (Figure 2B). To determine the composition of the tubiform and filamentous structures, we incubated these sections with antibodies to cell markers and with Masson stain. We found that the tubiform structures were positive for CD34 (Figure 2C), whereas the filamentous structures were positive for Masson stain (Figure 2D). These findings indicated that the tubiform structures were blood vessels and the filamentous structures were collagen fibers.

### *Formation of biliary casts is not related to inflammatory response*

HE staining showed large numbers of neutrophils and other inflammatory cells on the edge of the biliary casts; however, the boundaries were clear without crossover, and no inflammatory cells were present within the biliary casts (Figure 2E). Scanning electron microscopy showed no evidence of bacteria or bacterial debris on the surface of the biliary casts.





**Figure 1** Morphology of biliary casts. A: Cordlike, columnar and dendritic shapes of biliary casts within the biliary ductal system; B: A biliary cast in the shape of an irregular sheet composed of imbricated accumulation ( $\times 10000$ ); C: A biliary cast in the shape of a honeycombs with porous structures and adherent crystalline substances ( $\times 10000$ ); D: A biliary cast in the shape of filamentous structures ( $\times 10000$ ).

### Immune rejection is not significantly related to biliary cast formation

Acute rejection generally occurs 1 to 3 wk after liver transplantation. To determine the relationship between immune rejection reactions and biliary cast formation, we incubated the biliary cast samples with antibodies to CD3, CD5, CD68, and CD79a. None of the biliary casts was positive for any of these markers, indicating that these biliary casts did not contain T-lymphocytes, B-lymphocytes and macrophages.

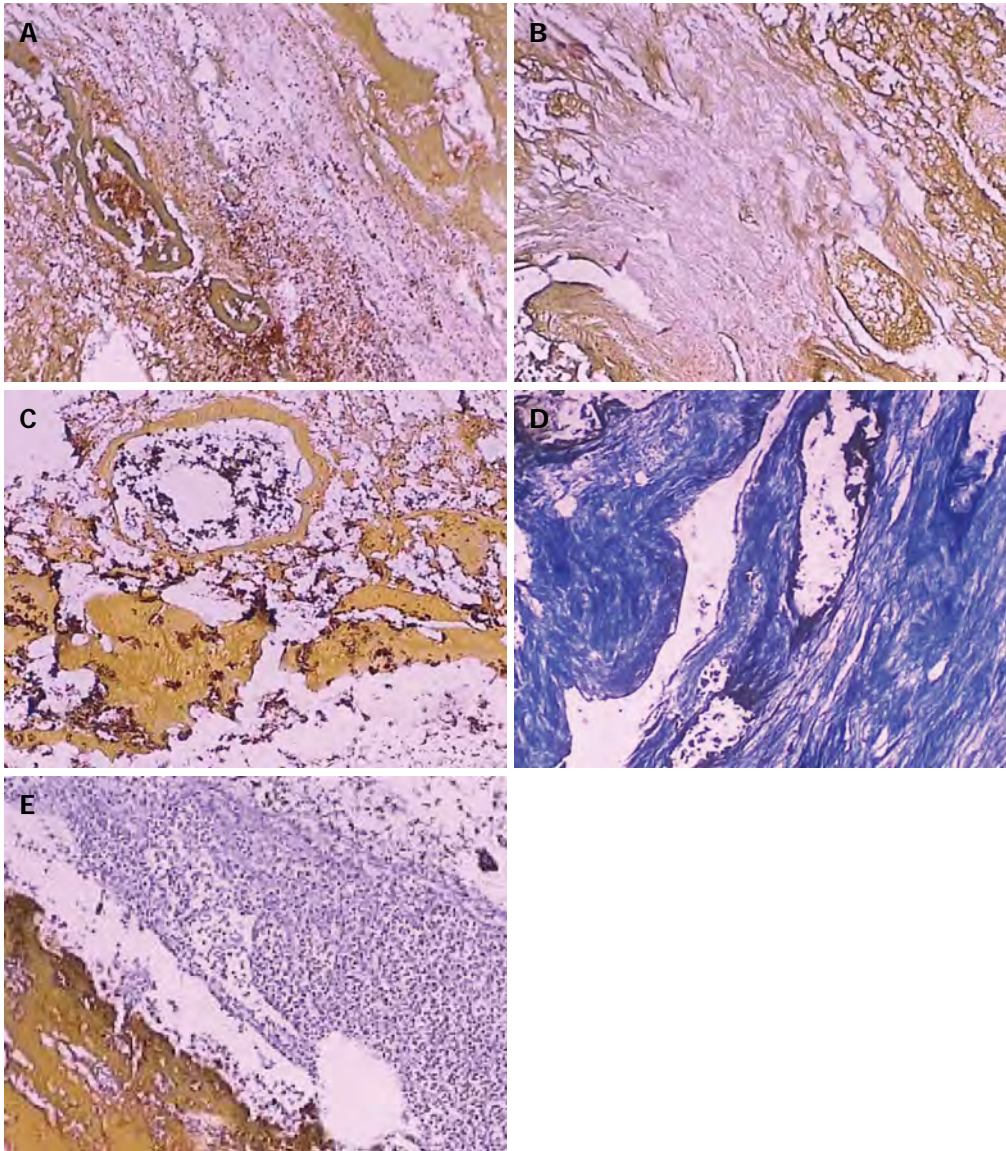
## DISCUSSION

Physically and morphologically, biliary casts appear as dark, hardened material in the shape of bile ducts within the biliary ductal system, but differ from bile duct stones. Scanning electron microscopy showed that biliary casts appear in a variety of forms, including irregular sheets composed of imbricated accumulations; honeycombs with a porous structure and adherent crystalline substances; and filamentous structures. Although bile duct stones and biliary casts have a similar microstructure<sup>[10]</sup>, their mechanism of formation differs significantly. Biliary casts that form after liver transplantation are not caused by a single pathogenic factor, but may be associated with late functional rehabilitation, biliary strictures and obstruction, acute rejection, recurrent cholangitis, cold and warm ischemia times, hepatic ischemia and reperfusion injury<sup>[3,11-13]</sup>.

Bilirubin has been reported to be the primary component of biliary casts (approximately 10%-50%), followed by bile acid synthesis products and cholesterol, with protein comprising only 5%-10%<sup>[14]</sup>. In comparison, we observed large amounts of bilirubin, as well as blood vessels and collagen fibers, consistent with our earlier findings. Choledochoscopy showed a large number of flocs in bile duct cellulose 5 mo after transplantation, with histopathological examination showing that these flocs were composed of cellulose, bile duct epithelial cells and necrotic inflammatory cells. These elements then become structureless, with biliary casts observed in the bile ducts 9 mo after transplantation<sup>[15]</sup>. The presence of blood vessels and collagen fibers in the biliary casts was related to injury to the bile duct mucosa. The extent of bile duct injury during orthotopic liver transplantation differs, with cold preservation/reperfusion injury being the most important initiator of bile duct tree injuries and vessel plexus damage. Bile duct injury may, therefore, be associated with microcirculatory disturbances surrounding the bile ducts<sup>[16]</sup>; however, the specific mechanisms underlying bile duct injury require further investigation.

Acute rejection after liver transplantation generally occurs 1 to 3 wk postoperatively. Typical clinical symptoms include unexplained fever, loss of appetite, poor spirit, liver pain, progressively deepening jaundice, and elevated bilirubin and transaminase. The diagnosis mainly depends on liver puncture biopsy and pathology. Biliary casts and acute rejection after transplantation have





**Figure 2** Histological and immunohistochemical examination of biliary casts. A: Histological examination of a biliary cast, showing tubiform structures (HE staining  $\times 100$ ); B: Histological examination of a biliary cast, showing filamentous structures (HE staining  $\times 100$ ); C: A biliary cast with tubiform structures positive for CD34 (brown color,  $\times 100$ ); D: A biliary cast with filamentous structures positive for collagen fibers (Masson staining  $\times 100$ ); E: A biliary cast showing peripheral positivity for neutrophils and other inflammatory cells (HE staining,  $\times 100$ ).

a similar time of onset and similar clinical symptoms. However, biliary casts generally form at least 1 mo after transplantation<sup>[17]</sup>. Liver recipients with high serum concentrations of soluble major histocompatibility complex class I related chain A (sMICA) tend to develop BCS more easily than recipients with normal post-transplant sMICA concentrations<sup>[18]</sup>. We hypothesized that the formation of biliary casts was related to acute rejection and that T lymphocytes, B lymphocytes and macrophages would be present in biliary casts. However, we found that these cells were absent from biliary casts arising after liver transplantation, similar to the findings in patients who underwent non-liver transplantation<sup>[19,20]</sup>. Therefore, our findings suggest that acute rejection after liver transplantation was not significantly associated with biliary cast formation.

Electron microscopic examination of cholesterol

calculi showed the presence of bacteria in the core and periphery of cholesterol stones, suggesting that bacteria may be involved in initiating the formation of cholesterol stones<sup>[21,22]</sup>. Patients with biliary casts usually have recurrent episodes of cholangitis. *Escherichia coli*, which has glucuronidase activity and can grow in cultures of biliary casts, can degrade conjugated bile acids and conjugated bilirubin, yielding free bile acids and free bilirubin, respectively. Free bile acids and free bilirubin are relatively insoluble and are not present in the bile of patients. Damage to the bile duct mucosa can result in their precipitation into biliary casts, suggesting that a number of factors, including infection, supersaturation with cholesterol and mucosal damage, may be involved in bile cast formation after liver transplantation<sup>[2]</sup>. To assess the relationship between bacteria and biliary casts, we evaluated biliary casts using scanning electron mi-

croscopy. However, neither bacteria nor bacterial debris was observed in the interior or surface of biliary casts. Large numbers of neutrophils were observed on the periphery of biliary casts, but the boundaries were clear and there were no neutrophils or similar cells within the mold. The multiplication of bacteria in an environment of poor bile drainage and cholestasis caused by biliary casts may therefore induce recurrent fever, obstructive jaundice and other complications. Biliary tract infections may be secondary pathological changes following biliary cast formation, rather than being the direct cause of mold formation. Therefore, when treating patients who experience complications after liver transplantation, anti-infectious agents may only alleviate the symptoms. The removal of the biliary casts may therefore be primary.

## COMMENTS

### Background

Biliary casts are infrequent complications after liver transplantation, resulting in various clinical symptoms. Although the associations between biliary casts and clinical treatment have been assessed recently, less is known about the associations between biliary casts and biochemical markers.

### Research frontiers

The current pathogenesis study of biliary casts after liver transplantation mostly concentrated on clinical aspects. Biliary casts were not caused by a single pathogenic factor, but may be associated with late functional rehabilitation, biliary strictures and obstruction, acute rejection, recurrent cholangitis, cold and warm ischemia times, hepatic ischemia and reperfusion injury.

### Innovations and breakthroughs

The results indicated that blood vessels and collagen fibers are present in biliary casts; however, bacteria and acute rejection are not clearly related to their formation, as evidenced by blood vessels positive for CD34 and collagen fibers with positive Masson staining, and the absence of T-lymphocytes, B-lymphocytes, macrophages and other inflammatory cells.

### Applications

These findings indicate that bile duct injury is clearly associated with biliary cast formation after liver transplantation; however, bacteria and acute rejection were not significantly related to their formation.

### Terminology

Biliary cast syndrome, first described in 1975, occurs less frequently than biliary sludge and stones, with an incidence of 2.5% after orthotopic liver transplantation. Orthotopic liver transplantation refers to a procedure in which a failed liver is removed from the patient's body and a healthy donor liver is transplanted into the same location. Biliary casts are infrequent complications after liver transplantation, resulting in various clinical symptoms.

### Peer review

The authors analyzed the pathogenesis of biliary casts after liver transplantation relative to their morphology and biochemical markers. These findings indicate that bile duct injury was clearly associated with biliary cast formation after liver transplantation, but that bacteria and acute rejection are not clearly related to their process of bile duct injury. Therefore, it is an interesting study. The analytical approaches are described in detail, and the results are impressive.

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## Amelioration of carbon tetrachloride-induced cirrhosis and portal hypertension in rat using adenoviral gene transfer of Akt

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Author contributions: Huang FZ, Liu XY and Wang YH participated in research design and other authors collectively contributed to the performance of laboratory measurements; Deng G, Huang XJ and Huang FZ were involved in data collection and analysis; and Deng G, Huang XJ and Luo HW wrote the manuscript.

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### Abstract

**AIM:** To investigate whether a virus constitutively expressing active Akt is useful to prevent cirrhosis induced by carbon tetrachloride (CCl<sub>4</sub>).

**METHODS:** Using cre-loxp technique, we created an Ad-myr-HA-Akt virus, in which Akt is labeled by a HA tag and its expression is driven by myr promoter. Further, through measuring enzyme levels and histological structure, we determined the efficacy of this Ad-myr-HA-Akt virus in inhibiting the development of cirrhosis induced by CCl<sub>4</sub> in rats. Lastly, using western blotting, we examined the expression levels and/or phosphorylation status of Akt, apoptotic mediators, endothelial nitric oxide synthase (eNOS), and markers for hepatic stellate cells activation to understand the underlying mechanisms of protective role of this virus.

**RESULTS:** The Ad-myr-HA-Akt virus was confirmed using polymerase chain reaction amplification of inserted

Akt gene and sequencing for full length of inserted fragment, which was consistent with the sequence reported in the GenBank. The concentrations of Ad-myr-HA-Akt and adenoviral enhanced green fluorescent protein (Ad-EGFP) virus used in the current study were  $5.5 \times 10^{11}$  vp/mL. The portal vein diameter, peak velocity of blood flow, portal blood flow and congestion index were significantly increased in untreated, saline and Ad-EGFP cirrhosis groups when compared to normal control after the virus was introduced to animal through tail vein injection. In contrast, these parameters in the Akt cirrhosis group were comparable to normal control group. Compared to the normal control, the liver function (Alanine aminotransferase, Aspartate aminotransferase and Albumin) was significantly impaired in the untreated, saline and Ad-EGFP cirrhosis groups. The Akt cirrhosis group showed significant improvement of liver function when compared to the untreated, saline and Ad-EGFP cirrhosis groups. The Hyp level and portal vein pressure in Akt cirrhosis groups were also significantly lower than other cirrhosis groups. The results of HE and Van Gieson staining indicated that Akt group has better preservation of histological structure and less fibrosis than other cirrhosis groups. The percentage of apoptotic cell was greatly less in Akt cirrhosis group than in other cirrhosis groups. Akt group showed positive HA tag and an increased level of phosphorylated Akt as well as decreased levels of Fas. In contrast, Caspase-3 and Caspase-9 levels in Akt group were significantly lower than other cirrhosis groups. Noticeable decrease of DR5 and  $\alpha$ -SMA and increase of phosphorylated eNOS were observed in the Akt group when compared to other cirrhosis groups. The NO level in liver was significantly higher in Akt group than other cirrhosis groups, which was consistent with the level of phosphorylated eNOS in these groups.

**CONCLUSION:** This study suggest that Ad-myr-HA-Akt virus is a useful tool to prevent CCl<sub>4</sub>-induced cirrhosis in rat model and Akt pathway may be a therapeutic target



for human cirrhosis.

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**Key words:** Adenovirus; Akt; Gene transfer; Apoptosis; Cirrhosis; Carbon tetrachloride; Rat

**Core tip:** In the present study, we have demonstrated for the first time that Ad-myr-HA-Akt virus was a useful tool to prevent carbon tetrachloride-induced cirrhosis in rat model. Our data obtained at different levels, from function to histological changes, apoptosis rate of hepatocytes, activation of hepatic stellate cells, deposition of collagen, portal vein pressure and NO level, which were all consistently and collectively supported the hypothesis that introduction of Ad-myr-HA-Akt virus inhibits the development of cirrhosis.

Deng G, Huang XJ, Luo HW, Huang FZ, Liu XY, Wang YH. Amelioration of carbon tetrachloride-induced cirrhosis and portal hypertension in rat using adenoviral gene transfer of Akt. *World J Gastroenterol* 2013; 19(43): 7778-7787 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7778.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7778>

## INTRODUCTION

It has been found that massive apoptosis of hepatocytes resulted in activation of hepatic stellate cells (HSC), which produce collagen and ultimately lead to the fibrosis and cirrhosis<sup>[1-5]</sup>. Accordingly, inhibiting hepatic apoptosis is considered as an important strategy to prevent cirrhosis<sup>[6-10]</sup>. Previous research showed that Akt plays a crucial role in preventing Fas signaling-mediated hepatic apoptosis, and that over-expression of Akt was capable of preventing hepatic apoptosis<sup>[11,12]</sup>. Therefore, we intended to establish a recombinant vector carrying *Akt* gene to inhibit cirrhosis in a rat model.

Adenovirus can effectively express genes of interest. Accordingly it has been widely used as vector for gene therapy<sup>[13-18]</sup>. Recently, Cre-loxp system has been used to create replication-defective virus and successfully used in creating therapeutic virus with recombinant gene<sup>[19-22]</sup>. In the present study, using cre-loxp technique, we created an Ad-myr-HA-Akt virus, in which Akt is labeled by a Hemagglutinin (HA) tag and its expression is driven by myr promoter. We further determined that this Ad-myr-HA-Akt virus was capable of inhibiting the development of cirrhosis induced by carbon tetrachloride (CCl<sub>4</sub>). Lastly, we examined the expression level and/or phosphorylation status of Akt, apoptotic mediators, endothelial nitric oxide synthase (eNOS), and markers for HSC activation. Our results indicated that introduction of Ad-myr-HA-Akt virus was able to prevent hepatocyte apoptosis and subsequent cirrhosis in a rat CCl<sub>4</sub>-induced cirrhosis model.

## MATERIALS AND METHODS

### Preparation of recombinant adenoviral vector carrying Akt

According to the method that has been published<sup>[23]</sup>, a replication-defective adenovirus vector was constructed in HEK293 cell with co-transfection of pCD316 plasmid, pBHGloxΔE1 and 3Cre (Invitrogen, United States). The details have been previously published. The vector of interest was selected with polymerase chain reaction (PCR) amplification of inserted *Akt* gene using the following primer pair as show: F: 5'-GGGAATTCATGGGATGCGTGTGTAGC-3'; R: 5'-GGGGATCCTCAGGCCGTGCCGCTGGCCGAGTA-3'. The PCR thermal condition consisted of 94 °C 5 min, 30 cycles of 94 °C 30 s, 58 °C 30 s, 72 °C 1 min, and a final extension at 72 °C 5 min. The amplified gene was further confirmed with DNA sequencing (Sunbiotech Co., Ltd, ABI 3730, Beijing). adenoviral enhanced green fluorescent protein (Ad-EGFP) was purchased from Stratagene (CA, United States) and used as a control vector for tracking the distribution of virus after introduced to animal. The concentration of recombinant adenovirus was measured with tissue culture infective dose method as previously reported<sup>[24]</sup>.

Plasmids were amplified in DH5a competent cells and purified using a commercial kit (Qiagen). Viruses were prepared in HEK293 cells (Qiagen, GmbH, Hilden, German), which contain the necessary gene for the virus package. Specifically, one day prior to the transfection, HEK293 cells were seeded at a concentration of  $10 \times 10^6$  in 10 mL complete dulbecco's modified eagle medium and cultured overnight. Upon transfection, 1 mg/mL plasmid stock was taken and adjusted into a volume of 1600 μL, 150 μL calcium phosphate was added to the same tube, mixed well and incubated at room temperature for 20 min. The resultant mixture was slowly added into HEK293 cell culture. The dish was gently moved and swirled to allow the even distribution of virus in culture. Cells were further cultured for 16 h. The supernatant was discarded and replaced with fresh medium. After a 2-d culture, cells were examined under a fluorescence microscope (E80i, Nikon, Japan) for the green fluorescent protein (GFP<sup>+</sup>) cells. Cells were harvested and lysed using freezing (-70 °C) and thawing (37 °C) twice. The resultant mixture was centrifuged at 10000 g for 10 min. The supernatant with virus was collected and stored at -70 °C for future infection of target cells.

### Rat cirrhosis model and experiment groups

Fifty male rats (weight  $220 \pm 20$  g) were purchased from Animal Center of Hunan Agriculture University (Hunan, China). Rats were housed under  $25 \pm 2$  °C and a 12-h light/dark cycle in microisolator cages. Ten rats were randomly selected and used for the normal control. The remaining 40 rats were subjected to the induction of cirrhosis using CCl<sub>4</sub> as previously reported<sup>[25]</sup>. These rats were further randomly divided into 4 groups ( $n = 10$  per

group). One group did not receive additional treatment, the other three groups received saline, Ad-EGFP, Ad-myr-HA-Akt ( $3.0 \times 10^{11}$  vp in 1 mL saline), respectively, *via* tail vein injection at 2 wk after cirrhosis induction. The treatment was repeated at 6 wk. Accordingly, the experiment groups of rats were defined as follows: normal control, untreated cirrhosis, saline cirrhosis, EGFP cirrhosis and Akt cirrhosis. All surgical procedures were completed in accordance with the guidelines on the care and use of laboratory animals for research purposes by the Central South University Xiangya Medical School's Animal Care and Use Committee. Mice were anesthetized with chloral hydrate (*iv*) for all surgical procedures.

### Hemodynamic and ultrasound parameters

Three days after treatment, five rats from each experimental groups (normal control group, untreated cirrhosis, saline cirrhosis, Ad-EGFP and Akt cirrhosis) were restricted from food with free access to water for 12 h. Rats were anesthetized by *iv* infusion of 2.5% chloral hydrate (50 gtt/min). Then, diameter (D) and peak velocity of blood flow (V) in portal was measured using ultrasound system (SIMENS Co., Ltd, Acuson Seguoia 512, Germany). The blood flow (Q) and congestion index (CI) was calculated using the following formulas:  $Q = 0.57\pi D^2/4V \times 60$ , and  $CI = \pi D^2/4V$ , respectively. Using a blood pressure device (RBP-1B, Sino-Japan friendship clinical medicine institute), the tail mean arterial pressure was recorded.

### Analysis of liver function and histological changes

Eight weeks after cirrhosis induction, 1 mL of blood from each rat was collected from portal vein and subjected to analysis for alanine aminotransferase (ALT); aspartate aminotransferase (AST) and albumin (ALB) levels using a automatic biochemical analyzer (Spotchem SP4430, Arkray, Kyoto, Japan). Using aseptic techniques, laparotomy was performed, the portal vein was exposed, and portal vein pressure was measured with catheterization. Then rats were sacrificed and the right lobe of the liver was removed and stored in liquid nitrogen for future analysis. Liver tissue sections were subjected to HE staining for cellular and tissue structure as well as Van Gieson (VG) staining for collagen deposition. Hepatic hydroxyproline (Hyp), which is the main constituent of collagen protein, was used to estimate the degree of hepatic fibrosis, was measured with a commercial kit following manufacture's protocol (Jiancheng Biological product institute, Nanjing, China). The stained sections were examined and photographed under a microscope equipped with a digital photograph acquiring system (E80i, Nikon, Japan). Hyp and liver function markers were measured with an automatic machine (Beckman LX20, United States).

### Flow cytometry analysis

Apoptosis of hepatic cells was detected using Annexin-V-fluorescein isothiocyanate (FITC)/Propidium iodide (PI) double staining and flow cytometry analysis. The

cells were harvested and resuspended in Annexin-V binding buffer and further incubated with 5  $\mu$ L of Annexin V-FITC and 10  $\mu$ L of PI for 10 min at room temperature in the dark, followed by cytometric analysis (EPICS XL, Beckman Coulter, United States) within 30 min of staining. All experiments were performed in triplicate.

### Western blotting

Using a protein extraction kit (Biovision, CA, United States), whole-cell extracts were prepared from frozen liver tissue. Protein concentration of the extracts was determined by the Bradford method with a kit purchased from Biovision (United States). Forty micrograms of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and were electronically transferred onto a polyvinylidene difluoride membrane (Roche Diagnostics, Mannheim, Germany). The membrane was blocked in a standard western blotting procedure. Briefly, the membrane was blocked with 7.5% milk in tris-buffered-saline with tween (TBST) buffer [20 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl, 0.05% Tween 20], then probed with a primary antibody [anti-Akt, anti-phospho-Akt-Ser-473 (p-Akt) Akt, p-Akt, Fas, DR5 and HA, purchased from Cell Signaling Technology (Beverly, MA, United States). After washing with TBST buffer, the membrane was further incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:20000, KPL, United States). The protein bands were visualized using an enhanced chemiluminescence detection system, LumiGLO (KPL, United States).  $\beta$ -actin (NeoMarkers, Fremont, CA, United States) was used as a loading control and normalization reference for quantification.

### Measurement of NO

Nitric acid reductase method was used to measure hepatic NO using a commercial kit (CST, United States), following the manufacture manual. Briefly, 1 g of rat liver tissue stored in the liquid nitrogen was thawed and homogenized at 4 °C in the saline at a concentration of 100 g/L. Following centrifuge (1000 r/min for 5 min), the supernatant was used for the nitric acid reductase reaction to measure product of  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , which indirectly represent the level of NO.

### Examination of transplanted GFP<sup>+</sup> cells

GFP<sup>+</sup> cells in the host were examined at 8 wk after induction of cirrhosis. Mice was euthanized and infused with normal saline until the liver became pale. The liver, spleen, heart, lung, brain and kidney were collected, cryostat sectioned at a thickness of 2  $\mu$ m, and examined for GFP<sup>+</sup> cells under a fluorescence microscope (E80i, Nikon, Japan). A fraction of the tissues from the above organs were also formalin-fixed, paraffin-embedded, continuously sectioned for H and E (hematoxylin and eosin) staining and histological analysis.

### Statistical analysis

The SPSS program (version 12.0, SPSS Inc., United

**Table 1** Portal vein diameter, peak velocity of blood flow, congestion index and blood flow in each experimental group

Group	n	D (mm)	V (cm/s)	CI	Q (mL/min)
Normal	5	1.13 ± 0.24	12.67 ± 0.64	0.0010 ± 0.0003	4.56 ± 1.86
Cirrhosis	20				
Untreated	5	1.81 ± 0.19 <sup>a</sup>	10.13 ± 0.68 <sup>a</sup>	0.0021 ± 0.0007 <sup>a</sup>	9.69 ± 2.58 <sup>a</sup>
Saline	5	1.83 ± 0.29 <sup>a</sup>	10.06 ± 0.72 <sup>a</sup>	0.0024 ± 0.0008 <sup>a</sup>	9.32 ± 2.83 <sup>a</sup>
EGFP	5	1.82 ± 0.27 <sup>a</sup>	9.98 ± 0.77 <sup>a</sup>	0.0020 ± 0.0006 <sup>a</sup>	8.97 ± 2.45 <sup>a</sup>
Akt	5	1.28 ± 0.32 <sup>a,c,e,g</sup>	11.39 ± 0.63 <sup>c,e,g</sup>	0.0013 ± 0.0004 <sup>c,e,g</sup>	5.11 ± 2.30 <sup>a,c,e,g</sup>

<sup>a</sup>*P* < 0.05 (*vs* Normal control); <sup>c</sup>*P* < 0.05 (*vs* Untreated group); <sup>e</sup>*P* < 0.05 (*vs* Saline group); <sup>g</sup>*P* < 0.05 [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups. D: Diameter; V: Peak velocity of blood flow; CI: Congestion index; Q: Blood flow.

**Table 2** Liver function parameter in each experiment group in each experimental group

Group	n	ALT (U/L)	AST (U/L)	ALB (g/L)	Hyp (μg/g)
Normal	5	23.5 ± 6.3	109.3 ± 6.1	33.1 ± 2.6	180.5 ± 12.5
Cirrhosis	20				
Untreated	5	277.6 ± 25.8 <sup>a</sup>	380.5 ± 16.9 <sup>a</sup>	22.7 ± 3.5 <sup>a</sup>	375.2 ± 17.3 <sup>a</sup>
Saline	5	290.7 ± 22.9 <sup>a</sup>	368.9 ± 23.8 <sup>a</sup>	24.7 ± 3.7 <sup>a</sup>	393.8 ± 22.3 <sup>a</sup>
EGFP	5	285.9 ± 27.3 <sup>a</sup>	374.4 ± 26.7 <sup>a</sup>	23.9 ± 2.9 <sup>a</sup>	388.5 ± 9.8 <sup>a</sup>
Akt	5	126.4 ± 5.8 <sup>a,c,e,g</sup>	202.5 ± 9.5 <sup>a,c,e,g</sup>	30.7 ± 4.8 <sup>a,c,e,g</sup>	245.9 ± 15.6 <sup>a,c,e,g</sup>

<sup>a</sup>*P* < 0.05 (*vs* Normal control); <sup>c</sup>*P* < 0.05 (*vs* Untreated group); <sup>e</sup>*P* < 0.05 (*vs* Saline group); <sup>g</sup>*P* < 0.05 [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; Hyp: Hepatic hydroxyproline.

States) was used for statistical analysis. Quantitative data were expressed as mean ± SD. Student *t* test and/or one-way Analysis of variance was used for group comparisons. The differences were considered significant when *P* < 0.05.

## RESULTS

### Preparation of Ad-myr-HA-Akt virus and transfer to rats

As detailed in the method, the Ad-myr-HA-Akt virus was confirmed using PCR amplification of inserted Akt gene and sequencing for full length of inserted fragment, which was consistent with the sequence reported in the GenBank. The concentrations of Ad-myr-HA-Akt and Ad-EGFP virus used in the current study were  $5.5 \times 10^{11}$  vp/mL. The virus was introduced to animal through tail vein injection. According to the control vector with expression of green fluorescent protein (GFP), the distribution of virus was mainly in the liver (Figure 1).

### Hemodynamic results of portal vein in different experimental groups

Five rats from each group (normal control group, untreated cirrhosis, saline cirrhosis, Ad-EGFP, and Akt cirrhosis groups) were subjected to measure portal vein diameter (D) and peak velocity of blood flow (V) and calculation of portal Q and CI (Figure 2). As shown in the Table 1, these parameters were significantly increased

**Table 3** Portal vein pressure, mean arterial pressure and heart rate in each experiment group at the end of the treatment

Group	n	PVP (mmHg)	MAP (mmHg)	HR
Normal	5	8.96 ± 1.46	83.5 ± 9.8	323 ± 73
Cirrhosis	20			
Untreated	5	16.01 ± 1.32 <sup>a</sup>	79.6 ± 14.2	339 ± 89
Saline	5	15.87 ± 1.40 <sup>a</sup>	80.1 ± 11.5	282 ± 101
EGFP	5	15.65 ± 1.18 <sup>a</sup>	82.9 ± 12.9	319 ± 78
Akt	5	9.23 ± 1.51 <sup>a,c,e,g</sup>	88.5 ± 17.6	289 ± 96

<sup>a</sup>*P* < 0.05 (*vs* Normal control); <sup>c</sup>*P* < 0.05 (*vs* Untreated group); <sup>e</sup>*P* < 0.05 (*vs* Saline group); <sup>g</sup>*P* < 0.05 [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups. PVP: Portal vein pressure; MAP: Mean arterial pressure; HR: Heart rate.

in untreated, saline and Ad-EGFP cirrhosis groups when compared to normal control. In contrast, these parameters in the Akt cirrhosis group were comparable to normal control group.

### Ad-myr-HA-Akt virus was able to preserve liver function and reduce portal hypertension

Eight weeks after cirrhosis induction, blood samples from portal vein were collected and subjected to the measurement of ALT, AST and ALB. As shown in the Table 2, compared to the normal control, the liver function was significantly impaired in the untreated, saline and Ad-EGFP cirrhosis groups. However, the Akt cirrhosis group showed significant improvement of liver function (lower levels of the four parameters) when compared to the untreated, saline and Ad-EGFP cirrhosis groups. Consistently, the Hyp level (Table 2) and portal vein pressure (Table 3) in Akt cirrhosis groups were also significantly lower than other cirrhosis groups (untreated, saline and Ad-EGFP). Of note, Mean arterial pressure and heart rate did not show significant differences among the groups.

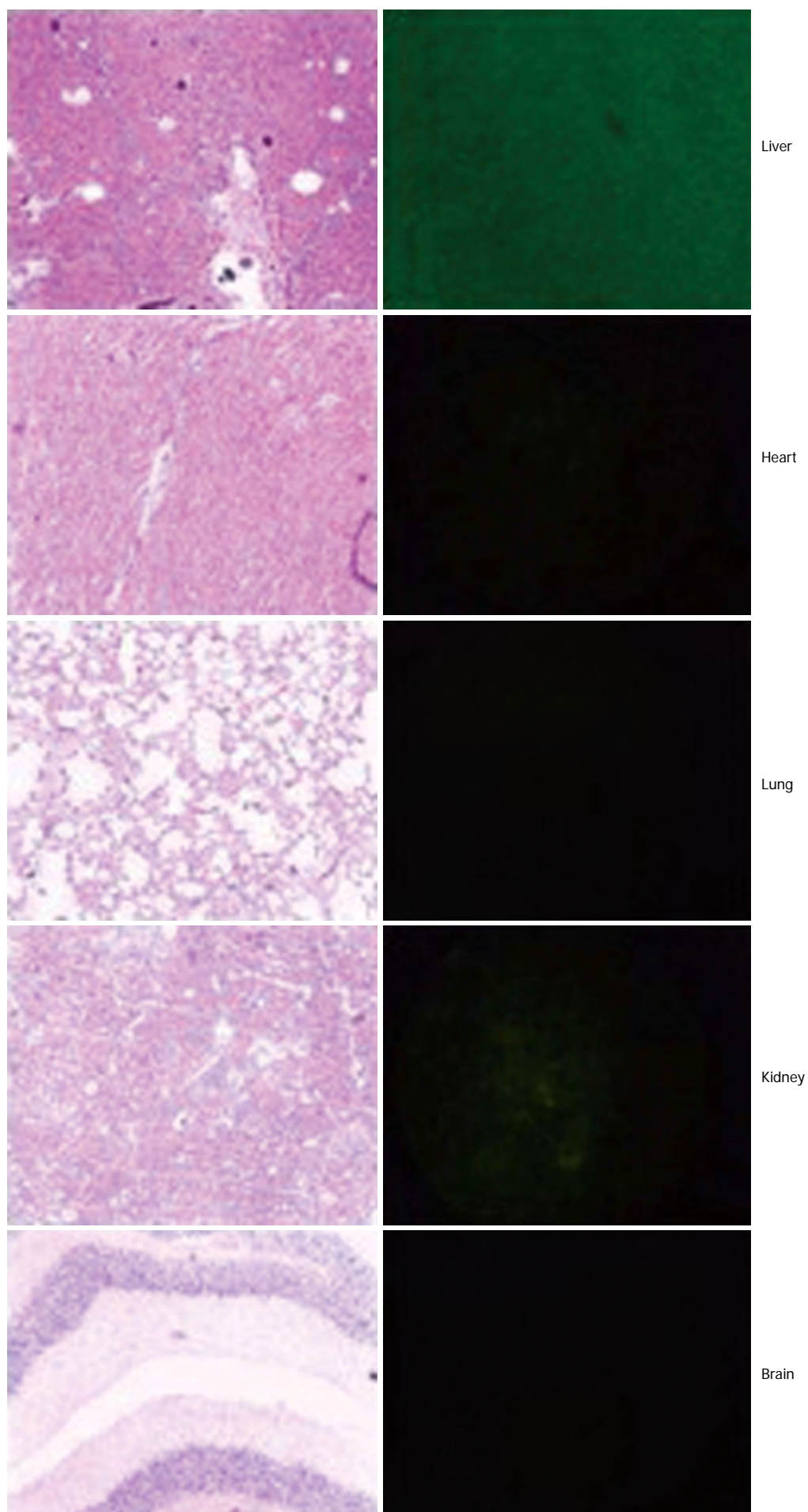
### Ad-myr-HA-Akt virus significantly reduced the liver fibrosis

To determine whether the above observations were derived from the histological changes of liver, we examined cellular and tissue structure and collagen deposition using HE and VG staining, respectively (Figure 3). Our results indicated that Akt group has better preservation of histological structure and less fibrosis than other cirrhosis groups. Collectively, these data supported that Ad-myr-HA-AKT virus was efficient in inhibiting the development of cirrhosis induced by CCl<sub>4</sub>.

### Ad-myr-HA-Akt virus inhibited apoptosis of hepatocytes

To confirm the reduction of liver fibrosis was related to the reduction of apoptosis of hepatocytes, we doubly stained hepatocytes with PI and Annexin V to detect the apoptosis rate in each experimental group. As shown in Figure 4, the percentage of apoptotic cell was greatly less in Akt cirrhosis group than in other cirrhosis groups (2.5%-3.9% reduction). These results suggested that







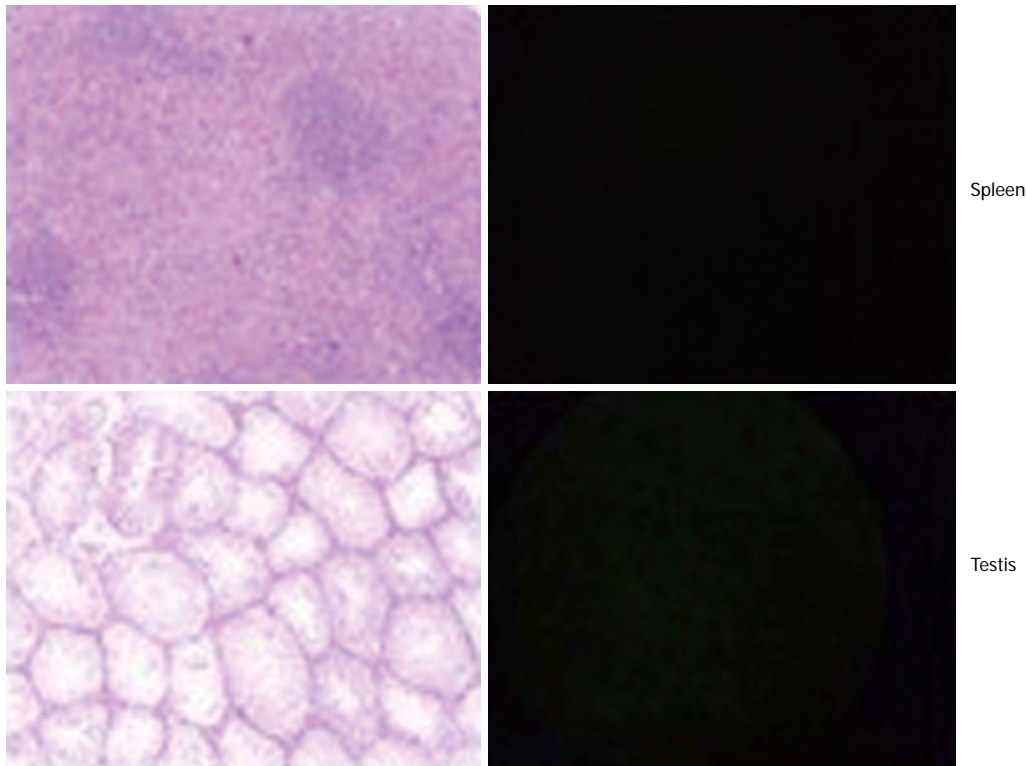


Figure 1 Tissue distribution of transferred viruses in the host. Representative micrographs from each organ (as designated) are shown for the presence of green fluorescent protein (GFP), which is a marker for the presence of the recombinant virus. GFP was primarily observed in the liver (top panel) and rarely present in other organs.

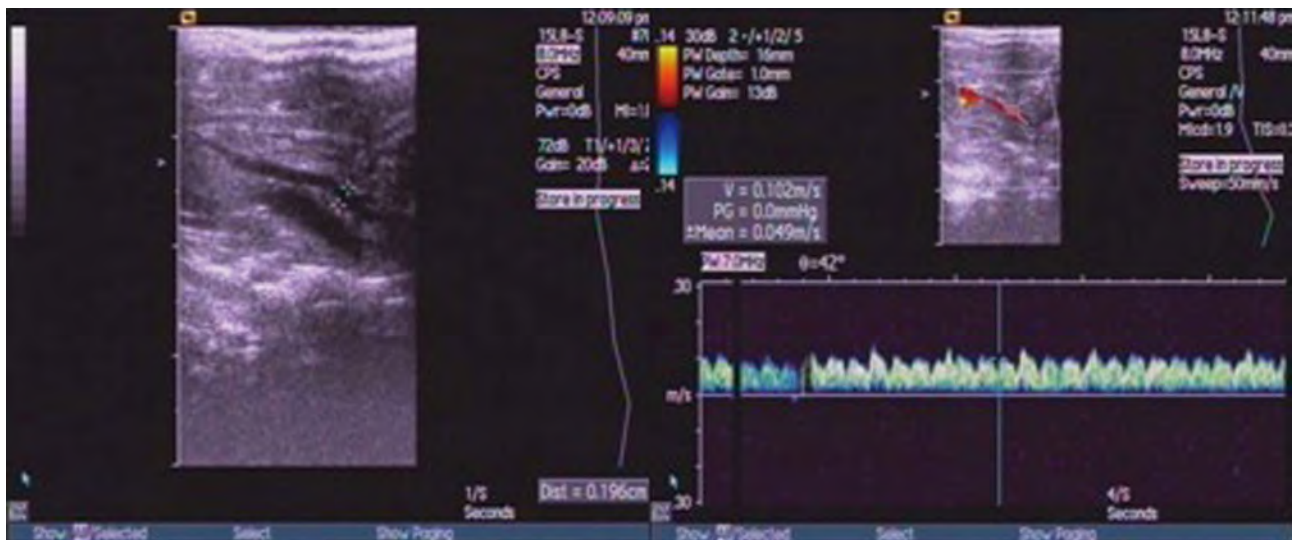


Figure 2 Measurement of hemodynamic parameter of portal vein. Representative graphs for identifying portal vein and measurement of hemodynamic parameters using ultrasound.

amelioration of liver function and fibrosis in Akt may be involved in the reduction of apoptosis of hepatocytes.

#### ***Ad-myr-HA-Akt virus inhibited apoptotic mediators***

Next, to confirm the apoptosis, we measured the expression levels of apoptotic mediators using Western blotting. As shown in Figure 5A, Akt group showed positive HA tag and an increased level of phosphorylated Akt

(p-Akt) as well as decreased levels of Fas. The levels of p-Akt and Fas are comparable in Akt cirrhosis group and normal control group. In contrast, Caspase-3 and Caspase-9 levels in Akt group were significantly lower than other cirrhosis groups (untreated, saline and Ad-EGFP groups). These results suggested that introduction of Ad-myr-HA-Akt virus resulted in the inhibition of Fas-mediated apoptotic pathway, which presumably led to the

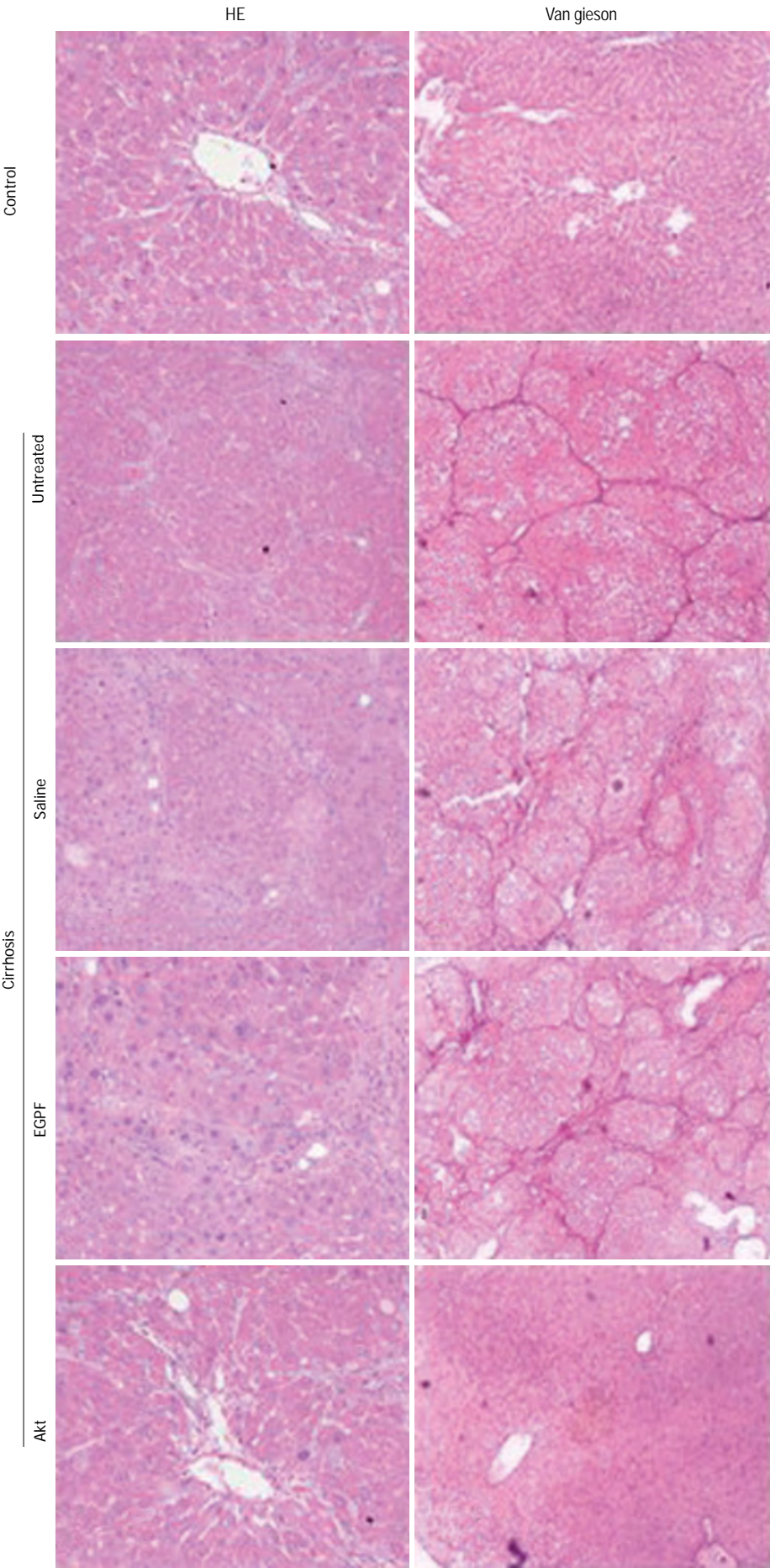


Figure 3 Histological changes in rat with cirrhosis induced by CCl<sub>4</sub>. Livers from rats in each experimental group were collected 8 wk after CCl<sub>4</sub> treatment, sectioned, and subjected to HE and van gieson staining for tissue structure and collagen deposition. While cirrhosis groups without treatment or received saline or ad-enhanced green fluorescent protein treatment show structure disruption and nodule formation as well as remarkable deposition of collagen, cirrhosis group with Akt virus transfer show well preserved tissue structure and less collagen, both of which are comparable to normal control (magnification × 100).



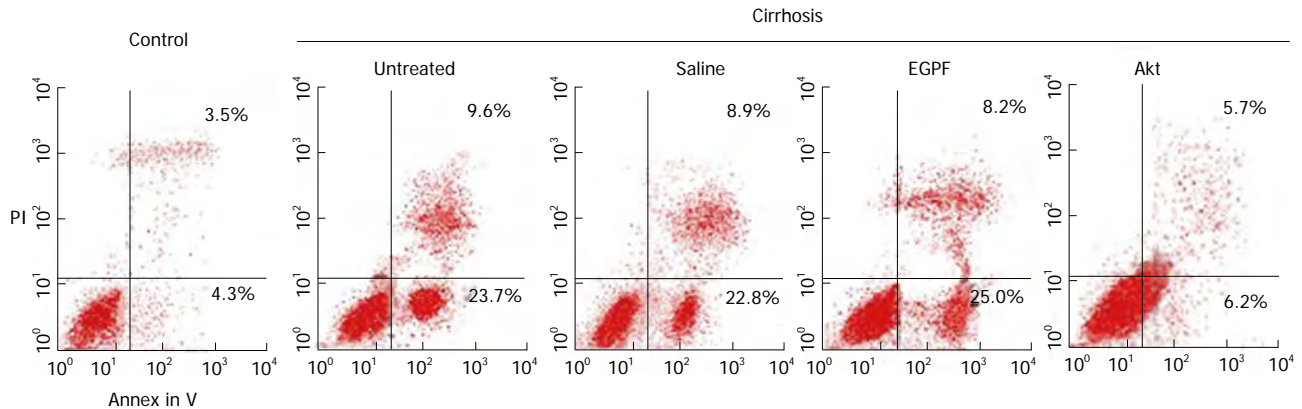


Figure 4 Flow cytometry analysis for hepatocyte apoptosis in each experimental group. Single hepatocytes from each experiment groups were prepared at 2 wk after CCl<sub>4</sub> treatment, and subjected to double staining of Propidium iodide (PI) and Annenin V to detect the dead and apoptotic cell. Representative data from each group are shown with designated name. Double positive cell with PI and Annenin V were considered as apoptotic dead cells and its percentage is listed in the right up-per quadrant of the plot.

Table 4 NO content in liver tissue

Groups	n	NO (μmol/g)
Normal	5	2.53 ± 0.61
Cirrhosis	20	
Untreated	5	1.20 ± 0.77 <sup>a</sup>
Saline	5	1.03 ± 0.87 <sup>a</sup>
EGFP	5	1.15 ± 0.58 <sup>a</sup>
Akt	5	2.38 ± 0.67 <sup>c,d,g</sup>

<sup>a</sup>P < 0.05 (*vs* normal control); <sup>c</sup>P < 0.05 (*vs* Untreated group); <sup>d</sup>P < 0.05 (*vs* Saline group); <sup>e</sup>P < 0.05 [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups.

reduction of the apoptosis of hepatocytes.

#### Ad-myr-HA-Akt virus inhibited activation of hepatic stellate cell and increased the levels of eNOS activity and NO production

To understand the mechanism underlying the hepatocyte apoptosis and fibrosis of liver, we further measured two markers (DR5 and α-SMA) for the activation of HSC and the levels of eNOS and its phosphorylation status that is correlated to the NO production. Noticeable decrease of DR5 and α-SMA (Figure 5A) and increase of phosphorylated eNOS (Figure 5B) were observed in the Akt group when compared to other cirrhosis groups (untreated, saline and Ad-EGFP). As shown in Table 4, the NO level in liver was significantly higher in Akt group than other cirrhosis groups (untreated, saline and Ad-EGFP), which was consistent with the level of phosphorylated eNOS in these group. Collectively, these results suggested that introduction of Ad-myr-HA-Akt virus blocked the activation of HSC and maintained NO level, which may subsequently reduced liver fibrosis and blood vessel resistance following the damage from CCl<sub>4</sub>.

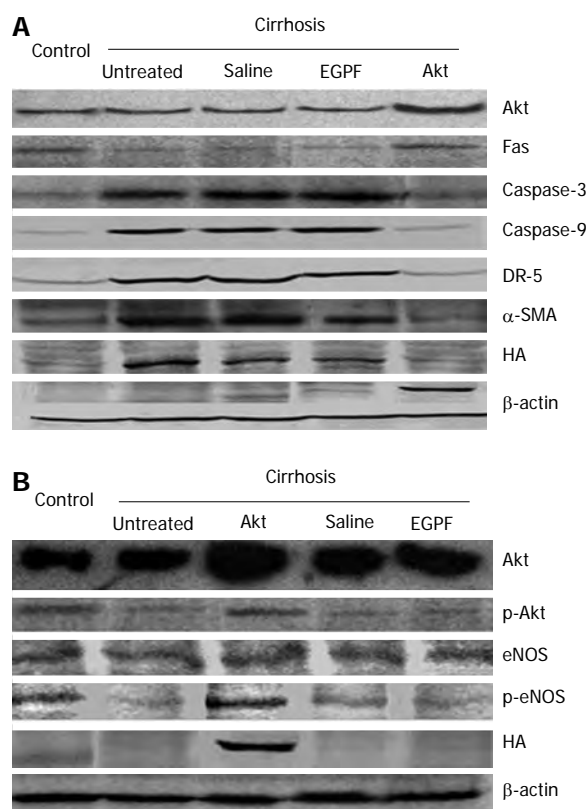
## DISCUSSION

Fas is one of the most important receptors on cell sur-

face to mediate apoptosis<sup>[26-29]</sup>. It has been shown that FasL-Fas pathway is an important cascade leading to hepatocyte apoptosis, which in turn activates HSC that produce collagen<sup>[11-5]</sup>. Song *et al*<sup>[30]</sup> showed that block of Fas signaling pathway could inhibit the development of cirrhosis. Akt plays important roles in regulating cell survival through inhibiting Fas-mediated apoptosis<sup>[11,12]</sup>. In the current study, we utilized the constitutive expression of active form of Akt to block cirrhosis induced by CCl<sub>4</sub>. The efficacy of this virus was firstly confirmed at the level of liver function. Furthermore, using multiple approaches, we examined whether the transfer of Akt *via* this virus in liver could result in the molecular alterations that favor the survival of hepatocyte and/or disfavor the fibrosis. Our data indicated that Ad-myr-HA-Akt virus was a useful tool to prevent CCl<sub>4</sub>-induced cirrhosis in rat model.

Encouragingly, the data obtained at different levels, from function to histological changes, apoptosis rate of hepatocytes, activation of HSC, deposition of collagen, portal vein pressure and NO level, which were all consistently and collectively supported the hypothesis that introduction of Ad-myr-HA-Akt virus inhibits the development of cirrhosis. First, all measured parameters for liver function were consistent with reduced hepatocyte apoptosis: the introduction of Akt virus led to increased expression of Akt and its phosphorylation, decreased expression of apoptotic mediators (Caspase-9 and Caspase-3) and ultimately preserved liver functions (enzyme levels). Second, the portal vein pressure was consistent with the histological structure as well as NO levels. The introduction of Akt virus led to reduction of formation of liver nodules, portal vein pressures and increased level of NO, which is directly correlated with vasodilation. Third, the level of specific marker (Hyp) for the liver fibrosis was consistent with the amount of deposition of collagen and the expression of α-SMA and DR5, markers for activated HSC.

While the introduction of Ad-myr-HA-Akt virus led to the rescue of phosphorylated Akt level as well as in-



**Figure 5** Western blotting analysis for apoptotic mediators and activation of hepatic stellate cells and endothelial nitric oxide synthase level. **A:** Apoptotic mediators and activation of HSC; Liver tissue from each group of rats were homogenized and subjected to measurement for the levels of Akt, phosphorylated Akt (p-Akt), Fas, Caspase-9 and Caspase-3, and HSC activation markers, DR5 and  $\alpha$ -SMA, as designated in the figure. **B:** eNOS level; Liver tissues from each group of rats were homogenized and subjected to the measurements for Akt, pAkt, eNOS and phosphorylated eNOS (p-eNOS). Blotting of HA was used to confirm the expression of recombinant HA-Akt protein, and  $\beta$ -actin was used for the loading control. Data are representative of 3 experiments. HSC: Hepatic stellate cells; eNOS: Endothelial nitric oxide synthase.

hibition of apoptotic pathway in the liver of rat cirrhosis model. However, several issues remain to be addressed regarding the application of this virus for the treatment for cirrhosis. First, the efficacy of this form of virus in other type of cirrhosis, especially those chronically developed remains to be determined. In addition, regarding the development of the cirrhosis, it was thought that HSC activation plays a crucial role in producing collagen<sup>[1-5]</sup>. While we speculate that reduced HSC activation in Akt group may be a subsequent outcome of reduced hepatocyte apoptosis, it is unknown whether the introduction of Akt-virus has a direct effect on HSC activation. Furthermore, we observed that rats in the cirrhosis groups without treatment or received saline or Ad-EGFP treatment showed a noticeable reduction of phosphorylated Akt level, suggesting that the damage of hepatocytes following CCl<sub>4</sub> treatment may also be involved in the disruption of Akt signaling pathway. Lastly, one of the risks of the constitutive activation of Akt is the increased susceptibility to tumorigenesis. We could not conduct a long-term study to determine the time of virus clearance and evalu-

ate the risk of tumor development. As future directions, we will further determine the application of this virus in other forms of cirrhosis and assess its side effects.

## ACKNOWLEDGEMENTS

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## COMMENTS

### Background

Massive apoptosis of hepatocytes result in activation of hepatic stellate cells (HSC), which produce collagen and ultimately lead to the fibrosis and cirrhosis. Inhibiting hepatic apoptosis is considered as an important strategy to prevent cirrhosis. Akt plays a crucial role in preventing Fas signaling-mediated hepatic apoptosis, and that over-expression of Akt was capable of preventing hepatic apoptosis.

### Research frontiers

The progress in understanding mechanisms of cirrhosis brings the development of effective therapies closer to reality. Points of therapeutic intervention may include: (1) removing the injurious stimuli; (2) suppressing hepatic inflammation; (3) down-regulating stellate cell activation; and (4) promoting matrix degradation. The future prospects for effective treatment are more promising than ever for the millions of patients with chronic liver disease worldwide.

### Innovations and breakthroughs

To date, there have been a number of studies regarding the therapeutic implication of hepatic cirrhosis. However, the research about the strategy to block apoptotic signaling pathway are limited. In this study, the authors created an Ad-myr-HA-Akt virus and determined the efficacy of this virus in inhibiting the development of cirrhosis induced by CCl<sub>4</sub> in rats. The authors confirmed that that introduction of Ad-myr-HA-Akt virus was not only able to ameliorate the liver cirrhosis but also to reduce the portal vein pressure.

### Applications

These results could be the basis for further studies to understand the pathogenesis of hepatic cirrhosis. The conclusion suggest that Akt pathway may be a therapeutic target for human cirrhosis.

### Terminology

HSC, also known as perisinusoidal cells or Ito cells are pericytes found in the perisinusoidal space of the liver. Substantial evidence now exists to recognize HSCs as the main matrix-producing cells in the process of liver cirrhosis. Liver injury of any etiology will ultimately lead to activation of HSCs, which undergo transdifferentiation to fibrogenic myofibroblast-like cells.

### Peer review

Authors demonstrated that Akt improved liver histology and function and reduced portal venous pressure, liver apoptosis and collagen levels is useful for accurate understanding the progress of hepatic cirrhosis and also give a feasible target for the therapy of cirrhosis.

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## Insulin-like growth factor-1 induces lymphangiogenesis and facilitates lymphatic metastasis in colorectal cancer

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### Abstract

**AIM:** To investigate the expression of insulin-like growth factor-1 (IGF-1)/insulin-like growth factor-1 receptor (IGF-1R) in colorectal cancer (CRC) tissues and to analyze their correlation with lymphangiogenesis and lymphatic metastasis.

**METHODS:** Immunohistochemistry was used to evaluate IGF-1 and IGF-1R expression and lymphatic vessel density (LVD) in 40 CRC specimens. The correlation between IGF-1/IGF-1R and LVD was investigated. Effects of IGF-1 on migration and invasion of CRC cells were examined using transwell chamber assays. A LoVo cell xenograft model was established to further detect the role of IGF-1 in CRC lymphangiogenesis *in vivo*.

**RESULTS:** Elevated IGF-1 and IGF-1R expression in CRC tissues was correlated with lymph node metastasis ( $r = 0.715$  and  $0.569$ , respectively,  $P < 0.05$ ) and tumor TNM stage ( $r = 0.731$  and  $0.609$ ,  $P < 0.05$ ). A higher LVD was also found in CRC tissues and was correlated with lymphatic metastasis ( $r = 0.405$ ,  $P < 0.05$ ). A positive correlation was found between LVD and IGF-1R expression ( $r = 0.437$ ,  $P < 0.05$ ). Transwell assays revealed that IGF-1 increased the migration and invasion of CRC cells. *In vivo* mouse studies showed that IGF-1 also increased LVD in LoVo cell xenografts.

**CONCLUSION:** IGF-1/IGF-1R signaling induces tumor-associated lymphangiogenesis and contributes to lymphatic metastasis of CRC.

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**Key words:** Colorectal cancer; Insulin-like growth factor-1; Insulin-like growth factor-1 receptor; Lymphangiogenesis; Lymphatic metastasis

**Core tip:** Insulin-like growth factor-1 (IGF-1) and its receptor, insulin-like growth factor-1 receptor (IGF-1R), are frequently overexpressed in many types of tumors including colorectal cancer. A recent study (Björndahl *et al*, 2003) showed that both IGF-1 and IGF-2 could potentially stimulate lymphatic vessel growth in the mouse cornea. However, equivalent evidence on IGF-1 in solid tumors is lacking. Here, we show that IGF-1/IGF-1R signaling induces tumor-associated lymphangiogenesis *in vivo* and contributes to lymphatic metastasis of colorectal cancer. Findings from the present study provide further evidence to support the involvement of IGF-1/IGF-1R signaling in lymphangiogenesis in solid tumors.

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Zhou SZ. Insulin-like growth factor-1 induces lymphangiogenesis and facilitates lymphatic metastasis in colorectal cancer. *World J Gastroenterol* 2013; 19(43): 7788-7794 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7788.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7788>

## INTRODUCTION

Lymphatic metastasis is one of the most common metastatic routes of colorectal cancer (CRC). When cancer spreads, it is much harder to treat successfully. The presence or absence of lymph node involvement is one of the most important factors that determine the long-term outcome of cancer patients<sup>[1-3]</sup>. There is a complex process that a tumor cell must go through before metastasis can occur<sup>[4,5]</sup>. Excessive formation of new lymphatic vessels in CRC is a key step in metastatic progression<sup>[5,6]</sup>. However, the factors triggering lymphangiogenesis and the detailed molecular mechanisms are poorly understood.

Insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) are frequently overexpressed in many types of tumors including CRC<sup>[7-10]</sup>. Increasing evidence suggests that amplified IGF-1/IGF-1R signaling not only is associated with an increased relative risk for cancer development, but also contributes to cancer cell survival, invasion, metastasis and resistance to chemotherapeutic drugs<sup>[7,10]</sup>. In addition to their roles in the development and progression of cancer, the IGF-1/IGF-1R signaling system can also induce lymphangiogenesis<sup>[11,12]</sup>. In a recent study, Björndahl *et al.*<sup>[11]</sup> reported that both IGF-1 and IGF-2 could potently stimulate lymphatic vessel growth in the mouse cornea. However, direct evidence showing the involvement of IGF-1/IGF-1R signaling in lymphangiogenesis in solid tumors is lacking.

In the present study, we found that IGF-1 and IGF-1R were significantly overexpressed in CRC tissues compared with adjacent normal tissues using an immunohistochemical (IHC) assay. Using a lymphatic endothelial-specific antibody marker D2-40<sup>[3,13]</sup>, we found that lymphatic vessel density (LVD) was significantly higher in CRC tissues. The levels of IGF-1, IGF-1R and LVD were all significantly correlated with lymphatic metastasis. In addition, a positive correlation was found between LVD and IGF-1R. These results suggest that the IGF-1/IGF-1R axis might promote lymph node metastasis of CRC by induction of lymphangiogenesis. To further explore its role in lymphangiogenesis, we created a LoVo cell (a human colon cancer cell line) xenograft model and showed that IGF-1 treatment resulted in an increase in the LVD *in vivo*. Together, our findings demonstrate that IGF-1/IGF-1R signaling can induce lymphangiogenesis in CRC and may facilitate lymphatic metastasis in CRC patients.

## MATERIALS AND METHODS

### Tissue samples

Forty CRC and adjacent normal tissue samples were

obtained from randomly selected patients undergoing surgical resection without preoperative neoadjuvant chemoradiotherapy between January 2011 and June 2011 at Shaoxing People's Hospital. Their average age was 68.5 years (range, 44 to 83 years). Of these patients, 9 had well-differentiated adenocarcinoma, 20 had moderately differentiated adenocarcinoma, and 11 had poorly differentiated adenocarcinoma. Twenty-five patients had stage I-II disease and 15 had stage III-IV disease according to the tumor-node-metastasis (TNM) classification defined by Union for International Cancer Control (UICC)<sup>[14]</sup>. D2 radical resection was performed in 3 patients and D3 radical resection was performed in 37 patients. The number of lymph nodes resected was 9-22.

### Immunohistochemical staining

Tissue sections were stained using the Envision System (DakoCytomation, Carpinteria, CA, United States) according to manufacturer's instructions. Mouse monoclonal antibodies against human IGF-1, IGF-1R, or D2-40 were obtained from Abcam (Cambridge, United Kingdom). Intensity of immunostaining signals was evaluated in 8 fields under a light microscope (Olympus Optical, Tokyo, Japan). Statistical analysis was carried out in accordance with a previous study<sup>[15]</sup>. In short, total staining of IGF-1 and IGF-1R were scored as the product of the staining intensity (on a scale of 0-3: negative = 0, weak = 1, moderate = 2, strong = 3) and the percentage of cells stained (0 = 0%, 1 = 1%-25%, 2 = 26%-50%, 3 = 51%-100%), which resulted in a scale of 0-9. Two independent pathologists scored each sample without prior knowledge of the patient's clinical information and outcome.

### Cell culture and IGF-1 treatment

The human CRC cell line LoVo was obtained from the American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin in a 5% CO<sub>2</sub> incubator at 37 °C. For IGF-1 treatment, media were replaced with fresh serum-free medium containing 50 ng/mL IGF-1 (PeproTech) when cells were grown to 30% confluency. An IGF-1 stock solution was prepared in PBS, and thus, PBS in serum-free medium was used as a control.

### Migration and invasion analysis

The migration and invasion of CRC cells were examined using non-coated or matrigel-coated transwell culture chambers (8 µm pore size, Corning, NY, United States) 48 h after IGF-1 treatment. For migration assays, 1 × 10<sup>5</sup> cells were seeded in the top non-coated chamber and incubated at 37 °C for 8 h. For invasion assays, 5 × 10<sup>4</sup> cells were seeded in the top Matrigel-coated (BD Biosciences, Bedford, MA, United States) chambers and incubated at 37 °C for 24 h. In both assays, cells were suspended in serum-free Roswell Park Memorial Institute (RPMI)-1640 medium in the upper chamber, and the lower chamber was filled with RPMI-1640 medium containing 5% FBS. After incubation, the top chambers were wiped with cot-



**Table 1** Expression of insulin-like growth factor-1 and insulin-like growth factor-1 receptor in colorectal cancer tissues

	<i>n</i>	IGF-1	<i>P</i> value	IGF-1R	<i>P</i> value
Tissue type			0.001		0.002
Adjacent normal tissues	40	1.4 ± 0.6		1.3 ± 1.2	
Cancer tissue	40	4.5 ± 2.5		4.7 ± 2.7	
Differentiation			0.012		0.001
Well	9	3.0 ± 1.4		2.1 ± 0.9	
Moderately	20	4.3 ± 2.6		4.6 ± 2.4	
Poorly	11	6.1 ± 2.1		6.6 ± 2.1	
Lymph node metastasis			0.003		0.002
Yes	15	6.8 ± 2.0		6.3 ± 2.2	
No	25	3.1 ± 1.5		3.5 ± 2.2	

IGF-I: Insulin-like growth factor-1; IGF-I R: Insulin-like growth factor-1 receptor.

ton wool to remove the non-migratory or non-invasive cells. Cells on the underside of the membrane were fixed in methanol for 30 min, stained with 0.1% crystal violet, and counted under a microscope (Eclipse TS100, Nikon, Japan).

### Xenograft and IGF-1 treatment

Four-week-old BALB/c nude mice (18–22 g) were used in this study. LoVo cells in the logarithmic phase of growth were adjusted to a density of  $5 \times 10^7$ /mL, and 0.2 mL of cell suspension was subcutaneously injected into the flank of the mice. On the second day after LoVo cell transplantation, the mice were randomized into either a treatment or a control group, with ten mice in each group. Referring to previous reports<sup>[16–19]</sup>, IGF-1 (50 µg/kg) was administered every day by intraperitoneal injection in the treatment group. The mice in the control group received the same amount of normal saline. Three weeks after treatment, the cancer tissues were dissected from the nude mice for measurement of LVD.

### Measurement of LVD

LVD in tissue sections was quantitatively analyzed using the EnVision system with the specific lymphatic endothelial cell marker, D2-40, which allows for accurate discrimination of lymphatic vessels from blood vessels<sup>[3,13]</sup>. Five fields with the most abundant lymphatic regions (hot spots) were chosen by light microscopy at 40 × magnification. The LVD was then assessed by counting all stained vessels at 200 × magnification. Single immunoreactive endothelial cells or endothelial cell clusters separated from other microvessels were counted as a vessel according to previous procedures<sup>[20]</sup>. The average number of lymphatic vessels in five selected fields was taken as the LVD on the slide.

### Statistical analysis

All statistical analyses were performed using SPSS (version 12.0, SPSS Inc, United States). Student's *t* test test, Mann-Whitney *U* test and  $\chi^2$  test were used for statistical analyses. Spearman's rank correlation was used for corre-

lation analysis. *P* values < 0.05 were considered statistically significant.

## RESULTS

### Up-regulation of IGF-1 and IGF-1R in colorectal cancer

Immunohistochemistry analysis was performed to examine the expression of IGF-1 and IGF-1R in CRC samples. IGF-1 and IGF-1R were weakly or negatively stained in tumor-adjacent normal tissues. In contrast, they were weakly or moderately stained in well-differentiated CRC tissues, and moderately or strongly stained in moderately and poorly differentiated CRC tissues (Table 1 and Figure 1). As expected, expression of IGF-1 was detected primarily in the cytoplasm and IGF-1R was detected on the membrane (Figure 1). Furthermore, the expression of IGF-1 and IGF-1R was correlated with lymph node metastasis ( $r = 0.715$  and  $0.569$ , respectively;  $P < 0.05$ ) and TNM stage ( $r = 0.731$  and  $0.609$ , respectively;  $P < 0.05$ ). These results indicate that elevated expression of IGF-1/IGF1R parallels CRC progression.

### Correlation between LVD and expression of IGF-1/IGF-1R

It was reported that LVD is an indicator of lymphangiogenesis<sup>[3,21]</sup>. D2-40 is a specific lymphatic endothelial cell marker for the evaluation of LVD in human cancers<sup>[3,13]</sup>. Using IHC, we found that D2-40 was localized in the cytoplasm and membrane of lymphatic endothelial cells (Figure 2). LVD was significantly higher in CRC tissue as compared to non-tumor tissue ( $11.45 \pm 3.03$  vs  $3.25 \pm 1.28$ ,  $P < 0.05$ ). LVD in tumor tissues with lymph node metastasis was higher than that in tissues without metastasis ( $12.67 \pm 4.09$  vs  $10.72 \pm 1.90$ ,  $P < 0.05$ ) (Table 2 and Figure 2). Lymphatic vessel invasion was also detected in CRC tissue (Figure 2B and C). There was a significant correlation between LVD and lymphatic metastasis ( $R = 0.405$ ,  $P < 0.05$ ). In addition, a statistically significant correlation was also found between LVD and IGF-1R ( $R = 0.437$ ,  $P < 0.05$ ). These results suggest that the IGF-1/IGF-1R system might promote lymph node metastasis of CRC through induction of lymphangiogenesis.

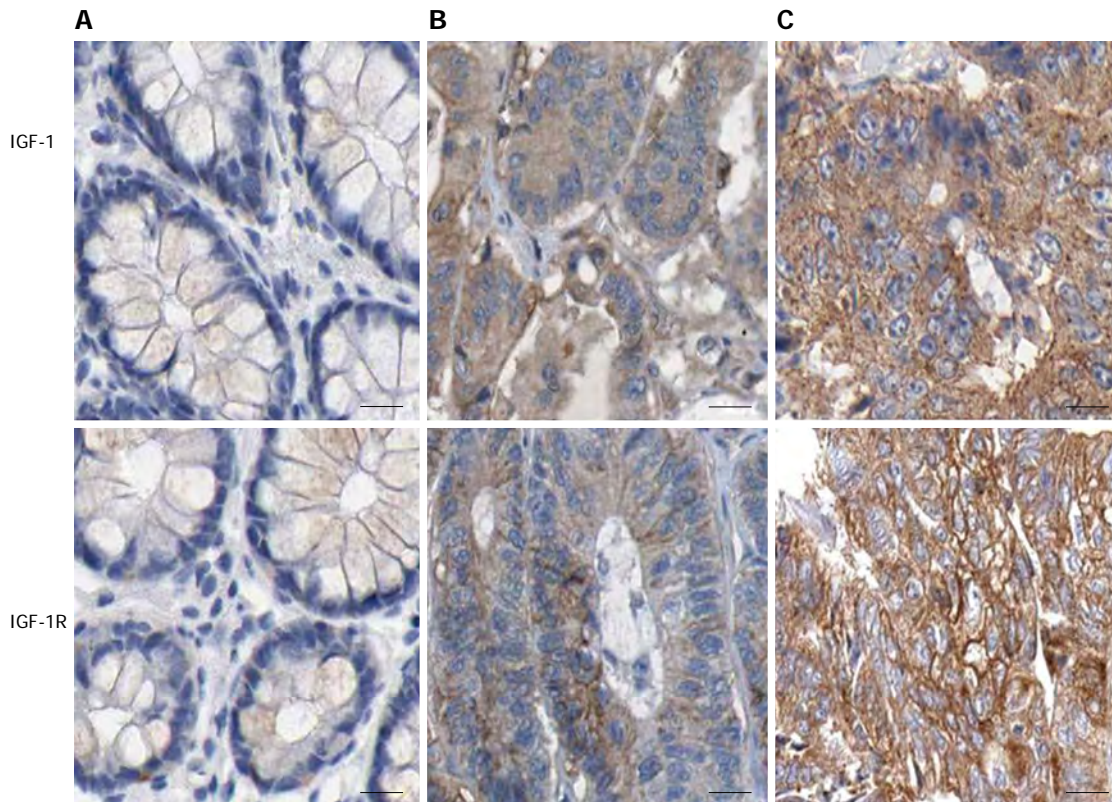
### IGF-1 increases migration and invasion of LoVo cells

In transwell migration assays, a significantly higher number of cells migrated through the chamber membrane after IGF-1 treatment ( $196.4 \pm 21.6$  vs  $96.4 \pm 11.2$ ,  $P < 0.05$ , Figure 3A). Similar results were obtained in the transwell invasion assays. Cells which migrated through the matrigel and chamber membrane increased in the IGF-1-treated group ( $163.6 \pm 19.4$  vs  $72.5 \pm 9.1$ ,  $P < 0.05$ , Figure 3B). These results indicated that IGF-1 increased the migration and invasion potential of CRC cells.

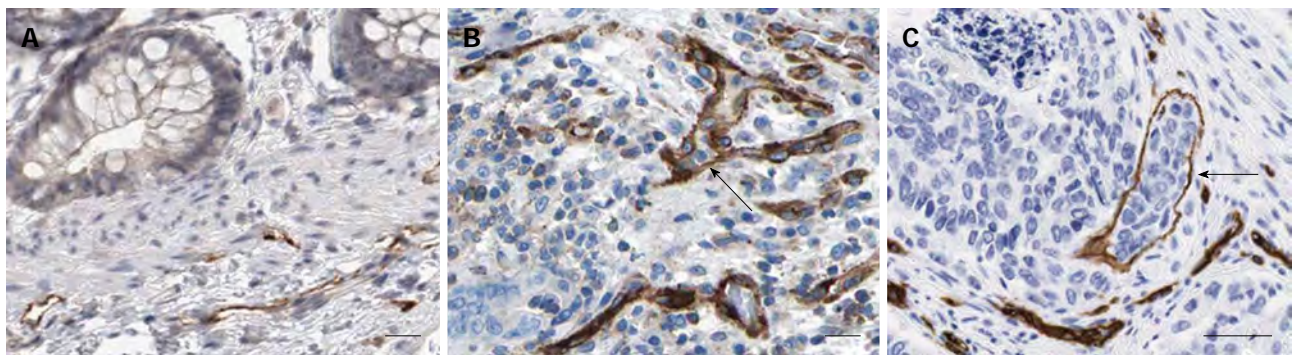
### Effects of IGF-1 on LVD of LoVo cell xenografts in nude mice

To assess whether IGF-1 affected lymphangiogenesis in CRC *in vivo*, LoVo cells were implanted subcutaneously





**Figure 1** Immunohistochemical staining of insulin-like growth factor-1 and insulin-like growth factor-1 receptor in colorectal cancer tissues. A: Tumor-adjacent normal tissues; B: Moderately differentiated CRC and C, poorly differentiated CRC ( $\times 200$ ). IGF-1: Insulin-like growth factor-1. IGF-1 R: Insulin-like growth factor-1 receptor; CRC: Colorectal cancer.



**Figure 2** Morphological features of D2-40 positive lymphatic vessels in colorectal cancer. A: Tumor-adjacent normal tissues; B and C: Colorectal cancer tissues ( $\times 200$ ). Lymphatic vessel invasion of tumor cells was also detected in B and C (black arrow). IGF-1: Insulin-like growth factor-1.

**Table 2** Lymphatic vessel density in colorectal cancer tissues

	<i>n</i>	LVD	<i>P</i> value
Tissue type			0.001
Adjacent normal tissues	40	$3.3 \pm 1.3$	
Cancer tissue	40	$11.5 \pm 3.0$	
Lymph node metastasis			0.032
Yes	15	$12.7 \pm 4.1$	
No	25	$10.7 \pm 3.9$	

LVD: Lymphatic vessel density.

into nude mice. Three weeks after daily IGF-1 or vehicle administration, tumor tissues were removed and measured. IHC analysis using anti-D2-40 antibodies revealed that LVD of LoVo cell xenografts was significantly higher in the IGF-1 group than in the control group ( $10.7 \pm 3.3$  vs  $6.4 \pm 2.9$ ,  $P < 0.05$ , Figure 4).

## DISCUSSION

The importance of lymph node metastasis in cancer pro-



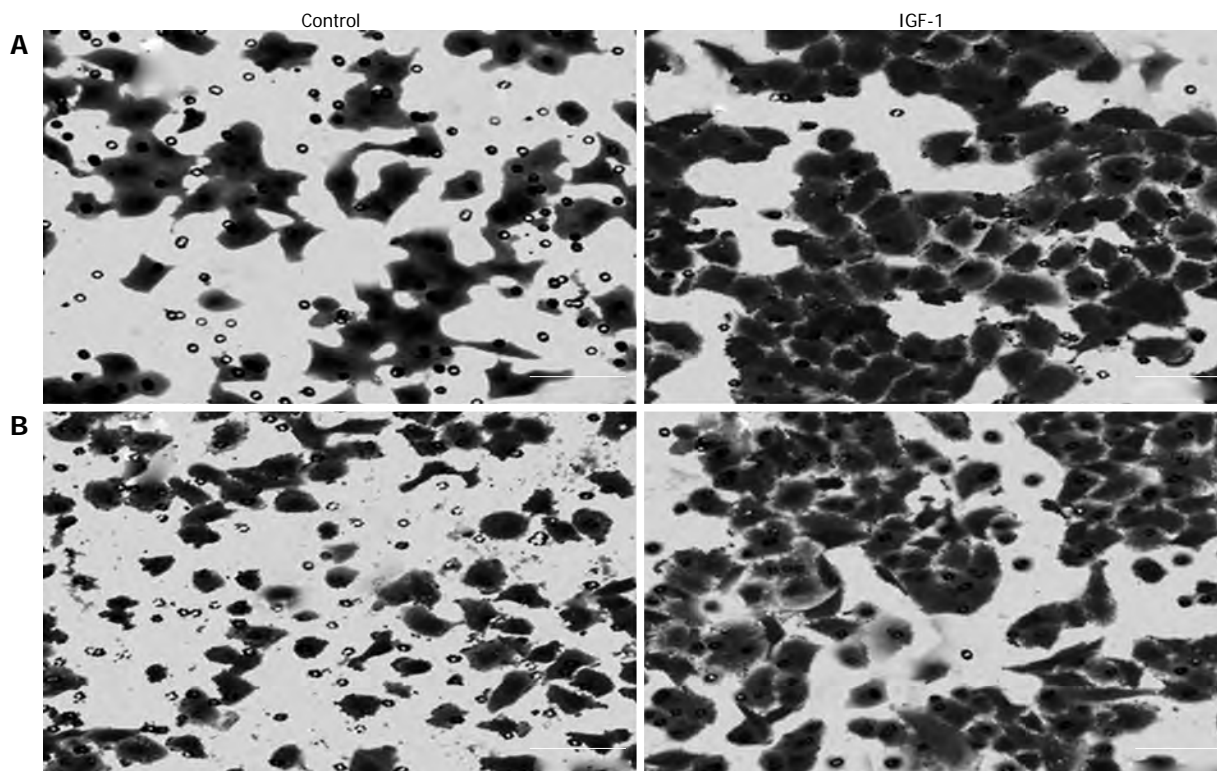


Figure 3 Effect of insulin-like growth factor-1 on the migration and invasion of LoVo cells after 48 h treatment. A: Cell migration; B: Cell invasion ( $\times 200$ ). IGF-1: Insulin-like growth factor-1.

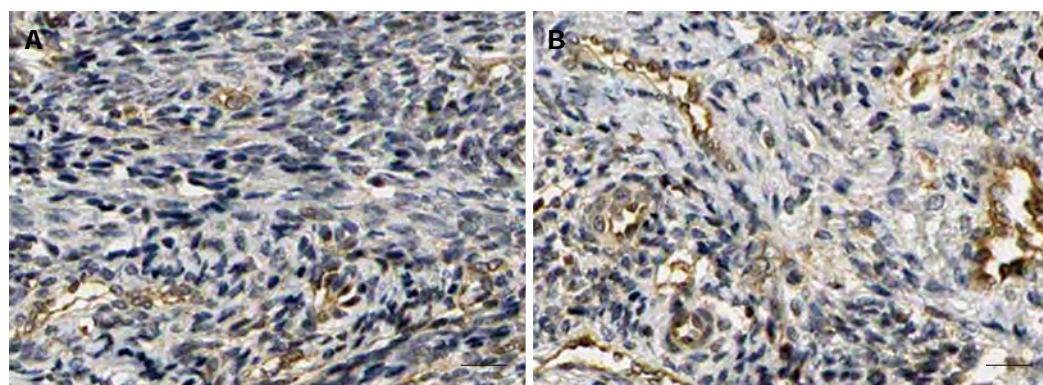


Figure 4 Effect of insulin-like growth factor-1 on lymphatic vessel density in LoVo cell xenografts in nude mice after treatment with insulin-like growth factor-1. A: Control group; B: 50  $\mu\text{g/kg}$  IGF-1 group ( $\times 200$ ). IGF-1: Insulin-like growth factor-1; IGF-1 R: Insulin-like growth factor-1 receptor.

gression has been well established and is considered one of the most important prognostic factors<sup>[1,2,22,23]</sup>. Recently, there is growing evidence that tumor lymphangiogenesis plays an important role in this process<sup>[5,6]</sup>. However, the detailed molecular mechanisms that regulate lymphangiogenesis remain largely unknown. In the present study, we have shown that IGF-1/IGF-1R signaling can induce tumor-associated lymphangiogenesis, and therefore, may facilitate further lymphatic metastasis of CRC.

IGF-1 and its receptor IGF-1R are frequently expressed in many solid tumors and have been implicated in cancer metastasis<sup>[7,24-26]</sup>. In 1999, Hakam *et al*<sup>[27]</sup> showed that IGF1-R plays a role in the evolution of colorectal adenoma to carcinoma and favors the metastasis of CRC.

In a recent study, Wu *et al*<sup>[28]</sup> reported that IGF-1 is critical in activating and sustaining an inflammatory response in the liver which is needed for CRC hepatic metastasis. In the present study, we found that IGF-1 and IGF-1R protein expression was elevated in CRC tissues, and their expression was correlated with clinical stage and lymphatic metastasis, which is consistent with the reported data.

Lymphangiogenesis, the formation of new lymphatic vessels, is a key process in lymphatic invasion and metastasis<sup>[29]</sup>. Numerous studies have shown that LVD is an indicator of lymphangiogenesis and represents a tool to determine the metastatic risk of neoplasia<sup>[3,21,29]</sup>. A study by Cacchi *et al*<sup>[1]</sup> revealed that CRC could induce lymphangiogenesis and a higher LVD could increase the

capability of cancer cells to invade the lymphatic system. In another study, Matsumoto *et al.*<sup>[30]</sup> reported that both LVD and lymphatic vessel invasion were related to an adverse outcome in CRC. Similar results were obtained in this study. In addition, lymphatic vessel invasion was also detected in CRC tissue by IHC staining. Moreover, a statistically significant correlation was found between LVD and the level of IGF-1R in CRC tissue. This suggests that IGF-1/IGF-1R signaling probably facilitates metastasis of CRC by inducing tumor-associated lymphangiogenesis.

Recently, a number of lymphangiogenic factors which stimulate the proliferation of lymphatic vessels and lymphangiogenesis have been identified, including vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, fibroblast growth factor 2, and platelet-derived growth factors<sup>[11,31]</sup>. Most of these lymphangiogenic factors were shown to be directly or indirectly regulated by IGF-1<sup>[11,12,32]</sup>. These findings prompted us to hypothesize that IGF-1/IGF-1R signaling promotes lymphangiogenesis. In 2005, Björndahl *et al.*<sup>[11]</sup> reported that both IGF-1 and IGF-2 could stimulate lymphatic vessel growth in the mouse cornea. However, to date, it is still unclear whether a similar correlation exists in solid tumors. Here, we showed that IGF-1 could also induce lymphangiogenesis in CRC. The spread of metastasis then occurs through the new lymphatic vessel system in the tumor.

Tumor lymphangiogenesis and metastasis to lymph nodes are a complex process and a number of growth factors are involved in these events. IGFs are particularly interesting regulators due to their multiple roles in promoting tumor growth<sup>[33-35]</sup>. Here, the *in vivo* studies provided direct evidence that the IGF-1/IGF-1R axis can also induce lymphangiogenesis in solid tumors. However, so far, it is not known whether the IGF-1/IGF-1R axis directly acts on lymphatic endothelial cells to induce lymphangiogenesis or indirectly *via* other growth factor/receptor systems. In addition, the detailed mechanisms underlying lymphangiogenesis associated with the IGF-1/IGF-1R system are largely unknown. Therefore, additional efforts are warranted to study the relationship between IGF-1/IGF-1R and lymphangiogenesis in cancer.

In summary, we found that the IGF-1/IGF-1R system can induce tumor-associated lymphangiogenesis and facilitate lymphatic metastasis of CRC. Findings from the present study provide further evidence supporting the involvement of IGF-1/IGF-1R signaling in lymphangiogenesis in solid tumors. In addition, we also found that increased expression of IGF-1 and IGF-1R is correlated with tumor differentiation in human CRC. Our findings and others suggest that the IGF-1/IGF-1R axis plays critical and diverse roles in promoting tumor progression. Taken together, the IGF-1/IGF-1R axis is a potentially useful target for the treatment of cancer and metastasis.

growth factor-1 (IGF-1) and insulin-like growth factor-1 receptor (IGF-1R) are frequently overexpressed in many types of tumors including colorectal cancer. In addition to their role in the development and progression of cancer, the IGF-1/IGF-1R signaling system can also induce lymphangiogenesis.

### Research frontiers

A recent study (Björndahl *et al.*, 2003) showed that both IGF-1 and IGF-2 potentially stimulated lymphatic vessel growth in the mouse cornea. However, equivalent evidence in solid tumors is lacking.

### Innovations and breakthroughs

The authors of this paper demonstrate that the IGF-1/IGF-1R system can induce tumor-associated lymphangiogenesis in colorectal cancer (CRC) and contributes to its lymphatic metastasis, thus providing a novel mechanism for lymphangiogenesis in solid tumors.

### Applications

The findings of this study are of value in further explaining the molecular mechanisms of lymphangiogenesis in CRC. The IGF-1/IGF-1R axis may be a potential target for the treatment of cancer and metastasis.

### Terminology

IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in child growth and continues to have anabolic effects in adults. The role of IGF-1 in promoting cancer has been investigated for many years. Increasing evidence suggests that IGF-1 not only is associated with an increased relative risk for the development of cancer, but also contributes to cancer cell survival, invasion, metastasis and resistance to chemotherapeutic drugs.

### Peer review

The authors examined the expression of IGF-1/IGF-1R in CRC tissues and its correlation with lymphangiogenesis and lymphatic metastasis. The study revealed that IGF-1/IGF-1R signaling system induces lymphangiogenesis in CRC and contributes to lymphatic metastasis of CRC. The results are interesting and provide further evidence that IGF-1/IGF-1R signaling is involved in lymphangiogenesis in solid tumors.

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## COMMENTS

### Background

Lymphangiogenesis plays an important role in lymphatic metastasis. Insulin-like



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## Identification of Annexin A1 protein expression in human gastric adenocarcinoma using proteomics and tissue microarray

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### Abstract

**AIM:** To study the differential expression of Annexin A1 (ANXA1) protein in human gastric adenocarcinoma. This study was also designed to analyze the relationship between ANXA1 expression and the clinicopathological parameters of gastric carcinoma.

**METHODS:** Purified gastric adenocarcinoma cells (GAC) and normal gastric epithelial cells (NGEC) were obtained from 15 patients with gastric cancer by laser capture microdissection. All of the peptide specimens were labeled as  $^{18}\text{O}/^{16}\text{O}$  after trypsin digestion. Differential protein expressions were quantitatively identified between GAC and NGEC by nanoliter-reverse-phase liquid chromatography-mass/mass spectrometry (nano-RPLC-MS/MS). The expressions of ANXA1 in GAC and NGEC were verified by western blot analysis. The tissue microarray containing the expressed ANXA1 in 75 pairs of gastric carcinoma and paracarcinoma specimens was detected by immunohistochemistry (IHC). The relationship between ANXA1 expression and clinicopathological parameters of gastric carcinoma was analyzed.

**RESULTS:** A total of 78 differential proteins were identified. Western blotting revealed that ANXA1 expression was significantly upregulated in GAC ( $2.17/1$ ,  $P < 0.01$ ). IHC results showed the correlations between ANXA1 protein expression and the clinicopathological parameters, including invasive depth (T stage), lymph node metastasis (N stage), distant metastasis (M stage) and tumour-lymph node metastasis stage ( $P < 0.01$ ). However, the correlations between ANXA1 protein expression and the remaining clinicopathological parameters, including sex, age, histological differentiation and the size of tumour were not found ( $P > 0.05$ ).

**CONCLUSION:** The upregulated ANXA1 expression may be associated with carcinogenesis, progression, invasion and metastasis of GAC. This protein could be considered as a biomarker of clinical prognostic prediction and targeted therapy of GAC.

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**Key words:** Gastric cancer; Annexin A1 protein; Pro-

teomics; Tissue microarray; Immunohistochemistry

**Core tip:** The anti-inflammatory protein Annexin A1 (ANXA1) mediates various important physiological and pathophysiological processes. Evidence has shown that ANXA1 is related to the development and progression of human multi-tumours. However, the ANXA1 expression in gastric adenocarcinoma of Chinese patients and the relationship between this protein and its clinicopathological parameters remain unclear. In the present study, the ANXA1 expression in gastric adenocarcinoma of Chinese patients was investigated by proteomics and western blot analysis. Authors examined 75 pairs of gastric adenocarcinoma and paracarcinoma tissues by tissue microarray to determine the presence of ANXA1 by immunohistochemistry. They found that ANXA1 expression was upregulated and involved in human gastric adenocarcinoma invasion and metastasis. Our findings suggested that ANXA1 might be used as a valuable biomarker in clinical diagnosis, prognostic prediction and targeted therapy of gastric cancer.

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## INTRODUCTION

Gastric cancer (GC) is a common digestive tract cancer because of the lack of early diagnosis strategies; a previous study revealed that GC cases are usually diagnosed when the disease is at an advanced stage<sup>[1]</sup>. On the basis of metastasis, recurrence and other causes, the treatment and prognosis of GC remain poor. Therefore, effective biomarkers of GC should be determined; the mechanism of incidence and development should also be investigated to promote early diagnosis, effective treatment and prognosis improvement of GC.

Annexin A1 (ANXA1) is a key member of the A subfamily and belongs to the multi-gene family of Annexins. ANXA1 exhibits calcium-mediated phospholipid binding properties and participates in many physiological and pathological processes. Further studies have shown that ANXA1 is abnormally expressed in various tumours. This abnormal expression is closely related to tumorigenesis, development, invasion and metastasis of human tumours. The expression of ANXA1 in tumours is tissue specific; for instance, a low ANXA1 expression is observed in oesophageal squamous cell carcinoma<sup>[2]</sup>, whereas a high expression is found in colorectal cancer<sup>[3]</sup>. The relationship between ANXA1 expression in GC and GC invasion as well as metastasis remains unclear. ANXA1 also exhibits a low expression in GC and negatively correlates with invasion and metastasis<sup>[4]</sup>. However,

other studies have revealed opposite results<sup>[5,6]</sup>.

We performed laser capture microdissection (LCM) to investigate the relation of ANXA1 expression in GC to the clinical parameters and to obtain purified gastric adenocarcinoma cells (GAC) and normal gastric epithelial cells (NGEC). <sup>18</sup>O/<sup>16</sup>O was used to label the digested peptides in the mixture of GAC and NGEC. Nanoliter-reverse-phase liquid chromatography-mass/mass spectrometry (nano-RPLC-MS/MS) was performed to identify and quantify the differentially expressed proteins. Nano-RPLC-MS/MS was also conducted to validate the results of proteomics. To verify the differential protein expression of ANXA1, we performed western blot. Immunohistochemistry (IHC) was performed to detect the expression of ANXA1 in 75 pairs of tissue microarray of GC tissues and paracarcinoma tissues. This study aimed to analyze the correlations of ANXA1 with clinical pathological parameters, including age, gender, differentiation degree, metastasis, invasion depth, tumour-lymph node metastasis (TNM) staging and tumour size (maximum diameter). This study also investigated the relations and possible mechanisms of the expression differences of ANXA1 protein in carcinogenesis, progression and prognosis of GC.

## MATERIALS AND METHODS

### Specimen collection and handling

Fifteen cases of GAC and paired gastric mucosa tissues were obtained from the First Affiliated Hospital of Xinjiang Medical University from June 2009 to October 2009 and used as the surgical resection specimens. Six female and nine male subjects aged 40-81 years (mean age of 56 years) and classified in TNM stages I to IV participated in this study. GC and paragastric mucosa tissues (located away from the primary tumour > 5 cm) with a size of approximately 1.0 cm<sup>2</sup> were obtained within 30 min of surgical resection. The tissues were washed immediately and repeatedly with normal saline to remove blood and other tissues. Afterwards, these tissues were stored at -80 °C in a refrigerator for future proteomics and western blot analysis. Informed consent was obtained from the patients to allow the collection and use of the samples. The procedures were also reviewed and approved by Xinjiang Medical Ethics Committee.

### LCM for target cells and protein sample preparation

Frozen sections of GAC and paragastric mucosa tissues (8-10 μm) were prepared, affixed on LCM-specific film slides, fixed with 75% ethanol and stained with methyl green dye (Sigma-Aldrich, United States). The LCM system (Leica AS, Germany) was manipulated to determine GAC and NGEC. Tissue cell lysate was added and the total proteins of the purified GAC and NGEC were extracted. 2D Quant Kit (Amersham Biosciences, Sweden) was used to determine the protein concentration. We performed 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to pre-separate the total proteins of cleaved GAC and NGEC with a sample

loading volume of 100 µg. The gel was then stained with Coomassie R-250. The parallel gel bands were cut to obtain 36 pairs of protein gel bands. The obtained bands were washed, bleached, dehydrated, reduced with dithiothreitol (Amersham Biosciences, Sweden), alkylated with IAA and vacuum dried by centrifugation. Trypsin (Promega Corporation, United States) was added to initiate digestion. The peptides were extracted, mixed and dried. Afterwards,  $^{18}\text{O}/^{16}\text{O}$  notation was performed by adding 8 µL of  $\text{H}_2^{18}\text{O}$  (or  $\text{H}_2^{16}\text{O}$ ; Huayi Isotope Corporation, Jiangsu) and 2 µL of acetonitrile (Sigma-Aldrich Inc., United States) to the mixed polypeptides, which also contained immobilized trypsin (Pierce, United States). The resulting mixture was then incubated at 37 °C for 24 h. At the end of the labelling reaction, 1 µL of formic acid was added to terminate the reaction.

### Protein identification and mass data analysis

The mobile phase solution A (0.1% formic acid in water) was added into Eppendorf tubes containing different peptide mixtures, shaken and centrifuged. The supernatant was extracted and added into a tapered bottom vial in the Ultimate FAMOS LC system autosampler. Isolation was performed using nano-RPLC-MS/MS instrument, in which an auxiliary pump was used to load the samples with mobile phase solution A at a flow rate of 20 µL/min for 10 min. The samples were then loaded in a pre-column and desalted. The pre-column was switched and connected to a capillary analytical column for gradient elution using the solvent gradient as follows: solution B [water/acetonitrile (v/v) containing 0.1% formic acid], 5% B, 0-10 min; 5%-90% B, 55 min; 90% B, 5 min; and 90%-0% B, 10 min. After equilibrium was reached for 15 min, separation was performed. Nanoliter analytical column flow rate was approximately 250 nL/min; therefore, the eluent could be placed directly in the Electrospray ionization ion Q-TOF mass spectrometer (Micromass Corporation, United Kingdom) for analysis. The standard peptide Glu-Fibrino peptide B was used as an external calibration standard of the mass spectrometer. The mass data provided peaklist files in Masslynx 4.0 software of the local Mascot 2.0 IPI database to search the protein database and identify the proteins. Quantitative analysis was performed in Masslynx according to the following procedures: the MS spectra containing the peptides used for quantification were obtained from the total ion chromatograph to integrate and form the spectrum of quantitative analysis;  $^{16}\text{O}/^{18}\text{O}$  ratio was then calculated according to Eq. (1)<sup>[7]</sup>.

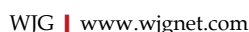
### Detection of differential ANXA1 expression levels by Western blot

Fifteen pairs of microdissected and purified GAC and NGEAC were added to the tissue lysate pre-cooled at 4 °C, vortexed and cleared in an ice bath for 30 min. Afterwards, the samples were centrifuged at 12000 r/min and 4 °C for 30 min. The supernatant (the total cellular protein) was then transferred to a new tube. The protein con-

centration was determined using Bradford method and the total protein was determined by separation *via* 10% PAGE at 100 V for approximately 2 h (loading volume of 40 µg). The protein was electronically transferred to a Polyvinylidene difluoride membrane. Rabbit anti-human ANXA1 antibody (1/500) was added and incubated at 4 °C overnight. HRP-labeled goat anti-mouse secondary antibody (1/2000) incubated at room temperature for 2 h was also added. Enhanced chemiluminescence reagent lightening, developing and fixing were conducted. The obtained images were scanned to calculate the relative expression levels of the differential proteins in Quantity One software.

### Immunohistochemical detection of the tissue microarray of ANXA1 expression

A total of 75 pairs of human GC tissue microarray (Shanghai Outdo Biotech Co., Ltd., China), including paired GC tissues and paraGC tissues were obtained from 50 males and 25 females aged 30-84 years (average age of 63.6 years). Among these subjects, 12 cases were in Phase I, 25 cases in Phase II, 32 cases in Phase III and 4 cases in Phase IV stage cases (according to the TNM classification Standard, 7<sup>th</sup> edition, developed by the International Union Against Cancer in 2009). The clinical pathological data were complete: the cases showing tumours that invaded the submucosa, muscularis, serosa and serosa were 6, 13, 46 and 10, respectively. A total of 34 cases showed high amounts of moderately differentiated adenocarcinoma and 41 cases showed low amounts of undifferentiated adenocarcinoma. No lymph node metastasis was observed in 30 cases, but lymph node metastasis was present in 45 cases. Distant metastasis was absent in 69 cases, but 6 patients exhibited distant metastasis. According to SP method and the manufacturer's instructions, tissue microarrays were subjected to conventional dewaxing hydration and retrieved using citrate antigen. Afterwards, 3%  $\text{H}_2\text{O}_2$ -formaldehyde was used to block endogenous peroxidase. ANXA1 primary antibody (1/100) was added and incubated at 4 °C overnight. The biolabelled secondary antibody and streptavidin-peroxidase solution were added. Each sample was washed with PBS and incubated at room temperature. The sample was then stained with DAB staining, restained with haematoxylin and eosin, dehydrated with graded alcohol and mounted using neutral gum. The primary antibody was replaced with PBS as the negative control sample; the known positive reaction chip was used as the positive control sample. IHC staining score was based on Formowitz comprehensive scoring method<sup>[8]</sup> and determined according to the staining intensity and percentage of positive cells in each section. Staining intensity was scored as follows: no staining, 0; pale yellow, 1; brownish-yellow, 2; and tan, 3. At least 10 high-power fields ( $\times 200$ ) were randomly selected for each point and at least 1000 cells were counted. Among the total number of cells, the following percentages were obtained: 5% positively-stained cells scored as 0; 5%-25% scored as 1; 26%-50% scored as 2; 51%-75% scored as 3;



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## RESULTS

The total proteins of highly homogeneous GAC and NGEC (> 90%) purified by LCM were determined to



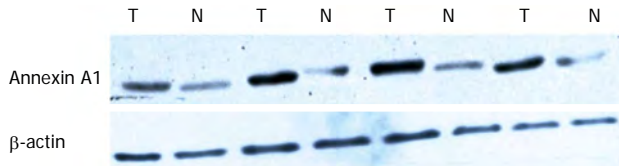


Figure 2 Expression of Annexin A1 protein in gastric adenocarcinoma cells and normal gastric epithelial cells by western blot analysis. T: Gastric adenocarcinoma cells; N: Normal gastric epithelial cells.

Table 1 Expression of annexin A1 protein in normal gastric epithelial cells and gastric adenocarcinoma cells

Group	n	Annexin A1 expression
NGEC	15	0.49 ± 0.082
GAC	15	1.06 ± 0.068 <sup>b</sup>

<sup>b</sup>*P* < 0.01 vs NGEC. GAC: Gastric adenocarcinoma cells; NGEC: Normal gastric epithelial cells.

separate by 1D SDS-PAGE, gel digestion, extraction and <sup>18</sup>O labelling. These proteins were then identified by nano-RPLC-MS/MS. According to the standard differential proteins (where 2 < <sup>18</sup>O/<sup>16</sup>O ratio < 0.5), a total of 78 differential proteins were identified, in which the expressions of 42 proteins, including ANXA1, ANXA2, ANXA4, Protein S100-A9, and HSP 90-α2 were up-regulated in GC. By contrast, the expressions of 36 proteins were downregulated in GC, including RKIP, ADP/ATP translocase 2 and L-lactate dehydrogenase B. These differentially expressed proteins function as metabolic enzymes, enzyme proteins, cytoskeletal proteins, and signal transduction proteins; other proteins also exhibit unknown functions, in which ANXA1 expression was 2.17 times higher in GAC than in NGEC (Figure 1).

#### Western blotting analysis identification of the differential expression of ANXA1

β-actin was used as the internal standard in western blot and the maximum gray value of the strip was set as 1. The other gray value was divided by the maximum gray value, and the obtained ratio corresponded to the relative protein expression level. The results showed that ANXA1 was upregulated at a higher extent in GAC than in NGEC (*P* < 0.01). The quantitative relationships calculated from the grayscale analysis were the same as the proteomic analysis results (Figure 2, Table 1).

#### ANXA1 protein expression in tissue microarray of GC and para-tissues

IHC was performed to detect 150 points of the tissue microarray in human GC. The results showed that ANXA1 protein was positively expressed mainly in the cytoplasm and the nuclei of GC tissues and paratissues (normal mucosa). Positive expression was also observed in the stroma. ANXA1 protein was highly expressed in GC. The negative, weakly positive, moderately positive and strongly positive expression rates in GC and para-

Table 2 Annexin A1 expression in normal gastric mucosa and gastric carcinoma by immunohistochemistry n (%)

Tissue type	n	Annexin A1 cases					W	P value
		-	+	++	+++	++++		
NGEC	75	37 (49.3)	25 (33.3)	13 (17.3)	0 (0)		4599	0
GAC	75	23 (30.7)	11 (14.7)	26 (34.7)	15 (20.0)			

*P* < 0.01 vs normal gastric mucosa group (NGEC). GAC: Gastric adenocarcinoma cells.

tissues were: 30.7%, 14.7%, 34.7% and 20.0% vs 49.3%, 33.3%, 17.3% and 0%, respectively (*w* = 4599.0, *P* < 0.01; Table 2) The ANXA1 expression was significantly related to the invasion depth, lymph node metastasis, distant metastasis and TNM staging (*P* < 0.01). By contrast, ANXA1 expression was not significantly related to age, gender, histological grade and tumour size (*P* > 0.05; Figure 3, Table 3).

## DISCUSSION

The mortality rate of GC ranks second among the mortality rates of malignant tumours and approximately 75 million people die of stomach cancer worldwide yearly<sup>[9]</sup>. The five-year survival rate of GC is only 20%-30%, and the five-year survival rate of radical resection of early GC can reach 90%-95%<sup>[10]</sup>. Molecular markers are considered as one of the most sensitive and effective indicators of tumour diagnosis, recurrence, metastasis and prognosis prediction. Therefore, the molecular markers closely associated with the development of GC and the relative pathogenesis should be determined in the early diagnosis of GC to improve treatment outcomes. Proteomics technology has an important function in the identification of tumour-related protein species in cancer development as well as in the discovery of tumour molecular markers and therapeutic targets. The present study employed simple LCM to solve problems about tumour proteomics heterogeneity, in which the purified cells exhibiting > 90% homogeneity were rapidly obtained. Using the advanced <sup>18</sup>O stable isotope labelling/MS quantitative proteomics technique, we set the normal gastric mucosa and GC tissue of humans as the targets in this study to screen and identify the differentially expressed proteins. A total of 78 differentially expressed proteins were found and 42 of such proteins were highly expressed in GC cell compared with NGEC. By contrast, 36 differentially expressed proteins were found at lower concentrations. These results provided information about differentially expressed proteins and the mechanism of GC carcinogenesis, development and screening of molecular markers.

During the screening of the identified differential proteins, ANXA1 was significantly upregulated in GC. ANXA1 is involved in various physiological and pathological processes, such as cell signal transduction, cell proliferation, differentiation and apoptosis as well as inflammation and immune response. Previous studies found that ANXA1 is upregulated in breast cancer<sup>[11]</sup>,

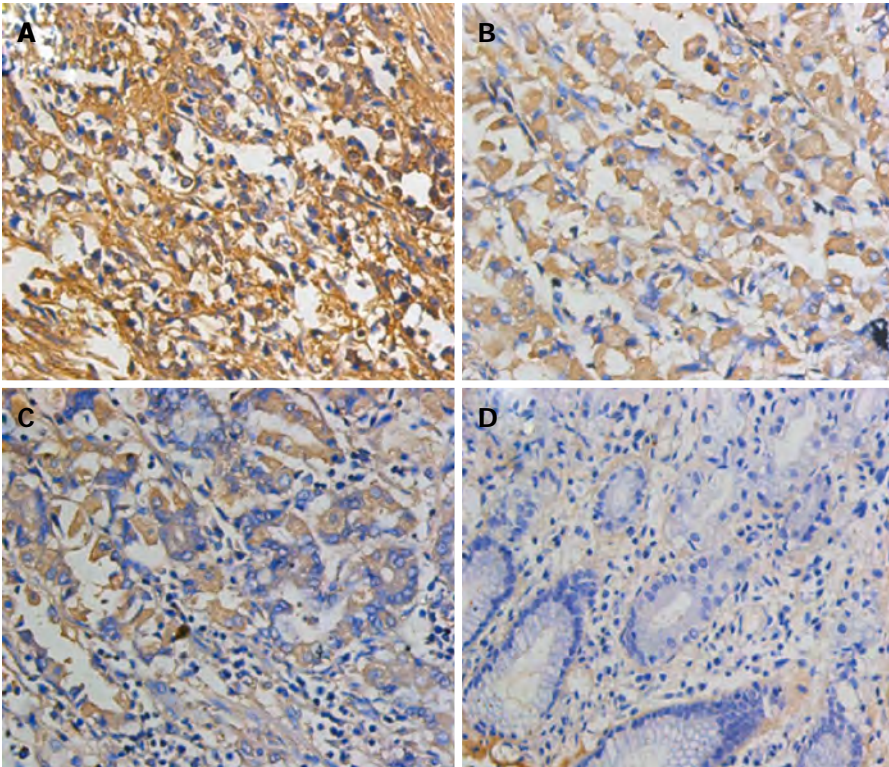


Figure 3 Representative immunohistochemical staining patterns of Annexin A1 expression in gastric carcinoma and normal gastric mucosa. A: Strongly positive staining; B: Moderately positive staining; C: Weakly positive staining; D: Negative staining (immunohistochemical staining × 400).

Table 3 Relationship between annexin A1 protein expression and clinicopathological parameters of gastric carcinoma <i>n</i> (%)							
Parametes	<i>n</i>	ANXA1 cases				Wilcoxon	<i>P</i> value
		-	+	++	+++		
Sex						1039.5	0.290
Male	50	15 (30.0)	7 (14.0)	17 (34.0)	11 (22.0)		
Female	25	8 (32.0)	4 (16.0)	9 (36.0)	4 (16.0)		
Age (yr)						840.0	0.394
≤ 60	24	7 (29.2)	5 (20.8)	10 (41.7)	2 (8.3)		
> 60	51	16 (31.4)	6 (11.7)	16 (31.4)	13 (25.5)		
Histological differentiation						1350.0	0.520
High	34	10 (29.4)	4 (11.8)	12 (35.3)	8 (23.5)		
Moderate/poor	41	13 (31.7)	7 (17.1)	14 (34.1)	7 (17.1)		
Invasive depth						514.5	0.008
T1-2	19	10 (52.6)	4 (21.1)	3 (15.8)	2 (10.5)		
T3-4	56	13 (23.2)	7 (12.5)	23 (41.1)	13 (23.2)		
N stage						929.0	0.017
N0	30	12 (40.0)	6 (20.0)	10 (33.3)	2 (6.7)		
N1-3	45	11 (24.4)	5 (11.1)	16 (35.6)	13 (28.9)		
M stage						348.5	0.017
M0	69	23 (33.3)	10 (14.5)	25 (36.2)	11 (15.9)		
M1	6	0 (0)	1 (16.7)	1 (16.7)	4 (66.7)		
TNM stage						1014.5	0.000
I - II	37	20 (54.1)	4 (10.8)	11 (29.7)	2 (5.4)		
III-IV	38	3 (2.6)	7 (10.5)	15 (42.1)	13 (44.7)		
Tumor size						1403.0	0.9735
≤ 5 cm	38	11 (28.9)	6 (15.8)	14 (36.8)	7 (18.4)		
< 5 cm	37	12 (32.4)	5 (13.5)	12 (32.4)	8 (21.6)		

ANXA1: Annexin A1; TNM: Tumour-lymph node metastasis.

lung cancer<sup>[12]</sup>, pancreatic cancer<sup>[13]</sup>, colorectal cancer<sup>[3,14]</sup> and bladder cancer<sup>[15]</sup>. By contrast, ANXA1 is downregulated in oral squamous cell carcinoma as well as nasopharyngeal, laryngeal and other head and neck cancer<sup>[16-18]</sup>,

oesophageal squamous cell carcinoma<sup>[2]</sup> and prostate cancer<sup>[19]</sup>. The dysfunction of ANXA1 is closely related to breast cancer, lung cancer, and pancreatic cancer as well as in other tumour invasion and metastasis. There-

fore, ANXA1 is considered as a risk factor affecting the survival of patients. Wang *et al.*<sup>[20]</sup> used an immunohistochemical method and found that ANXA1 expression is 39% higher in the stomach/gastroesophageal junction adenocarcinoma and closely correlated with the pathological staging and distant metastasis of tumour, *i.e.*, higher clinical stages, particularly in distant lymph node metastasis, correspond to higher ANXA1 expression levels; higher tumour recurrence rates correspond to lower survival rate. This result suggested that the upregulation of ANXA1 could be used as a prognosis indicator in the stomach-oesophageal junction adenocarcinoma. Currently, the expression and function of ANXA1 in GC remain controversial.

In this study, quantitative proteomics was performed to screen ANXA1, revealing that ANXA1 was expressed 2.17 times higher in GC than in normal gastric mucosa. Western blotting and IHC of the tissue microarray also revealed the same results as proteomics analysis. In particular, the results showed that ANXA1 was significantly expressed at a higher extent in GC tissues than in paratissues; further analysis about the relations of ANXA1 and clinical parameters of human GAC revealed that ANXA1 expression was upregulated in tumour-penetrating serosa and thus invaded the paratissues. This result is different from human GAC normally confined to the mucosa, submucosa and muscularis; human GAC also possibly invaded the tumour in the lower serosa. ANXA1 was also highly expressed in human GAC with lymph node metastasis and distant metastasis compared with human GAC without lymph node metastasis and distant metastasis. As TNM staging increased, the positive expression rate of ANXA1 increased. However, no relationship was observed among ANXA1, patient's age, gender, histological grade and tumour size. This result suggested that ANXA1 was closely related to the biological behaviour of human GAC and involved in the development, invasion and metastasis of human GAC. Currently, the mechanism by which ANXA1 functions in the biological behaviour of GC remains unclear. Cheng *et al.*<sup>[21]</sup> reported that ANXA1 can regulate GC invasion by mediating formyl peptide receptor (FPR)/extracellular signal-regulated kinase/integrin protein  $\beta$ -1 bind protein pathway; all of the three FPRs are involved in the regulation process. Kang *et al.*<sup>[11]</sup> further found that *ANXA1* gene can decrease the activity and protein expression of matrix metalloproteinase-9 (MMP-9) transcriptional promoter by inhibiting the activity of nuclear factor  $\kappa$ -B. MMP-9 also has an important function in GC invasion and metastasis. Vascular endothelial growth factor (VEGF) is another important factor regulating angiogenesis, and angiogenesis is important in tumour growth and metastasis. Pin *et al.*<sup>[22]</sup> found that VEGF-induced cell migration and angiogenesis of miR-196a can change the expression level of ANXA1, which is controlled by p38-ANXA1 signal conduction pathway. The function of *Helicobacter pylori* (*H. pylori*) infection in GC has also been demonstrated, showing that *H. pylori* can change the cellular ANXA1

localization<sup>[23]</sup>. This result suggests that ANXA1 may be involved in *H. pylori* infection-induced GC. Furthermore, these results can explain the possible mechanism by which ANXA1 participates in GAC biological behaviour to some extent.

A few reports about ANXA1 in GC have been published. For instance, Yapar *et al.*<sup>[4]</sup> conducted an immunohistochemical study and found that ANXA1 expressed in GC is 56.3% higher than that in paratissues; this expression is also positively correlated with tumour invasion and lymph node metastasis; a high ANXA1 expression suggests poor prognosis of GC. Jorge *et al.*<sup>[6]</sup> performed RT-PCR and immunohistochemistry, revealing that ANXA1 mRNA and protein expressions are increased in GC compared with normal gastric mucosa. The results of the present study are consistent with those in the aforementioned previous studies, although other studies have revealed contrasting results. In some studies, ANXA1 is downregulated in GC<sup>[4,24]</sup> and negatively correlated with tumour staging and lymph node metastasis<sup>[4]</sup>. The reasons may be described as follows: (1) different test conditions, antibodies, and test methods; and (2) different samples or different types and pathological staging parameters of GC samples. The intracellular ANXA1, which is mainly in the cytoplasm, is possibly redistributed at different stimulations. For example, ANXA1 likely enters the nucleus as induced by an epidermal growth factor (EGF) under oxidation conditions or heat shock; by contrast, ANXA1 is transferred to the membrane and then secreted out of the cells as stimulated by GC or phorbol-12-myristate-13-acetate<sup>[25,26]</sup>. Therefore, the upregulation and downregulation of ANXA1 in GC may be associated with different stages of GC pathology. Different levels of EGF and GC *in vivo* may possibly induce cellular ANXA1 relocation. ANXA1 may also be involved in GC occurrence and development *via* different pathways and mechanisms.

In summary, the present study provided valuable information to clarify GC pathogenesis. This study also presented the basic foundation to screen cancer biomarkers. After the differential proteins of GC were initially screened, the results suggested that identification and function *in vivo* and *in vitro* of some important differential proteins require further studies. Given that ANXA1 was also expressed in gastric stromal cells, ANXA1 expression in GC may be difficult to assess by IHC. Nevertheless, the study on immunohistochemical tissue microarray revealed the relationship between ANXA1 and GC clinicopathological parameters. We found that the upregulation of ANXA1 in human GAC was closely related to the depth of tumour invasion, lymph node metastasis, distant metastasis and TNM stage. The results also suggested that ANXA1 was possibly involved in tumour invasion and metastasis. The high expression of ANXA1 suggested poor prognosis of GC. The mechanism by which ANXA1 participated in the GC biological behaviour should be further studied. With the continuous development of this research, ANXA1 may be used in early



cancer detection, diagnosis and treatment. ANXA1 may also become an indicator of cancer prognosis and a new target of cancer therapy, thereby providing new ideas of GC diagnosis and treatment.

## COMMENTS

### Background

Gastric cancer (GC) is a common digestive tract cancer because of the lack of early diagnosis strategies; a previous study revealed that GC cases are usually diagnosed when the disease is at an advanced stage.

### Research frontiers

During the screening of the identified differential proteins, Annexin A1 (ANXA1) was significantly upregulated in GC. ANXA1 is involved in various physiological and pathological processes, such as cell signal transduction, cell proliferation, differentiation and apoptosis as well as inflammation and immune response. Previous studies found that ANXA1 is upregulated in breast cancer, lung cancer, pancreatic cancer, colorectal cancer and bladder cancer. By contrast, ANXA1 is downregulated in oral squamous cell carcinoma as well as nasopharyngeal, laryngeal and other head and neck cancer, oesophageal squamous cell carcinoma and prostate cancer.

### Innovations and breakthroughs

The study performed laser capture microdissection to investigate the relation of ANXA1 expression in GC to the clinical parameters and to obtain purified gastric adenocarcinoma cells (GAC) and normal gastric epithelial cells (NGEC).  $^{18}\text{O}/^{16}\text{O}$  was used to label the digested peptides in the mixture of GAC and NGEC. Nanoliter-reverse-phase liquid chromatography-mass/mass spectrometry was performed to identify and quantify the differentially expressed proteins.

### Applications

ANXA1 may be used in early cancer detection, diagnosis and treatment. ANXA1 may also become an indicator of cancer prognosis and a new target of cancer therapy, thereby providing new ideas of GC diagnosis and treatment.

### Peer review

This study is realistic significance to the GC, This manuscript is well written. The data is interesting and worthy for publication.

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## Is Dor fundoplication optimum after laparoscopic Heller myotomy for achalasia? A meta-analysis

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### Abstract

**AIM:** To compare the outcome of acid reflux prevention by Dor fundoplication after laparoscopic Heller myotomy (LHM) for achalasia.

**METHODS:** Electronic database PubMed, Ovid (Evidence-Based Medicine Reviews, EmBase and Ovid MEDLINE) and Cochrane Library were searched between January 1995 and September 2012. Bibliographic citation management software (EndNote X3) was used for extracted literature management. Quality assessment of random controlled studies (RCTs) and non-RCTs was performed according to the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 and a modification of the Newcastle-Ottawa Scale, respectively. The data were analyzed using Review

Manager (Version 5.1), and sensitivity analysis was performed by sequentially omitting each study.

**RESULTS:** Finally, 6 studies, including a total of 523 achalasia patients, compared Dor fundoplication with other types of fundoplication after LHM (Dor-other group), and 8 studies, including a total of 528 achalasia patients, compared Dor fundoplication with no fundoplication after LHM (Dor-no group). Dor fundoplication was associated with a significantly higher recurrence rate of clinical regurgitation and pathological acid reflux compared with the other fundoplication group (OR = 7.16, 95%CI: 1.25-40.93,  $P = 0.03$ , and OR = 3.79, 95%CI: 1.23-11.72,  $P = 0.02$ , respectively). In addition, there were no significant differences between Dor fundoplication and no fundoplication in all subjects. Other outcomes, including complications, dysphagia, postoperative physiologic testing, and operation-related data displayed no significant differences in the two comparison groups.

**CONCLUSION:** Dor fundoplication is not the optimum procedure after LHM for achalasia. We suggest more attention should be paid on quality of life among different fundoplications.

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**Key words:** Laparoscopic Heller myotomy; Dor fundoplication; Gastroesophageal reflux; Achalasia; Meta-analysis

**Core tip:** Laparoscopic Heller myotomy (LHM) is commonly used to treat achalasia and an antireflux procedure is added after LHM for prevention of gastroesophageal reflux (GER). However, there is no consensus on whether Dor fundoplication is the optimum procedure after LHM for the prevention of GER. We conducted this meta-analysis to assess Dor fundoplication com-

pared with non-fundoplication surgery or other types of fundoplication surgery for achalasia. The results indicated higher recurrence rate of clinical regurgitation and pathological acid reflux in Dor fundoplication indicating that Dor fundoplication is not the optimum procedure for the prevention of GER after LHM in achalasia patients.

Wei MT, He YZ, Deng XB, Zhang YC, Yang TH, Jin CW, Hu B, Wang ZQ. Is Dor fundoplication optimum after laparoscopic Heller myotomy for achalasia? A meta-analysis. *World J Gastroenterol* 2013; 19(43): 7804-7812 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7804.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7804>

## INTRODUCTION

Achalasia has generally been accepted as an autoimmune esophageal motility disorder resulting from the loss of inhibitory nerve endings in the myenteric plexus of the esophagus<sup>[1]</sup>. Pathophysiologically characterized by poor relaxation of lower esophageal sphincter (LES) and aperistalsis of the esophageal body, achalasia presents mainly relevant symptoms such as dysphagia, regurgitation, heartburn, and chest pain. The commonly used treatment of achalasia involves medicine therapy, endoscopic pneumatic dilation, and surgical myotomy with the aim of eliminating the high LES pressure. Previous studies have reported better long-term satisfaction with surgical myotomy than with drug medicine therapy or pneumatic dilation<sup>[2-4]</sup>. Kostic and colleagues in 2007 also demonstrated the superiority of laparoscopic Heller myotomy (LHM) to pneumatic dilation for achalasia patients<sup>[5]</sup>. As a result, LHM is routinely considered an option for achalasia patients.

However, although LHM has been previously demonstrated to have positive long-term outcomes for achalasia patients, gastroesophageal reflux (GER) after LHM is commonly regarded as one of the main failures of surgical treatment. For this reason, many surgeons suggest the addition of a fundoplication to LHM for the prevention of acid reflux, and anterior 180° Dor fundoplication is currently well recognized as the best choice<sup>[6]</sup>. Recently, in 2012, a review conducted by Mayo reconfirmed the efficacy of anti-acid reflux fundoplication following LHM both on pH monitoring and symptom relief; however, the clinical differences between Dor fundoplication and posterior 270° Toupet fundoplication have not been verified. In addition, the Mayo review provides limited evidence without pooling available data from the included studies<sup>[7]</sup>. Thus, it also remains controversial that Dor fundoplication is the optimum procedure for the prevention of postoperative GER after LHM in achalasia patients.

To address these issues, our team conducted the following meta-analysis to compare Dor fundoplication plus LHM with LHM alone (Dor-no group) and LHM

plus other types of fundoplication (Dor-other group), namely, 270° Toupet and 360° Nissen fundoplication. The assessed outcomes included: (1) the primary endpoints of postoperative GER, dysphagia, and perforation; and (2) the secondary endpoints of other symptoms, quality of life, operation-related data, complications, and postoperative physiologic testing.

## MATERIALS AND METHODS

This meta-analysis was conducted following the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 (updated March 2011) to ensure data quality (<http://www.cochrane.org/training/cochrane-handbook>).

### Search for studies

Electronic databases PubMed, Ovid (EBM Reviews, EmBase and Ovid MEDLINE) and Cochrane Library were searched. Moreover, previously published reviews on the topic of interest were obtained and checked. We traced the reference list of relevant articles and used Google Scholar to find potential studies. The search terms were as follows: combined terms of “fundoplication” and “achalasia” using [Mesh] or [Keyword]. The electronic search was up to September 2012 from January 1995 with no limitation on language.

### Study selection

Study designs included random controlled studies (RCTs), clinical controlled studies, cohort studies, case-control studies, and case series.

The inclusion criteria were as follows: (1) diagnosis of achalasia confirmed in an adult patient; (2) the surgical procedure compares Dor fundoplication with other fundoplication types (none, Toupet and Nissen); (3) laparoscopic Heller myotomy; and (4) available data for each comparison. We excluded: studies including (1) achalasia in children and pregnancy; (2) one type of fundoplication; (3) special surgical procedure such as anterior 120° wrap or Watson wrap<sup>[8]</sup>; and (4) studies lacking available data.

We imported the search results into bibliographic citation management software (EndNote X3). Two reviewers independently screened studies by reading titles and abstracts to roughly identify potential reports. The full texts of articles for all references identified as matching the inclusion criteria were obtained. Inclusion criteria were applied to the full texts. Disagreement was resolved through discussion and asking for advice from corresponding authors. The flow chart of study selection was made following the PRISMA statement (<http://prisma-statement.org/statement.htm>).

### Data extraction and quality assessment

Two reviewers independently extracted data from eligible studies, and any disagreement was adjudicated by discussion or consulting the corresponding author. Base-

**Table 1 Checklist of quality assessment and scoring of non-random controlled studies**

Checklist
Selection
Is the subject definition adequate or described? (if yes, one star)
Were the subjects representative of the total population? (one star, if truly or obviously; no stars if subjects were selected group or not described)
Comparability
Did the study have no differences between Dor fundoplication and no fundoplication or other types of fundoplication? Major factors for consideration were age, gender, symptoms, preoperative therapy (pneumatic dilation and botulin toxin injection), and preoperative diagnostic test (endoscopy parameter and barium swallow) (if yes, two stars; one star if there were no other differences between the two groups even if one or more of these five characteristics was not reported; no star was assigned if the two groups differed)
Outcome assessment
Clearly defined outcome of interest (if yes, one star)
Adequacy of follow-up (one star if less than 20% of achalasia patients lost to follow-up, otherwise no stars)

line information included first author, published year, fundoplication type, study design, region, numbers of cases, and mean age among other parameters. Furthermore, the following outcome data were extracted: (1) the primary outcomes of GER-related clinical regurgitation and pathological acid reflux, dysphagia, and perforation; and (2) the secondary endpoints included other symptoms, quality of life, operation-related data (operation time and hospital stay time), complications, postoperative physiologic testing (LES pressure, DeMeester score and percent total time  $\text{pH} \leq 4$ ).

Quality assessment of RCTs was performed by two reviewers according to the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 based on the following aspects: random sequence generation, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias. Three bias levels including low risk, high risk and unclear were assigned to every study aspect. Studies with more “low risk” bias assignments were recognized as superior. For non-random controlled studies, a modification of the Newcastle-Ottawa Scale (NOS)<sup>[9,10]</sup> was used as an assessment tool for selection, comparability and outcome assessment. Out of a total of six scores, studies valued more than four stars were recognized as being moderate to high quality. The detailed checklist is shown in Table 1.

### Statistical analysis

The data were analyzed using Review Manager (Version 5.1). OR or RD and MD were used for analyzing dichotomous data and continuous data, respectively. Heterogeneity was measured with the  $I^2$  index and  $P$  value. A random effect model was used when  $I^2 > 50\%$ . Otherwise, a fixed-effect model was considered. SD was estimated by a formula when only a range was reported: Estimate SD = Range/4 ( $15 < n < 70$ ); Range/6 ( $n > 70$ )<sup>[11]</sup>. The value of  $P < 0.05$  was considered to indicate statistical significance. Sensitivity analysis was performed by sequentially omitting each study.

## RESULTS

### Characteristics of pooled studies

A total of 731 potential abstracts were identified in

the primary search of the electronic databases. A flow diagram of the detailed selection process is shown in Figure 1. Finally, 6 studies (2 RCTs and 4 non-RCTs), including a total of 523 achalasia patients, compared Dor fundoplication with other types of fundoplication (Toupet and Nissen fundoplication) after LHM, and 8 studies (3 RCTs and 5 non-RCTs), including a total of 528 achalasia patients, compared Dor fundoplication with no fundoplication after LHM<sup>[12-24]</sup>. In the 5 RCTs, two reported the same population group but differed in the main outcomes. Thus, we just extracted useful data integrated from both articles<sup>[19,23]</sup>. In addition, in the 8 non-RCTs, two studies were conducted by the same research group, who reported on the achalasia population with short- and long-term outcomes, and we chose the latter for our meta-analysis<sup>[15,24]</sup>. In one non-RCT, we divided the pooled data into two comparisons from the three reported subgroups<sup>[20]</sup>. In terms of non-Dor fundoplication, surgical fundoplication included 2 studies that used Nissen fundoplication, 4 studies with Toupet fundoplication and no fundoplication was used in the other studies. Table 2 offers the baseline characteristics of all studies.

### Quality judgments of studies

In the pooled studies, 5 were RCTs, and 8 were non-RCTs. We used two methods to assess the quality of RCTs and non-RCTs, respectively. Table 3 lists the quality of RCTs according to the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0. All the studies described the random sequence generation method used. Two studies generated sequence using a permuted block size of 4. Two used computer-generated random numbers, and 1 used a random number table generated in Microsoft Excel. In regards to allocation concealment, 3 studies used sealed opaque envelopes, 1 used Random Allocation Software version 1.0, and 1 study was unclear about the allocation concealment method used. In term of blinding, double blinding is difficult, and risks were judged by whether the outcome was likely influenced by the lack of blinding. In the 5 RCTs, only two trials reported double blinding of all recruited patients and researchers involved in the evaluation. Concerning selective reporting, although the protocol of each study was unavailable, the published outcomes included all the outcomes detailed in the method. Other sources of bias



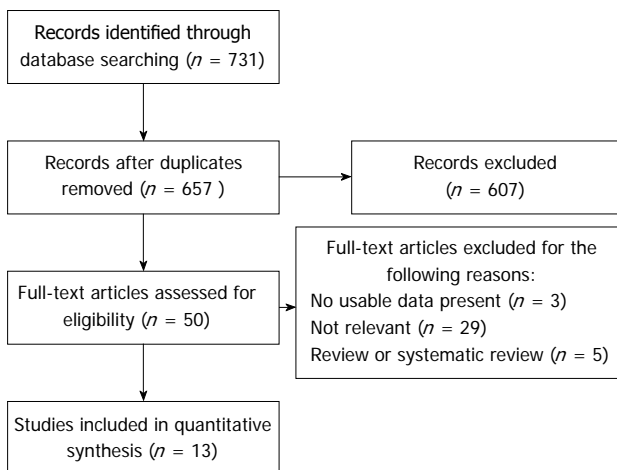
**Table 2 Basic characteristics of all pooled studies in the meta-analysis (Dor-other/no group)**

Ref.	Patients (n)		Follow-up (mean $\pm$ SD or range)		Age (mean $\pm$ SD or range)	Type of fundoplication in control group	Study design	Country
	Dor group	Control group	Dor group	Control group				
Di Martino <i>et al</i> <sup>[13]</sup> , 2011	30	26	24	24	42.8 $\pm$ 14.7	Other <sup>1</sup>	Prospective	Italy
Oelschlager <i>et al</i> <sup>[15]</sup> , 2003	52	58	46 (1-85)	16 (1-38)	42.6 $\pm$ 15.5	Other <sup>2</sup>	Retrospective	United States
Rawlings <i>et al</i> <sup>[17]</sup> , 2012	36	24	12	12	48.8 $\pm$ 13.0	Other <sup>2</sup>	RCT	United States
Rebecchi <i>et al</i> <sup>[18]</sup> , 2008	72	72	125 (60-168)	125 (60-168)	49 (11-80)	Other <sup>1</sup>	RCT	Italy
Richardson <i>et al</i> <sup>[20]</sup> , 2006	18	20	37 (2-97)	37 (2-97)	69 (15-80)	Other <sup>2</sup>	Retrospective	United States
Wright <i>et al</i> <sup>[24]</sup> , 2007	52	63	46 $\pm$ 24	45 $\pm$ 17	42.5 (15.4)	Other <sup>2</sup>	Retrospective	United States
Dempsey <i>et al</i> <sup>[12]</sup> , 2004	22	29	39 $\pm$ 22	26 $\pm$ 19	47.5 (12.6)	No	Retrospective	United States
Finley <i>et al</i> <sup>[14]</sup> , 2007	71	24	6.9 $\pm$ 3.5	6.9 $\pm$ 3.5	47.9 (16-84)	No	Retrospective	Canada
Ramacciato <i>et al</i> <sup>[16]</sup> , 2005	17	15	12	12	42.0 (14-77)	No	Retrospective	Italy
Richards <i>et al</i> <sup>[19]</sup> , 2004	22	21	6	6	50 $\pm$ 12.7	No	RCT	United States
Richardson <i>et al</i> <sup>[20]</sup> , 2006	18	14	37 (2-97)	37 (2-97)	69 (15-80)	No	Retrospective	United States
Simić <i>et al</i> <sup>[21]</sup> , 2010	36	22	36	36	49.6 $\pm$ 29.2	No	RCT	Serbia
Tapper <i>et al</i> <sup>[22]</sup> , 2008	75	99	8.4 $\pm$ 12.0	48.7 $\pm$ 34.6	47.0 $\pm$ 16.8	No	Prospective	United States
Torquati <i>et al</i> <sup>[23]</sup> , 2006	22	21	NA	NA	50 $\pm$ 12.7	No	RCT	United States

<sup>1</sup>Other: Nissen fundoplication; <sup>2</sup>Other: Toupet fundoplication. NA: Not available; RCT: Random controlled trial.

**Table 3 Quality assessment of random controlled studies in the meta-analysis based on the Cochrane Handbook version 5.1.0**

Ref.	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other sources of bias
Rawlings <i>et al</i> <sup>[17]</sup> , 2012	Low	Unclear	High	Low	Low	Low	Unclear
Rebecchi <i>et al</i> <sup>[18]</sup> , 2008	Low	Low	High	Low	Low	Low	Unclear
Richards <i>et al</i> <sup>[19]</sup> , 2004	Low	Low	Low	Low	Low	Low	Unclear
Simić <i>et al</i> <sup>[21]</sup> , 2010	Low	Low	High	High	Unclear	Low	Unclear
Torquati <i>et al</i> <sup>[23]</sup> , 2006	Low	Low	Low	Low	Low	Low	Unclear

**Figure 1 Flow diagram of meta-analysis study selection process.**

were unclear in the included RCTs.

In term of the 8 non-RCTs, Table 4 lists the evaluation stars of each study followed by the modified NOS. In the selection of patients, one study included patients without continuity, which could hardly represent the total population<sup>[22]</sup>. Three studies (two studies reported the same patients group) received no stars in the domain of adequacy of follow-up, with a follow-up of 63.7%<sup>[20]</sup> and 30% (postoperative manometer), respectively<sup>[15,24]</sup>. Overall, all studies were evaluated as being moderate to high quality.

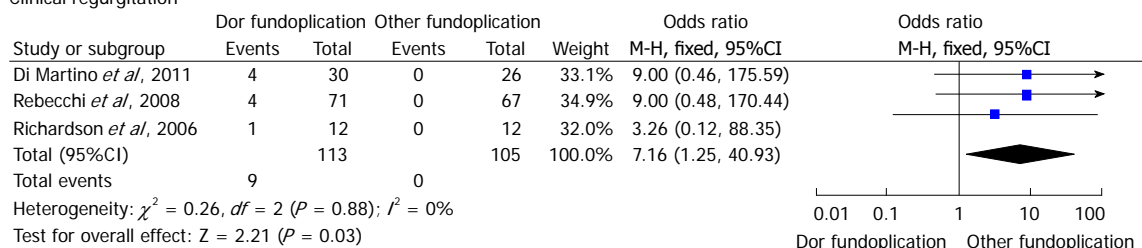
### Outcomes in the Dor-other group

**Primary endpoints:** With respect to clinical regurgitation, 3 non-RCTs reported the available number of achalasia patients<sup>[13,18,20]</sup>, and a fixed-effect model was used in the subgroup meta-analysis. Dor fundoplication was appraised to have a significantly higher recurrence rate of clinical regurgitation compared with other types of fundoplication (OR = 7.16, 95%CI: 1.25-40.93,  $P$  = 0.03 and heterogeneity  $I^2$  = 0%) (Figure 2A). One study, which did not have an available number of achalasia patients, reported no significant difference in regurgitation frequency score ( $P$  = 0.546)<sup>[24]</sup>.

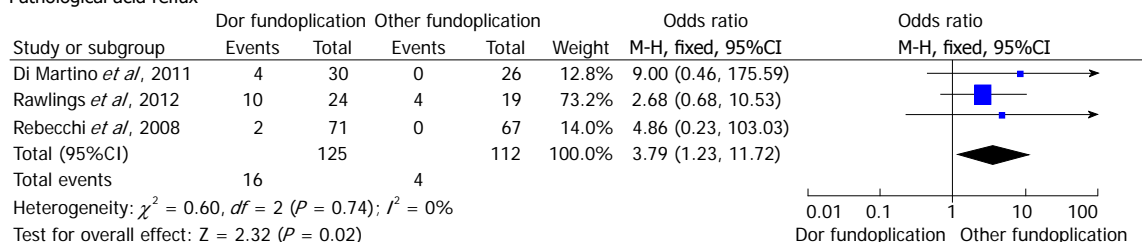
In the pathological acid reflux analysis, 1 RCT and 2 non-RCTs were pooled, and a fixed-effect model was used<sup>[13,17,18]</sup>. The odds ratio was 3.79 in the Dor fundoplication group compared with the other fundoplication group (95%CI: 1.23-11.72,  $P$  = 0.02 and heterogeneity  $I^2$  = 0%).

Perforation was estimated in 2 RCTs and 1 non-RCT, and a fixed-effect model was used. The subgroup analysis indicated no significant differences in the Dor-other group (RD = -0.00, 95%CI: -0.04-0.04,  $P$  = 0.94, heterogeneity  $I^2$  = 0%) (Figure 2A).

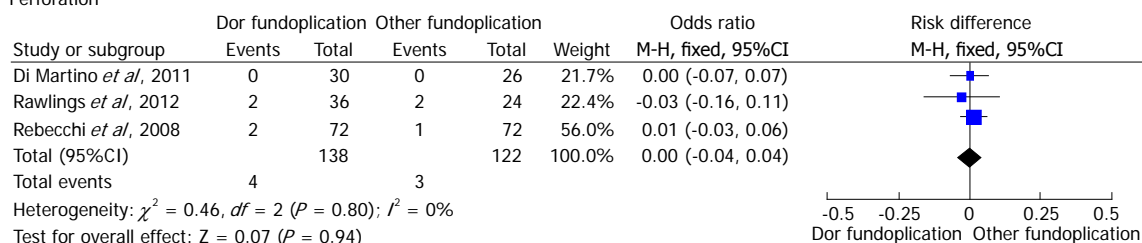
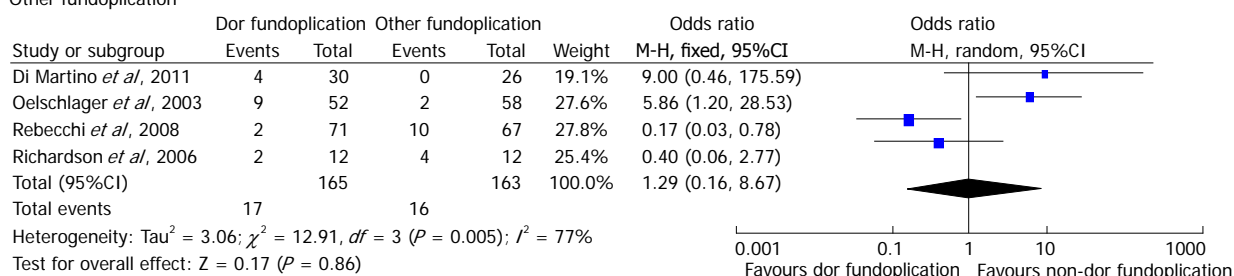
Considering dysphagia, no significant symptom relief benefit was found for Dor fundoplication compared with other types of fundoplication, and a random-effect model was used (OR = 1.19, 95%CI: 0.16-8.67,  $P$  = 0.86 and heterogeneity  $I^2$  = 77%) (Figure 2B). One study that lacked information on the number of acha-

**A** Clinical regurgitation

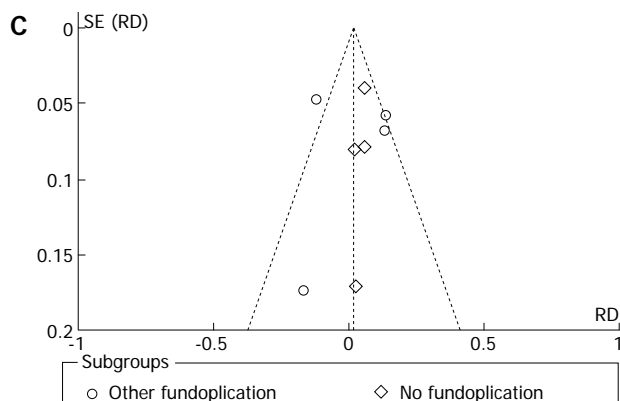
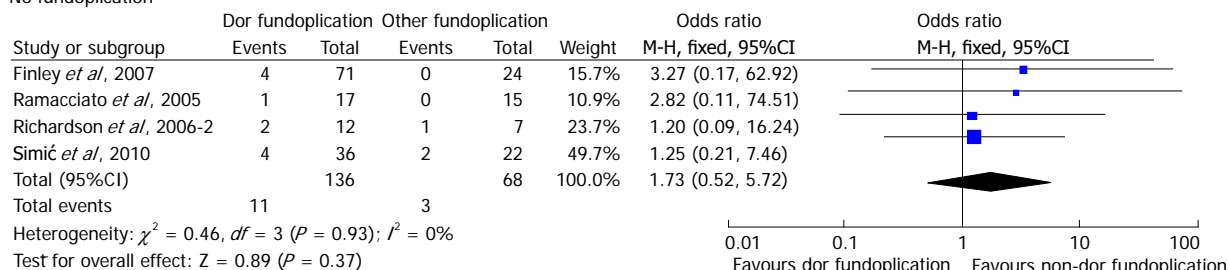
## Pathological acid reflux



## Perforation

**B** Other fundoplication

## No fundoplication



**Figure 2 Forest plot.** A: Forest plot of the major outcomes in the Dor-other group; B: Forest plot of dysphagia symptoms in both the Dor-other and Dor-no groups; C: Funnel plot of dysphagia symptoms in both the Dor-other and Dor-no groups.

**Table 4** Quality assessment of non-random controlled studies in the meta-analysis based on modified Newcastle-Ottawa Scale judgment

Ref.	Selection		Comparability	Outcome assessment		Quality judgment
	1	2	3	4	5	
Dempsey <i>et al</i> <sup>[12]</sup> , 2004	*	*	**	*	*	*****
Di Martino <i>et al</i> <sup>[13]</sup> , 2011	*	*	**	*	*	*****
Finley <i>et al</i> <sup>[14]</sup> , 2007	*	*	*	*	*	*****
Oelschlager <i>et al</i> <sup>[15]</sup> , 2003	*	*	*	*	--	****
Ramacciato <i>et al</i> <sup>[16]</sup> , 2005	*	*	*	*	*	*****
Richardson <i>et al</i> <sup>[20]</sup> , 2006	*	*	**	*	--	*****
Tapper <i>et al</i> <sup>[22]</sup> , 2008	*	--	*	*	*	****
Wright <i>et al</i> <sup>[24]</sup> , 2007	*	*	*	*	--	****

**Table 5** Pooled outcomes of random controlled studies and non-random controlled studies for postoperative physiological testing and operation-related data

	Studies (n)	Participants		Test of heterogeneity		MD (95%CI)	P value for effect size
		Dor group	Control group	I <sup>2</sup>	P value		
LES pressure							
Dor-other group	2	47	43	94%	< 0.0001	-1.02 (-9.90, 7.86)	0.82 <sup>1</sup>
Dor-no group	2	58	43	65%	0.09	1.97 (-0.93, 4.86)	0.18 <sup>2</sup>
DeMeester score							
Dor-other group	2	40	39	48%	0.17	-7.13 (-18.37, 4.12)	0.21 <sup>2</sup>
Dor-no group	1	21	18	Not applicable		-25.00 (-58.40, 8.40)	0.14
Percent total time pH ≤ 4							
Dor-other group	4	154	142	63%	0.05	0.96 (0.00, 1.91)	0.05 <sup>1</sup>
Dor-no group	1	21	18	Not applicable		-7.20 (-13.34, -1.06)	0.02
Surgery time							
Dor-other group	3	138	122	14%	0.31	-5.37 (-7.71, -3.03)	< 0.00001 <sup>2</sup>
Dor-no group	2	39	36	0%	0.35	24.14 (7.21, 41.08)	0.005 <sup>2</sup>
Hospital stay time							
Dor-other group	4	171	176	94%	< 0.00001	0.10 (-0.59, 0.80)	0.77 <sup>1</sup>
Dor-no group	1	22	21	Not applicable		0.00 (-0.15, 0.15)	1.00

<sup>1</sup>Random-effect model; <sup>2</sup>Fixed-effect model. RCT: Random controlled trial.

lasia patients reported no significant difference in the dysphagia frequency score and a significant difference in the dysphagia severity score ( $P = 0.465$  and  $P = 0.003$ , respectively)<sup>[24]</sup>. No publication bias was observed in the funnel plot of studies when reporting dysphagia in the two comparisons (Figure 2C).

**Secondary endpoints:** In regards to the other symptoms, one study reported bloating, chest pain, and heartburn recurrence frequency score without any significance difference in the Dor-other group<sup>[24]</sup>. With regard to quality of life, one multicenter RCT assessed the outcome using an SF-36 questionnaire and ten health-related domains<sup>[17]</sup>. No significant score difference was observed in the Dor fundoplication group compared with the other fundoplication group in five and seven domains of the total ten domains, respectively. Another prospective study in the Dor-other group reported that the SF-36 score ranged from 0-100, and the two compared types of fundoplication scored  $70.5 \pm 4.06$  and  $72.3 \pm 4.53$  each with a  $P$  value  $> 0.5$ <sup>[13]</sup>.

Postoperative physiologic testing including LES pressure, DeMeester score, and percent total time pH  $\leq 4$  displayed no obvious significant difference in the Dor-other group. Considering the relatively high heteroge-

neity, the random effect model was applied in all three outcomes. In the subgroup analysis of surgery time, Dor fundoplication took significantly less time than the other types of fundoplication, and the estimated hospital stay time was not different in the comparison group. The details are shown in Table 5.

In addition to perforation, other complications were described as follows: Di Martino *et al*<sup>[13]</sup> reported intra-operatively 1 mucosal tear and 2 cervical subcutaneous emphysema occurrences and postoperatively reported 2 pulmonary atelectasis occurrences in the Dor fundoplication group, intra-operatively 1 pneumothorax occurrence, and postoperatively 1 urinary retention occurrence in the other fundoplication group. Wright *et al*<sup>[24]</sup> reported 1 urinary retention occurrence in the Dor fundoplication group compared with none in the other fundoplication group. Another 2 studies reported no complications in either group<sup>[17,18]</sup>.

### Outcomes in the Dor-no group

**Primary endpoints:** With respect to clinical regurgitation, two non-RCTs were validly pooled without displaying any significant difference in the Dor-no group (OR = 0.51, 95%CI: 0.09-2.92,  $P = 0.32$  and heterogeneity  $I^2 = 0\%$ )<sup>[16,20]</sup>. A fixed-effect model was applied in this

analysis. Two studies without an available number of achalasia patients reported no significant differences of dysphagia severity score<sup>[14,22]</sup>.

In the pathological acid reflux analysis, one RCT indicated that Dor fundoplication was associated with an obviously lower pathological acid reflux rate than no fundoplication (OR = 0.11, 95%CI: 0.02-0.59,  $P = 0.01$ )<sup>[19]</sup>.

Perforation was estimated in 1 RCT and 3 non-RCTs, and a fixed-effect model was used<sup>[12,14,16,19]</sup>. Subgroups were evaluated with no significant differences in Dor-no group (RD = 0.02, 95%CI: -0.04-0.07,  $P = 0.59$  and heterogeneity  $I^2 = 0\%$ ) observed.

Considering dysphagia, no significant symptom relief benefit was found for Dor fundoplication compared with no fundoplication (OR = 1.73, 95%CI: 0.52-5.72,  $P = 0.37$  and heterogeneity  $I^2 = 0\%$ ), and a fixed-effects model was used (Figure 2B). Of two studies that did not provide the number of patients, one reported significantly less severe dysphagia in the Dor fundoplication group<sup>[22]</sup>, and the other reported no difference<sup>[12]</sup>.

**Secondary endpoints:** Concerning other symptoms, there was no obvious difference in heartburn in the Dor-no group in two studies<sup>[12,24]</sup> and a lower recurrence rate in Dor fundoplication compared with no fundoplication in one study<sup>[22]</sup>. No significant difference in chest pain was found in 2 studies<sup>[12,22]</sup>, and 1 study reported vomiting without difference and a lower choking rate in the Dor fundoplication group compared with the no fundoplication group<sup>[22]</sup>. With regard to symptom satisfaction, Dempsey reviewed the consecutive patients with 86% satisfaction in the Dor-no group, which was not significantly different than the Dor group<sup>[12]</sup>. Tapper and colleagues selectively reviewed their patients and found a slightly higher satisfaction rate in the no fundoplication group compared with the Dor group (89% and 75%, respectively)<sup>[22]</sup>.

Similar to the results in the Dor-other group, postoperative physiologic testing and hospital stay time displayed no obvious significance in the Dor-no group. In addition, Dor fundoplication took significantly more time than no fundoplication on surgery time ( $P = 0.005$ ). Complications were rarely reported in the Dor-no group, except for 2 studies mentioning no complications in this comparison<sup>[14,19]</sup>.

## DISCUSSION

Since the description of minimal invasive treatment for achalasia by Shimi *et al.*<sup>[25]</sup>, LHM has gained world-wide popularity and is increasingly regarded as the standard treatment for achalasia by surgeons and gastroenterologists. Furthermore, the routine application of fundoplication following LHM has been identified as useful for protection of postoperative GER<sup>[4,6,26]</sup>. Dor fundoplication, with the advantage of a simple procedure and covering of the mucosa, is being accepted as the first-line type of fundoplication for achalasia in most regions. However, some opponents of Dor fundoplication have

reported no significant benefit with regard to the clinical outcomes when Dor fundoplication was added to LHM, and they recommend posterior or even total fundoplication, such as posterior 270° Toupet fundoplication and total 360° Nissen fundoplication, be added to LHM for better long-term outcome<sup>[12,27]</sup>. Thus, there is no consensus on whether Dor fundoplication is the optimum procedure after LHM for achalasia.

Our group conducted this meta-analysis to provide evidence for fundoplication choice on achalasia surgery. Finally, we found a significantly higher clinical and pathological acid reflux rate in for Dor fundoplication than for other types of fundoplication, although no significant difference was found between Dor fundoplication and no fundoplication. Our results contradict the conventional concept that Dor fundoplication after LHM is the optimal choice for achalasia. However, caution should be taken care to explain the pooled results because of the limitations of our study.

Postoperative GER was the main evaluation used to assess the efficacy of Dor fundoplication for achalasia. GER includes clinical regurgitation symptoms and pathological acid reflux, which was defined as more than 4.2% total time per 24-h period for which  $\text{pH} \leq 4$  or as a DeMeester score of  $\geq 18$  for 24 consecutive hours. Our results demonstrate that Dor fundoplication provides no beneficial clinical regurgitation palliation compared with fundoplication, and, in addition, it leads to a significantly higher clinical regurgitation rate than the other types of fundoplication examined. These results may be explained by the fact that Dor fundoplication may add less resistance than Toupet or Nissen fundoplication, which allows acid to flow through the loose esophagogastric junction more easily. In addition, though the pH testing in two comparison groups indicated no significant difference, the clinical regurgitation symptoms result is supported by the pathological acid reflux outcome, which also demonstrates that Dor fundoplication resulted in more acid reflux than the other fundoplication types. It should be noted that our pooled clinical regurgitation results exclude one study without available data that might affect the outcome<sup>[24]</sup>.

With regard to dysphagia, Dor fundoplication displayed no significant dysphagia relief difference compared with either the other types of fundoplication or no fundoplication. Campos and colleagues suggested that dysphagia relief is independent of whether a fundoplication is performed<sup>[2]</sup>. Their conclusion is consistent with our pooled outcome that Dor fundoplication did not produce a lower recurrence of dysphagia than the other types of fundoplication, and we also found no obvious difference in postoperative dysphagia when comparing the Dor and Dor-no groups. The relative lack of change in the mucosa fibrosis around the dissected esophagus between the different types of fundoplication may explain this finding, although we have found no significant difference on LES pressure. As the Heller muscle is dissected whether LHM alone or LHM plus fundoplication



is performed, the pressure changes associated with the follow-up procedure may not be significantly different between fundoplication types. Furthermore, a study designed by Rohof on efficacy of treatment for achalasia indicates distensibility of the esophagogastric junction should recommend as better parameter of treatment for achalasia rather than LES pressure<sup>[28]</sup>.

As the most dangerous and latest complication, perforation is the main outcome we focus on. In the pooled outcomes, we failed to find that Dor fundoplication had a lower perforation rate when used for achalasia treatment. As previous studies have reported, perforation was highly related with perioperative therapy, especially pneumatic dilation, and the occurrence is more predicted by the number and duration of dilations<sup>[29]</sup>. The restricted number of pooled studies and small participant size might decrease the power of these outcomes. In addition, just the fact that observation studies were included may be somewhat responsible for these results.

The surgery time differences can be easily explained by the fact that the more complex surgical procedures and difficulties associated with the other types of complex fundoplication surgeries require more time to perform than Dor fundoplication. The recovery of achalasia patients accounts for many factors: disease itself, surgery, and complications, among others. Our pooled hospital stay time outcome indicates that surgery type has little influence on recovery. Because of the relatively skillful clinicians and the standardized nature of the surgical procedures, no perioperative surgery-related death was found for any surgical type.

Finally, in the sensitivity analysis, the primary pooled estimation of the outcomes is consistent with that of the sensitivity analysis when one study was extracted out, and this result may indicate our pooled results had good quality.

There are some limitations in this meta-analysis: (1) some indirect data acquirement methods were used, such as when dealing with the SD from range; (2) relatively high heterogeneity of data was estimated for the secondary outcomes, especially in postoperative physiological testing. This may be derived from differences in technology used in different regions and countries; (3) RCTs and non-RCTs were pooled for some outcomes because of the lack of available data and studies; and (4) though, we searched for studies without language limitation, the pooled studies were all published in English, which may be responsible for part of the observed heterogeneity.

In summary, we identified a significantly higher recurrence rate of clinical regurgitation and pathological acid reflux for Dor fundoplication than for other types of fundoplication after LHM for achalasia, although no significant difference was found between Dor fundoplication and no fundoplication. Therefore, we conclude that Dor fundoplication after LHM is not the optimum procedure for achalasia and suggest that more attention should be paid on quality of life among different fundoplication approaches.

## COMMENTS

### Background

Achalasia is generally regarded as an autoimmune esophageal motility disorder resulting from the loss of inhibitory nerve endings in the myenteric plexus of the esophagus, and laparoscopic Heller myotomy (LHM) is commonly used as the main surgical treatment. However, although LHM has been previously shown to have positive long-term outcomes for achalasia patients, gastroesophageal reflux (GER) after LHM is often one of the main failures of treatment.

### Research frontiers

In recent years, anterior 180° Dor fundoplication has been recommended after LHM for the prevention of acid reflux. However, LHM alone or LHM plus other types of fundoplication (e.g., posterior 270° Toupet and total 360° Nissen fundoplication) have also been reported to have different benefits compared with LHM plus Dor fundoplication. Thus, there is no consensus on whether Dor fundoplication is the optimum procedure after LHM for the prevention of GER.

### Innovations and breakthroughs

Dor fundoplication did not display any obvious benefit in relation to dysphagia and other symptoms versus non-fundoplication or other types of fundoplication surgery. Conversely, the pooled Dor fundoplication results indicated a higher recurrence rate for clinical regurgitation and pathological acid reflux compared with other types of fundoplication (95%CI: 1.25-40.93, and  $P = 0.03$  and 95%CI: 1.23-11.72, and  $P = 0.02$ , respectively), although no significant difference was found between Dor fundoplication and no fundoplication. The results of this meta-analysis indicate that Dor fundoplication after LHM should not be routinely recommended for achalasia.

### Applications

This present meta-analysis demonstrates that Dor fundoplication after LHM is not the optimum procedure for achalasia. To prevent postoperative GER, complex types of fundoplication, such as Toupet and Nissen fundoplication, may be added after LHM for the treatment of achalasia.

### Peer review

LHM is commonly used to treat achalasia, but GER is a frequent side effect, and an antireflux surgical technique is normally used. The aim of this meta-analysis was to assess Dor-fundoplication compared with non-fundoplication surgical techniques and other types of surgical fundoplication. The paper is well designed and demonstrates the difficulty in assessing and standardizing the published data. The paper is slightly difficult to read, but the graphs and tables facilitate comprehension. This meta-analysis gives the readers relevant reliable data for the selection of fundoplication surgical techniques after LHM.

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## Mesenteric vein thrombosis in a patient heterozygous for factor V Leiden and G20210A prothrombin genotypes

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### Abstract

Mesenteric venous thrombosis (MVT) is a rare but life threatening form of bowel ischemia. It is implicated in 6%-9% of all cases of acute mesenteric ischemia. The proportion of patients with primary (or idiopathic) MVT varies from 0% to 49%, with a decrease in frequency secondary to more recent availability of newer investigations for hypercoagulability. The presence of factor V Leiden (FVL) and prothrombin G20210A mutations (PGM) have been well documented in these cases. However, there have been scarce case reports describing MVT in heterozygotes of both these mutations occurring simultaneously and its implications on long term management. Our case describes acute MVT in a previously asymptomatic young patient with no prior history of venous thromboembolism. The patient was found to be heterozygous for FVL and PGM and treated with lifelong anticoagulation with warfarin (goal international normalized ratio: 2-3) and avoidance of hormonal contraceptives.

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**Key words:** Mesenteric vein thrombosis; Prothrombin

gene; Factor V Leiden; Heterozygous; Anticoagulation; Oral contraceptives

**Core tip:** The common presence of two thrombophilic defects increases the thrombotic risk several folds above the risk of a single defect and these tend to occur at an earlier age as seen in our case. Also the risk of recurrent thrombosis is significantly increased among these heterozygotes. Indefinite anticoagulation with oral anti-coagulants (goal International Normalized Ratio = 2-3) is recommended for high risk patients like our case with thrombosis at unusual sites (*e.g.*, mesenteric vein), and heterozygosity for both factor V Leiden and prothrombin G20210A mutations. These patients should avoid any hormonal therapy and family members should be screened for underlying prothrombotic condition.

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### INTRODUCTION

Mesenteric venous thrombosis (MVT) is a rare but life threatening form of bowel ischemia, responsible for 6%-9% of all acute mesenteric ischemia. The presence of factor V Leiden (FVL) and prothrombin G20210A mutations (PGM) have been well documented in these cases. However, there have been scarce case reports describing MVT in heterozygotes of both these mutations occurring simultaneously and its implications on long term management.

### CASE REPORT

A 22-year-old Caucasian female presented to the emer-



gency department with progressively worsening, colicky left upper quadrant abdominal pain for 4 d with radiation to her back, associated with some nausea. She denied any history of fever or changes in bowel movements. Past medical history was significant for Polycystic Ovarian syndrome treated with oral contraceptives. She denied personal or family history of thrombosis. Physical exam was significant for tenderness on left upper quadrant of abdomen without guarding, rigidity or rebound tenderness. Complete blood count and electrolytes were within normal limits. A computed tomography (CT) scan of the abdomen revealed possible superior mesenteric vein (SMV) thrombosis without evidence of bowel ischemia (Figure 1). Patient was started on enoxaparin (1 mg/kg twice daily) with bridging to warfarin. Subsequent CT angiography confirmed the initial diagnosis of SMV thrombosis. Surgical intervention was not indicated due to lack of bowel ischemia. Workup for possible vasculitis, including antinuclear antibody, Anti-neutrophil cytoplasmic antibody, titers of hepatitis B, hepatitis C, and Human Immunodeficiency Virus screening were all negative. Hypercoagulable evaluation including protein-C and S, antithrombin III, anticardiolipin antibodies were all negative. However she was found to have heterozygous mutations for both prothrombin G20210A and FVL. She was discharged on warfarin with a therapeutic level of anticoagulation. She was advised to have lifelong anticoagulation with warfarin [goal International Normalized Ratio (INR) = 2-3] and avoidance of hormonal contraceptives.

## DISCUSSION

MVT is a rare but potentially life threatening cause of mesenteric ischemia with high recurrence rates<sup>[1,2]</sup>. It is implicated in 6%-9% of all cases of acute mesenteric ischemia<sup>[1,3,4]</sup>. Predisposing conditions including myeloproliferative disorders, neoplasia, hereditary hemorrhagic telangiectasia, paroxysmal nocturnal hemoglobinuria, inherited thrombophilias, oral contraceptive pill (OCP) use, pancreatitis, recent abdominal surgery or local intraabdominal infections can be identified in most patients<sup>[5]</sup>. When no underlying etiology is identified, MVT is described as primary or idiopathic. The proportion of patients with primary (or idiopathic) MVT varies from 0% to 49%, with a decrease in frequency secondary to more recent availability of newer investigations for hypercoagulability. Abdominal pain is the most common symptom, especially with acute thrombosis, whereas chronic MVT usually manifests as portal hypertension or diagnosed incidentally by imaging. The increasing use of CT for the investigation of abdominal pain and anticoagulation for the treatment of acute MVT have improved outcomes in these patients<sup>[6]</sup>. Surgery and bowel resection may occasionally be needed for patients with bowel infarction, perforation, and peritonitis. The management of patients with chronic MVT is aimed at reducing complications of portal hypertension<sup>[4,6]</sup>.

The present case is of interest in that acute MVT was



**Figure 1** Contrast-enhanced computed tomography scan. It shows decreased attenuation within the superior mesenteric vein (arrow), immediately below the portal confluence, compatible with venous thrombosis.

the initial presentation in a patient with combined heterozygosity for FVL mutation and the G20210A prothrombin gene variation in the face of oral contraceptive use. The association of each of these mutations with thrombotic disease has been well established. Among Caucasian patients presenting with an initial episode of idiopathic deep venous thrombosis, 12%-20% will be found to be heterozygous for the FVL mutation and 6% heterozygous for the prothrombin G20210A gene variation as compared to 6% and 2% respectively, in asymptomatic Caucasian controls<sup>[7]</sup>. A recent retrospective study by Amitrano *et al*<sup>[5]</sup> noted a high prevalence of thrombophilic genotypes (75%): FVL (25%), prothrombin G20210A gene (25%), and MTHFR prothrombotic defects (50%) in patients with acute mesenteric vein thrombosis. Double heterozygotes of FVL mutation and the prothrombin G20210A gene variation have been shown to be associated with a greater risk of venous thrombosis than either defect alone. Also the age at the first episode of venous thromboembolism in double heterozygotes was significantly younger than those without both gene defects (34.7 years *vs* 40.6 years;  $P < 0.01$ ) in observational and meta-analytic studies. Finally, use of OCPs was associated with a significantly increased risk of thrombosis over those not using them (OR = 16.97, 95%CI: 3.95-72.80)<sup>[8-11]</sup>.

It seems plausible that in our case, MVT was induced by OCPs on the background of her hematological disorders leading to a hypercoagulable state. The common presence of two thrombophilic defects increases the thrombotic risk several folds above the risk of a single defect and these tend to occur at an earlier age which was also seen in our case<sup>[10]</sup>. Also the risk of recurrent thrombosis is significantly increased among these heterozygotes. Indefinite anticoagulation with oral anticoagulants (with goal INR = 2-3) is recommended for high risk patients like our case with thrombosis at unusual sites (*e.g.*, mesenteric vein), and heterozygosity for both FVL and PGM<sup>[9,12,13]</sup>. These patients should avoid any hormonal therapy including OCPs due to increased risk of blood clots. It may also be advised to screen the family members for underlying prothrombotic condition, even with a



first episode of idiopathic venous thrombosis<sup>[7,11]</sup>.

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## Development of enterohepatic fistula after embolization in ileal gastrointestinal stromal tumor: A case report

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revealed an enterohepatic fistula between the liver and distal ileum. The fistula was treated surgically by segmental resection of the distal ileum and unlooping of the liver mass.

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**Key words:** Gastrointestinal stromal tumor; Enterohepatic fistula; Therapeutic embolization; Bleeding; Ileal gastrointestinal stromal tumor

**Core tip:** Gastrointestinal stromal tumor (GIST) with fistula is a rare condition, however, it can be seen during treatment. Herein we report a case of an enterohepatic fistula that occurred after therapeutic embolization of liver mass originated from ileal GIST.

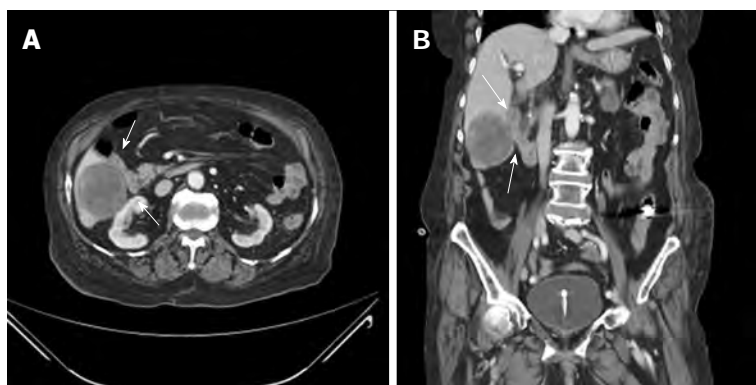
### Abstract

Gastrointestinal stromal tumor (GIST) is a rare mesenchymal tumor of the gastrointestinal tract that has been associated with the formation of fistulas to adjacent organs in few case reports. However, GIST with enterohepatic fistula has not been reported. Here we report the case of an enterohepatic fistula that occurred after embolization of a liver mass originating in the distal ileum. An 87-year-old woman was hospitalized for melena. On initial conventional endoscopy, a bleeding focus in the gastrointestinal tract was not found. Because of massive hematochezia, enteroscopy was performed through the anus. A protruding, ulcerative mass was found in the distal ileum that was suspected to be the source of the bleeding; a biopsy sample was taken. Electrocoagulation was not successful in controlling the bleeding; therefore, embolization was performed. After embolization, the patient developed a high fever and severe abdominal tenderness with rebound tenderness. Follow-up abdominopelvic computed tomography

Lee YH, Koo JS, Jung CH, Chung SY, Lee JJ, Kim SY, Hyun JJ, Jung SW, Choung RS, Lee SW, Choi JH. Development of enterohepatic fistula after embolization in ileal gastrointestinal stromal tumor: A case report. *World J Gastroenterol* 2013; 19(43): 7816-7819 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7816.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7816>

### INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor originating in the digestive tract. GISTs are thought to arise from the interstitial cells of Cajal in the normal myenteric plexus<sup>[1]</sup>. GISTs most commonly occur in the stomach (60%), followed by the jejunum and ileum (30%), duodenum (5%), colon and rectum (5%), and, rarely, the esophagus and appendix<sup>[2]</sup>. However, they account for less than 1% of all gastrointestinal (GI) tumors<sup>[3]</sup>. It is estimated that up to 6000 new



**Figure 1 Initial abdominopelvic computed tomography findings.** Enhanced computed tomography shows a 5.7 cm × 5.2 cm heterogeneous enhancing liver mass connected with a small mass of the distal ileum (arrows). A: Axial view; B: Coronal view.



**Figure 2 Large protruding mass on the distal ileum (enteroscopic findings).** A large protruding mass with ulceration was found on the distal ileum.



**Figure 3 Angiographic findings (post-embolization state).** Angiography shows that the hepatic mass (arrows) was not supplied by the right hepatic artery after embolization.

cases are diagnosed in the United States every year<sup>[4,5]</sup>.

Before the late 1990s, these mesenchymal tumors arising in the GI tract were most often classified as smooth muscle tumors or neural tumors. In the 1990s, investigators noted similarities between GIST cells and the interstitial cells of Cajal, a group of cells located in the muscularis propria and around the myenteric plexus throughout the GI tract. These tumors may not cause any symptoms unless they are in a certain location or grow to a certain size. GISTs are often found because they cause bleeding into the GI tract. Other symptoms can result from the mass effect of the tumor causing abdominal discomfort, nausea, vomiting or early satiety. The critical determinants of GIST behavior include tumor size, mitotic rate, and location<sup>[6]</sup>. The liver is the most common site of metastasis from GISTs, and liver metastases are a major determinant of patient survival<sup>[7,8]</sup>. Complications such as abscess formation, fistulae or perforation are rare but can be seen during treatment<sup>[9,10]</sup>.

Here we describe the case of a patient with ileal GIST with an enterohepatic fistula caused by tumor necrosis after therapeutic embolization for the control of active bleeding.

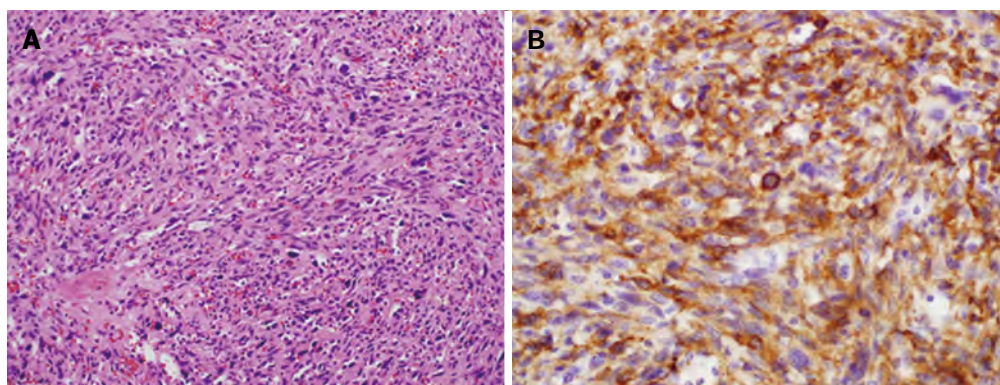
## CASE REPORT

An 87-year-old woman was admitted to the emergency department because of melena that occurred the day before. She had been taking antihypertensive medica-

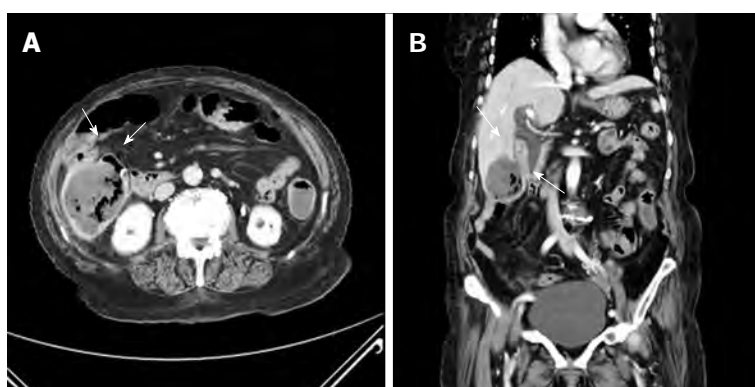
tion for 7 years and was not taking aspirin or other antiplatelet agents. She had a history of cholecystectomy, total abdominal hysterectomy, and right hemicolectomy due to GI bleeding. At admission, her vital signs were stable; laboratory findings showed anemia with a serum hemoglobin concentration of 7.8 g/dL and leukocytosis with a white blood cell count of 14000/mm<sup>3</sup>. A definite bleeding focus was not found on upper endoscopy and total colonoscopy. On a subsequent capsule endoscopy, a small amount of fresh blood was seen in the small bowel but the exact bleeding site was not identified. Abdominopelvic computed tomography (CT) showed a 5.7 cm × 5.2 cm heterogeneous enhancing mass in segments V and VI of the liver (Figure 1) adjacent to the distal ileum and multiple ill-defined low-attenuation lesions that were thought to represent metastases.

On the 9<sup>th</sup> day of hospitalization, she showed massive hematochezia. Single-balloon enteroscopy was performed through the anus, and a large protruding mass with central ulceration, which was suspected to be the bleeding focus, was found on the distal ileum (Figure 2). After an enteroscopic biopsy of the ileal mass was taken, active bleeding occurred that was not controlled by enteroscopic electrocoagulation. The patient refused surgical treatment for the active ileal bleeding. Therapeutic angiography was performed; however, no extravasation of the contrast was found in the inferior mesenteric or superior mesenteric arteries. Because a liver mass adja-





**Figure 4** Microscopic findings (Endoscopic biopsy specimen in the ileum). A: Microscopic image of the specimen demonstrating spindle cells (HE,  $\times 200$ ); B: The tumor cells were strongly positive for c-KIT (c-KIT,  $\times 400$ ).



**Figure 5** Abdominopelvic computed tomography findings after embolization. Internal necrosis and direct communication (arrows) with the small bowel were identified in the liver mass. A: Axial view; B: Coronal view.

cent to the small bowel had been seen on the abdominopelvic CT, it was suspected that the protruding mass in the distal ileum originated from the suspected malignant liver mass. Based on the assumptions that the bleeding lesion originated from the hepatic mass and was supplied by the hepatic artery, a branch of the right hepatic artery was embolized, and there was no evidence of additional bleeding in the GI tract (Figure 3).

The biopsy specimen showed that the ileal mass was a GIST with more than 20 cells undergoing mitosis per 20 high-power fields (Figure 4). After the embolization, the patient developed fever and right upper quadrant pain. On repeated abdominopelvic CT 5 d after embolization, newly developed internal necrosis and a direct communication with distal ileum were identified in the previously seen liver mass (Figure 5).

Segmental resection of the distal ileum and unlooping of the liver mass were undertaken, and fistula formation between the liver mass and the distal ileum was found intraoperatively. The gross specimen of the resected ileum showed a large fistula orifice that connected with the adjacent liver (Figure 6). Histopathologic examination of the surgical specimen obtained from the resected distal ileum and adjacent liver mass showed that both specimens were identical with GIST on immunohistochemical staining. After the operation, the patient refused chemotherapy and was transferred to a convalescent hospital. She died 6 mo later of multiple organ failure after recurrent GI sepsis.

## DISCUSSION

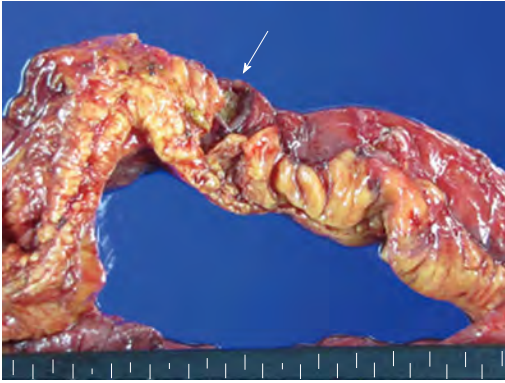
GISTs with intraperitoneal rupture or organ invasion have rarely been reported<sup>[11]</sup>. Nonetheless, an enterocolic fistula can be caused by prior surgery, malignancy, and infection<sup>[12,13]</sup>. In similar situations, albeit very rarely, enterohepatic fistulas can develop with GISTs.

We presented a case of an enterohepatic fistula that occurred after embolization of a metastatic liver mass from a malignant GIST. An active bleeding site was not found on initial upper endoscopy and colonoscopy. Abdominopelvic CT demonstrated a 5.7 cm liver mass adjacent to the small bowel.

On initial workup, there was no evidence suggesting that the hepatic mass was the source of the GI bleeding. After enteroscopy by the anal approach because of the patient's massive hematochezia, we found an exophytic mass that was thought to be connected with the hepatic mass and the cause of the bleeding. Therefore, embolization of the right hepatic artery supplying the liver mass was performed. However, within 24 h of arterial embolization, the patient developed fever and right upper quadrant abdominal pain, indicating that fistula formation between the liver mass and distal ileum occurred.

As the patient had undergone several previous abdominal operations, we assumed there was a strong possibility that intraabdominal adhesions were present, specifically between the small bowel and liver. High mitotic rates ( $> 20$  per 20 high power field) also suggested





**Figure 6** Gross findings of resected segment of the distal ileum. It showed a large fistula orifice (arrow) which connected with the adjacent liver, as proven by food material in the liver mass.

that the tumor was likely to be invasive. Therefore, it was highly probable that the primary ileal tumor directly invaded the adjacent liver and then developed multiple hepatic metastases. Embolization of the right hepatic artery was performed to control the patient's massive hematochezia, and necrosis of the hepatic tumor gradually organized into a fistula.

There have been several cases of GIST with abscess formations, perforations, or various fistulas<sup>[9,14-17]</sup>. However, GIST with a hepatic fistula is a unique presentation that has not been reported to our knowledge.

Watanabe *et al.*<sup>[9]</sup> presented a similar case of GIST with a vesicocutaneous fistula during treatment with sunitinib. Sunitinib has anti-tumor and anti-angiogenic effects that cause necrosis, similar to our case in which arterial embolization induced visceral necrosis, with a resulting fistula in both cases.

In conclusion, we have reported a case of an enterohepatic fistula that occurred after embolization of a metastatic liver mass in an ileal GIST. It is important that physicians consider the possible complications, such as fistula, perforation and abscess formation, during treatment of highly invasive GISTs.

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## Unusual early-stage pancreatic sarcomatoid carcinoma

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poor prognosis. Its pathogenesis has not been elucidated. We herein report a case of an early-stage SCP involving successful treatment and a good prognosis. The patient was a 48-year-old Chinese man with a 5-mo history of vague abdominal pain. Ultrasonography revealed a 93 mm × 94 mm × 75 mm mass of mixed echogenicity in the tail of the pancreas. Laboratory test results were within the normal range, with the exception of an obviously increased pretreatment neuron-specific enolase level. The plasma transforming growth factor (TGF)β1 and interleukin-11 levels were obviously increased according to enzyme-linked immunosorbent assay. Microscopically, the excised tumor tissue comprised cancer cells and mesenchymal cells. Immunohistochemical analysis was positive for α-1-antichymotrypsin, pan-cytokeratin, cytokeratin 19, cytokeratin 8/18, and vimentin and negative for CD68 and lysozyme. The pathogenetic mechanism of this case shows that TGFβ1 may regulate the epithelial-to-mesenchymal transition in SCP. With early eradication of the tumor and systemic therapy, this patient has been alive for more than 3 years without tumor recurrence or distant metastasis. This case is also the first to show that TGFβ1 may regulate the epithelial-to-mesenchymal transition in early-stage SCP.

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**Key words:** Sarcomatoid carcinoma of the pancreas; Transforming growth factorβ1; Epithelial-to mesenchymal transition; Interleukin-11; Vimentin

**Core tip:** We herein report a case of an early-stage sarcomatoid carcinoma of the pancreas (SCP) involving successful treatment and a good prognosis. The plasma transforming growth factor (TGF)β1 and interleukin-11 levels were obviously increased according to enzyme-linked immunosorbent assay. Immunohistochemical analysis was positive for pan-cytokeratin, cytokeratin 8/18, and vimentin and negative for CD68 and lysozyme. The pathogenetic mechanism of this case shows that TGFβ1 may regulate the epithelial-to-

### Abstract

Sarcomatoid carcinoma of the pancreas (SCP) is a very rare pathological type of carcinoma that usually has a

mesenchymal transition in SCP. This case is also the first to show that TGF $\beta$ 1 may regulate the epithelial-to-mesenchymal transition in early-stage SCP.

Ren CL, Jin P, Han CX, Xiao Q, Wang DR, Shi L, Wang DX, Chen H. Unusual early-stage pancreatic sarcomatoid carcinoma. *World J Gastroenterol* 2013; 19(43): 7820-7824 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i43/7820.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i43.7820>

## INTRODUCTION

Microscopically, sarcomatoid carcinoma of the pancreas (SCP) comprises mostly anaplastic cells and is strikingly sarcoma-like in appearance<sup>[1]</sup>. SCP may originate from many different organs, such as the pancreas, lung, liver, and esophagus<sup>[2-8]</sup>. Confirmation of this disease is often based on the pathological diagnosis. Advanced radiographic studies are also good tools with which to support the diagnosis of sarcomatoid carcinoma<sup>[2]</sup>. Early diagnosis and eradication of the tumor is important for a better prognosis of malignant sarcomatoid carcinomas.

It has been proposed that during malignant progression, carcinoma cells undergo an epithelial-to-mesenchymal transition (EMT), which is a vital step in the formation of pancreatic ductal adenocarcinoma (PDAC)<sup>[9]</sup>. The etiology of SCP is unknown. The *EML4-ALK* fusion gene was reportedly involved in the development of a sarcomatoid carcinoma of the lung<sup>[10]</sup>. *ALK* gene amplification is a nonrandom and clonally related event in a subset of pulmonary sarcomatoid carcinomas, but its biologic rationale deserves further investigation<sup>[11]</sup>. The mechanism of the formation of SCP and its metastasis remains unknown.

## CASE REPORT

A 48-year-old Chinese man suffered from vague abdominal pain for 5 mo. He had no evidence of jaundice, hematuria, vomiting, or fever, but abdominal swelling and chest distress were present. He had no smoking or drinking habits, and no history of malignant or other diseases. Ultrasonography revealed a 93 mm  $\times$  94 mm  $\times$  75 mm mass of mixed echogenicity in the tail of the pancreas (Figure 1). Computed tomography (CT) showed displacement of the retroperitoneal organs by the mass (Figure not shown).

Laboratory test results, including a blood count, serum biochemistry, and urinalysis, were within the normal ranges. The levels of 11 common serum tumor markers, including CA19-9, CEA, and CA242, were normal, except that NSE was obviously increased before any treatment (Table 1). The plasma transforming growth factor (TGF) $\beta$ 1 and Interleukin (IL)-11 levels were higher than those of the healthy controls, patients with PDAC, and

**Table 1 Pretreatment serum tumor markers**

Tumor markers	Index	Normal range
CA19-9 (KU/L)	1.21	< 35.00
CA242 (KU/L)	1.14	< 20.00
CA125 (KU/L)	11.71	< 35.00
CA15-3 (KU/L)	2.32	< 35.00
NSE (ng/mL)	23.42	< 13.00
CEA (ng/mL)	0.24	5
Ferritin (ng/mL)	161.47	< 322.00
$\beta$ -HCG (MIU/mL)	0.12	< 3.00
AFP (ng/mL)	1.07	< 20.00
Free-PSA (ng/mL)	0.32	< 1.00
PSA (ng/mL)	1.53	< 5.00
HGH (ng/mL)	0.36	< 7.50

CA: Cancer antigen; NSE: Neuron-specific enolase; CEA: Carcinoembryonic antigen;  $\beta$ -HCG:  $\beta$ -human chorionic gonadotropin; AFP:  $\alpha$ -fetoprotein; PSA: Prostate-specific antigen; HGH: Human growth hormone.

**Table 2 Plasma transforming growth factor $\beta$ 1 and interleukin-11 in sarcomatoid carcinoma before any treatment**

Tumor (pg/mL)		Index (median)	n
TGFβ1	SCP	35688	1
	PDAC	10475 (5142-30865)	20
	PanINs	7949 (6655-11404)	10
	HC	6865 (3272-22463)	11
IL-11	SCP	58	1
	HC	22 (10-42)	11

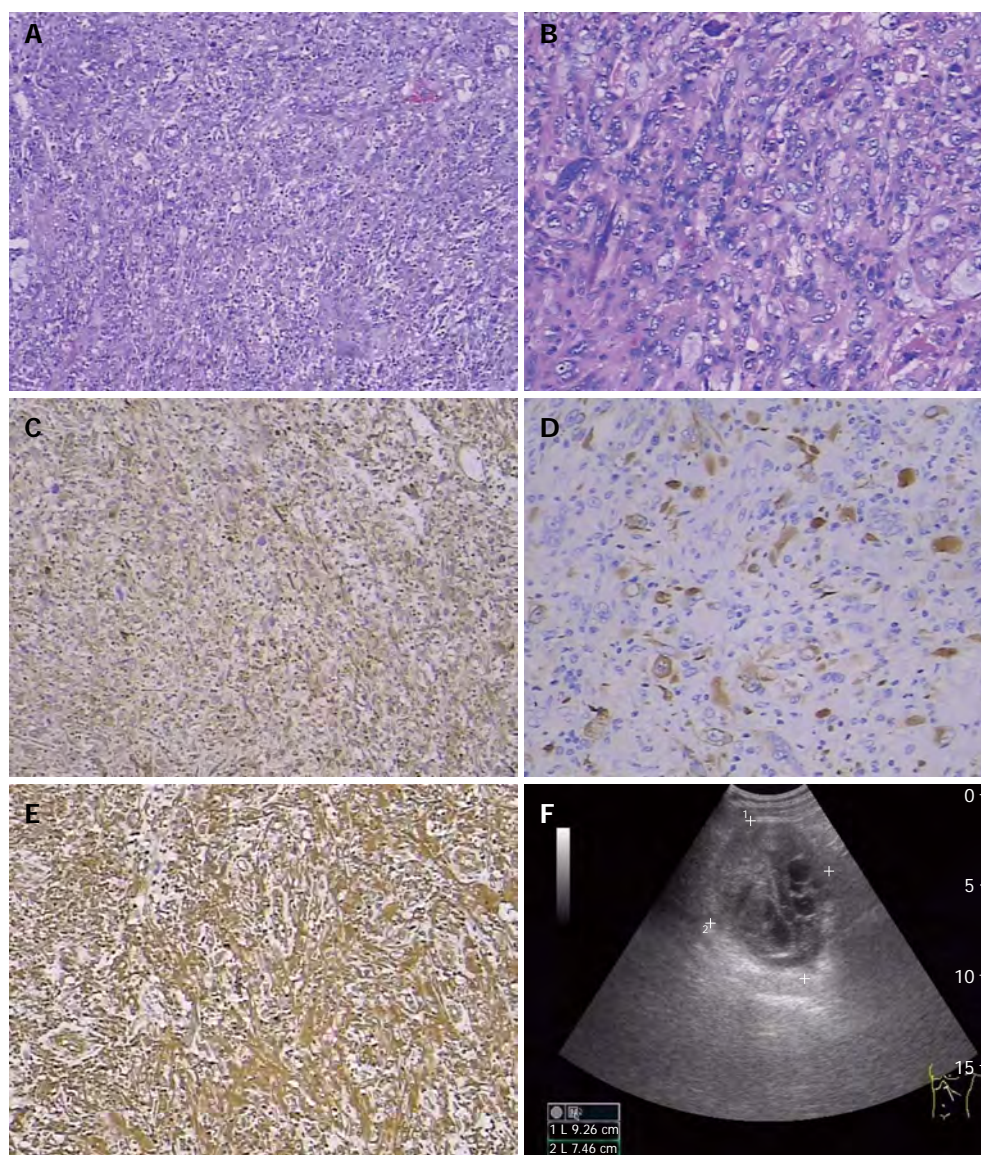
SCP: Sarcomatoid carcinoma of the pancreas; HC: Healthy control; PDAC: Pancreatic ductal adenocarcinoma; PanINs: Pancreatic intraepithelial neoplasias; TGF: Transforming growth factor; IL-11: Interleukin-11.

patients with pancreatic intraepithelial neoplasias (PanINs) (Table 2).

Surgery was performed, and the tumor was completely resected. The mass measured 10 cm  $\times$  8 cm  $\times$  3.5 cm and had cystic features after the excision. The section containing the solid tumorous tissue was pale in color. Microscopically, the excised tumor tissue comprised cancer cells and mesenchymal cells, with dispersion of atypical cells and obvious karyokinesis. Some were fusiform in shape and some were multinucleated giant cells (Figure 1A and B). Therefore, SCP was pathologically diagnosed. The neighboring lymph nodes and incisional margin were free of tumor cells. Immunohistochemical study results showed that the tumor cells were positive for vimentin,  $\alpha$ -1-antichymotrypsin (AACT), cytokeratin 19, cytokeratin 18 (Figure 1C), and pan-cytokeratin (Figure 1D) and negative for CD68 and lysozyme (data not shown). Thus, an early-stage SCP was diagnosed.

The preoperative diagnosis was cystadenoma in the tail of the pancreas. Seven months after surgical excision, there was no evidence of tumor recurrence or metastasis. Digital subtraction angiography interventional chemotherapy was then implemented. Gemcitabine (1.4 g), oxaliplatin (150 mg), and floxuridine (1.0 g) were intravenously injected *via* the superior mesenteric artery and celiac trunk artery. After 28 mo of follow-up, a





**Figure 1** Hematoxylin and eosin stained sections, immunohistochemical test and ultrasonography diagnosis. A: Histologic findings of the tumor; the morphology of sarcomatoid carcinoma of the pancreas (SCP) is shown (hematoxylin and eosin,  $\times 100$ ); B: Microscopically, the excised tumor tissue comprised cancer cells and mesenchymal cells, with dispersion of atypical cells and obvious karyokinesis (hematoxylin and eosin,  $\times 200$ ); C: Widely diffuse immunohistochemical staining for the epithelial marker cytokeratin 18 ( $\times 100$ ); D: Heterogeneous immunohistochemical staining for the epithelial marker pan-cytokeratin (Pan-CK) ( $\times 100$ ); E: Widely diffuse immunohistochemical staining for the mesenchymal marker vimentin ( $\times 100$ ); F: Ultrasonography revealed a 93 mm  $\times$  94 mm  $\times$  75 mm mass of mixed echogenicity in the tail of the pancreas.

routine check-up and CT scan revealed that the patient was in good condition and free of tumor recurrence and metastasis. Because the patient had the opportunity to be treated in the early stage of the disease, he is in good condition and has been alive for more than 3 years without tumor recurrence or metastasis.

## DISCUSSION

Sarcomatoid carcinoma is a rare and very aggressive malignant tumor comprising a mixture of carcinomatous and sarcomatous elements<sup>[12]</sup>. Areas of spindle cells arranged in a storiform pattern were present<sup>[13]</sup>. The tumor demonstrated cellular patterns similar to those present in tumors of mesenchymal origin in the case. In the

present case, many cells undergoing heterotypic division were seen in the tissue specimen, and karyokinesis was frequent. Some cells were fusiform in shape, and some were pleomorphic giant cells. This change into pleomorphic giant cells was the most frequent sarcomatoid transformation encountered<sup>[1]</sup>. Compared with ordinary pancreatic carcinomas, malignant giant cell tumors of the pancreas appear to have a distinctive behavior characterized by local invasiveness, a reluctance to metastasize, and a more favorable prognosis when resected<sup>[1]</sup>. Immunohistochemical study results showed that the tumor cells were positive for vimentin, AACT, pan-cytokeratin, cytokeratin 19, and cytokeratin 8/18 and negative for CD68 and lysozyme. Some authors reported that the tumor cells in sarcomatoid carcinoma were positive for



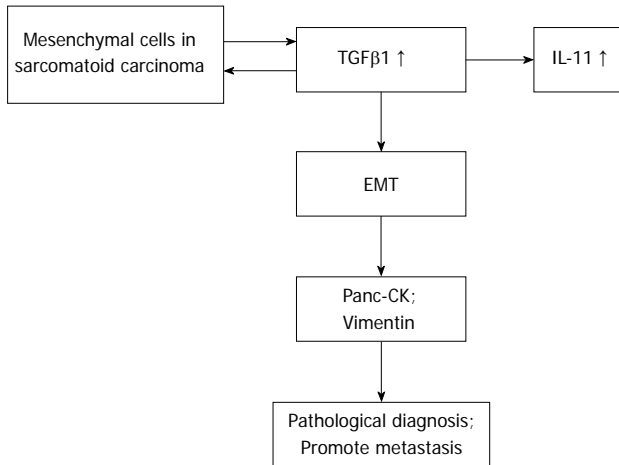


Figure 2 Mechanism of transforming growth factor $\beta$ 1 regulating the epithelial-to-mesenchymal transition of sarcomatoid carcinoma of the pancreas. TGF: Transforming growth factor; EMT: Epithelial-to-mesenchymal transition; IL-11: Interleukin-11.

CK, S-100 protein,  $\alpha$ 1-antitrypsin,  $\alpha$ 1-chymotrypsin, anti-CA19-9, and SMA. No cells were positive for vimentin or desmin<sup>[14]</sup>. Vimentin-positive tumor cells are of mesenchymal origin in sarcomatoid carcinoma and are also seen in inflammatory myofibroblastic tumor of the prostate, another rare malignant disease<sup>[15]</sup>. In the present case, both epithelial and mesenchymal markers were positive. The process of EMT may play an important role in the formation of SCP. TGF $\beta$  signaling plays a dual role in oncogenesis. TGF $\beta$  can sometimes function as a tumor suppressor gene that inhibits the proliferation of normal epithelial cells, while in other tumor types it functions as an oncogenic gene. This dual function implies that the activity of TGF $\beta$  is highly dependent on the cellular context, pathological type, and specific environment<sup>[16-21]</sup>. In this case, the TGF $\beta$ 1 level was markedly higher than those in patients with PDAC and PanINs and in HCs. TGF $\beta$ 1 may regulate the EMT pathway in pancreas cells and promote the formation of SCP. The plasma IL-11 level in the present patient was obviously higher than that of the healthy controls. IL-11 is a TGF $\beta$  target gene. IL-11 stimulates the production of the osteoclastogenic factors RANKL and granulocyte macrophage-colony stimulating factor in osteoblasts. Induction of IL-11 and CTGF expression by TGF $\beta$  is mediated by the SMAD pathway<sup>[22]</sup>. TGF $\beta$ 1 could be an important driving force during the sarcomatoid transdifferentiation of clear cell renal cell carcinoma<sup>[23]</sup>. The combination of early diagnosis of sarcomatoid carcinoma, eradication of the tumor, and systemic therapy may provide a chance of a good prognosis. Whether postoperative patients in the early tumor stage require chemotherapy may be controversial. High levels of NSE, TGF $\beta$ 1, and IL-11 in the serum or plasma may help in the early diagnosis of SCP. TGF $\beta$ 1 may play an important role in tumor metastasis (Figure 2) and some papers support our hypothesis<sup>[24,25]</sup>. In view of the complex biologic behavior of SCP, continued real-time monitoring of the clinical course of the

disease is strongly recommended.

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## Is liver biopsy necessary in the management of alcoholic hepatitis?

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### Abstract

Acute alcoholic hepatitis (AAH) is characterised by deep jaundice in patients with a history of heavy alcohol use, which can progress to liver failure. A clinical diagnosis of AAH can be challenging to make in patients without a clear alcohol history or in the presence of risk factors for other causes of acute liver failure. Other causes of acute on chronic liver failure such as sepsis or variceal haemorrhage should be considered. Liver biopsy remains the only reliable method to make an accurate diagnosis. However, there is controversy surrounding the use of liver biopsy in patients with AAH because of the risks of performing a percutaneous biopsy and limitations in access to transjugular biopsy. We review the existing literature and find there are few studies directly comparing clinical and histological diagnosis of AAH. In the small number of studies that have been conducted the correlation between a clinical and histological diagnosis of AAH is poor. Due to this lack of agreement together with difficulties in accessing transjugular liver biopsy outside tertiary referral centres and research institutions, we cannot advocate universal biopsy for AAH but there remains a definite role for liver biopsy where there is clinical diagnostic doubt or dual pathology. It

also adds value in a clinical trial context to ensure a homogeneous trial population and to further our understanding of the disease pathology. Further prospective studies are required to determine whether non-invasive markers can be used to accurately diagnose AAH.

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**Key words:** Alcoholic hepatitis; Liver biopsy; Diagnosis; Prognosis; Transjugular liver biopsy

**Core tip:** Acute alcoholic hepatitis (AAH) is a clinical syndrome of jaundice and coagulopathy in a patient with a recent history of heavy alcohol consumption. Clinical diagnosis is challenging and transjugular liver biopsy remains the gold standard. Here we discuss the literature which demonstrates there is a lack of agreement between clinical and histological diagnosis. This, together with limited availability of transjugular liver biopsy makes it impossible to advocate universal biopsy in all suspected cases of AAH. We suggest further research is conducted to prospectively compare histological and clinical parameters and to develop a reliable and accurate non-invasive diagnostic tool.

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### INTRODUCTION

Acute alcoholic hepatitis (AAH) is a severe manifestation of alcoholic liver disease and is associated with a high short term mortality of 35% if untreated<sup>[1]</sup>. The clinical syndrome is characterised by a history of excessive alco-

hol consumption (> 80 g ethanol/d in males and > 60 g ethanol in females) and a recent onset of deep jaundice, which can lead to progressive liver failure. Symptoms are usually non-specific such as fatigue, weakness and anorexia but there is usually tender hepatomegaly and often fever, ascites and encephalopathy<sup>[2,3]</sup>. These features can also be seen in many hazardous drinkers making AAH challenging to diagnose clinically. Histology remains the gold standard in diagnosing AAH with well described features of steatosis, hepatocyte injury and neutrophil infiltration<sup>[4,5]</sup>. However, there are difficulties in access to transjugular liver biopsy and subsequent expert histopathology review limiting its utility.

Indeed, there is controversy over whether histology is essential in the diagnosis of AAH with the American Association for the Study of Liver Disease and European Association for the Study of the Liver offering different guidance<sup>[6,7]</sup>. Here, we discuss the existing evidence regarding liver biopsy in the management of AAH.

## DIFFERENTIAL DIAGNOSIS OF AAH

Obtaining an accurate alcohol history is notoriously difficult but especially so in patients with potential AAH who are often unable to provide an accurate history due to symptoms of encephalopathy or acute alcohol withdrawal. Where there is uncertainty regarding recent heavy alcohol consumption as much evidence as possible should be obtained from relatives and friends or failing that primary or secondary care records. In all situations it is important to consider the differential diagnosis of acute liver failure including acute viral hepatitis, autoimmune hepatitis, Wilson's disease and drug induced liver injury. These factors can also co-exist in patients with heavy alcohol consumption, most commonly hepatitis C infection, which has been reported as a co-factor in up to 25% in one cohort<sup>[8]</sup>. Although clues to the diagnosis can be ascertained from the history and laboratory tests, in complex cases or where there is diagnostic doubt a liver biopsy often supplements the clinical information.

Studies which included histological diagnosis as an entry requirement have shown a variation in the prevalence of cirrhosis in patients with AAH from 65%-95%<sup>[9,10]</sup>. Therefore a minority of patients may present with AAH without features of chronic liver disease making it important to exclude other causes of acute liver failure.

However, in patients with chronic liver disease the key challenge in making a diagnosis of AAH is in differentiating it from other causes of acute on chronic liver failure (ACLF).

## ACUTE ON CHRONIC LIVER FAILURE

ACLF is an increasingly recognised entity which, although not formally defined, has been the subject of 2 recent consensus meetings<sup>[11,12]</sup>. Both groups describe the condition as an acute deterioration in a patient with chronic liver disease associated with jaundice and coagulopathy<sup>[12]</sup>

and high 3-mo mortality due to multiorgan failure<sup>[11]</sup>. In patients with alcohol-related cirrhosis AAH can be the precipitating cause of ACLF but other precipitants must be excluded especially gastrointestinal haemorrhage and sepsis of any source.

A recently published large multicentre prospective observational study was conducted to help establish diagnostic criteria for ACLF among European patients<sup>[13]</sup>. In 197 patients with alcohol-related liver disease (ALD) who met the criteria for ACLF, alcohol consumption within the preceding 3 mo was considered the precipitating event in 69 (35%) but because only small numbers underwent liver biopsy a histological diagnosis of alcoholic steatohepatitis (ASH) could not be made in these cases. The other commonest precipitants were bacterial infection and gastrointestinal haemorrhage.

Further information can be obtained from studies in patients with ACLF who underwent liver biopsy. In a series of 68 patients with acute decompensation of ALD 36 had a clinical diagnosis of AAH but only 18 of these (50%) had corresponding histological features of ASH, while a further 13 (19%) had histological ASH without clinical AAH<sup>[14]</sup>. In a separate study of 54 ALD patients admitted to hospital with ACLF a precipitating cause could only be identified in 30 (56%): 13 due to alcohol, 12 sepsis and 5 variceal bleeds<sup>[15]</sup>.

These studies demonstrate that AAH is not always the cause of ACLF in patients with ALD; other causes of acute decompensation must be sought.

## LIVER BIOPSY IN AAH PATIENTS

ASH, originally defined by an international consensus group, was described as the presence of steatosis, hepatocyte injury (ballooning and apoptosis) and polymorphonuclear infiltration<sup>[5]</sup>. Additional features of Mallory-Denk bodies and intraparenchymal cholestasis are observed but not necessary for diagnosis<sup>[16]</sup>. However, these characteristic changes of ASH can be seen in patients with ALD without the clinical syndrome of AAH or even active alcohol consumption. As described above 13 out of 68 (19%) patients with acute decompensation of ALD had histological ASH without corresponding clinical AAH<sup>[14]</sup>. ASH has also been noted in explant tissue from patients transplanted for ALD who were presumed to be abstinent. In 1 study ASH was noted in 32 of 148 (22%) explants from ALD patients including 25 who declared abstinence from alcohol for more than 6 mo<sup>[17]</sup>. A Spanish group reported 36 out of 68 (53%) explants from ALD patients had ASH, which was not associated with a reduced survival<sup>[18]</sup>. Therefore, it is important to be clear about terminology: we recommend the use of ASH to apply to the histological diagnosis while AAH should refer to the clinical syndrome.

In patients with severe AAH, many with deranged coagulation and significant ascites, the risks of performing a percutaneous liver biopsy are increased and a transjugular route is required. This is a well described and safe to per-

form procedure<sup>[19]</sup> which should be considered standard practice in hepatology centres<sup>[20]</sup>. In a systematic review of over 7500 transjugular liver biopsies minor bleeding (not requiring blood transfusion) and major complications were similar to the percutaneous approach (6.5% and 0.6% respectively) and death was rare at 0.09%<sup>[21]</sup>. No specific subgroup analysis was performed in those with coagulopathy as the indication but in 183 patients with congenital coagulopathy there was no mortality and the minor and major haemorrhagic complication rates were similar to the whole group (6% and 0.5% respectively). Sufficient biopsy material allowed a histological diagnosis to be made in 96.1% of samples with a median number of 2.7 passes<sup>[21]</sup>. In 132 patients presenting with AAH transjugular biopsy allowed accurate histological interpretation in 100% of cases with a mean length of 19 mm of tissue<sup>[22]</sup>.

Unfortunately little attention has been paid to the timing of biopsy in AAH, which is usually unreported in clinical trials. Early biopsy, as is the practice of several liver centres (median time of 3 d in 1 centre)<sup>[22]</sup>, may be more sensitive in confirming the diagnosis of AAH. Further studies are required to establish the optimal timing of liver biopsy.

Interpreting a liver biopsy specimen requires appropriate expertise and experience with access to specialist histopathologists but there still remains interobserver error. In patients with severe AAH and background cirrhosis this error has been shown to be minimal in one study with a high degree of concordance between 2 histopathologists ( $\kappa = 0.77$ )<sup>[23]</sup>. However, this was based in a specialist hepatology centre with expert liver pathologists and was lower in patients without cirrhosis ( $\kappa = 0.65$ ).

Access to transjugular liver biopsy is variable and generally only available in tertiary referral centres and academic institutions. Transferring patients between centres only to obtain a liver biopsy is logistically challenging, may increase the risk to the patient and is associated with additional costs.

## HISTOLOGICAL SCORES TO DETERMINE PROGNOSIS

There is evidence that some of the histological characteristics as well as liver expressed soluble factors, such as chemokines and interleukins, can be used to predict clinical outcome from AAH. This could assist clinical decision making and guide treatment choices. Steatosis < 20% is an independent predictor of poor outcome<sup>[24]</sup> and polymorphonuclear cell infiltrate is associated with severity of AAH<sup>[25]</sup> and is correlated with 1 year survival<sup>[10]</sup>. Liver tissue interleukin-8, a potent neutrophil chemoattractant, correlates with neutrophil infiltration and biochemical markers of outcome<sup>[26,27]</sup>. Intercellular adhesion molecule-1, a leukocyte adhesion molecule associated with T helper cell recruitment to sites of inflammation, is elevated in AAH versus fatty liver and its level correlates with histological hepatocellular damage<sup>[28]</sup>. CXC family chemokine expression correlates with prognosis<sup>[25]</sup>

and CCL2 is elevated in AAH<sup>[29]</sup>. However, a histological scoring system combining these multiple parameters has not been developed and many of these individual predictors have not been validated nor are they routinely used outside a research setting. A histological severity score including K8/18 staining (a marker for hepatocyte ballooning) shows good accuracy for predicting 90-d survival but has not yet been validated in a second cohort<sup>[14]</sup>. Only 1 validated AAH histology score has been published in abstract form, finding that fibrosis stage, polymorphonuclear infiltrate, cholestasis and the presence of megamitochondria predicted 90 d mortality<sup>[30]</sup>. Further studies in this area are required to establish a reliable and reproducible histological scoring system that predicts clinical outcome.

## USING CLINICAL SCORES TO DIAGNOSE AAH

An accurate non-invasive clinical test for AAH remains elusive. There are a host of different clinical scoring systems in the literature which have been developed to determine severity and likely benefit from glucocorticoid therapy (modified discriminant function<sup>[31]</sup>), prognosis (Glasgow Alcoholic Hepatitis Score<sup>[32]</sup>) or response to glucocorticoids (Lille score<sup>[33]</sup>). Little work has been conducted to examine how accurate these clinical scores are by comparing them to histological data. An abstract describing a literature review of 39 randomized controlled trials (RCTs) in AAH (11 of which had histological ASH as an entry criteria) suggested that overall 84.5% had histological ASH but this could be enriched to 96% if a minimum bilirubin level of 80  $\mu\text{mol/L}$  was used<sup>[34]</sup>. Another study (also published only as an abstract) found that 70% of patients with a clinical diagnosis of AAH with an MDF  $\geq 32$  had histological confirmation of ASH<sup>[35]</sup>. Further work to prospectively compare clinical and histological diagnosis of AAH is needed and to establish whether non-invasive methods of diagnosing AAH can be used accurately. One such study is currently underway in a United Kingdom RCT (STOPAH; ISRCTN reference number: ISRCTN88782125), which intends to compare histological and clinical parameters as part of a secondary analysis. This pragmatic study, which aims to reflect everyday United Kingdom clinical practice, includes all patients with a clinical diagnosis of severe AAH (defined as recent onset jaundice with a bilirubin > 80  $\mu\text{mol/L}$  and heavy alcohol consumption within the last 2 mo of > 80 g/d in men and > 60 g/d in women with a discriminant function  $\geq 32$ ) without the requirement for a biopsy. Liver histology from the subgroup that has a biopsy as part of an institution's standard clinical care will be compared to clinical parameters using this robust clinical definition of AAH.

## CONCLUSION

Whilst it is clear that liver biopsy remains the recommended gold standard to diagnose ASH<sup>[6]</sup> and has the



potential to be used as a prognostic indicator, opinion remains divided on its practical clinical utility. Liver biopsy in this group of patients is as safe to obtain by the transjugular route and provides sufficient tissue to make a histological diagnosis compared to the percutaneous route. It is mandatory where there is diagnostic uncertainty and is useful where cirrhosis is not clinically suspected. However, the widespread clinical utility of liver biopsy is limited by the lack of provision of transjugular liver biopsy in non-specialist centres and cannot therefore be recommended to form part of routine practice outside these centres. In addition, the timely specialist interpretation that is essential to make the diagnosis of ASH is also generally restricted to specialist centres. Efforts need to be made to improve clinical diagnostic accuracy and studies are required to prospectively compare histological and clinical features in patients with AAH. There is the suggestion that accuracy can be improved by using a high cut-off of bilirubin  $> 80 \mu\text{mol/L}$ <sup>[34]</sup> but this also requires prospective validation, which may be provided by the STOPAH clinical trial.

Liver biopsy in AAH plays an important role in a research context: it can improve the homogeneity of the study population and reduce the risk of type II error. It allows researchers to study the mechanisms of alcohol mediated liver damage and identify new targets for therapy. However, including only patients with histological ASH may not actually reflect the patient population we treat in everyday clinical practice but only a highly selected subgroup. Until a reliable non-invasive diagnostic method for the clinical syndrome of AAH has been developed and validated, clinical trials should continue to include patients defined by a robust clinical definition of AAH.

In summary, we recommend the clinical use of transjugular liver biopsy in patients with severe AAH only where there is irresolvable diagnostic uncertainty and as a research tool to further our understanding of the mechanisms and pathology of the disease.

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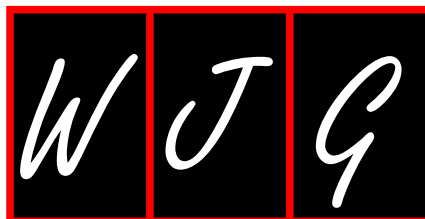
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## Improvement analysis of article quality in *World Journal of Gastroenterology* during 2008-2012

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The number of countries of origin of *WJG* authors was 65, 66, 61, 65 and 60 for the period 2008-2012. Authors from 66 countries cited a total of 3194 of the 4409 papers, and these citations were found in 1140 journals.

**CONCLUSION:** The results suggest that *WJG* has stayed on the track of normal international publication and all the indices of this journal are stable and reasonable.

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**Key words:** Author analysis; Bibliometrics; *World Journal of Gastroenterology*; Science Citation Index

### Abstract

**AIM:** To understand the changes and development of *World Journal of Gastroenterology (WJG)* in recent years.

**METHODS:** The Journal Citation Report (JCR) and SCI-E database of the ISI Web of Knowledge were used to search the articles and data of related indices in *WJG* during 2008-2012. Bibliometric methods were used for statistical analysis of the author's degree of collaboration, collaboration rate, the first author's publications, high-productivity authors, the authors' origins in each year; the distribution of the countries and journals of the authors citing *WJG* papers was also analyzed. In addition, the indices related to this journal in each year were compared with the data from 6 SCI journals in the field of gastroenterology in the 2012 volume.

**RESULTS:** A total of 4409 papers in *WJG* were examined in this study. For the period 2008-2012, the self-citation rate was 8.59%, 6.02%, 5.50%, 4.47% and 5.21%. Of a total of 3898 first authors, 3526 published 1 paper, 291 published 2 papers, 59 published 3 papers, and 22 published 4 or more papers. The origin of *WJG* authors covered the six continents, and the majority came from Asia, Europe and North America.

**Core tip:** A total of 4409 articles were examined to explore the development of *World Journal of Gastroenterology (WJG)* during 2008-2012. Based on analysis of the relevant indices, this study not only discussed the development and changes of *WJG* in recent years, but also the characteristics of the published papers and the authors' origins. Furthermore, we performed analyses involving several journals of gastroenterology. The results show that all the indexes of this journal are stable and reasonable, and *WJG* has developed into one of the important journals in the field of gastroenterology.

Yang H, Chen YX. Improvement analysis of article quality in *World Journal of Gastroenterology* during 2008-2012. *World J Gastroenterol* 2013; 19(44): 7830-7835 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/7830.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.7830>

### INTRODUCTION

Journal quality evaluation is an important subject of concern to both editors and readers. Although the evaluation indexes are frequently the citation data of the papers

in a given journal such as the total citation frequency, impact factor and so on, paper publication data for the journal, such as the number of papers and authors' origins can also reflect the journal's academic status in the relevant disciplines. *World Journal of Gastroenterology* (WJG) is an English journal founded in 1995 and published by Baishideng Publishing Group. In 2005 and 2008, 2 papers analyzed the multiple indexes of WJG for the periods of 1998-2004 and 2001-2007, respectively<sup>[1,2]</sup>. Following the above 2 papers, this study compared and analyzed the various indexes of the papers published in WJG in each year from 2008-2012 and the citations of these papers. We also selected 6 internationally renowned journals of gastroenterology including *American Journal of Gastroenterology*, *BMC Gastroenterology*, *Gastroenterology*, *Journal of Clinical Gastroenterology*, *Journal of Gastroenterology* and *Scandinavian Journal of Gastroenterology* for comparative analysis of the relevant indexes with WJG. Based on analysis of the above indexes, this study intended to determine not only the development and changes of WJG in the past several years, but also the characteristics of the published papers and the origins of the authors of this journal.

## MATERIALS AND METHODS

The Journal Citation Report (JCR) of ISI Web of Knowledge<sup>[3]</sup> and SCI-E<sup>[4]</sup> database were employed. The JCR database was searched to identify the number of references, the number of self-citations, the self-citation rate, and other indicators in WJG during 2008-2012. The SCI-E database was retrieved to identify the papers included in WJG every single year from 2008 to 2012; in addition, the relevant items including Title, Author, Source, Document Type, Times Cited, and Addresses were analyzed. Bibliometric methods were utilized for statistical analysis of the author's degree of collaboration, the collaboration rate, the first author's productivity, high-production authors, the authors' geographic areas and/or country related to this journal in each year; the distribution of the countries and journals for the authors citing WJG papers was also analyzed. In the meantime, the 2012 issues of *American Journal of Gastroenterology*, *BMC Gastroenterology*, *Gastroenterology*, *Journal of Clinical Gastroenterology*, *Journal of Gastroenterology*, *Scandinavian Journal of Gastroenterology* and WJG were retrieved and compared. The comparative indexes included the number of annual publications, the author's degree of collaboration, the collaboration rate, the number of countries of origin for all the authors, the proportion of papers written by native authors, the impact factor in 2012, discipline ranking and self-citation rate. Meanwhile, comparative analysis with WJG was carried out to determine the relative performance of various indexes of WJG.

## RESULTS

### Basic situation of WJG in 2008-2012

WJG published 48 issues yearly in 2008-2012, and during the period SCI-E indexed 1200, 964, 916, 762 and 1008

WJG items in the respective years, giving a total inclusion of 4850 items; the included 5 types of items were article, review, editorial, letter and biography. The number of indexed articles and reviews was 1112, 863, 813, 677 and 944 in the respective years, a total of 4409 papers. The results and conclusion of our research are from the analysis of these 4409 papers. Table 1 lists the number of references, the average number of references in each paper, the number of self-citations in the journal, the average number of self-citations and the self-citation rate.

### Description of the authors of WJG papers between 2008 and 2012

There were 26600 authors from 4409 papers. Table 2 lists the distribution of the number of co-authors (mono-authorship and co-authorship), and 3898 were first authors; 3526 (90.46% of 3898 first authors) published 1 paper, 291 (7.47%) 2 papers, 59 (1.51%) 3 papers, 11 (0.28%) 4 papers, and 11 (0.28%) 5 or more papers. Table 3 shows the authors who published 5 or more papers.

### Distribution of author's geographic area and main country

According to the 6 continents geographically, the authors' addresses were mainly located in Asia, Europe and North America (Figure 1). Table 4 lists the number of papers published by authors of the top 15 countries. Of the top 15 countries, there were 5 countries in Asia, 7 in Europe, 2 in North America and 1 in South America.

### Distribution of the countries and journals for authors citing WJG papers

Authors from 66 countries cited 3194 of the papers (72.44%), with a total of 19872 citations. The authors from the United States of America were the top and responsible for 4716 citations (23.73% of the total); authors from China ranked second and were responsible for 3088 citations (15.54%); the third was Japan with 1617 citations (8.14%). The top 15 countries were responsible for 18889 citations (95.06% of the total cited) (Figure 2). These citations were from 2083 journals, and the top 15 of these journals gave 3373 citations (16.97% of the total) (Table 5).

### Comparison of the relevant data of gastroenterological journals

The data of the JCR database can be used to analyze the citation status of journals, we can evaluate the quality of the journals in each discipline. The Gastroenterology and Hepatology category of JCR 2012 Science Edition included 74 journals, and mean value of impact factor of these journals was 3.115. The 7 representative journals are *American Journal of Gastroenterology*, *Gastroenterology*, *BMC Gastroenterology*, *Journal of Clinical Gastroenterology*, *Journal of Gastroenterology*, *Scandinavian Journal of Gastroenterology*, and WJG; of these, 3 journals are from North America, 2 from Europe, and 2 from Asia. Table 6 lists the number of papers, author's degree of collaboration,

**Table 1** Literature indexes for papers published in *World Journal of Gastroenterology* between 2008 and 2012

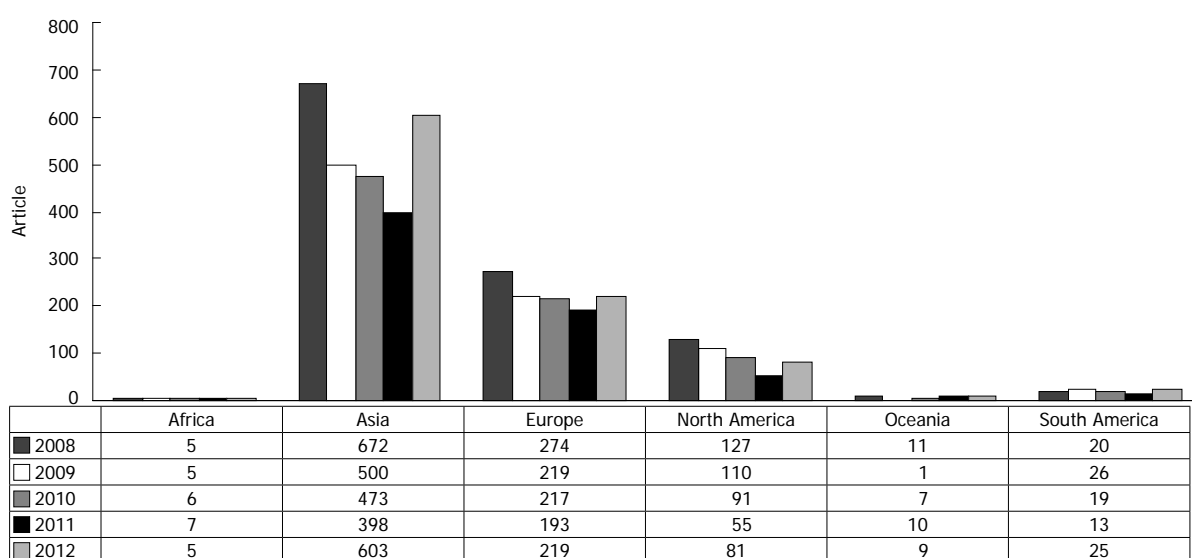
Year	No. of papers	No. of references	Average No. of references	No. of self-citations in the journal	The mean No. of self-citations in each paper	Self-citation rate
2008	1112	40485	36.41	930	0.84	8.59%
2009	863	29458	34.13	767	0.89	6.02%
2010	813	29624	36.44	832	1.02	5.50%
2011	677	25878	38.22	758	1.12	4.47%
2012	944	37947	40.20	918	0.97	5.21%

**Table 2** Co-author collaboration status in *World Journal of Gastroenterology* in 2008-2012

Year	Distribution of number of co-author articles											Total (articles)	Authors	Cooperation degree	Cooperation rate
	1	2	3	4	5	6	7	8	9	10	≥ 11				
2008	49	96	91	147	154	167	111	121	63	42	71	1112	6501	5.85	95.59%
2009	28	66	100	102	139	107	107	74	46	38	56	863	5072	5.88	96.76%
2010	28	62	77	84	113	108	108	74	53	46	106	813	5037	6.20	96.56%
2011	21	46	63	77	103	87	100	96	29	25	30	677	4034	5.96	96.90%
2012	31	67	86	122	95	137	111	91	57	57	90	944	5956	6.31	96.72%
Total	157	337	417	532	604	606	537	456	248	208	353	4409	26600	6.03	96.44%

**Table 3** Authors with 5 or more publications in *World Journal of Gastroenterology* between 2008 and 2012

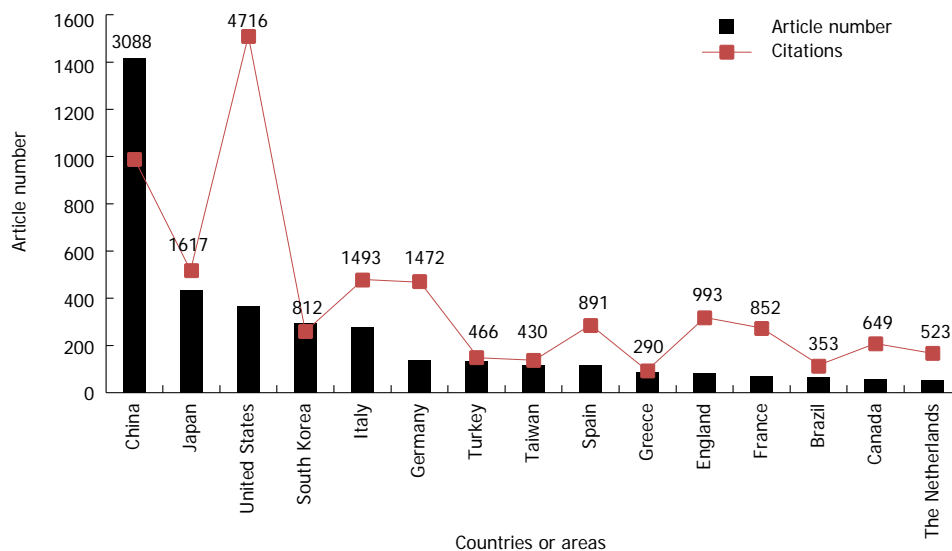
Author	Institute	No. of papers of first authors	No. of papers of the communicating authors	No. of cited papers	Citation frequency
Freeman, Hugh James	Univ British Columbia Hosp, Canada	22	22	22	133
Tarantino, Giovanni	Univ Naples Federico II, Med Sch Naples, Italy	7	9	7	42
Ishikawa, Toru	Saiseikai Niigata Daini Hosp, Japan	7	7	5	20
Akbulut, Sami	Diyarbakir Educ and Res Hosp, Turkey	7	8	5	18
Hirasaki, Shoji	Sumitomo Besshi Hosp, and Kubo Hosp, Japan	7	8	6	27
Sporea, Ioan	Univ Med and Farm Timisoara, Romania	5	5	5	52
Katsinelos, Panagiotis	Cent Hosp, Greece	5	5	4	19
Lohsiriwat, Varut	Mahidol Univ, Siriraj Hosp, Thailand	5	5	5	48
Terada, Tadashi	Shizuoka City Shimizu Hosp, Japan	5	5	3	41
Sun, Long	Xiamen Univ, Affiliated Hosp 1, China	5	0	4	37
Lee, Tae Hoon	Soon Chun Hyang Univ, Coll Med, Cheonan Hosp, South Korea	5	2	4	9

**Figure 1** Distribution of *World Journal of Gastroenterology* authors among the continents between 2008 and 2012.



**Table 4** Distribution of the top 15 countries or regions in *World Journal of Gastroenterology* during 2008-2012

Country name	2008	2009	2010	2011	2012	Total	Percentage
China	361	276	290	240	365	1532	34.75%
Japan	121	74	80	52	107	434	9.84%
United States	94	79	74	49	69	365	8.28%
South Korea	60	62	45	51	74	292	6.62%
Italy	54	53	53	60	56	276	6.26%
Germany	34	26	28	28	20	136	3.08%
Turkey	48	33	19	19	12	131	2.97%
Spain	31	35	13	16	21	116	2.63%
Greece	30	15	18	15	7	85	1.93%
England	23	18	16	9	17	83	1.88%
France	20	12	14	8	13	67	1.52%
Brazil	15	12	12	8	19	66	1.50%
Canada	23	13	9	3	11	59	1.34%
Netherlands	17	9	12	7	9	54	1.22%
Thailand	13	5	11	6	11	46	1.04%
Total	1305	998	984	811	1176	3742	84.87%

**Figure 2** Comparison between the countries of authors citing *World Journal of Gastroenterology* papers of 2008-2012 and the countries of authors publishing *World Journal of Gastroenterology* papers.**Table 5** Main journals citing *World Journal of Gastroenterology* papers published between 2008 and 2012 *n* (%)

No.	Name of the citing journals	Quantity
1	<i>World J Gastroenterol</i>	1131 (5.69)
2	<i>PLoS One</i>	430 (2.16)
3	<i>Dig Dis Sci</i>	198 (1.00)
4	<i>Gastrointest Endosc</i>	180 (0.91)
5	<i>J Gastroenterol Hepatol</i>	171 (0.86)
6	<i>Aliment Pharmacol Ther</i>	147 (0.74)
7	<i>Inflamm Bowel Dis</i>	147 (0.74)
8	<i>J Hepatol</i>	138 (0.70)
9	<i>Hepatogastroenterology</i>	137 (0.69)
10	<i>Endoscopy</i>	122 (0.62)
11	<i>Eur J Gastroenterol Hepatol</i>	120 (0.60)
12	<i>Hepatology</i>	120 (0.60)
13	<i>Gastroenterology</i>	114 (0.57)
14	<i>Am J Gastroenterol</i>	110 (0.55)
15	<i>Scand J Gastroenterol</i>	108 (0.54)
	Total	3373 (16.97)

author's collaboration rate, geographical distribution of the authors, proportion of articles contributed by domestic authors, 2012 impact factor (IF), discipline ranking of the journal by IF, and self-citation rate for these 7 journals in 2012.

## DISCUSSION

The publishing frequency of *WJG* was stable during 2008-2012, without significant changes in the annual number of papers published and the average number of papers in each issue. The average number of references in each paper increased gradually while the self-citation rate decreased gradually year by year. When compared with 2004, the average number of references in each paper in 2012 increased by 8.9<sup>[1]</sup>, while the self-citation rate per article decreased by 0.73<sup>[1]</sup> in 2012; all the indexes were in a satisfactory state.

The degree of collaboration increased slightly while the

**Table 6 Data comparisons of the 7 representative gastroenterology journals in 2012**

Journal name	Articles published in 2012	Cooperation degree in 2012	Cooperation rate in 2012	Geographical distribution of authors	Ratio of articles contributed by domestic authors	2012 impact factor	Ranking of discipline impact factors	Self-citation rate in 2012
<i>Am J Gastroenterol</i>	190	7.50	94.74	32	United States 45.16	7.553	7	3.36
<i>BMC Gastroenterol</i>	165	7.35	98.79	36	China 21.21	2.11	42	2.6
<i>Gastroenterology</i>	278	10.55	97.48	44	United States 44.25	12.821	1	2.2
<i>J Clin Gastroenterol</i>	154	6.41	96.10	24	United States 38.96	3.203	23	3.1
<i>J Gastroenterol</i>	140	9.53	98.57	22	Japan 76.43	3.788	17	3.34
<i>Scand J Gastroenterol</i>	179	6.40	98.88	35	Sweden 21.79	2.156	40	3.29
<i>World J Gastroenterol</i>	944	6.31	96.72	58	China 35.67	2.547	34	5.21

collaboration rate decreased slightly during 2008-2012. The collaboration degree for each respective year was 5.85, 5.88, 6.20, 5.96 and 6.31; the mean collaboration degree was 6.03 and increased by 0.15 when compared with 5.88 during 2001-2007<sup>[2]</sup>. The collaboration rate for each year was 95.59%, 96.76%, 96.56%, 96.90% and 96.72%; the mean collaboration rate was 96.44%. In contrast with the slight increase in collaboration degree during 2008-2012, the collaboration rate during this period decreased by 1.22% when compared with the 97.66% during 2001-2007.

The origin of the authors diversified and the proportion of authors with 1 paper increased, but the high-productivity authors did not increase. During 2008-2012, 3526 authors published 1 paper in *WJG* accounting for 90.46% of the total authors, and 22 authors published 4 papers or more accounting for 0.56%. The core author group of this journal has yet to increase.

The number of author geographic areas increased: the origin of *WJG* authors became increasingly diversified; the authors came from 87 countries across the 6 continents of the world. Asia, Europe and North America were the main origins of the authors; and the proportion of authors from Asia was relatively stable; the number of papers contributed by authors from Asia was 672 (60.60%), 500 (58.07%), 473 (58.18%), 398 (58.88%) and 603 (63.88%) in respective years. The number of authors from Europe and North America changed little in each year and only decreased slightly in 2012. The number of papers contributed by authors from North America was slightly higher when compared with the data during 2006-2007<sup>[2]</sup> but the number was slightly lower during 2011-2012. The number of author countries exceeded 60 in each year; these authors came from a total of 87 countries and/or regions. The origin of the first authors of *WJG* became increasingly diversified and the number of originating countries increased when compared with the data of the period of 2001-2007.

The distribution of the author countries tended to be balanced: authors from the top 15 countries published 3742 (84.87%) papers. The proportion of papers published by Chinese authors showed an annual incremental trend, which coincided with an overall increase in the number of scientific publications in China. The ranking of the top 15 countries have changed; the contemporary top 5 countries were China, Japan, United States, South

Korea and Italy. The international trend in the origin of *WJG* authors increased significantly.

During 2008-2012, 72.44% of all *WJG* papers were cited; although the time factor of 2012 may be responsible for the relatively lower number of citations, and therefore affected the citation rate of *WJG*, but the rate reflected the fairly satisfactory quality of *WJG* papers. The authors citing these papers were distributed among 66 countries or regions; American authors were ranked first and accounted for 23.73%, while Chinese authors were ranked second and accounted for 15.54%. The significant impact of *WJG* around the world was evidenced by the fact that 1140 journals cited *WJG* papers, and the distribution of the citing journals was dispersed widely.

The changes in *WJG* in the past 5 years were compared with another 6 journals of gastroenterology at the same time. *WJG* had the highest number of annual publications; authors' degree of collaboration in *WJG* was slightly lower than that of the other 6 journals, while the collaboration rate was within a reasonable range; the origin of *WJG* authors was the most diversified, and has gradually expanded from the predominant Chinese author group at the early stage to Asia and even the entire world across the 6 continents. Based on the data in JCR 2012, *WJG* ranked fifth of the 7 journals of gastroenterology; its self-citation rate declined and all other indexes were fairly reasonable.

In summary, *WJG* is attracting the attention of gastroenterologists globally, with authors scattered among 87 countries across the 6 continents. It has become a stage for gastroenterologists around the world to demonstrate their research findings. The author's geographic areas and countries are widely distributed, and all the indexes of this journal are stable and reasonable. *WJG* has embarked onto the track of normal internationalized publication, although it is still necessary to cultivate the core author group for the journal to establish its stable research characteristics.

## COMMENTS

### Background

The paper publication data of a journal can reflect a journal's academic status in the relevant disciplines, and the evaluation of journal quality is paid more and more attention by both editors and readers. In 2005 and 2008, two papers analyzed the multiple indices of *World Journal of Gastroenterology (WJG)* for the periods 1998-2004 and 2001-2007. Therefore, it was necessary to analyze the

relevant indexes and make an objective evaluation of journal quality in recent years.

### Research frontiers

Although the evaluation indexes are frequently the citation data of the papers in a given journal such as the total citation frequency, impact factor and so on, the paper's publication data of the journal such as the number of papers and author's origin can also reflect the journal's academic status in the relevant disciplines.

### Innovations and breakthroughs

Based on analysis of the relevant indexes, the authors only studied the development and changes of *WJG* during 2008-2012, but also the characteristics of the published papers and the origin of the authors of this journal.

### Applications

The results indicate that *WJG* has become an international publication, and gastroenterologists around the world can report their research findings in this journal.

### Terminology

The impact factor of a journal is a measure of the citations to science and social science journals, and is frequently used as a proxy for the importance of a journal to its field, with journals with higher impact factors deemed to be more important than those with lower ones.

### Peer review

Commendable effort. Much needed analysis to establish the position of *WJG* in the field of gastroenterology and hepatology. In this interesting paper, authors

performed analyses involving several journals of gastroenterology. All aspects of comparison are presented sufficiently. Statistical analysis of the study made clear and demonstrate that *WJG* has developed into one of the important journals in the field of gastroenterology.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Non-coding RNAs in hepatitis C-induced hepatocellular carcinoma: Dysregulation and implications for early detection, diagnosis and therapy

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Author contributions: Hou W conceived the topic, reviewed the literature and wrote the manuscript; Bonkovsky HL reviewed the literature and revised this paper critically.

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techniques, a significant number of non-coding RNAs (ncRNAs) associated with HCC, particularly caused by HCV infection, have been found to be differentially expressed and to be involved in pathogenesis of HCV-associated HCC. In this review, we focus on recent studies of ncRNAs, especially miRNAs and lncRNAs related to HCV-induced HCC. We summarize those ncRNAs aberrantly expressed in HCV-associated HCC and highlight the potential uses of ncRNAs in early detection, diagnosis and therapy of HCV-associated HCC. We also discuss the limitations of recent studies, and suggest future directions for research in the field. miRNAs, lncRNAs and their target genes may represent new candidate molecules for the prevention, diagnosis and treatment of HCC in patients with HCV infection. Studies of the potential uses of miRNAs and lncRNAs as diagnostic tools or therapies are still in their infancy.

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## Abstract

Hepatitis C virus (HCV) infection is one of main causes of hepatocellular carcinoma (HCC) and the prevalence of HCV-associated HCC is on the rise worldwide. It is particularly important and helpful to identify potential markers for screening and early diagnosis of HCC among high-risk individuals with chronic hepatitis C, and to identify target molecules for the prevention and treatment of HCV-associated-HCC. Small non-coding RNAs, mainly microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) with size greater than 200 nucleotides, are likely to play important roles in a variety of biological processes, including development and progression of HCC. For the most part their underlying mechanisms of action remain largely unknown. In recent years, with the advance of high-resolution of microarray and application of next generation sequencing

**Key words:** MicroRNA; Long non-coding RNAs; Non-coding RNAs; Hepatitis C virus; Hepatocellular carcinoma

**Core tip:** Regulatory non-coding RNAs (ncRNAs), mainly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are likely to play important roles in a variety of biological processes, including development and progression of hepatitis C-induced hepatocellular carcinoma (HCC). In this review, we focus on recent studies of ncRNAs, especially miRNAs and lncRNAs associated with hepatitis C virus (HCV)-induced HCC. We summarize those ncRNAs aberrantly expressed in HCV-induced HCC and highlight the potential of these ncRNAs to aid in early detection, diagnosis and therapy of HCV-induced HCC. Further, we discuss the limitations of current studies, and suggest future directions for research in the field.



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## INTRODUCTION

Non-coding RNAs (ncRNAs) are transcribed RNA molecules with little or non-protein coding capacity; they represent approximately 97% of RNAs in higher eukaryotic organisms. ncRNAs include structural or house-keeping ncRNAs such as transfer RNA, ribosomal RNA, small nuclear RNA and small nucleolar RNA, as well as regulatory ncRNAs, which function to regulate gene expression. Based on transcript size, regulatory ncRNAs are classified into two major groups, small ncRNAs such as microRNAs (miRNAs), approximately 22 nucleotides (nt) in length, and long non-coding RNAs (lncRNAs) with sizes longer than 200 nt (Figure 1). Based upon a large number of experimental studies carried out over the past decades or two, it is now generally well-accepted that miRNAs play an important role in the regulation of gene expression primarily through post-transcriptional destabilization, translational repression of target mRNAs which bear complementary sites, or a combination of these two mechanisms<sup>[1-4]</sup>. With the development of next generation sequencing (NGS) techniques, a growing number of lncRNAs have been identified, characterized and functionally annotated<sup>[5,6]</sup>. lncRNAs are still among the least well-understood of transcripts. Several lines of evidence have suggested that lncRNAs are biologically functional rather than transcriptional “noise”<sup>[5,6]</sup>. Thus, lncRNAs have recently enjoyed increased and deserved attention, although the underlying mechanisms by which they function remain largely unexplored and unifying theories regarding their actions are still vague. ncRNAs including miRNAs and lncRNAs have been reported to be associated with cancer, including hepatocellular carcinoma (HCC), a highly prevalent and deadly cancer because of its frequent recurrence and/or metastasis.

HCC is among the most frequent forms of cancer worldwide, and its incidence is increasing rapidly. This increase is related to several factors. Chief among these are chronic hepatitis B and C (CHC) infections, and fatty liver disease. Indeed, hepatitis C virus (HCV) infection is one of the leading underlying causes of HCC, increasing the risk for HCC development by nearly 17-fold compared to healthy individuals<sup>[7,8]</sup>. In recent decades and especially in recent years, HCC incidence has increased sharply, and has been attributed largely to HCV infection. HCV-induced HCC typically develops in the setting of cirrhosis (advanced chronic liver diseases), although it does also occur in the absence of cirrhosis. Similarly, the development of HCC has been observed in mice expressing HCV transgenes in the absence of appreciable

hepatic inflammation and fibrosis, suggesting that HCV infection is likely to have direct and unique cancer-promoting effects, which may be different from other carcinogenic factors such as those due to hepatitis B virus (HBV) and fatty liver disease. Understanding and insight into unique ncRNAs involved in HCV-induced HCC may suggest new approaches for diagnosis, prevention and treatment of HCV-induced HCC. To date, there have been few reports on differentially expressed lncRNAs in HCV-induced HCC. In this review, we will summarize recent studies regarding ncRNAs related to HCV-induced HCC. We will then address the potential utility of these ncRNAs in early detection, diagnosis and therapy of HCV-associated HCC. Finally, we will discuss the limitations of current knowledge, and suggest future directions for research in this field.

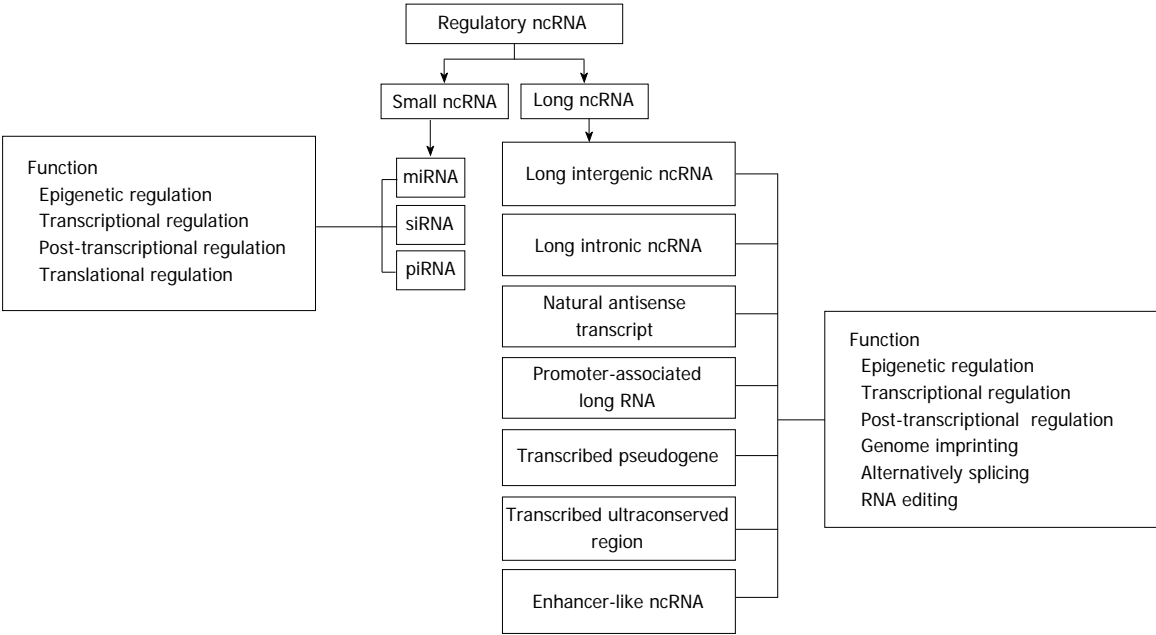
## DYSREGULATED NCRNAS IN HCV-INDUCED HCC

### microRNAs

miRNAs regulate gene expression primarily through post-transcriptional repression<sup>[3,4,9,10]</sup>. Sequence complementarity in the 6-8 base pair “seed regions” at the end of miRNA-mRNA heteroduplexes seem to determine the specificity of miRNA-target RNA interactions<sup>[11]</sup>. miRNAs are likely to play significant roles in the development and progression of cancers, including HCC<sup>[12,13]</sup> and HCV replication<sup>[9,14-16]</sup>. Identification and characterization of dysregulated miRNAs specific to HCV-induced HCC in tissue- and biofluid-based studies are important and helpful to reveal therapeutic targets or diagnostic markers, in particular, molecular signatures for the detection and early diagnosis of HCC among HCV patients in high-risk groups. The miRNAs reported differentially expressed in HCV-induced HCC are summarized in Tables 1 and 2, and we now summarize their known biological functions, and the molecular mechanisms and pathways in which they might be involved.

### Up-regulated miRNAs

miRNAs profiling studies in HCV-induced HCCs compared with paired controls have found that a number of miRNAs are significantly elevated in HCV-induced HCCs, compared with normal controls. miR-1269 is the most increased in HCV-associated HCCs in contrast to normal livers, HCV-associated cirrhosis, or HBV-associated liver failure. Up-regulation of miR-1269 has also been found in other cancers such as breast cancer<sup>[17]</sup>, colorectal cancer<sup>[18]</sup> and laryngeal squamous cell carcinoma (LSCC), one of the most common head and neck malignancies with no significant difference between tumors with and without lymphatic metastasis, suggesting that miR-1269 did not affect metastasis of LSCC. Thereto date there have been few, if any, reports on function and role of miR-1269 in HCC. Nevertheless, the increased expression of miR-1269 in HCV-induced HCC when compared with controls suggests that this miRNA may have an on-



**Figure 1 Classification of regulatory non-coding RNA and function in gene regulation.** Regulatory non-coding RNA (ncRNAs) are divided into two major groups based on transcript size, small ncRNAs such as microRNA, small interfering RNA and piwi-interacting RNA (piRNA), as well as long ncRNAs with size greater than 200 nt. Both small ncRNA and long ncRNA have important regulatory function in gene expression. miRNA: microRNA; siRNA: small interfering RNA; piRNA: piwi-interacting RNA.

Table 1 Summary of microRNAs significantly up-regulated in hepatitis C virus-induced hepatocellular carcinoma				
ncRNAs	Chromosomal location	Differential expression level	Clinical relevance	Ref.
Liver miRNAs				
miR-1269	4q13.2	15.7-fold, HCV-associated HCC (n = 9) <i>vs</i> normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-224	Xq28	10.7-fold, HCV-associated HCC (n = 9) <i>vs</i> normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-452	Xq28	10.1-fold, HCV-associated HCC (n = 9) <i>vs</i> normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-224-3p	Xq28	8.1-fold, HCV-associated HCC (n = 9) <i>vs</i> normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-221	Xp11.3	3.7-fold, HCV-associated HCC (n = 9) <i>vs</i> normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-122	18q21.31	> 2-fold, HCV-associated HCC (n = 43) <i>vs</i> normal livers (n = 3), P < 0.01; HCV associated dysplastic nodules (n = 9) <i>vs</i> normal livers (n = 3), P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
miR-100	11q24.1	> 2-fold, HCV-associated HCC (n = 43) <i>vs</i> normal livers (n = 3); HCV associated dysplastic nodules (n = 9) <i>vs</i> normal livers (n = 3)	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
miR-10a	17q21.32	> 2-fold, HCV-associated HCC (n = 43) <i>vs</i> normal livers (n = 3); HCV associated dysplastic nodules (n = 9) <i>vs</i> normal livers (n = 3)	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
Urinary miRNAs				
miR-625	14q23.3	> 3-fold, HCV-associated HCC (n = 32) <i>vs</i> normal urine samples (n = 12), P < 0.05	Potential marker for the detection/early diagnosis of HCC among high-risk HCV patients	[33]
miR-532	Xp11.23	> 3-fold, HCV-associated HCC (n = 32) <i>vs</i> normal urine samples (n = 12), P < 0.05	Potential marker for the detection/early diagnosis of HCC among high-risk HCV patients	[33]
miR-618	12q21.31	> 3-fold, HCV-associated HCC (n = 32) <i>vs</i> normal urine samples (n = 12), P < 0.05	Potential marker for the detection/early diagnosis of HCC among high-risk HCV patients	[33]

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; miRNAs: MicroRNAs; ncRNAs: Non-coding RNAs.

**Table 2** Summary of microRNAs down-regulated in hepatitis C virus-induced hepatocellular carcinoma

ncRNAs	Chromosomal location	Differential expression level	Clinical relevance	References
Liver miRNAs				
miR-199a-5p	19q13.3	7.2-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-199a-3p	19q13.3	6.9-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-199b-3p	19q13.3	6.2-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-214	1q24.3	5.5-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-198	19p13.3	Approximately 5-fold, HCV-associated HCC ( $n = 43$ ) <i>vs</i> normal livers ( $n = 3$ ), $P < 0.01$ ; HCV-associated Dysplastic nodules ( $n = 9$ ) <i>vs</i> normal livers ( $n = 3$ ), $P < 0.01$	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
miR-139-3p	11q13.4	4.6-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-139-5p	11q13.4	4.4-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-424-3p	Xq26.3	3.9-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-125a-5p	19q13	3.7-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-130a	11q12.1	2.9-fold, HCV-associated HCC ( $n = 9$ ) <i>vs</i> normal livers ( $n = 12$ ) and other liver diseases [HCV-associated cirrhosis ( $n = 10$ ), HBV-associated accurate liver failure ( $n = 4$ )], $P < 0.05$	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-145	5q32	> 2-fold; HCV-associated HCC ( $n = 43$ ) <i>vs</i> normal livers ( $n = 3$ ); HCV associated dysplastic nodules ( $n = 9$ ) <i>vs</i> normal livers ( $n = 3$ )	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
Urinary miRNAs				
miR-516-5p	19q13.42	> 3-fold, HCV-associated HCC ( $n = 32$ ) <i>vs</i> normal urine samples ( $n = 12$ ), $P < 0.05$	Potential marker for early diagnosis of HCC among high-risk HCV patients	[33]
miR-650	22q11.22	> 3-fold, HCV-associated HCC ( $n = 32$ ) <i>vs</i> normal urine samples ( $n = 12$ ), $P < 0.05$	Potential marker for early diagnosis of HCC among high-risk HCV patients	[33]

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; miRNAs: MicroRNAs; ncRNAs: Non-coding RNAs.

cogenic role in HCV-induced HCC.

Interestingly, miR-224, miR-224-3p and their precursor are significantly up-regulated in HCV-associated HCCs compared to normal livers, HCV-associated cirrhosis and HBV-associated liver failure. miR-452, also significantly up-regulated in HCV-induced HCC, was recently shown to be coordinately expressed with its neighboring miR-224 in HCC through epigenetic mechanisms<sup>[19]</sup>. The DNA that encodes miR-224 is located on the X-Chromosome. miR-224 has been reported to be a cancer-related miRNA, including in HCC. Wang *et al.*<sup>[20]</sup> identified and validated the apoptosis inhibitor 5 (API-5) as a specific target gene for miR-224. Additionally, SMAD family member 4 (*SMAD4*) has been identified as another target gene for miR-224. Over-expression of miR-224 increases the concentration of SMAD4 protein in murine granulosa cells, while SMAD4 RNA levels remain unchanged, suggesting a post-transcriptional role

for miRNA-224<sup>[21]</sup>. It is likely that miR-224 plays a role in cell proliferation, migration, invasion, and anti-apoptosis in HCC, and is involved in hepatocarcinogenesis by directly binding to its validated gene targets such as *API-5*, *SMAD4*, *etc.*<sup>[20,22,23]</sup>.

miR-122, a liver specific miRNA, is the most abundant miRNA expressed in hepatocytes (accounting for approximately 70% of total miRNAs) and the most extensively studied miRNA in liver diseases. miR-122 has major effects on several enzymes of cholesterol metabolism<sup>[24,25]</sup>. Unexpectedly, miR-122 was also shown to be required for HCV replication<sup>[14,16]</sup>. The effects of miR-122 depend upon the context and location of its cognate seed sequence binding sites. The sites in the 5' region are mostly associated with up-regulation of expression, whereas those in the 3' untranslated region are mostly associated with repression of expression<sup>[26]</sup>. miR-122 exerts several functions in the HCV life cycle<sup>[27,28]</sup>. Recent

studies have shown that miR-122 acts to protect HCV genome from degradation, and therefore stabilizes HCV RNA by decreasing activity of the cytosolic exonuclease Xrn1<sup>[27,28]</sup>. The role of miR-122 in HCC has been controversial. Some but not all studies suggest that miRNA-122 is preserved and increased specifically in HCV-associated HCC<sup>[12,29,30]</sup>. Nevertheless, a decrease in expression of miR-122 or undetectable miRNA-122 in human hepatoma cell lines such as HepG2 and Hep3B cell has been observed<sup>[31]</sup>. In parallel with these observations, overexpression of miR-122 inhibits anchorage-independent growth, migration, invasion and tumor formation in nude mice<sup>[31]</sup>. This needs to be further studied in the future.

### Down-regulated miRNAs

The miR-199 family members including miR-199a-5p, miR-199a-3p and miR-199b are the most down-regulated miRNAs in HCV-induced HCC compared to normal livers, HCV-associated cirrhosis and HBV-associated liver failure<sup>[32]</sup>. miR-199a/b-3p is the third most highly expressed miRNA in the liver<sup>[34]</sup>, and was also found to be consistently decreased in HCC patients with HBV infection<sup>[34]</sup> and alcohol consumption<sup>[35]</sup>. Its decrement significantly correlates with poor survival of HCC patients<sup>[34]</sup>. Down-regulation of miR-199a-3p results in a pronounced increase in cell proliferation while overexpression miR-199a-3p inhibits cell proliferation by imposing G<sub>1</sub> cell cycle arrest<sup>[36]</sup>. The target mRNAs for the miR-199a-3p have been predicted using bioinformatic approaches and validated experimentally. For example, mammalian target of rapamycin (mTOR) has been identified as one of important targets for miR-199a-3p binding. Through negative regulation of oncogenic mTOR, miR-199a-3p inhibits tumor proliferation<sup>[37]</sup>.

miR-214 has been reported to be down-regulated by 5.5-fold in HCV-induced HCC compared to normal livers, HCV-associated cirrhosis and HBV-associated liver failure<sup>[32]</sup>. The down-regulation of miR-214 has been reported in HCC<sup>[20,38,39]</sup> and other cancers such as cervical cancer, whereas increase in miR-214 was found to significantly reduce growth of Hela cells<sup>[40]</sup>. In addition, reduced level of miR-214 is associated with invasion, stem-like traits and early recurrence of HCC<sup>[41]</sup>. Re-expression of miR-214 significantly suppressed the growth of HCC cells *in vitro* and reduced the tumorigenicity *in vivo*<sup>[41]</sup>. In the same study, the enhancer of zeste homologue (EZH2) and  $\beta$ -catenin (CTNNB1) were identified and validated as two functional target mRNAs of miR-214. Silencing miR-214 increased stem-like cells through activation of CTNNB1. Furthermore, the up-regulation of EZH2, CTNNB1 and the down-regulation of E-cadherin (CDH1), known to inhibit cell invasion and metastasis in HCC patients, correlated with earlier recurrent HCC and were independent predictors of poor survival.

### LncRNAs

The discovery of lncRNAs ushered in a new and exciting area of study, although at the time lncRNAs were first

found, they were considered to be merely transcriptional “noise”<sup>[5]</sup>. Recently, with fast development and application of NGS techniques, the numbers of lncRNAs continues to grow at a rapid pace, and it is increasingly clear that lncRNAs are a new class of regulators of gene expression, being involved in diverse biological processes and human diseases such as cancer. The association of lncRNAs with HCC has been studied and summarized<sup>[42]</sup>, although the mechanisms whereby effects of the lncRNAs are realized are largely unknown. Analysis of the differentially expressed lncRNAs in HCCs (underlying etiology not specified) have revealed that a number of lncRNAs such as HOTAIR<sup>[43-46]</sup>, HEIH<sup>[47]</sup>, MVIH<sup>[48]</sup>, MALAT-1<sup>[49]</sup>, HULC<sup>[50-52]</sup>, H19<sup>[53-55]</sup>, CUDR<sup>[56]</sup>, YIYA<sup>[57]</sup>, lncRNA-Dreh<sup>[58]</sup>, lncRNA-LET<sup>[59]</sup> and MEG3<sup>[60,61]</sup> are associated with HCC. Most of these lncRNAs are up-regulated in HCCs, but less expressed or undetectable in normal controls. HCC patients with HOTAIR expression had significantly poorer prognoses and larger primary tumor sizes than those without HOTAIR expression<sup>[46]</sup>. Moreover, introduction of HOTAIR into human liver cancer cells promoted more rapid proliferation compared to controls<sup>[46]</sup>. Functional gene annotation analysis of TUC338 indicated predominant effect on genes involved in cell growth in both human and murine cells, suggesting that TUC338 plays a critical role in regulation of transformed cell growth and in the pathobiology of HCC<sup>[62]</sup>. Lai *et al.*<sup>[49]</sup> found up-regulation of MALAT-1 in both liver cancer cell lines and HCC patient samples. HCC patients with high level of MALAT1 had a significantly increased risk of tumor recurrence after liver transplantation. MVIH was identified to be related to frequent microvascular invasion and higher tumor-node-metastasis stages as well as to decreased overall survival. In addition, in mouse models it promoted tumor growth and intrahepatic metastasis by activating angiogenesis<sup>[48]</sup>. The expression level of HEIH in HBV-induced HCCs is significantly associated with recurrence and is an important independent prognostic factor for survival<sup>[47]</sup>. Further studies indicated that HEIH plays a key role in the regulation of zeste homologue 2 (EZH2) and that this association was required for the repression of EZH2 target genes, suggesting that HEIH is an oncogenic lncRNA that promotes tumor progression<sup>[47]</sup>. Thus far, few studies have been focused on lncRNAs specific to HCV-induced HCC although HCV infection is one of the major causes of HCC, and HBV and HCV cause hepatocarcinogenesis by different mechanisms.

## CLINICAL IMPLICATIONS FOR DIAGNOSIS AND THERAPY

miRNAs, lncRNAs and their target genes comprise a large and still growing number of candidate molecules for the prevention, diagnosis and treatment of HCC in patients with HCV infection, and studies of the potential use of miRNAs and lncRNAs as therapeutic or diag-



nostic approaches is still in its infancy. In this section, we mainly discuss clinical potentials of miRNAs and lncRNAs for HCV-induced HCC diagnosis and therapy.

### **miRNAs and lncRNAs to aid in diagnosis of HCV-induced HCC**

The biomarkers currently available for screening and early diagnosis of HCC, including serum alpha-fetoprotein (AFP), des-gamma-carboxyprothrombin, and AFP-L3 fraction or assaying cells from tissue biopsy by needle aspiration and surgical resection, suffer from numerous limitations<sup>[63,64]</sup>. Most patients chronically infected with HCV are asymptomatic for many years, and the average time to develop HCC after onset of HCV infection is about 28 years. The long latency period between initial HCV infection and development of HCC provides an important time window of opportunity for individuals to be monitored for disease progression and intervention. Therefore, the development of more reliable markers for diagnosis of HCC at an early stage and better approaches for HCC screening and early detection are urgently needed. The recent study from Abdalla *et al.*<sup>[33]</sup> to identify urinary miRNAs as biomarkers specific for early detection of HCV-induced HCC, appears to be attractive and promising. The significantly up-regulated and down-regulated urinary miRNAs as listed in Tables 1 and 2 can be considered as promising candidate miRNA urinary markers for the early detection and diagnosis of HCV-induced HCC among high-risk HCV patients (Genotype 4). Of the identified miRNAs, miR-618 was found to have a sensitivity of 64% and a specificity of 68% for detecting HCC among HCV-positive individuals, whereas the sensitivity and specificity of urinary miR-650 were 72% and 58%, respectively. Also worthy of note, miR-618/650 in tandem improved the specificity to 75%, greater than the traditional methods based on serum levels of AFP. The urinary miRNAs signatures found in this study may be of great value and applied for the early diagnosis of HCC, before the onset of disease in high-risk patients infected with HCV. However, it is noted that this study was carried out in patients infected with HCV genotype 4, the most prevalent HCV genotype in Egypt. The potential for their use in the early diagnosis of HCC caused by different HCV genotypes other than genotype 4 needs further investigation and independent confirmation. So far, few studies have been reported regarding on lncRNAs signatures in biofluids specific to HCV-induced HCC, which may represent an exciting area for future exploration.

### **miRNAs and lncRNAs for HCV-induced HCC therapy**

Recent studies have suggested the exciting possibility that ncRNAs may represent a novel therapeutic strategy for human diseases. The miR-122 antagonist, miravirsin, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that sequesters mature miR-122 in a highly stable heteroduplex, already has shown promising results in phase 2a clinical trials at seven international sites. In this clinical study, Janssen

*et al.*<sup>[65]</sup> evaluated the safety and efficacy of miravirsin in 36 patients with chronic HCV genotype 1 infection. This landmark study demonstrated that the use of miravirsin produced prolonged dose-dependent reductions in HCV RNA with no evidence of development of viral resistance. Meanwhile, targeting a number of other miRNAs such as miR-33a/b for the treatment of atherosclerosis<sup>[66,67]</sup>, miR-208/449 for chronic heart failure, miR-34 and the let-7 miRNA for cancer, await entering clinical trials. In addition, Coelho *et al.*<sup>[68]</sup> has recently demonstrated a new therapeutic approach to transthyretin amyloidosis by RNA interference (RNAi). In this phase I clinical trial, a potent antitranssthyretin small interfering RNA was encapsulated in lipid nanoparticles, delivered to human hepatocytes, and resulted in a significant reduction of transthyretin, establishing proof of concept for RNAi therapy<sup>[68]</sup>.

As summarized and discussed earlier in this review, many of the significantly dysregulated cellular miRNAs as listed in Tables 1 and 2, and currently found to be involved in the modulation of cell growth, apoptosis and invasiveness, can be considered as potential therapeutic targets for HCV-induced HCC therapy. The overexpression of these specific mature miRNAs can be achieved by synthetic miRNAs mimics or expression vectors. When inhibition of the selected miRNAs is desirable, antagomir or antisense oligos complementary to the specific miRNAs can be used. However, the introduction of the miRNA-based agents into clinical trials and the development of new therapeutic agents are hampered by a number of factors. The major road block is still the big challenge of developing a small animal model used in biomedical research to understand roles of ncRNAs in the pathogenesis of HCV-induced HCC. Among nonhuman species, only chimpanzees have thus far been capable of being infected with HCV, and disease in them is generally relatively mild. Most recently, Dorner *et al.*<sup>[69]</sup> has reported a breakthrough and milestone in development of a genetically humanized mouse model for HCV research, in which the entire HCV life cycle can be completed and immune system is fully functioning. This genetically humanized mouse model will allow us to gain new insights into not only an important biology of HCV but also carcinogenesis of HCC caused by HCV. Additional challenging factors remain which slow the progress of the miRNA-based agents into clinical trials and new drugs. The dysregulated miRNAs in HCV-induced HCC as discussed earlier in this review were identified in individual studies, lacking consensus among the different reports, having been attributed to the differences among miRNA probe, staging and grade of malignancy of the tumor and different HCV genotypes. Therefore, there is still a long way to go before the miRNA-based therapy can be used in the clinic in the prevention and treatment of HCV-induced HCC.

## **CONCLUSION**

As we summarized and discussed in this review, the re-

cent findings of roles of ncRNAs in not only regulating HCV life cycle but also their contribution to pathogenesis of HCV-induced HCC have been remarkable, despite the fact that we may have unveiled only a small portion of the very large number of ncRNAs; this is probably especially true for lncRNAs. We are only beginning to understand the nature and extent of the involvement of lncRNAs in disease. Recently, a number of lncRNAs have been found to be aberrantly regulated in cancer, including HCC<sup>[42-48,53-55,57,59-62]</sup>. However, at the present time there are no reports on aberrantly lncRNAs exclusively associated with HCV-induced HCC, and thus remain a large unexplored and undefined area, which may allow us to better understand the role of lncRNAs in the pathogenesis of HCV infections and HCC and to identify better therapeutic targets and more sensitive diagnostic markers.

The intracellular ncRNAs that were significantly altered in HCV-induced HCC have been proposed to be potential molecular targets for therapy to combat HCV and HCV-induced HCC. Furthermore, over the past years, extracellular ncRNAs, particularly ncRNAs in exosomes, have risen to be promising as biomarkers with diagnostic or prognostic value. Exosomes (30-100 nm in diameter) are one of the class of microvesicles found in biofluids, including blood, ascites fluid, urine, culture media of cell cultures, *etc.* Exosomes carry with them various nucleic acids, including ncRNAs (*e.g.*, miRNAs and lncRNAs) and proteins from their cells of origin, which allow us to achieve access to molecular information about their cell-of-origin without biopsying or destroying the actual cells themselves<sup>[70-80]</sup>. This is of particular importance because direct cellular biopsy may be difficult or otherwise unattainable for screening high-risk populations, such as screening for HCC in CHC patients. It is highly anticipated that future studies on exosomal ncRNAs in different stages of HCC in HCV patients, which reflect the stepwise carcinogenic process from preneoplastic lesions to HCC may unveil better and reliable markers to aid in HCC early diagnosis among CHC patients and tracking of disease progression, which may directly benefit patients affected with HCC. Furthermore, there is a possibility that ncRNAs in exosomes may be taken by hepatocytes as a part of the cell-to-cell communication to spread HCV and promote carcinogenic signal transduction among hepatocytes, and therefore studies on exosomal ncRNAs associated with HCV-induced HCC may suggest novel molecular targets to help prevent and treat HCV-induced HCC patients.

In conclusion, the recent discovery of ncRNAs ushered in exciting and novel area to explore. Meanwhile, challenges to investigators are obvious: With respect to ncRNAs in HCV-induced HCC, in-depth knowledge on functional roles for ncRNAs in HCV-induced HCC through well-designed studies are required to shed light on the molecular pathways of carcinogenesis and to aid in truly exploiting the potential of ncRNAs to serve as molecular targets or markers with a real value. Would it

be possible in the future to use exosomal ncRNAs from body fluids such as blood, ascites or urine, for screening and early diagnosis of HCC among HCV patients at high-risk? Could we successfully slow or prevent the development of HCC in HCV patients using selected ncRNA antagonists or mimics as novel approaches?

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Hepatitis C virus control among persons who inject drugs requires overcoming barriers to care

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HCV treatment for PWID, a pressing need exists to develop strategies to engage these individuals into HCV care. In this article, we propose several strategies that can be pursued in an attempt to engage PWID into HCV management. We advocate that multidisciplinary approaches that utilize health care practitioners from a wide range of specialties, as well as co-localization of medical services, are strategies likely to result in increased numbers of PWID entering into HCV management. Pursuit of HCV therapy after stabilization through drug treatment is an additional strategy likely to increase PWID engagement into HCV care. The full impact of direct acting antivirals for HCV will only be realized if innovative approaches are pursued to engage all HCV infected individuals into treatment.

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**Key words:** Treatment of hepatitis C; Viral infection; Human immunodeficiency virus; Hepatitis C virus coinfection; Persons who inject drugs; Obstacles to treatment

## Abstract

Despite a high prevalence of hepatitis C virus (HCV) infection, the vast majority of persons who inject drugs (PWID) have not engaged in HCV care due to a large number of obstacles. Education about the infection among both PWID and providers remains an important challenge as does discrimination faced by PWID in conventional health care settings. Many providers also remain hesitant to prescribe antiviral therapy due to concerns about adherence and relapse to drug use resulting in reinfection. Presently, however, as a result of improvements in treatment efficacy combined with professional society and government endorsement of

**Core tip:** Despite persons who inject drugs (PWIDs) representing the majority of the hepatitis C virus (HCV) disease burden, few receive treatment for HCV. Barriers to treatment uptake exist at multiple levels. Co-localization of HCV management with substance abuse facilities may result in greater treatment uptake for PWID.

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## INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic liver disease affecting more than 120 million people worldwide<sup>[1,2]</sup> and at least 3.2 million in the United States<sup>[3-5]</sup>. Among HCV-exposed individuals, up to 80% will develop chronic infection that can ultimately lead to hepatic fibrosis, cirrhosis, hepatocellular carcinoma and death<sup>[6]</sup>. The prevalence of cirrhosis is estimated to increase from 25% in 2010 to 45% by 2030 in untreated patients with chronic HCV infection, and liver-related deaths are projected to increase by 175% over the next decade<sup>[7]</sup>. Currently, HCV is the leading indication for liver transplantation in the United States<sup>[8]</sup>.

As the virus is most effectively transmitted via blood, injection drug use is currently the primary route of HCV transmission in the United States and other developed countries. Among persons who inject drugs (PWID), estimated HCV prevalence ranges from 30% to 70%, depending on frequency and duration of use, while incidence ranges from 16% to 42% per year<sup>[9,10]</sup>. Additionally, up to 20% of human immunodeficiency virus (HIV)-infected PWID in the United States are co-infected with HCV<sup>[11]</sup>. A recent study predicted that for a PWID population with 20% baseline chronic HCV prevalence, treatment rates of 5, 10, 20 or 40 per 1000 annually can lead to a 15%, 30%, 62% and 72% reduction in prevalence after 10 years, respectively<sup>[12]</sup>. The same authors have also estimated that novel treatments, expected to result in viral clearance rates of 90%, can halve HCV prevalence of 25%, 50%, and 65% within 15 years with treatment rates of 15, 40, or 76 per 1000 PWIDs annually<sup>[13]</sup>. Therefore, addressing HCV infection among PWID is a crucial step toward its successful control and prevention.

Despite the fact that PWID represent the majority of the HCV disease burden in developed countries, only 21%-65% have been evaluated for HCV, with less than 20% of evaluated patients receiving treatment<sup>[14-16]</sup>. Moreover, while the majority (> 70%) of PWID initially express willingness to undergo HCV treatment, only a minor percentage (1%-6%) actually receives therapy<sup>[14,16,17]</sup>. A variety of factors limit enrollment of PWID into HCV care and treatment. Identification of these barriers is therefore a key step toward formulating interventions to increase access to HCV care for PWID. Our goal in this article is to highlight the obstacles to providing HCV care to PWID and to propose interventions by which these barriers can be overcome.

## BARRIERS TO HCV TREATMENT IN PWID

Obstacles to providing HCV care to PWID emanate from patients, health care providers and the health care system<sup>[18]</sup> (Table 1). One of the most important patient level obstacles to receiving care is lack of HCV-related knowledge resulting in a low perceived need for treatment. Between 65%-75% of HCV-infected patients are unaware of their status<sup>[19]</sup>. While many patients are aware that treatment for HCV exists, few are cognizant that it

**Table 1 Most common barriers to engagement of persons who inject drugs into care for hepatitis C virus infection**

Domain	Specific barrier
Patient-level	Low perceived treatment need
	Fear of side effects
	Lack of knowledge of serostatus
	Fear of liver biopsy
	Needles may promote relapse
	Coexisting mental health diagnosis
Physician-level	Lack of insurance, poverty, low socioeconomic status
	Concerns about reinfection
	Biases against PWID
	Adherence concerns
	Dual diagnoses
Health system-level	Navigation can be complex
	Mistrust between PWID and medical community
	High cost of HCV treatment
	Stigmatization in health care venues

HCV: Hepatitis C virus; PWID: Persons who inject drug.

is curative. Some PWID are reluctant to undergo liver biopsy, an invasive procedure that has been frequently required prior initiation of HCV treatment. The presence of needles that are required for interferon injection might also be an obstacle to treatment in some persons who previously injected drugs. Additionally, many PWID perceive treatment-related side effects to be worse than the virus itself. Finally, mistrust of the health care system and difficulty keeping medical appointments may also contribute to PWID's unwillingness to initiate HCV therapy<sup>[14]</sup>. PWID are also more likely to be uninsured, have limited access to health care services, be affected by poverty, and have reduced social support<sup>[20]</sup>.

Provider barriers also contribute to low rates of treatment provision to PWID. Patients who report injecting drugs are less likely to be referred for HCV evaluation and less likely to receive HCV treatment<sup>[21,22]</sup>. Many health care providers remain hesitant to treat patients with a history of drug use due to concerns about adherence to the therapeutic regimen. Some providers avoid treatment of PWID due to the misconception that reinfection occurs at a high level following relapse to injection drug use<sup>[23]</sup>. Finally, people with drug addiction have been perceived as challenging patients because they are more likely to be dually diagnosed with psychiatric co-morbidities, such as depression and anxiety, compared to non-addicted individuals<sup>[24]</sup>.

The health care system itself may pose numerous obstacles to HCV treatment of PWID. The United States health care system is complex and the referral and scheduling process, as well as insurance and payment issues, can be difficult to navigate. Long-seated, distrusting relations between PWID and the medical community have contributed to feelings of stigmatization among those seeking HCV treatment. PWID often experience health care providers as judgmental, unresponsive to their medical needs, and disdainful, all of which serve as systemic barriers to care.

Finally, high cost of HCV therapy is another treatment barrier. For example, the estimated total cost of

telaprevir-based therapy, including the cost of side effect management, can be as high as \$147000<sup>[25]</sup>. Although this problem is not specific to PWID, it certainly affects them to a greater extent compared to general population, particularly as PWID are more likely to be uninsured and to have less financial resources.

Excluding PWID from HCV treatment contradicts current recommendations issued by several United States governmental and relevant professional organizations. Governmental bodies, including the Institute of Medicine (IOM)<sup>[26]</sup> and the Department of Health and Human Services (HHS)<sup>[27]</sup>, now advocate for increased awareness and resources to address the issue of disparities in HCV treatment for PWID. Professional organizations such as the American Association for the Study of Liver Disease (AASLD)<sup>[28]</sup>, have stated in their guidelines that PWID should be treated for HCV. Yet despite these recommendations, PWID are frequently excluded from therapy by the health care system.

## OVERCOMING THE OBSTACLES TO HCV TREATMENT FOR PWID

Through advances in HCV management, we are now experiencing partial resolution of the obstacles to HCV treatment among PWID. The rapid acceleration of HCV treatment toward an all oral regimen with improved efficacy and fewer adverse effects will likely result in the elimination of the liver biopsy as a requirement to initiate treatment. Additionally, the avoidance of needle exposure associated with interferon injection would eliminate anxiety among persons who no longer inject drugs. The onus now moves toward strategy development to address other obstacles in the management of HCV in PWID.

As patient-related obstacles can derive from misconceptions and lack of HCV-related knowledge, appropriately designed educational interventions could prove beneficial in promoting HCV care and treatment. Unfortunately, while nationwide surveys in the United States have documented that most opioid agonist treatment (OAT) facilities provide at least some form of HCV education<sup>[29,30]</sup>, patients infrequently avail themselves of these opportunities<sup>[31]</sup>. Increased awareness of potential benefits of such programs and the addition of patient incentives, such as financial compensation or travel stipends, might increase participation. Peer support groups, directed by treatment-experienced patients, could encourage treatment acceptance and provide emotional support through shared treatment experiences. Support from mental health and allied health professionals to assist with procurement of social and mental health services, temporary disability, accessing Medicaid, and obtaining transportation, may potentially increase involvement in HCV treatment. These interventions can be incorporated into an individualized treatment plan to maximize adherence rates and successful outcome achievement.

Other obstacles to provision of HCV care and treatment result from lack of HCV-related knowledge and

misconceptions among health professionals regarding PWID. These barriers may be overcome by provider education about PWID or by close collaboration between health care providers from diverse specialties<sup>[32]</sup>. Involvement of a multidisciplinary team consisting of representatives of hepatology, addiction medicine, generalists, and mental health experts in the treatment of HCV for PWID has been shown to result in increased treatment efficacy<sup>[32]</sup>. Besides direct interaction for the purposes of patient care, mentoring programs conducted between HCV specialists, substance abuse treatment staff, and peers could increase knowledge and build the skills necessary to treat this population. Mentoring programs could be conducted in person or via telemedicine.

A recent meta-analysis demonstrated that HCV treatment outcomes among PWID were improved among those treated for opioid addiction compared to untreated individuals<sup>[32]</sup>. In addition, rates of successful treatment outcomes for PWID were shown to be almost identical to outcomes achieved in registration trials<sup>[32,33]</sup>. However, while occasional drug use does not impact on adherence, treatment completion or treatment efficacy, frequent drug use (daily or every other day) does<sup>[34]</sup>. Consequently, successful outcomes for HCV are more likely to be achieved if PWID who inject frequently are initially stabilized for their addiction and subsequently undergo HCV therapy.

By co-localizing both HCV preventive and treatment services at venues where PWID receive care for drug addiction, uptake of HCV services might increase. For example, due to annual HCV serologic testing in some OAT facilities, HCV-infected patients have been more readily identifiable. At present, however, offsite referral to HCV specialty-care clinics is a common practice among drug treatment providers<sup>[29,35]</sup>. However, its effectiveness is limited as the majority of referred patients often fail to schedule or appear at appointments<sup>[14,36,37]</sup>. Yet, OAT facilities that do offer on-site HCV evaluation and treatment have achieved improved outcomes<sup>[38-41]</sup>. Similar findings have been previously reported for HIV-infected PWID, many of whom voluntarily use primary care services if they are offered onsite in OAT facilities<sup>[42]</sup>. Unfortunately, a recent study of substance abuse treatment programs affiliated with academic medical centers conducted through the National Drug Abuse Treatment Clinical Trials Network found a significant lack of comprehensive HCV counseling, testing, and treatment both on-site or by referral<sup>[43]</sup>. The same programs, however, offered significantly more HIV/AIDS-related health services<sup>[44]</sup>.

OAT facilities that do offer integrated HCV care programs may also provide comprehensive on-site primary care services administered by health care providers with training in diverse disciplines including infectious diseases, hepatology, addiction medicine, and mental health<sup>[45-47]</sup>. Many of these programs also offer active case management and have diverse staff consisting of physicians, physician assistants, nurse practitioners, nurses, counselors, and social workers. To improve adherence, some programs utilize directly observed therapy as well



as offering counseling sessions, motivational interviewing, peer-based support groups, and HCV-related education<sup>[45,47-50]</sup>. Improvement over offsite referral has also been achieved through an integrated model combining addiction medicine physicians with hepatologists in a viral hepatitis clinic<sup>[51]</sup>.

Finally, overcoming the financial obstacles for HCV treatment will not be easy, especially in developing countries. In the United States, health care reform will promote integration of specialty services into primary care, promote prevention, and will likely provide an opportunity for development of innovative models for previously medically-marginalized populations such as PWID. In contrast, in developing countries, pharmacy assistance programs will most likely be necessary in order to enable patients to access novel HCV treatments.

## PARALLELS BETWEEN HIV AND HCV

The issue of increasing awareness and funding for HCV treatment among PWID has many similarities to HIV; indeed, HIV treatment is often touted as one of the great medical successes of our time. As the gravity of the emerging HIV epidemic became apparent in the 1980s, national attention and subsequent funds were directed toward combating the infection. Although similarities exist between both viral infections, so do important differences. For example, HCV is curable in a majority of cases while HIV presently requires costly lifelong treatment. Prevention activities among PWID that have been highly effective in controlling HIV have not been as effective in the control of HCV, largely due to limited funding and advocacy<sup>[52]</sup>. Additionally, the ultimate consequences of HCV infection, such as development of end-stage liver disease, hepatic decompensation, or hepatocellular carcinoma leading to liver transplant and subsequent lifelong immunosuppression, are largely preventable through screening and subsequent treatment. With implementation of improved therapies, the HCV field hopes to achieve the same levels of success accomplished by the HIV field.

## CONCLUSION

As many HCV-infected PWID acquired the virus decades ago, they suffer from cirrhosis and other complications of end-stage liver disease with increasing prevalence. Therefore, strategies to increase HCV care and treatment among PWID are critically needed. Achieving higher treatment rates among this population will require overcoming existing barriers at the patient, provider, and institutional levels. Co-localization of HCV management with substance abuse treatment may be a strategy that could facilitate HCV diagnosis as well as promote treatment acceptance and adherence. This approach would reduce the prevalence of end-stage liver disease, viral transmission, and HCV-associated mortality. Additionally, early identification and treatment of HCV infection

is more cost-effective compared to management of end-stage liver disease<sup>[53]</sup>. Tremendous advances are presently occurring in the HCV field, and we hope that PWID will be included in these changes.

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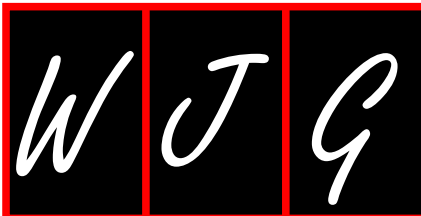
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WJG 20th Anniversary Special Issues (2): Hepatitis C virus

## Between Scylla and Charybdis: The role of the human immune system in the pathogenesis of hepatitis C

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### Abstract

Hepatitis C virus (HCV) frequently elicits only mild immune responses so that it can often establish chronic infection. In this case HCV antigens persist and continue to stimulate the immune system. Antigen persistence then leads to profound changes in the infected host's immune responsiveness, and eventually contributes to the pathology of chronic hepatitis. This topic highlight summarizes changes associated with chronic hepatitis C concerning innate immunity (interferons, natural killer cells), adaptive immune responses (immunoglobulins, T cells, and mechanisms of immune regulation (regulatory T cells). Our overview clarifies that a strong anti-HCV immune response is frequently associated with acute severe tissue damage. In chronic hepatitis C, however, the effector arms of the immune system either become refractory to activation or take over regulatory functions. Taken together these changes in immunity may lead to persistent liver damage and cirrhosis. Consequently, effector arms of the immune system will not only be considered with respect to antiviral defence but also as pivotal mechanisms of inflammation, necrosis and progression to cirrhosis. Thus, avoiding Scylla - a strong, sustained antiviral immune response with initial tissue damage - takes the infected host to virus-triggered immunopathology,

which ultimately leads to cirrhosis and liver cancer - the realm of Charybdis.

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**Key words:** Natural killer cells; CD4<sup>+</sup> T helper cells; Regulatory T cells; Interferon; Hepatitis C; Hepatic stellate cells; Hepatocytes; Immunoglobulin; Retinoic acid inducible gene-1; Toll like receptors

**Core tip:** This topic highlight on the immunopathogenesis of chronic hepatitis C addresses changes in innate immunity (interferons and natural killer cells), adaptive immunity and immunoregulation (regulatory T cells). Our review provides a succinct but comprehensive overview and presents the concept, that effective antiviral immunity is associated with pronounced acute liver damage, while during chronic infection the arms of immunity will acquire new functions, which will cause and maintain tissue damage. Thus, the immune response becomes part of the mechanisms that eventually lead to progressive inflammation, liver cirrhosis and death in chronic hepatitis C.

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### INTRODUCTION

Scylla and Charybdis were two immortal and irresistible sea monsters in Greek mythology believed to live on either side of the Strait of Messina between Sicily and Italy. Scylla was a six-headed supernatural creature - probably



reflecting a shoal that devoured whatever came within her reach, and Charybdis was a whirlpool off the coast of Sicily. Avoiding Charybdis meant passing too close to Scylla and vice versa. According to Homer, the Greek hero Odysseus opted for Scylla when passing the strait, and had to sacrifice six of his companions rather than to risk the loss of his vessel in the whirlpool. Thus, being “between Scylla and Charybdis” means to be forced to make a choice between two equally unpleasant evils.

This allegory matches the challenge of the human immune system when defending against a viral infection, such as hepatitis C which has a high potential to establish chronic persistence. On one hand a strong and efficient immune response rapidly clears the virus; accepting the risk of severe tissue damage from immune-mediated destruction. On the other hand a less vigorous response allows for viral persistence and facilitates a low-level smoldering inflammation, which eventually results in progressive liver disease and ultimately death of the individual. In line with this analogy, acute self-limited hepatitis C is frequently associated with symptomatic disease and jaundice, while chronic hepatitis C often establishes in the absence of any characteristic symptoms<sup>[1,2]</sup>. Studies in various expression systems (cell culture or transgenic mice) indicate that hepatitis C virus (HCV) is not directly cytopathic, and viral replication may occur in the absence of any detectable inflammatory reaction<sup>[3,4]</sup>. On the other hand, chronic hepatitis C is associated with liver cell damage and intrahepatic inflammatory infiltrates. Of note, hepatocellular damage coincides with the onset of an immune response during acute infection but not with that of viral replication<sup>[5]</sup>. Thus, activation of the immune response is a pivotal factor for the pathogenic processes in hepatitis C leading to progressive tissue injury. Ultimately, hepatic inflammation and progressive fibrosis in chronic hepatitis C may result in cirrhosis and carry a high risk for hepatocellular carcinoma.

## BASIC FACTS

HCV is a hepacivirus of the *Flaviviridae* family. Its genome consists of a single strand positive sense RNA. After cell entry the viral genome is translated into a single polyprotein which is co- and post-translationally cleaved into structural and non-structural proteins by host peptidases and two virus-encoded proteases. Replication involves generation of an antigenomic replication intermediate, and probably intermediate double-stranded RNA (ds-RNA) products, which can trigger intracellular pattern recognition receptors. The new viral genomes are packaged into viral particles by the viral non-structural proteins, which then are released from hepatocytes in association with host lipoproteins. Thus, HCV circulates in blood as a lipoprotein-coated virus<sup>[6]</sup>. During replication HCV is sensed by pattern recognition receptors (PRRs) in the host cell which detect pathogen-associated molecular patterns within viral products. This process then leads to coordinated activation of innate

and adaptive immune responses. Both arms of the immune response work together in an integrated fashion to recognize and defend against HCV infection.

Innate responses to HCV comprise both cellular responses, such as recognition of non-self by various types of natural killer (NK) cells and humoral components, such as induction of a variety of cytokines, especially interferons. These various elements of innate immunity act in a highly integrated fashion as do innate and adaptive immune responses. Thus, development of adaptive B and T cell immunity is shaped by the initial innate responses, in particular interferons and other inflammatory and immunoregulatory cytokines that are induced by viral invasion<sup>[7]</sup>. However, despite these immune defences, hepatitis C becomes chronic in about 70%-80% of acute infections<sup>[8]</sup>. Failing immunity and continued viral persistence lead to sustained inflammatory host responses which then become the key mechanism for tissue injury in chronic hepatitis C.

## INNATE IMMUNITY IN HEPATITIS C

Three types of PRRs are known to detect HCV: (1) the retinoic acid inducible gene- I (RIG- I)-like receptors, RIG- I and melanoma differentiation antigen 5, which sense viral RNA in the cytosol; (2) toll-like receptors (TLRs), such as TLR3, which detects ds-RNA fragments in the endosomal compartment; and (3) the non-traditional pattern recognition receptor protein kinase R (PKR), which binds ds-RNA binding and upon activation promotes interaction with mitochondrial antiviral signaling protein (MAVS) to trigger innate immunity<sup>[9]</sup>.

RIG- I signaling is initiated by binding of the HCV PAMP RNA which consists of an exposed 5'triphosphate and the 3'poly-U/UC-rich untranslated region of the HCV RNA<sup>[10,11]</sup>. These regions are located at opposite ends of the viral genome but are brought together by intra-genomic interactions. In this configuration the viral RNA comes into close contact with RIG- I and induces conformational changes of RIG- I. RIG- I activation leads to the formation of a multi-component complex with MAVS (also termed interferon beta promoter stimulator protein 1 or card adaptor inducing interferon beta, cardiff). Finally, the interferon signaling cascade results in the activation of multiple transcription factors, such as interferon-regulatory factor-3 (IRF-3) and nuclear factor kappa B and production of multiple pro-inflammatory cytokines<sup>[12]</sup>.

HCV dsRNA intermediates, which occur late in HCV replication, have been identified as ligands for TLR3<sup>[13]</sup>. TLR3 signals are transmitted by the adaptor molecule TIR-domain-containing-adaptor-inducing-interferon- $\beta$  (TRIF) and also lead to production of interferons and pro-inflammatory cytokines<sup>[14]</sup>. TLR3 mediated interferon and cytokine responses are considered a secondary innate immune defense after initial RIG- I activation to establish an antiviral state and trigger T cell recruitment in HCV infection.

The ligand for PKR is the structured RNA at the internal ribosomal entry site (IRES) of HCV RNA<sup>[15,16]</sup>. Binding of HCV RNA induces phosphorylation of the  $\alpha$ -subunit of the eukaryotic initiation factor 2 (eIF2 $\alpha$ ). In addition, RNA binding also triggers a kinase-independent signal transduction cascade involving MAVS which finally activates interferon- $\beta$  and interferon-stimulated genes (ISGs)<sup>[9,16]</sup>.

Although HCV can be detected effectively by RIG- I, TLR3 and PKR, it frequently establishes chronic persistence in up to 80% of patients, because it has evolved several mechanisms to counter-act innate immunity. The multi-functional HCV NS3/NS4A protease is a key component of the HCV evasion strategy from innate immunity. Studies in Huh-7 cells indicate that HCV initially activates the RIG- I pathway which is shut down as infection progresses and NS3/NS4 abundance increases<sup>[17]</sup>. In addition to proteolytically processing the HCV polyprotein, NS3/NS4A can block RIG- I signaling, because it cleaves MAVS from intracellular membranes<sup>[18-21]</sup>. This cleavage prevents signal transduction, abrogates interferon induction and facilitates progression to chronic infection. However, other hepatotropic viruses, such as hepatitis A virus also encode proteases that can cleave MAVS but in general do not become chronic<sup>[22]</sup>. Thus, MAVS cleavage alone is not sufficient for viral chronicity. Nevertheless, cleavage of MAVS has been demonstrated in the livers of patients with chronic hepatitis C, and patients with cleaved MAVS revealed reduced interferon pathway activation, although this inverse correlation was rather weak<sup>[23]</sup>.

The NS3/NS4A protease can also cleave TRIF<sup>[24]</sup>, the adaptor protein of the TLR3 pathway, and the relative abundance of this protein is reduced after HCV infection, probably as a result of degradation following its cleavage by NS3/NS4A<sup>[25]</sup>. Although details are insufficiently understood at present, blocking of the TLR3 pathway by HCV also seems to contribute to establishing chronic infection. TLR3-independent sensing of RNA which signals *via* TRIF has also been described, and is likewise blocked by NS3/NS4A targeting of TRIF<sup>[26]</sup>. Finally HCV proteins E2, NS3, NS4A and NS5 provide several strategies to interfere both with PKR signaling and PKR-regulated inhibition of translation<sup>[27-29]</sup>. However, these interactions are complex and the exact mechanisms how they support HCV persistence are still unclear.

Continued triggering of PRR pathways in chronic hepatitis C is likely to contribute to immunopathology, such as hepatic inflammation, fibrosis progression and HCV-associated malignancy. In this context it is interesting to note that HCV proteins core and NS3 also trigger TLR 1-2 and 2-6 dimers<sup>[30,31]</sup>, and there is evidence from genetic epidemiology and functional *in vitro* studies that HCV-TLR interactions might contribute to hepatic fibrogenesis and cirrhosis<sup>[32,33]</sup>, development of liver cancer<sup>[34]</sup>, HCV-associated autoimmunity and B cell lymphoma<sup>[35]</sup>.

## INTERFERONS

HCV recognition by PRRs ultimately leads to induction of antiviral cytokines termed interferons (IFNs). Type I IFNs (several interferons- $\alpha$  and interferon- $\beta$ ) bind to the ubiquitously expressed type I interferon receptor, while type III IFNs [IFN- $\lambda$ 1 alias interleukin (IL)-29, IFN- $\lambda$ 2 alias IL-28A, IFN- $\lambda$ 3 alias IL-28B] have their own receptor consisting of the IL10R2 chain (IL-10 receptor beta chain) and a unique IFN- $\lambda$  receptor chain with a limited expression mainly on hepatocytes<sup>[36,37]</sup>. The type II interferon IFN- $\gamma$  has its own IFN- $\gamma$  receptor. All IFN receptors transmit signals from the cell surface to the nucleus *via* the Jak-STAT pathway to activate interferon stimulated genes (ISGs). Specifically type I and III IFNs induce IFN-stimulated gene factor 3 consisting of phosphorylated STAT1 and 2 proteins and IRF9 which activate the IFN-stimulated response elements (ISRE) of multiple genes contributing to antiviral activity<sup>[38-40]</sup>.

IFN signaling is regulated by suppressors, such as suppressor of cytokine signaling and ubiquitin specific peptidase 18 (USP18) which provide important negative feed-back loops<sup>[41-43]</sup>. USP18 is a protease cleaving ISG15 from its target proteins, also including STAT1<sup>[44]</sup>. ISG15 is conjugated to STAT1 by the sequential action of several enzymes<sup>[45]</sup>. This so-called ISG-ylation and its de-conjugation by USP18 modify signal transduction pathways and immune responsiveness<sup>[46,47]</sup>. However, recently it has been recognized that USP18 suppresses IFN-signaling independently from its de-conjugating activity by interfering with the interaction between Jak1 and the type I IFN receptor<sup>[48]</sup>. USP18 is a major mediator of unresponsiveness to type 1 IFNs in liver cells<sup>[49]</sup>. However, it does not inhibit signal transduction of type II and III IFNs<sup>[50]</sup>.

Activation of the endogenous IFN system in the liver exerts little anti-HCV activity, and it has been well established that patients with high activation of the endogenous IFN system respond poorly to IFN $\alpha$  based therapies<sup>[51-55]</sup>. It has been proposed that expression of HCV proteins inhibits binding of activated STATs to ISRE<sup>[56]</sup>, and Jak-STAT signaling was found to be inhibited both in HCV transgenic mice and liver biopsies from patients with hepatitis C<sup>[57,58]</sup>. Beyond that, phosphorylation and activation of STAT3 is involved in the antiviral IFN activity<sup>[59]</sup>, and STAT3 expression was found to be reduced in HCV-infected livers<sup>[60]</sup>. Indeed, HCV core protein can prevent STAT3 phosphorylation<sup>[57,60,61]</sup>, and this has been associated with HCV resistance to IFN- $\alpha$ <sup>[62]</sup>. Next, HCV-induced PKR activation inhibits cap-dependent translation of antiviral host proteins at the ribosomes owing to phosphorylation of eIF2 $\alpha$  while production of HCV proteins is not impaired, because translation occurs *via* an IRES-dependent mechanism<sup>[63]</sup>. Of note, most studies on endogenous ISG induction in hepatitis C were based on steady state mRNA level measurements rather than determination of protein concentrations<sup>[51-55]</sup>. Finally, HCV proteins might directly inhibit ISG antiviral

effector functions apart from their inhibition of ISG translation. This concept is supported by experimental evidence from knock-out mice which demonstrated that expression of the USP18 leads to a long-term refractory state towards IFN $\alpha$  stimulation<sup>[49]</sup>. Likewise, strong USP18 expression was found in many hepatocytes of patients with chronic hepatitis C and high endogenous IFN activity, when histological specimens were studied<sup>[64]</sup>. At present the cellular sources and involved types of IFNs that maintain long-term ISG expression in chronic hepatitis C are still a matter of debate. IFN- $\lambda$ s are strong candidates as triggers of ISG induction in patients with chronic hepatitis C and endogenous activation of the IFN system, because, unlike all other IFN types, IFN- $\lambda$  mRNA is readily detected in liver biopsies<sup>[53]</sup>, and their action is not inhibited by USP18<sup>[50]</sup>.

In patients with chronic hepatitis C endogenous ISG induction varies considerably between individuals, and this variability, as well as differential responsiveness to exogenous IFN- $\alpha$  is attributed to a combination of viral and host factors. For instance, difficult-to-treat HCV genotypes 1 and 4 induce high levels of endogenous IFN expression in hepatocytes resulting in an IFN-insensitive state that attenuates treatment responses<sup>[65]</sup>. Of note, endogenous ISG induction in Kupffer cells, the resident liver macrophages, is also a strong predictor of treatment responsiveness<sup>[66]</sup>. However, the relationship between baseline ISG induction and treatment outcome is opposite to that observed in hepatocytes: Virtually all non-responders lack baseline induction of ISGs whereas strongly induced ISG expression is found in responders<sup>[67]</sup>. This finding suggests that ISG induction in Kupffer cells may have a protective role for the host concerning both spontaneous HCV elimination and treatment outcomes.

Apart from viral factors genome-wide association studies have identified single nucleotide polymorphisms (SNPs) upstream of the *IFNL3* gene on chromosome 19q13, which are associated with outcomes of HCV infection both under IFN-based therapy of chronic hepatitis C<sup>[68-70]</sup> and disease evolution during acute HCV infection<sup>[71,72]</sup>. Although some initial studies failed to find a relationship between the SNPs and *IFNL3* mRNA expression<sup>[71,73]</sup>, it has meanwhile become clear that SNPs in this region alter IFN- $\lambda$  expression levels<sup>[53,68,70,74-76]</sup>, and the unfavorable minor alleles result in less *IFNL3* expression. Thus, it is quite unlikely that these SNPs simply reflect linkage disequilibrium with some other gene. However, the molecular and cellular mechanisms that underlie this association between outcomes of HCV infection and the *IFNL3* gene locus are not yet understood. It has been proposed that the unfavorable *IFNL3* variants may lead to compromised innate immune functions in particular with respect to natural killer cell activity<sup>[77,78]</sup>. However, given the fact that NK cells do not express type III IFN receptors this hypothesis needs refining<sup>[79]</sup>. In addition, a dinucleotide polymorphism upstream of the *IFNL3* gene has been described, which

can create or disrupt an alternative open reading frame giving rise to a new gene, termed *IFNL4*<sup>[80,81]</sup>. Although it has been proposed that loss of *IFNL4* expression should be protective against HCV, it is as yet not clear if the putative *IFNL4* gene product plays any role for differential immune responses to HCV infection.

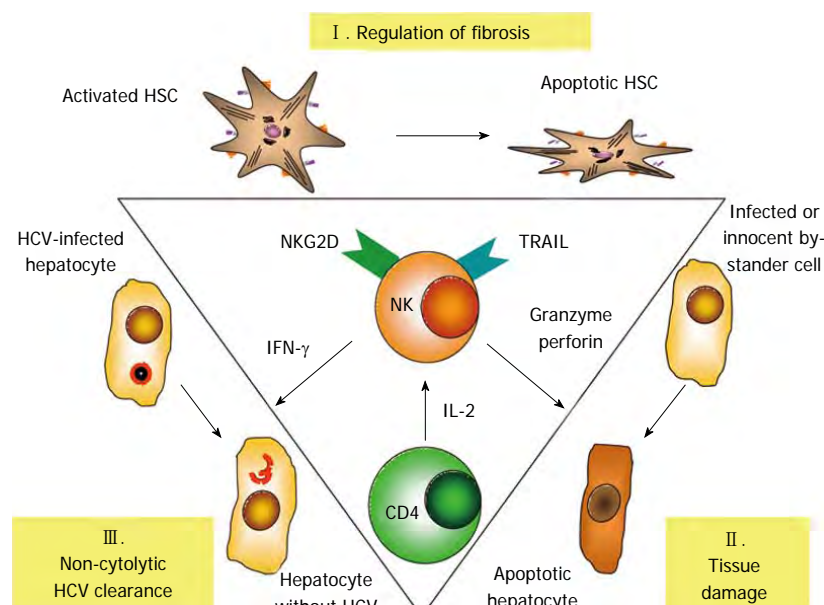
## NATURAL KILLER CELLS

NK cells constitute a first line of defence against viral infections. They rapidly recognize and lyse virus-infected cells, inhibit viral replication but also exert immune-regulatory functions. NK cells constitute approximately 30% of resident lymphocytes in a normal liver, and may account for as many as 60% of lymphocytes in HCV infection<sup>[82]</sup>.

Activation of natural killer cells results from the integration of multiple activating and inhibitory signals *via* specific receptors. The most important NK cell receptors (and their cognate ligands) comprise the killer immunoglobulin-like receptor (KIR) family (ligands: HLA-A, -B and -C), the CD94-NKG2A/C complex (ligand: HLA-E), NKG2D (ligands: MIC-A and MIC-B and others) and the natural cytotoxicity receptors NKp30, NKp44 and NKp46<sup>[83]</sup>. In addition, part of these receptors also exerts immune-regulatory functions in subsets of T lymphocytes. NK cells are activated, when there is a relative reduction of inhibitory signals, *e.g.*, down-regulated MHC class I expression on virus-infected cells, or a relative increase in signals from activating receptors, *e.g.*, binding of antibody-coated antigens<sup>[84]</sup>. However, conventional MHC class I expression is not substantially reduced in hepatitis C, and it has been proposed that NK cell functions might be altered by binding of HCV-derived peptides to non-polymorphic restriction molecules, such as HLA-E<sup>[85,86]</sup>. NK cells are recruited to inflammatory sites by a variety of chemokines and can also be stimulated by cytokines, such as IFN- $\alpha$  and ILs 8, 12, 15 and 18<sup>[87]</sup>.

Activated NK cells with potent de-granulation and substantial cytokine production have been described in acute HCV infection<sup>[88,89]</sup>, and there is accumulating evidence to suggest that NK cells play an important role in the antiviral immune response to hepatitis C and later on also in the immune-mediated pathogenesis of chronic hepatitis C. NK cells can inhibit HCV replication *in vitro* both by IFN- $\gamma$  mediated non-cytolytic as well as granzyme/perforin and TRAIL-mediated cytotoxic mechanisms<sup>[90]</sup>. While HCV-infected hepatocytes up-regulate expression of TRAIL receptors<sup>[91]</sup>, *in vivo* IFN $\gamma$ -mediated clearance of HCV might be more important than direct cytotoxicity, because cytolytic elimination of all HCV-infected hepatocytes would lead to extensive liver damage<sup>[92]</sup>. Multi-functional NK cells are also detectable early after HCV exposure in health-care workers and iv drug users, who do not proceed to develop acute hepatitis; suggesting a potentially protective role of NK cells in early HCV infection<sup>[93,94]</sup>. Further support for a pro-





**Figure 1** Central role of natural killer cells in the pathogenesis of hepatitis C. Natural killer (NK) cells regulate fibrosis by killing of activated hepatic stellate cells (HSC), which trigger NK cell activation *via* natural killer cell receptor with extracellular C-type lectin domains (NKG2D) signalling. The release of granzyme/perforin and cytotoxic cytokines, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induce tissue damage. Interferon- $\gamma$  (IFN- $\gamma$ ) released from NK cells can clear hepatitis C virus (HCV) infection from infected hepatocytes without cytolysis. On the other hand NK cell activity is critically dependent on sufficient supply with interleukin 2 (IL-2) from CD4<sup>+</sup> T cells.

protective role of NK cells in HCV infection comes from genetic studies, where genes encoding the inhibitory receptor KIR2DL3 and its ligand human leucocyte antigen group 1 (HLA-C1) seem to favour both spontaneous and treatment-induced elimination of HCV<sup>[95,96]</sup>. Since the affinity between inhibitory KIR2DL3 and HLA-C1 is weaker than all other combinations, it is reasonable to assume that a lower threshold of activation is needed to trigger KIR2DL3 NK cell responses in HLA-C1 homozygous individuals<sup>[88,97]</sup>. Finally, NK cells can become more activated upon IFN-based therapy and may contribute to HCV elimination by TRAIL-mediated cytotoxic mechanisms<sup>[98]</sup>. Interestingly, responsiveness in this setting again depends on the endogenous IFN- $\alpha$  activation state, since a rapid first phase HCV decline is associated with strong induction of STAT1 phosphorylation, whereas non-responders exhibit reduced STAT1 induction<sup>[99]</sup>. Chronic exposure of NK cells to IFN- $\alpha$  results in preferential STAT1 over STAT4 phosphorylation, which is associated with increased STAT1-dependent cytotoxicity but reduced STAT4-dependent IFN- $\gamma$  production<sup>[99-101]</sup>. These findings correspond to NK cell phenotypes and functional differentiation seen at later stages in IFN- $\alpha$  responders and non-responders<sup>[100,102]</sup>, although patients who achieve a sustained virological response also exhibit substantial NK cell cytotoxicity<sup>[103]</sup>.

NK cells in chronic hepatitis C have been reported to also express altered patterns of NK receptors (Figure 1). Although reported patterns are somewhat inconsistent and may vary between peripheral blood and the liver, altered expression on NK cells has been reported for receptors NKp30, NKp44, NKp46, NKG2A, NKG2C, NKG2D and CD122<sup>[100,104-107]</sup>. In addition, NK cells ex-

press the tetraspanin CD81, a co-receptor of HCV, and *in vitro* binding of the HCV envelope 2 (E2) protein to CD81 has been shown to block antiviral functions of NK cells and to alter their migratory behaviour<sup>[108-111]</sup>. However, the experimental setting of these studies involved cross-linking of HCV E2 on plastic plates, whereas NK cells exposed to intact virions did not exhibit altered functionality<sup>[112]</sup>. Thus, it remains to be elucidated if cross-linking of CD81 by HCV E2 affects functions of NK cells to facilitate chronic infection. A particularly interesting NK cell receptor is NKp46, since it is considered a major activating receptor in hepatitis C, which also has a role in the regulation of adaptive immunity: High expression of NKp46 defines a NK cell subset with high cytotoxic activity and IFN- $\gamma$  production that accumulates in the liver in chronic hepatitis C<sup>[113,114]</sup>. Of note, recently Pembroke *et al.*<sup>[115]</sup> confirmed intrahepatic enrichment of NKp46<sup>+</sup> NK cells in chronic hepatitis C and reported a high (> 80%) frequency of NKp46<sup>+</sup> cells in the liver to be associated with pronounced inflammation in histology. Another important finding of this study was the observation, that expression of NKp46 could predict responses to IFN therapy. Patients with chronic hepatitis C, who successfully cleared their HCV infection, had lower mean frequencies of activated NKp46<sup>+</sup> NK cells than patients who did not respond to therapy. The possible identification of NKp46 as a marker of both IFN-un-responsiveness and hepatic inflammation bears some similarity to the paradoxical relationship between IFN-un-responsiveness and high baseline ISG expression and may be linked to chronic endogenous interferon exposure. On the other hand, unlike Pembroke *et al.*<sup>[115]</sup> the group of Golden-Mason<sup>[113]</sup> reported increased NKp46



expression in white female Americans as opposed to male African-Americans and proposed that a high proportion of functionally active NKp46+ NK cells could explain their higher response to IFN therapy. Thus, the precise role of NKp46+ NK still remains elusive.

Finally, NK cell-mediated cytotoxicity against hepatic stellate cells (HSC) may contribute to the regulation of intrahepatic fibrosis in hepatitis C. HSC store vitamin A, reside in the space of Disse, and produce extracellular matrix proteins upon activation, *e.g.*, upon TLR stimulation, exposure to cytokines or reactive oxygen species<sup>[116]</sup>. HSC activation leads to trans-differentiation into myofibroblasts, which in the mouse also alters the balance in the expression between activating and inhibitory NK cell receptor ligands, so that they become target cells for NKG2D-, TRAIL- and granzyme-mediated killing by NK cells<sup>[117,118]</sup>. NKG2D- and TRAIL-mediated killing by NK cells has now also been reported for human HSC in chronic hepatitis C<sup>[119]</sup>, and CXCR3 + CD56<sup>Bright</sup> as well as NKp46+ NK cells express particularly high cytotoxic capacity against HSC in chronic hepatitis C<sup>[114,120]</sup>. Importantly, when other processes, such as CD4 T cell depletion in HIV/HCV co-infection interfere with the regulation of hepatic fibrosis by NK cells, this may result in accelerated fibrosis progression<sup>[121]</sup>.

## ADAPTIVE IMMUNITY IN HEPATITIS C

A coordinated immune response involving both antibodies and T cell responses is normally required for efficient adaptive immunity. However, in hepatitis C the role of antibodies is complex: Circulating antibodies against structural and non-structural components are generated in virtually all patients irrespective from the outcome of HCV infection. A rapid induction of neutralizing antibodies early in the course of hepatitis C has been demonstrated to contribute to HCV clearance<sup>[122]</sup>, but broad antibody responses usually occur at the stage of chronic infection and are not neutralizing<sup>[123,124]</sup>. Neutralizing antibodies frequently recognize the HCV envelope proteins<sup>[125-127]</sup>. However, these proteins have a high degree of mutational diversity, so that antibody responses are frequently directed against only a single strain or are easily evaded by viral mutations<sup>[124]</sup>. It is also quite likely that glycosylation of HCV proteins and the close association of the virus with lipoproteins further prevent antibody recognition. HCV antibodies are not required to clear HCV infection as has been demonstrated in patients with hypo-gammaglobulinemia<sup>[128]</sup>. HCV antibodies gradually disappear after successful HCV elimination<sup>[129]</sup>. Conversely, there is circumstantial evidence that HCV-specific cellular immune responses can protect individuals at high risk for hepatitis C without seroconversion<sup>[123,130,131]</sup>. Thus, adaptive cell-mediated immunity is considered a key mechanism for resolution of primary HCV infection<sup>[132]</sup>. Cell-mediated immunity involves CD8<sup>+</sup> cytolytic T lymphocytes (CTL), which recognize linear HCV peptides of 8 to 11 amino acids in length

bound to self HLA class I molecules, and CD4<sup>+</sup> T helper lymphocytes, which respond to longer viral peptides bound to class II molecules. Single source outbreaks further support a clear relationship between distinct HLA types and the outcome of HCV infection: patients with HLA-A3, HLA-B27, and HLA-B57 exhibit greater chances to develop protective immunity, thus strengthening the importance of effective antigen presentation and the generation of efficient antigen-specific T cell responses for immune control of HCV infection<sup>[133-136]</sup>.

## T CELL RESPONSES

The most conclusive experiments to suggest an important role for T cells in protective immunity against HCV stem from chimpanzee experiments: Depletion of CD8<sup>+</sup> T cells in animals, which had recovered from previous hepatitis C, resulted in prolonged viraemia, and viral clearance was correlated to recovery of HCV-specific CD8<sup>+</sup> T cells<sup>[137]</sup>. Likewise, depletion of CD4<sup>+</sup> T cells resulted in abrogation of a previously protective immune response<sup>[138]</sup>. In acute hepatitis C strong HCV-specific CTL<sup>[139,140]</sup> and TH1 type CD4<sup>+</sup> T helper cell responses<sup>[141]</sup> have consistently been reported to be closely associated with a self-limited course of HCV infection. Moreover, several groups have reported an inverse relationship between the strength of the CTL response and HCV viral loads<sup>[142-144]</sup> further suggesting that in principle it is possible for cellular immunity to control HCV infection<sup>[145]</sup>. A substantial proportion of individuals who ultimately develop chronic hepatitis C also generate HCV-specific CD4(+) and CD8(+) T cell responses during the early acute phase of infection and may transiently gain some control over HCV<sup>[140,146-149]</sup>. However, early T cell responses decline to almost undetectable levels later on, and initial control over HCV replication is lost. If present, HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells are detected at only low frequency in peripheral blood although they are somewhat enriched in the liver<sup>[150,151]</sup>. Thus, chronic hepatitis C is characterized by a progressive functional exhaustion and ultimately loss of HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>[152,153]</sup>.

Exhausted T cells exhibit a couple of characteristic abnormalities: They show increased expression of inhibitory receptors, such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain-containing molecule 3, corresponding to up-regulated expression of their cognate ligands in the liver<sup>[154-159]</sup>. Conversely, functional recovery of HCV-specific T cells can be achieved experimentally by the combined blockade of CTLA-4 and PD-1 signalling<sup>[157,160]</sup>.

HCV replicates by an RNA-dependent RNA polymerase which has a high error rate and consequently generates considerable genomic diversity of HCV and T cell escape mutations. Mutations that affect CD8<sup>+</sup> T cell epitopes and proteasomal processing have been observed in several HCV single source outbreaks<sup>[161-163]</sup>.

Due to the exhausted state of T cells new epitope variants rarely elicit strong CD8<sup>+</sup> T cell responses at this stage of infection, and consequently further escape mutation to secondary epitopes are selected infrequently in man and the chimpanzee<sup>[146,164,165]</sup>. Protective T cells seem to target epitopes that do not allow for escape mutations owing to the associated loss of viral replication fitness<sup>[133-135]</sup>. Conversely, T cells that are not stimulated any more after HCV viral escape, do not show features of exhaustion<sup>[166]</sup>. Thus, prolonged exposure appears to be the mechanism that leads to T cell dysfunction in chronic hepatitis C, and T cell exhaustion in hepatitis C seems to follow the same pattern as has been first described in mice for lymphocytic choriomeningitis virus (LCMV) infection<sup>[167,168]</sup>. In this model, persistent high level viremia can be established in susceptible mouse strains by pathogenic virus variants. Initially, mice develop a robust T cell response but fail to eliminate the virus and subsequently exhibit a gradual decline of CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses. T cells undergo T cell exhaustion in this model, and first lose production of IL-2, a cytokine which supports T cell proliferation. Then, cytotoxicity and production of tumour necrosis factor alpha and IFN- $\gamma$  are lost sequentially. Finally, intracellular expression of pro-apoptotic factors, such as Bcl2-interacting mediator (Bim), is up-regulated both in the LCMV model and hepatitis C<sup>[169]</sup>. In analogy to the LCMV model, virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses decline in chronic hepatitis C but full exhaustion with deletion of antigen-specific CD8 T cells does not occur, because at least *in vitro* T cell responses can be rescued.

## REGULATORY T CELLS

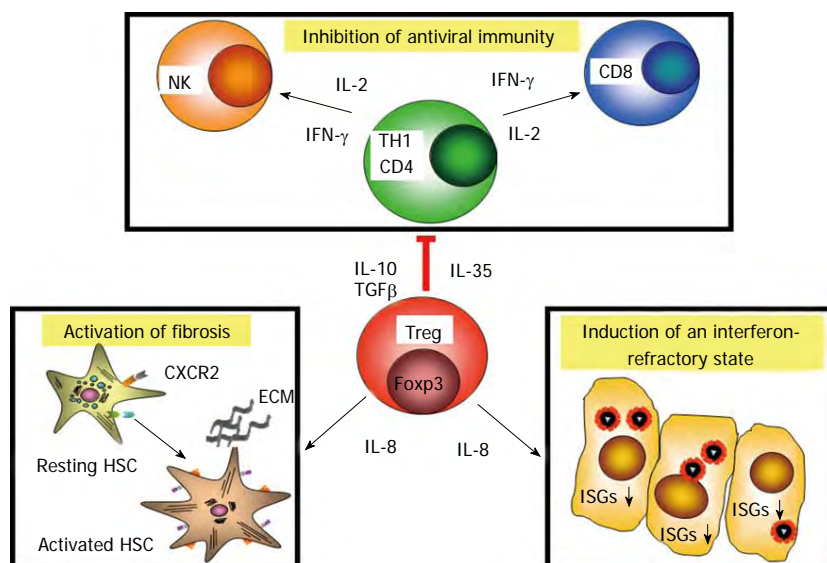
Recently CD8<sup>+</sup> T cells have been reported in the livers of patients with chronic hepatitis C which were considered to represent CD8<sup>+</sup> regulatory T cells, because they secrete IL-10 and suppress *in vitro* proliferation of liver-derived T cells<sup>[170]</sup>. In general, regulatory T cells (Tregs) actively control induction and activity of other immune cells by suppressing their functional activity *via* contact-dependent mechanisms and by release of immunosuppressive cytokines, such as IL-10 and transforming growth factor beta. The major cell type with these properties constitutes CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>-</sup> T cells, which express the transcription factor Foxp3 (forkhead box P3). They can be divided into thymus-derived natural regulatory T cells, that prevent autoreactivity to self-antigens and induced regulatory T cells, that are generated in the peripheral immune system as a regulatory response to antigenic stimulation. Foxp3<sup>+</sup> Tregs were rarely detected in acute hepatitis C<sup>[171]</sup> and they are also not found in patients who had managed to resolve HCV infection<sup>[172]</sup>, suggesting that effector T cells in acute and self-limited hepatitis C are not under active suppression by Tregs. In chronic hepatitis C, however, numbers of CD4<sup>+</sup> Tregs were increased in the peripheral blood of patients, and depletion of CD4<sup>+</sup> CD25<sup>+</sup> T cells was as-

sociated with increased numbers and function of CD8<sup>+</sup> T cells in *in vitro* assays<sup>[173-176]</sup>. Such regulatory T cells may reduce inflammatory activity and are considered to contribute importantly to preventing immune-mediated pathology in chronic hepatitis C. Functional analysis of regulatory T cell clones generated from patients with chronic hepatitis C revealed that Tregs were directed against HCV antigens and showed the same pattern of HLA class II restriction and epitope specificity as effector T cells<sup>[172]</sup>. Importantly, Treg clones from chronic hepatitis C inhibited *in vitro* proliferation and IFN- $\gamma$  production of autologous reporter T cells *via* release of inhibitory cytokines, such as IL-10 and IL-35. Of note, intrahepatic regulatory T cells in chronic hepatitis C also produced substantial amounts of IL-8, and isolated Tregs as well as Treg clones activated fibrogenic genes of hepatic stellate cells *in vitro*<sup>[177]</sup>. High intrahepatic IL-8 mRNA levels in chronic hepatitis C have been linked with progression of fibrosis<sup>[178,179]</sup> and CD4<sup>+</sup> Tregs are enriched in the liver<sup>[175,177,180-182]</sup>. Moreover, some but not all studies also reported a correlation between numbers of intrahepatic Tregs and the stage of fibrosis. Beyond that, IL-8 counter-acted the antiviral activity of IFN- $\alpha$  in the replicon model by down-regulation the expression of ISGs<sup>[183,184]</sup>. Moreover, *in vitro* studies suggest that part of the superior antiviral activity in IFN/ribavirin combination therapy may be due to preferential inhibition of Tregs by ribavirin<sup>[185]</sup> (Figure 2).

Thus, once the immune system has failed to clear HCV infection, regulatory T cells in chronic hepatitis C seem to exert multiple different effects: they dampen inflammatory responses associated with reduced antiviral activity of the immune system, facilitate HCV persistence, and also contribute to the regulation of fibrosis in the liver.

## CONCLUSION

When an individual becomes infected with HCV, the immune system has to make a choice between Scylla and Charybdis. If it takes a course close to Scylla, it generates strong antiviral immune responses, which eliminates virus infected liver cells by the combined action of its several innate and adaptive defense mechanisms. This may cause extended liver damage and eventually liver failure. To avoid this risk, immune responses may be softer. Then, the virus has a chance to escape from control by immunity, and functions of innate and adaptive immune mechanisms become diverted owing to continued antigenic stimulation. An inflammatory state is induced, which, however, is refractory to stimulation by antiviral cytokines, and NK cells as well as cells in the adaptive immune system take over regulatory functions. Necro-inflammatory and pro-fibrotic activities maintained by diverted immune responses inevitably take a course towards Charybdis, and may ultimately result in liver cirrhosis, liver cancer and death of the individual. Thus, the immune system holds the steer to find the way between



**Figure 2 Multiple activities of hepatitis C virus-specific regulatory T cells in chronic hepatitis C.** Regulatory T cell (Tregs) inhibit antiviral immunity *via* release of immunosuppressive factors, such as interleukin 10 (IL-10), transforming growth factor beta (TGF- $\beta$ ) and interleukin 35 (IL-35). Tregs in hepatitis C are also differentiated towards interleukin 8 (IL-8) production. Release of IL-8 binds to its receptors, such as CXCR2 on hepatic stellate cells (HSC), which become activated and produce extracellular matrix (ECM) components. IL-8 down-regulates interferon-stimulated genes in infected cells and induces an interferon (IFN)-refractory state, which also counter-acts antiviral immunity. ISGs: Interferon-stimulated genes.

Scylla and Charybdis.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Tumor necrosis factor- $\alpha$ inhibitors and chronic hepatitis C: A comprehensive literature review

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## Abstract

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibitors are known to increase reactivation of concurrent chronic hepatitis B, but their impact on the hepatitis C virus (HCV) is controversial. Some conditions of immunosuppression, such as liver transplantation, typically cause an increase in the rate of HCV evolution. Inhibition of TNF- $\alpha$ , a cytokine involved in the apoptotic signaling pathway of hepatocytes infected by HCV, could potentially increase viral replication. Currently available clinical data appear to contradict this hypothesis. A review of medical literature revealed that a total of 216 patients with HCV were exposed to one or more treatments with TNF- $\alpha$  inhibitors, with a median observation time of 1.2 years and 260 cumulative patient-years of exposure. Only three cases of drug withdrawal due to suspected HCV liver disease recrudescence were reported. Treatment with TNF- $\alpha$  inhibitors in patients with HCV infection appears to be safe in the short term, but there are insufficient data to assess their long-term safety. Universal screening for HCV before beginning treatment with TNF- $\alpha$  inhibitors is currently controversial. The presence of HCV is not a contraindication to therapy with TNF- $\alpha$  inhibitors, with the exception of cirrhotic pa-

tients. In cases of cirrhosis, the benefit/risk ratio should be evaluated at the individual level. Prior to treatment with TNF- $\alpha$  inhibitors, patients with HCV should be referred to a hepatologist to determine the necessity of hepatic disease assessment, using liver biopsy or non-invasive methods, and the potential indication for antiviral therapy. In patients with HCV infection who are treated with TNF- $\alpha$  inhibitors, liver function monitoring every three months is advised.

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**Key words:** Infliximab; Etanercept; Adalimumab; Hepatitis C virus; Rheumatoid arthritis; Inflammatory bowel disease; Psoriasis

**Core tip:** Our review summarizes data on patients with hepatitis C exposed to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibitors, thus building a stronger safety profile than previously reported. A comprehensive paragraph on the pathway of TNF- $\alpha$  in hepatitis C virus (HCV) and an overview on immune-mediated damage induced by TNF- $\alpha$  inhibitors (cryoglobulins, autoimmune hepatitis) have been also included. Some controversies regarding the universal screening and monitoring of HCV-RNA were also addressed.

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## INTRODUCTION

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a cytokine involved

in the pathogenesis of inflammatory diseases and in the immune-mediated response to infections, especially against intracellular pathogens. Drugs targeting and inhibiting the biological activity of TNF- $\alpha$ , such as infliximab, etanercept and adalimumab, are increasingly used for the treatment of immune-mediated diseases such as rheumatoid arthritis, inflammatory bowel diseases and psoriasis<sup>[1]</sup>. TNF- $\alpha$  inhibitors increase susceptibility to new or reactivation of concurrent infections. Thus, before its use for therapy, a screening for tuberculosis (with chest radiography and an interferon-gamma release assay) and certain viral infections such as hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus, and herpes virus is recommended<sup>[2]</sup>.

The potential risk of reactivation of HBV infection during TNF- $\alpha$  inhibitor therapy is well established. Animal studies have demonstrated that TNF- $\alpha$  plays a key role in clearing HBV from infected hepatocytes by synergizing with interferons (IFNs) in the suppression of viral replication<sup>[3,4]</sup>. TNF- $\alpha$  inhibitors can increase HBV replication and reactivate chronic hepatitis, both during and after discontinuation of treatment. It is worth noting that many patients receiving TNF- $\alpha$  inhibitors have been previously or simultaneously treated, even for long periods, with other immunosuppressant agents that further increase the risk of HBV reactivation<sup>[5]</sup>. Hepatitis reactivation has been reported in twenty-three hepatitis B surface antigen (HBsAg)-positive patients treated with TNF- $\alpha$  inhibitors in the absence of prophylaxis (inactive carriers or with unrecognized HBsAg seropositivity), including 9 cases of fulminant hepatitis, 4 deaths and 1 liver transplantation. Furthermore, three HBsAg-negative, hepatitis B core antibody (Anti-HBc)-positive patients presented HBsAg seroreversion followed by a hepatitis flare-up after administration of TNF- $\alpha$  inhibitors<sup>[6]</sup>. The protocol that is currently recommended, borrowed from other clinical situations of pharmacologically induced immunosuppression, includes prophylaxis with lamivudine of all inactive carriers during and for 6-12 mo following therapy with TNF- $\alpha$  inhibitors and quarterly monitoring of HBsAg in HBsAg-negative anti-HBc positive patients<sup>[7,8]</sup>.

In the context of HCV infection, the potential risk of reactivation of infection during therapy with TNF- $\alpha$  inhibitors is controversial. Several clinical reports have shown that chronic hepatitis C usually evolves rapidly in some conditions associated with immunosuppression, such as co-infection with human immunodeficiency virus, hypogammaglobulinemia, and after bone marrow transplantation and, above all, liver transplantation<sup>[9]</sup>. In various other circumstances, *e.g.*, following chemotherapy, hepatitis flare-up does not occur during immunosuppression or after its suspension<sup>[10]</sup>. The inhibition of TNF- $\alpha$ , a cytokine involved in the apoptotic signaling pathway of hepatocytes infected by HCV, could potentially increase viral replication and worsen the course of chronic hepatitis<sup>[11]</sup>. In this review, we present an overview of the relationship between the TNF- $\alpha$  pathway and HCV, summarize the available evidence regarding the safety of TNF- $\alpha$  inhibi-

tor usage in patients with HCV and provide suggestions for the management of therapy in this clinical setting.

## TNF- $\alpha$ PATHWAY IN CHRONIC HCV INFECTION

The role of TNF- $\alpha$  in chronic HCV infection is not well understood. Serum levels of TNF- $\alpha$  and its soluble receptors (sTNF-R55 and sTNF-R75) are significantly higher in HCV-infected patients than in healthy subjects<sup>[12]</sup>. Serum levels of TNF- $\alpha$  correlate with serum transaminase levels, histological activity and fibrosis, but not with serum HCV RNA levels or viral genotype<sup>[13,14]</sup>. Laboratory studies have indicated that the HCV core protein has the potential to inhibit the TNF- $\alpha$ -mediated apoptotic signaling pathway, providing a selective advantage for HCV replication and avoidance of the host antiviral defense mechanism<sup>[15]</sup>. Thus, further suppression of TNF- $\alpha$  by biological drugs may pose a potential threat of excessive viral replication and worsening of chronic HCV infection. In contrast, some studies have postulated that the baseline overexpression of TNF- $\alpha$  is associated with reduced cell capability to respond to IFN signaling and, consequently, to reduced viral clearance<sup>[16]</sup>. Zein *et al.*<sup>[17]</sup> conducted a controlled, double-blind, randomized, placebo trial assessing the effects of etanercept as adjuvant therapy to IFN alfa-2b for 24 wk plus ribavirin in patients with chronic hepatitis C. The 19 patients treated with etanercept achieved sustained virologic response at a significantly higher rate compared to the 25 controls, and treatment was associated with decreased incidence of the most common side effects associated with IFN and ribavirin. This phase II study supported the assumption that etanercept may restore TNF-induced CD4<sup>+</sup> cell impairment and enhance antiviral effects of IFN and ribavirin combination therapy. Large studies of the effects of adjuvant etanercept on therapy with pegylated IFN and ribavirin are currently lacking.

Infliximab is a recombinant human-murine chimeric immunoglobulin-G1 (IgG1) antibody that specifically binds both soluble and membrane-bound precursor forms of TNF- $\alpha$ . Etanercept is a dimeric fusion protein that consists of the extracellular ligand-binding portion of the human 75 kDa TNF receptor linked to the Fc portion of the human IgG1, and binds only soluble TNF- $\alpha$ . Adalimumab is a human-derived recombinant IgG1 monoclonal antibody that binds to TNF- $\alpha$  and blocks the interaction between soluble TNF- $\alpha$  and cell-surface TNF receptors<sup>[18]</sup>. The limited data that are currently available are not sufficient for the assessment of the potential specific differences between the drugs regarding the effect on viral replication.

## CLINICAL EVIDENCE OF THE SAFETY OF TNF- $\alpha$ INHIBITORS IN PATIENTS WITH HCV

We performed a comprehensive review of reports pub-



**Table 1** Safety of tumor necrosis factor- $\alpha$  inhibitors in patients with hepatitis C virus

Drug	Patients with HCV infection ( <i>n</i> )	Mean follow-up (yr)	Patients/yr exposure	Elevation in AST/ALT serum level > 3 ULN	Elevation in HCV-RNA (> 1 log above baseline)	Drug withdrawal due to liver toxicity
Etanercept	153	1.14	174.49	3 <sup>1</sup>	5	2
Infliximab	40	1.59	63.64	2	4	1
Adalimumab	23	0.97	22.43	0	0	0

<sup>1</sup>Elevation of transaminases without concomitant increase of HCV viremia in two cases. HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ULN: Upper limit of normal.

lished in English between January 2000 and August 2013; patients were evaluated for the following variables: disease, comorbidities, TNF- $\alpha$  inhibitors, previous HCV treatment, concomitant immunosuppressive drugs, liver function tests, HCV-ribonucleic acid (HCV-RNA), histopathological liver findings (when available), complications and outcomes. Patients with HCV are excluded from participation in controlled clinical trials with TNF- $\alpha$  inhibitors. Next, available data regarding the safety of TNF- $\alpha$  inhibitors in patients with hepatitis C, as derived from several case reports and small retrospective cohort studies in the field of rheumatology, dermatology and gastroenterology, in addition to the already mentioned trial of Zein *et al*<sup>[17]</sup>, were evaluated. These findings come from various clinical contexts, in terms of differing uses of concomitant immunosuppressive drugs (in most cases dermatologists tend to employ TNF- $\alpha$  inhibitors in monotherapy, while gastroenterologists and rheumatologists prescribe them in combination with other immunosuppressants), pre-treatment selection of HCV patients, monitoring protocols and differences in the threshold used for discontinuing treatment with TNF- $\alpha$  inhibitors. Furthermore, most of the evidence concerns the measurement of transaminases and viral load, with few reports including a histological evaluation before and after treatment.

Total of 216 patients with hepatitis C were treated with one or more TNF- $\alpha$  inhibitors, with a median observation time of 1.2 years and 260 cumulative patient-years of treatment, a measure of exposure that includes all patients treated and normalizes the different durations of treatment to one year (Table 1)<sup>[19-58]</sup>. The majority of the available safety data concern etanercept. Clinical evidence suggests that the role of TNF- $\alpha$  in the control of HCV replication is modest. Currently, only three cases of drug withdrawal due to clinical suspicion of a worsening of HCV liver disease have been reported. The viral load in most cases remains stable or decreases, and it is difficult to confidently attribute the few cases of serum HCV-RNA increase > 1 log above the baseline value to treatment with TNF- $\alpha$  inhibitors, considering the well-known virological profile of HCV, which shows spontaneous fluctuations > 1 log of HCV-RNA level in 5%-10% of patients<sup>[59]</sup>. Overall, TNF- $\alpha$  inhibitors do not increase transaminase levels or viral load in the short term in patients with hepatitis C. Furthermore, the administration of these drugs has allowed the concomitant use of IFN in some patients with hepatitis C in whom IFN had been previously discontinued due of a worsening of concur-

rent immunomediated diseases such as psoriasis, rheumatoid arthritis or other arthritis. In regard to the long-term safety of TNF- $\alpha$  inhibitors and their impact on the progression of liver fibrosis, the limited available data do not allow for the assessment of this issue. Another area of uncertainty is related to their use in cirrhotic patients; only two cases of patients with cirrhosis who received TNF- $\alpha$  inhibitors have been reported, by Zein and Abdelmalek<sup>[17,36]</sup>, and both cases were without significant side effects.

Another potential concern is the possibility of immune-mediated liver damage induced by TNF- $\alpha$  inhibitors. Emergence of serum auto-antibodies is a common observation in patients treated with TNF- $\alpha$  inhibitors and presents an additional concern in patients with hepatitis C. In the absence of HCV infection, the auto-antibodies induced by such treatments are usually non-organ specific [anti-double-stranded-DNA (dsDNA), rheumatoid factors, anti-cardiolipin] and belong to the IgM class<sup>[60,61]</sup>. Vauloup *et al*<sup>[62]</sup> prospectively evaluated the induction of circulating auto-antibodies during therapy with TNF- $\alpha$  inhibitors in patients with HCV and observed induction of anti-nuclear and anti-dsDNA antibodies, but no induction of anti-tissue antibodies (anti-smooth muscle and anti-liver/kidney/microsome type 1), even in patients with actively replicating chronic hepatitis C. Induction of cryoglobulinemia was also a possibility, and HCV-related mixed cryoglobulinemia usually includes an IgM component. Auto-antibodies emerging during treatment with TNF- $\alpha$  inhibitors are usually clinically silent, possibly due to the low avidity of antibodies to their antigen. Seventeen cases of TNF- $\alpha$ -induced hepatitis without known past history of liver disease have been reported in the literature<sup>[63-78]</sup>. The majority of these cases are secondary to infliximab and resemble autoimmune hepatitis type 1 due to an increased prevalence among females, the more common elevation of autoantibodies related to autoimmune hepatitis type 1 (anti-nuclear, anti-smooth-muscle or anti-dsDNA), the presence of interface hepatitis at liver biopsy, and the strong response to steroid therapy. Some of these patients were subsequently able to tolerate etanercept, suggesting a different potential of the two drugs in inducing immune-mediated liver damage. Indeed, among patients with HCV infection, only one case of granulomatous hepatitis not associated to a rise of serum HCV-RNA, diagnosed after 7 mo of therapy with etanercept, has been reported<sup>[79]</sup>. A TNF- $\alpha$  blockade induces a cytokine imbalance that is rarely responsible

for inducing pulmonary, cutaneous, eye and even hepatic sarcoidosis. Overall, the incidence of autoimmune hepatitis induced by TNF- $\alpha$  inhibitors appears to be low and does not represent a contraindication in the treatment of patients with chronic hepatitis C.

Although observations of transaminase elevation have been documented in the package inserts of TNF- $\alpha$  inhibitors, no direct link between these drugs and liver toxicity has been established to date, with the exception of one single case of acute hepatitis during infliximab treatment, in which the liver biopsy showed signs of toxic damage (intralobular necrosis, ceroid-containing Kupffer cells)<sup>[80]</sup>. For this reason, TNF- $\alpha$  inhibitors present an attractive alternative therapy in some patients with autoimmune diseases, such as psoriasis or rheumatoid arthritis, which are routinely treated with other drugs with well established, likely more severe liver toxicity profiles (cyclosporine, acitretin, methotrexate, leflunomide).

## CLINICAL MANAGEMENT OF TNF- $\alpha$ INHIBITORS IN PATIENTS WITH HCV

Many guidelines recommend screening by means of serum anti-HCV antibodies in all patients undergoing therapy with TNF- $\alpha$  inhibitors, emphasizing that a definitive decision on the safety of TNF- $\alpha$  inhibitors in cases of chronic HCV infection has not been made<sup>[81-83]</sup>. A study conducted in Ireland, a country with a low prevalence of HCV (< 1%), including 215 patients with psoriasis treated with TNF- $\alpha$  inhibitors documented a single case of positivity for antibodies to HCV with undetectable serum HCV-RNA. The authors concluded that, in areas with low prevalence of HCV infection, universal screening should be replaced by targeted screening based on the individual risk factors of each patient<sup>[84]</sup>. Other guidelines state that universal screening should not be definitively recommended, as the risk of HCV reactivation under immunosuppressive drugs appears to be very low<sup>[85,86]</sup>. Before beginning treatment with TNF- $\alpha$  inhibitors, assays for serum alanine aminotransferase (ALT), gamma-glutamyl-transferase and total bilirubin are recommended, bearing in mind that approximately 30% of patients with chronic HCV infection show persistently normal ALT levels<sup>[87]</sup>. In cases of anti-HCV positivity, assessment of HCV-RNA, HCV genotype, cryoglobulins, complete blood count, total protein, albumin, total cholesterol, prothrombin time, creatinine, and urine exam, as well as a liver ultrasound, are also recommended.

TNF- $\alpha$  inhibitors in patients with HCV are not contraindicated, provided that monitoring of liver function tests is performed every three months during treatment. Currently, there is uncertainty in the standards for viral load monitoring (quarterly or only in case of serum transaminase increase). Due to the absence of data regarding cirrhotic patients, TNF- $\alpha$  inhibitors should be used with caution in compensated patients, while they are contraindicated in patients with decompensated liver disease, considering the extremely high risk of potentially fatal severe

infections. In cases of reactivation of hepatitis, patients should be referred to a hepatologist for a differential diagnosis and to consider the potential for TNF- $\alpha$  inhibitor treatment withdrawal.

## CONCLUSION

TNF- $\alpha$  inhibitor treatment in patients with HCV appears to be safe in the short term, but there are insufficient data to assess their long-term safety. A potential concern related to the administration of these drugs is the induction of immune-mediated reactions that potentially involve the liver (cryoglobulinemic syndrome or autoimmune hepatitis), but the incidence of such reactions appears to be low. Universal screening for HCV before beginning treatment with TNF- $\alpha$  inhibitors is currently controversial. The presence of HCV is not a contraindication to therapy with TNF- $\alpha$  inhibitors, except in cirrhotic patients, in whom the benefit/risk ratio should be evaluated at the individual level before treatment is initiated. Before administration of TNF- $\alpha$  inhibitors, patients with HCV should be referred to a hepatologist for the evaluation of the liver disease stage through liver biopsy or non-invasive methods and the potential for antiviral therapy. Liver function tests are advised for patients with HCV at a frequency of every three months during treatment with TNF- $\alpha$  inhibitors.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Relationships between lymphomas linked to hepatitis C virus infection and their microenvironment

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## Abstract

The relationships between lymphomas and their microenvironment appear to follow 3 major patterns: (1) an independent pattern; (2) a dependent pattern on deregulated interactions; and (3) a dependent pattern on regulated coexistence. Typical examples of the third pattern are hepatitis C virus (HCV)-associated marginal zone lymphomas (MZLs) and mucosa-associated lymphoid tissue lymphomas. In these lymphomas, a regulated coexistence of the malignant cells and the microenvironmental factors usually occurs. At least initially, however, tumor development and cell growth largely depend on external signals from the microenvironment, such as viral antigens, cytokines, and cell-cell interactions.

The association between HCV infection and B-cell lymphomas is not completely defined, although this association has been demonstrated by epidemiological studies. MZL and diffuse large B-cell lymphoma are the histotypes most frequently associated with HCV infection. Many mechanisms have been proposed for explaining HCV-induced lymphomagenesis; antigenic stimulation by HCV seems to be fundamental in establishing B-cell expansion as observed in mixed cryoglobulinemia and in B-cell lymphomas. Recently, antiviral treatment has been proved to be effective in the treatment of HCV-associated indolent lymphomas. Importantly, clinically responses were linked to the eradication of the HCV-RNA, providing a strong argument in favor of a causative link between HCV and lymphoproliferation.

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**Key words:** Hepatitis C virus-infection; B-cell lymphomas; Marginal zone lymphoma; Mucosa-associated lymphoid tissue lymphomas; Diffuse large B-cell lymphomas; Microenvironment

**Core tip:** The relationships between lymphomas and their microenvironment appear to follow 3 major patterns: (1) an independent pattern; (2) a dependent pattern on deregulated interactions; and (3) a dependent pattern on regulated coexistence. The association between hepatitis C virus infection and B-cell lymphomas is not completely defined, although this association has been demonstrated by epidemiological studies.

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## INTRODUCTION

Genetic alterations and abnormal microenvironmental factors are involved in tumor development, cell growth and disease progression. Inflammatory cells and soluble mediators, *i.e.*, cytokines and chemokines, are essential microenvironmental factors that sustain cell growth and invasion, induce angiogenesis and suppress anti-tumor immune functions<sup>[1]</sup>.

In multidimensional studies on hematolymphoid malignancies, a relevant clinical role of the tumor microenvironment has recently emerged, bringing new knowledge and suggesting new ideas and targets for treatment<sup>[2-5]</sup>.

The relationships between lymphomas and their microenvironment appear to follow 3 major patterns: (1) an independent, largely autonomous pattern; (2) a dependent on deregulated interactions pattern; and (3) a dependent on regulated coexistence pattern<sup>[2]</sup>. A typical example of the first pattern is Burkitt lymphoma where all tumor cells proliferate because of permanent *MYC* gene activation. A typical example of the second pattern is classic Hodgkin lymphoma, where Reed-Sternberg cells escape the regulated cell growth- and proliferation-control. Typical examples of the third pattern are Hepatitis C virus (HCV)-associated marginal zone lymphomas (MZLs) and mucosa-associated lymphoid tissue (MALT) lymphomas. In this pattern, a regulated coexistence of the malignant cells and the microenvironment is reminiscent of the pattern that the normal counterpart B cells engage in with their respective microenvironment. At least initially, tumor development and cell growth largely depend on external signals from the microenvironment, such as viral antigens, cytokines, and cell-cell interactions<sup>[6]</sup>.

HCV infection is a worldwide problem. There are important regional differences in the prevalence of HCV infection: the lowest rates are reported in Northern Europe while prevalence estimates exceed 2% in Italy, Japan, Egypt and southern parts of United States<sup>[7]</sup>. Among the carcinogenic viruses recognized by the recent International Agency for Research on Cancer (IARC) monograph Epstein-Barr virus (EBV), human papilloma virus (HPV), human T-lymphotropic virus type I (HTLV-1), and Kaposi sarcoma-associated herpesvirus (KSHV) play a direct role in carcinogenesis encoding oncoproteins which are able to promote cellular transformation<sup>[8,9]</sup>. Conversely, HCV and *Helicobacter pylori* appear to have an indirect role, by inducing a chronic inflammation<sup>[10]</sup>. Hepatitis B virus (HBV) has both a direct and indirect role in promoting hepatocellular carcinoma (HCC); as a matter of fact, chronic HBV carriers can develop HCC without developing cirrhosis.

## PATHOGENETIC ASPECTS

HCV infection is the cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) (Table 1). HCV infection has been also associated to a spectrum of extra-hepatic lymphoproliferative disorders including

mixed cryoglobulinemia (MC)<sup>[11]</sup>, the most well defined disorder associated with HCV infection, monoclonal gammopathies<sup>[12]</sup> and B-cell lymphomas<sup>[13]</sup>. HCV infection has been associated with B-cell low grade indolent lymphoma, especially of marginal zone origin, as well as with aggressive lymphomas, mainly diffuse large B-cell lymphomas (DLBCL) (Table 2). Authoritative studies have demonstrated that in HCV-infected patients with indolent lymphomas, eradication of HCV with antiviral treatment (AT) could directly induce lymphoma regression, providing a strong argument in favor of a causative link between HCV and lymphoproliferation<sup>[14]</sup>.

## INFLAMMATORY MICROENVIRONMENT

The liver is the main target of HCV infection and the major site of inflammatory events, including recruitment of inflammatory cells.

Occurrence of HCV enrichment in intrahepatic inflammatory infiltrates supports the notion that HCV is directly involved in the emergence and maintenance of these B-cell expansions<sup>[15]</sup>. Intrahepatic B-cell clonalities are invariably associated with extrahepatic manifestations of HCV infection associated B-cell lymphomas.

## HCV AND LYMPHOMAGENESIS

According to the recent IARC monograph on biological agents and carcinogenesis, the association between HCV infection and B-cell lymphomas is not completely defined<sup>[9]</sup>. This association has been demonstrated by epidemiological studies in highly endemic geographical areas<sup>[16]</sup>. The role of HCV infection in lymphomagenesis may be related to the chronic antigenic stimulation of B-cell response, similar to the well characterized induction of gastric MALT lymphoma development by *Helicobacter pylori* chronic infection<sup>[17]</sup>. In fact, chronic HCV infection may sustain a multi-step evolution from MC to overt low grade lymphoma and eventually to high-grade lymphoma<sup>[17]</sup>. During this process, additional genetic aberrations may induce independence from antigenic stimulation. The clonal component of MC is often an IgM with a rheumatoid factor activity that mirrors the expansion of a B-cell monoclonal population not only in bone marrow but also in liver. It has also been suggested that HCV antigens (NS3 and E7 or E2) can play a role in lymphomagenesis<sup>[18,19]</sup>. Recently, it has been published that HCV-related cryoglobulins (either IgM and IgG) are mainly directed against core and NS3 proteins<sup>[20]</sup>. Chromosomal alterations could also play a role in development of HCV-related lymphoproliferative disorders: for instance, MC with or without lymphoma is characterized by translocation t(14; 18) with the overexpression of the antiapoptotic *bcl-2* gene leading to prolonged B-cell survival<sup>[21]</sup>.

Importantly, cytokines and chemokines (IFN $\gamma$ , TNF $\alpha$ , CXCL13 and BAFF in MC<sup>[16]</sup>, as well as osteopontin<sup>[22]</sup>) are involved in the mechanisms of HCV-

**Table 1 Biological agents assessed by the International Agency for Research on Cancer Monographs Working Group<sup>[9]</sup>**

Group-1 agent	Cancers on which sufficient evidence in humans is based	Other sites with limited evidence in humans	Established mechanistic events
Epstein-Barr virus	Nasopharyngeal carcinoma, Burkitt lymphoma, Immune-suppression-related non-Hodgkin lymphoma, Extranodal NK/T-cell lymphoma (nasal type), Hodgkin lymphoma	Gastric carcinoma <sup>1</sup> Lympho-epithelioma-like carcinoma <sup>1</sup>	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
Hepatitis B virus	Hepatocellular carcinoma	Cholangiocarcinoma <sup>1</sup> , Non-Hodgkin lymphoma <sup>1</sup>	Inflammation, liver cirrhosis, chronic hepatitis <sup>2</sup>
Hepatitis C virus	Hepatocellular carcinoma, Non-Hodgkin lymphoma <sup>1</sup>	Cholangiocarcinoma <sup>1</sup>	Inflammation, liver cirrhosis, liver fibrosis
Kaposi sarcoma herpes virus	Kaposi sarcoma <sup>1</sup> , Primary effusion lymphoma <sup>1</sup>	Multicentric Castleman's disease <sup>1</sup>	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
Human immunodeficiency virus, type 1	Kaposi sarcoma, Non-Hodgkin lymphoma, Hodgkin lymphoma <sup>1</sup> , Cancer of the cervix <sup>1</sup> , anus <sup>1</sup> , conjunctiva <sup>1</sup>	Cancer of the vulva <sup>1</sup> , vagina <sup>1</sup> , penis <sup>1</sup> , Non-melanoma skin cancer <sup>1</sup> , Hepatocellular carcinoma <sup>1</sup>	Immunosuppression (indirect action)
Human papillomavirus type 16 (For the other types see Table 2)	Carcinoma of the cervix, vulva, vagina, penis, anus, oral cavity, oropharynx and tonsil	Cancer of the larynx	Immortalization, genomic instability, inhibition of DNA damage response, anti-apoptotic activity
<i>Helicobacter pylori</i>	Non-cardia gastric carcinoma, Low-grade B-cell mucosa-associated lymphoid tissue gastric lymphoma <sup>1</sup>		Inflammation, oxidative stress, altered cellular turnover, changes in gene expression, methylation, mutation

<sup>1</sup>Newly identified link between virus and cancer. In red are highlighted the lymphoid proliferations; <sup>2</sup>HBV has both a direct and indirect role in promoting hepatocellular carcinoma. Modified and adapted from Bouvard *et al*<sup>[8]</sup>.

**Table 2 Hepatitis C virus-associated indolent and aggressive lymphoid proliferations**

Subtype	Variant	Specific lymphoma sites
Monoclonal B-cell lymphocytosis		
Tissue based monoclonal B cell and plasma cell proliferations of uncertain type		
Lymphoplasmocytic lymphoma/WM		
Chronic lymphocytic disorders (non CLL)		
MZL	Splenic MZL	
	Nodal MZL	
	MALT	Gastric
		Extranodal non gastric
		Salivary gland
		Skin
		Orbit
		Liver
Diffuse large B-cell lymphoma		

WM: Waldenström's macroglobulinemia; CLL: Chronic lymphocytic leukemia; MZL: Marginal zone lymphoma; MALT: Mucosa-associated lymphoid tissue.

induced lymphoproliferation.

## HCV INFECTION AND SPECIFIC LYMPHOMAS

Within indolent lymphoma subtypes, the association with HCV infection has been best characterized in MZLs. Other infectious agents have been involved in the pathogenesis of specific types of MZLs. For examples *Helicobacter pylori*, *Borrelia burgdorferi*, and *Chlamydia psittaci* have been involved in MALT lymphomas arising

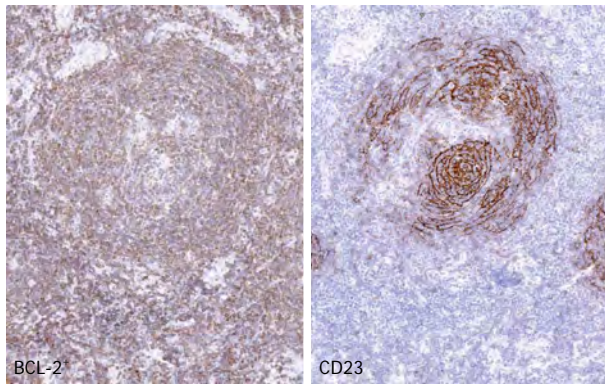
in stomach, skin and orbit, respectively<sup>[9]</sup>. Conversely, chronic stimulation by HCV plays a role in development of splenic marginal zone lymphoma (SMZL) and primary nodal marginal zone lymphoma. Primary nodal marginal zone lymphoma is a distinct clinical-pathological subtype characterized by exclusive primary lymph node localization in the absence of extranodal site of involvement (Figure 1).

Splenic and nodal MZL are indolent B-cell lymphomas corresponding to post-germinal center memory B cells that are supposed to derive from marginal zone<sup>[23,24]</sup>. These entities share some morphologic and pathogenic features, but have distinctive clinical presentation, immunophenotype and molecular abnormalities. Histologically, when the marginal zone B cells surround normal follicles with benign mantle zones as a third outer layer, they produce a marginal zone pattern<sup>[25]</sup>. As the marginal zone cells extend outwards into the interfollicular areas, they form confluent clusters resulting in an interfollicular pattern or a diffuse pattern in the absence of any follicles at later stages of the disease<sup>[25]</sup>. The marginal zone cells may also grow inwards into the follicles and produce either partial or complete follicular colonization<sup>[25]</sup> (Figure 1).

Gastric and non-gastric extranodal MZL are typically indolent diseases of middle and advanced age; disseminated disease is present in nearly one-third of cases. Interestingly, three specific MALT lymphoma sites showed an elevated prevalence of HCV infection: salivary glands, skin and orbit<sup>[26]</sup>. The association of HCV infection and salivary glands lymphoma has been clearly demonstrated<sup>[27]</sup>.

Moreover, a study on B-cell lymphoma in patients with Sjögren's syndrome and HCV infection reported an





**Figure 1 Nodal marginal zone lymphoma.** The panel shows an example of nodal marginal zone lymphoma (MZL) with follicular colonization. BCL-2<sup>+</sup> neoplastic cells surround and colonize the germinal center, whereas CD23 highlights the disrupted follicular dendritic cell meshwork. Images were acquired with the Olympus Dot. Slide Virtual microscopy system using an Olympus BX51 microscopy equipped with PLAN APO 2x/0.08 and UPLAN SApo 40x/0.95 objectives.

elevated occurrence of parotid involvement and a high proportion of MALT lymphomas with primary extra-nodal involvement (exocrine glands, liver, and stomach) (Table 2)<sup>[28]</sup>.

Beside MZLs, also LPL/Waldenström's macroglobulinemia (WM) has been associated to HCV infection. However, this association is not completely defined. B-cell chronic lymphoproliferative disorders are defined as the miscellaneous category of lymphoproliferative disorders distinct from chronic lymphocytic. Association of these entities with HCV is not clear. Interestingly, monoclonal B-cell lymphocytosis (MBL), a pre-clinical condition characterized by an expansion of clonal B cells in the absence of frank lymphocytosis, was identified in nearly 30% of HCV-positive subjects with a significantly higher frequency than in the general population.

Recently, a retrospective study of B-cell lymphoproliferative disorders associated with HCV infection found two poorly described groups of cases. The first featured disseminated MZL without splenic MZL features, defying the current MZL classification; the other consisted of monoclonal B lymphocytes in the peripheral blood, bone marrow or other tissues, with no clinical or histological evidence of lymphoma. This pattern requires proper identification in order to avoid the misdiagnosis of the lymphoma<sup>[29]</sup>.

Despite the classical association of HCV with indolent lymphoma, aggressive lymphoma, in particular DLBCL, are emerging as diseases linked to HCV infection. Patients affected by HCV-associated DLBCL display specific presentation with respect to HCV-negative DLBCL. In particular, residual signs of low-grade lymphoma and extranodal disease such as spleen are more frequently detected in HCV-associated DLBCL cases in comparison with HCV-negative DLBCL<sup>[9]</sup>. Unlike indolent B-cell lymphoma, AT seems not to play a central role in the first-line approach for HCV-associated DLBCL, because lymphoma cells are most likely to be independent from

chronic antigenic stimulation due to the acquisition of additional oncogenic lesions. For this reason, HCV-associated DLBCL patients have to be treated with anthracycline-based chemotherapy coupled with rituximab.

## CONCLUSION

Many epidemiological studies have provided evidence that HCV infection is associated with development of indolent and aggressive B-cell lymphoma<sup>[30,31]</sup>. However, the causal association between HCV infection and B-cell lymphomas is not completely defined. The similarities shared by rearranged Ig genes present in B cells from patients with type II MC and malignant B-cells from HCV-positive patients affected by B-cell lymphoma support the possibility that the antigens that promote type II MC and B-cell lymphoma in HCV-positive patients are the same<sup>[32,33]</sup>. These similarities also suggest that type II MC may be a precursor of B-cell lymphoma<sup>[34]</sup>. Type II MC probably plays a central role in the development of B-cell lymphoma in HCV-positive patients with Sjögren's syndrome<sup>[35]</sup>.

Three hypothetical models have emerged to understand the molecular mechanisms of HCV-associated lymphoma development: (1) continuous external stimulation of lymphocyte receptors by viral antigens and consecutive proliferation; (2) direct role of HCV replication and expression in infected B-cells; and (3) permanent B-cell damage, *e.g.*, mutation of tumor suppressor genes, caused by a transiently intracellular virus ("hit and run" theory)<sup>[9,36]</sup>. Other non exclusive hypotheses have been proposed over the past two decades. These hypotheses have variously emphasized the important role played by chromosomal aberrations, cytokines, or microRNA molecules<sup>[37]</sup>. However, the mechanisms by which B-cell lymphomas are induced by HCV remain the subject of debate.

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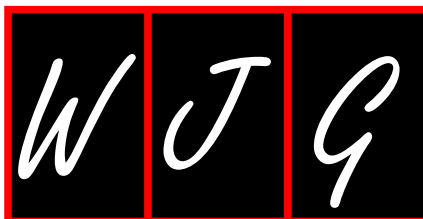
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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Burden of pediatric hepatitis C

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## Abstract

Hepatitis C virus (HCV) is a major health burden infecting 170-210 million people worldwide. Additional 3-4 millions are newly-infected annually. Prevalence of pediatric infection varies from 0.05%-0.36% in the United States and Europe; up to 1.8%-5.8% in some developing countries. The highest prevalence occurs in Egypt, sub-Saharan Africa, Amazon basin and Mongolia. HCV has been present in some populations for several centuries, notably genotypes 1 and 2 in West Africa. Parenteral anti-schistosomal therapy practiced in the 1960s until the early 1980s had spread HCV infection throughout Egypt. Parenteral acquisition of HCV remains a major route for infection among Egyptian children. Insufficient screening of transfusions, unsterilized injection equipment and re-used needles and syringes continue to be major routes of HCV transmission in developing countries, whereas vertical transmission and adolescent high-risk behaviors (*e.g.*, injection drug abuse) are the major routes in developed countries. The risk of vertical transmission from an infected mother to her unborn/newborn infant is approximately 5%. Early stages of

HCV infection in children do not lead to marked impairment in the quality of life nor to cognitive, behavioral or emotional dysfunction; however, caregiver stress and family system strain may occur. HCV slowly progresses to serious complications as cirrhosis (1%-2%) and hepatocellular carcinoma (HCC) especially in the presence of risk factors as hemolytic anemias, obesity, treated malignancy, and concomitant human immune deficiency and/or hepatitis B virus co-infection. HCV vaccine remains elusive to date. Understanding the immune mechanisms in patients who successfully cleared the infection is essential for vaccine development. The pediatric standard of care treatment consists of pegylated interferon- $\alpha$  2a or b plus ribavirin for 24-48 wk. The new oral direct acting antivirals, approved for adults, need further evaluation in children. Sustained virologic response varies depending on the viral load, genotype, duration of infection, degree of aminotransferase elevation, adiposity and single nucleotide polymorphisms of interleukin (IL)-28B locus. The goals of treatment in individual patients are virus eradication, prevention of cirrhosis and HCC, and removing stigmatization; meanwhile the overall goal is decreasing the global burden of HCV. *IL-28B* polymorphisms have been also associated with spontaneous clearance of vertically acquired HCV infection. The worldwide economic burden of HCV for children, families and countries is estimated to be hundreds of millions of US dollars per year. The United States, alone, is estimated to spend 199-336 million dollars in screening, monitoring and treatment during one decade. The emotional burden of having an HCV infected child in a family is more difficult to estimate.

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**Key words:** Hepatitis C virus; Burden; Genotypes; Cost; Pediatrics

**Core tip:** Hepatitis C virus (HCV) is a worldwide health burden infecting up to 5.8% of children in some developing countries with thousands of annual new



infections. HCV vaccine is illusive, but understanding immune mechanisms in patients who cleared infection may be crucial. The pediatric standard of care treatment is pegylated interferon- $\alpha$ 2 plus ribavirin for 24-48 wk. The new oral direct acting antivirals need further evaluation in children. Interleukin-28B polymorphisms have been associated with treatment response and spontaneous clearance of vertical HCV infection. The worldwide economic burden of HCV is estimated to be hundreds of millions United States dollars/year. The emotional burden is difficult to estimate.

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## INTRODUCTION AND EPIDEMIOLOGY

Hepatitis C virus (HCV) is a small, enveloped, positive-sense, single-stranded RNA virus of the *Flaviviridae* family<sup>[1]</sup>. HCV was first cloned in 1989 after more than 6 years of work to extract the virus from infected patients by a group of scientists from California in the United States<sup>[2]</sup>. HCV infection is recognized nowadays as a disease of global importance<sup>[3]</sup>. It is considered a major health and economic burden in adults as well as children in both developing and developed countries<sup>[3,4]</sup>.

Viral hepatitis is the most common cause of liver disease in the world. Acute infections with their sequelae are responsible for 1-2 million deaths/year. Of them 54000 deaths are due to acute HCV infection<sup>[4]</sup>. After acute infection with HCV, as many as 50%-85% of patients fail to clear the virus resulting in chronic infection with 350000 deaths/year and 955000 disability due to related complications such as cirrhosis and liver cancer<sup>[5]</sup>.

A recent systematic review found that globally between 1990 and 2005, the prevalence of people with anti-HCV has increased from 2.3% to 2.8%<sup>[4]</sup>. It is estimated that approximately 210 million individuals, *i.e.*, approximately 3% of the world population, are chronically infected with HCV<sup>[3,6]</sup> and 3-4 millions are newly infected each year<sup>[3]</sup>. Available data indicate that infection with HCV varies considerably by country and region, and the true burden of disease is not well known in many countries, because the capacity is often limited for collecting epidemiologic data<sup>[7]</sup>. The prevalence may vary markedly from one geographic area to another and even within the population assessed<sup>[8]</sup>. The highest prevalence of HCV is in Sub-Saharan Africa (5.3%), followed by the Eastern Mediterranean (4.6%), Western Pacific (3.9%) and South-Eastern Asia (2.15%) regions. Europe is thought to have the lowest prevalence of HCV (1.03%). In North America, prevalence is also low and estimated at 1.6% in the United States and 0.8% in Canada<sup>[9]</sup>.

The study carried out by Uhanova *et al*<sup>[9]</sup> on the epidemiology of HCV in a North American population from

the Canadian province of Manitoba, revealed several important findings: First, the diagnosis of HCV appears to have peaked in 1998 and has been relatively stable thereafter; second, the prevalence of HCV continued to increase amongst both men and women (4.6-fold during the 12-year period of the study). Overall, 84% of all subjects diagnosed since 1991 were alive in 2002, supporting the evidence of the growing burden of HCV; third, with the exception of young adults, males were 1.7 times more often infected than females; fourth, HCV infections were more common in urban centers.

Egypt has the highest worldwide prevalence with 9% countrywide rate; and up to 50% rates in certain rural areas due to specific modes of infection<sup>[10]</sup>. Prevalence in healthy Egyptian children is reported, by our group and others, to range from 1.4% to 5.8%<sup>[11,12]</sup>. Parenteral anti-schistosomal therapy, practiced in the 1960s until the early 1980s, had had a major role in the spread of HCV throughout Egypt<sup>[13]</sup>.

## GLOBAL HCV GENOTYPE DISTRIBUTION

By phylogenetic analysis, 6 distinct genotypes of HCV (denoted 1 to 6) and more than 100 subtypes (denoted 1a, 2c, 3d, 6f, *etc.*) have been described. Each genotype differs in its amino acid sequence by 31%-34%<sup>[14]</sup>. Genotypes 1-3 have a worldwide distribution, whereas 4 is found principally in Egypt, the Middle East and black Africa, 5 in South Africa, and 6 in Asia<sup>[15]</sup>.

Genotype 1 (subtypes 1a and 1b) is by far the most prevalent genotype worldwide, with a higher prevalence of 1b in Europe and 1a in the United States. Genotype 3a is highly prevalent in European intravenous drug abusers<sup>[16]</sup>. This group is currently experiencing an increasing incidence and prevalence of infections related to HCV genotype 4 as well. Genotype 2 is found in clusters in the Mediterranean region<sup>[17]</sup>. Molecular clock analyses suggest that HCV strains have been present in some populations in their respective geographical regions for at least several centuries, notably genotypes 1 and 2 in West Africa and genotype 6 in Southeast Asia<sup>[18]</sup>.

## METHODS OF TRANSMISSION

Prior to the 1990s, the principal routes of HCV infection were via blood transfusion, unsafe injection procedures, and intravenous drug abuse. These modes of acquisition are estimated to account for approximately 70% of cases in industrialized countries. Epidemiological evidence shows that a wave of HCV infection occurred in the 1945-1965 period (baby boomers) in Western countries, as there was an increase in the use of injections, blood products and illicit drugs following World War II<sup>[1]</sup>. Screening of blood products for HCV by means of enzyme immunoassays and now, in an increasing number of countries, by nucleic acid testing (NAT) has virtually eradicated transfusion-transmitted HCV. Currently, new HCV infections are primarily due to intravenous or nasal drug abuse, and to a lesser degree to unsafe medical or

surgical procedures. Parenteral transmission via tattooing or acupuncture with unsafe materials is also implicated in occasional transmissions<sup>[8]</sup>. The risk of heterosexual transmission is low, while recent data indicate that promiscuous male homosexual activity is related to HCV infection<sup>[19]</sup>.

In developing countries, insufficient screening of blood, blood products and parenteral exposure, continue to be the major causes of HCV transmission and are still reported among Egyptian children<sup>[20]</sup>. Unsafe use and re-use of injection equipment in hospitals is still a threat in many parts of Africa<sup>[21]</sup>. Intra-familial transmission may occur, but specific immune responses may be protective against house-hold infection in some children<sup>[22]</sup>.

At present the vertical, maternal-neonatal or perinatal transmission is the most common route of pediatric HCV infection<sup>[23]</sup>. Worldwide, it has been estimated that 60000 HCV-infected infants are born yearly<sup>[24]</sup>. Mother-to-infant vertical transmission of HCV is reported to occur in approximately 5% of cases (with a range of 3%-10%), mostly in the late intrauterine period, at delivery or in the peri-natal period<sup>[1,24]</sup>. Many factors have been reported to influence the transmission rate<sup>[25]</sup>, including maternal high viral load<sup>[26-28]</sup>, labor duration, newborn gender, HCV genotype<sup>[29]</sup>, human immuno-deficiency virus (HIV) co-infection<sup>[24]</sup>, amniocentesis<sup>[30,31]</sup>, fetal scalp monitoring<sup>[32]</sup>, prolonged rupture of membranes<sup>[32,33]</sup> and fetal anoxia around the time of delivery<sup>[28]</sup>.

The role of elective cesarean section to reduce mother-to-infant transmission rates is debated and controversial<sup>[33]</sup> and the guidelines of the European Association for the Study of the Liver (EASL) do not recommend cesarean section to prevent HCV vertical transmission<sup>[8]</sup>. Breast feeding is not considered to be contraindicated in women who are infected with HCV<sup>[34,35]</sup>. In spite that the majority of HCV-infected women do not transmit the virus to their offsprings, maternal uncertainty and guilt always surround possible transmission. Cost-effectiveness analysis based on available epidemiologic data indicates that screening of all pregnant mothers for HCV infection is not cost-effective<sup>[36]</sup>, however high risk mothers should be screened<sup>[25]</sup>.

## NATURAL HISTORY OF INFECTION

In some patients, HCV infection is a self-limited disease and HCV RNA becomes undetectable in most of these cases within 3 to 4 mo after the onset of acute infection<sup>[37]</sup>. Symptoms and signs following acute HCV infection are mild and usually non-specific; and fulminant HCV has not been reported in childhood<sup>[38]</sup>.

Unfortunately spontaneous clearance of HCV occurs only in a minority of cases as 54%-86% of adult patients establish a chronic infection<sup>[39]</sup>. Many chronically infected patients do not know that they have been infected with HCV because infection is largely asymptomatic<sup>[40]</sup>. In the approximately 86% of infected patients who develop a chronic infection, HCV progresses insidiously with 10%-20% progressing to cirrhosis and approximately 7%

of cirrhotic patients developing HCC<sup>[41]</sup>.

Little is known about the characteristics of chronic HCV infection in children. Children rarely require liver transplantation for HCV infection. In the United States, only 133 children were transplanted for chronic HCV infection between 1988 and 2009<sup>[25]</sup>. To date, HCC is extremely uncommon in children with HCV infection<sup>[25]</sup>. Only 2 cases have been reported in children<sup>[42]</sup> and two further cases who had acquired chronic infection in childhood presented as young adults<sup>[43]</sup>, however other unreported cases may exist. HCC complicating HCV infection may develop in the absence of cirrhosis<sup>[44,45]</sup>, a finding of potential importance to pediatric patients<sup>[25]</sup>. Progression of liver affection depends on the viral load, serum aminotransferase levels, gender, ethnicity, obesity, toxins, environmental factors and co-morbid risk factors such as hemolytic anemias, treated malignancy, immunosuppression, and concomitant HIV or hepatitis B virus infection, or genetic factors *e.g.*, single-nucleotide polymorphisms (SNPs) of interleukin (IL)-28B gene locus<sup>[46]</sup>.

Chronic HCV infection in children is associated with a variety of histological patterns of liver disease, generally not as severe as in adults. Indeed in many children, liver biopsy may disclose no obvious histological changes or only mild inflammation and fibrosis. Nevertheless, significant fibrosis or cirrhosis may occur<sup>[47]</sup>. Reports on the histological features and progression of hepatitis C in children are scarce but are generally milder than in adults<sup>[48]</sup>. In 1997, Kage *et al.*<sup>[49]</sup> reported, in a cohort of Japanese children with chronic HCV infection, that liver histopathology presents the same lesions as in adults such as lymphoid aggregates, sinusoidal lymphocytosis and steatosis<sup>[48]</sup>. The stage of fibrosis in this cohort was mild, with only a 3.6% prevalence of bridging fibrosis with architectural distortion, and no cases of cirrhosis<sup>[48]</sup>. This seemingly mild course is in contrast with the findings in some of the earlier clinical reports in which the prevalence of cirrhosis was found to be up to 14%<sup>[50-52]</sup>. In a North American study carried out by Badizadegan *et al.*<sup>[48]</sup>, the characteristic histopathological lesions occurred with approximately the same frequencies in children as have been reported in adults. Necroinflammatory activity was generally mild. Portal fibrosis was present in 78% of the specimens, including fibrous portal expansion (26%), bridging fibrosis (22%), bridging fibrosis with architectural distortion (22%), and cirrhosis (8%). Centrilobular pericellular fibrosis, which has not been previously reported in the context of chronic HCV infection in adults or children, was also a prominent feature in their series, occurring with a similar frequency as steatosis or portal lymphoid aggregates/follicles. They suggested that in spite of mild histological necroinflammatory activity in general, the stage of fibrosis in children can be severe in spite of relatively short duration of infection<sup>[48]</sup>.

## EXTRAHEPATIC MANIFESTATIONS OF HCV

Chronic HCV infection may cause numerous extrahepat-

ic manifestations. Up to 40%-74% of patients with HCV infection develop at least one extrahepatic manifestation during their life time. The disorder with strongest link to HCV infection in adults is mixed cryoglobulinemia<sup>[46]</sup>. Other common symptoms are peripheral polyneuropathy, Raynaud's syndrome and sicca-like symptoms. Seven and a half to 10% of the patients develop a B-cell lymphoma at some point<sup>[53-55]</sup>. The clinically most relevant manifestation of mixed cryoglobulinemia is membranoproliferative glomerulonephritis, which appears in 30%-36% of the cases and significantly increases mortality<sup>[54,55]</sup>. Membranoproliferative glomerulonephritis may occur in children with chronic HCV infection, but unlike in adults, neither cryoglobulinemia nor lymphoma has yet been reported in children<sup>[25]</sup>.

Another important extrahepatic manifestation of HCV infection is the involvement of the central nervous system. About 20%-80% of the patients with chronic HCV infection develop fatigue at some point independent from the severity of hepatitis. Fatigue often is the predominant complaint of the patients and might reduce the quality of life to a large extent. Patients may also develop depression or a general cognitive impairment irrespective of the stage of liver disease<sup>[56]</sup> which may be linked to HCV-induced neuro-inflammation and brain dysfunction<sup>[57]</sup>. These observations raise the issue of learning impairment in children with chronic HCV<sup>[25]</sup>.

Impaired quality of life, potentially severe enough to have a negative effect on learning, has been reported in children with chronic HCV infection including developmental delay, learning disorders, and cognitive deficits less severe than those of attention deficit hyperactivity disorder but still reflecting decreased executive function<sup>[58,59]</sup>.

## COSTS OF INFECTION

Vietri *et al.*<sup>[40]</sup> studied the burden of HCV in Europe and found that HCV patients compared to healthy controls have more impairment in work and non-work activities, and more annual physician visits per patient. Work-productivity impairment due to HCV costs over €7500 per employed patient per year<sup>[40]</sup>. Health-related quality of life was lower among HCV patients. Treatment-naïve HCV patients reported higher work impairment and more frequent physician visits. Each treatment-naïve HCV infected patient incurred €934 in direct costs. Employed treatment-naïve patients reported higher productivity loss per year<sup>[40]</sup>. In comparison, in the United States, Menzin and his group estimated that it costs \$4956/patient in the year following the diagnosis of advanced liver disease secondary to HCV which were largely driven by inpatient costs<sup>[60]</sup>.

There are no precise estimates of the true costs of HCV for a child and family. In one country like the United States, it is likely that several thousand children per year need treatment costing several thousand dollars/child; and it is estimated that in one decade, 26 million dollars will be spent in screening, 117-206 million dollars

in monitoring, 56-104 millions dollars in treatment and the total cost would be about 199-336 million. Worldwide, global costs would be millions of dollars/year<sup>[61]</sup>.

Treatment of a child which results in virus eradication is highly cost-effective because of the higher costs of the long-term consequences of untreated HCV cirrhosis and/or HCC. The small numbers of liver transplants for children with HCV performed each year cost several million dollars. The emotional costs of having an HCV infected child are more difficult to estimate for the child and family; but are real<sup>[61]</sup>.

## PREVENTION

The reduction of global morbidity and mortality related to chronic HCV infection should be a concern to public health authorities, and primary, secondary and tertiary prevention activities should be implemented and monitored in each country, with precise targets set to be reached. A working group was created to assist the World Health Organization in estimating the global burden of disease associated with HCV infection<sup>[3]</sup>. Public awareness of the transmission and prevention of HCV is crucial in decreasing the incidence and prevalence of the disease. Public and physician education in various forms is therefore extremely important. There is a need for implementing evidence-based international guidelines for preventing and managing hepatitis C in children worldwide<sup>[62]</sup>.

One of the major hurdles in the eradication/reduction of the burden of HCV is the lack of hepatitis C vaccine. An effective HCV vaccine remains elusive to date. HCV has been difficult to target with a vaccine because it has many different strains. In addition, HCV mutates rapidly and exists as a complex family of mutated viruses within each infected individual (quasispecies) allowing the infecting virus to escape control by the immune system. This makes it difficult to identify which part of the virus should be targeted for developing a vaccine<sup>[62]</sup>.

Viral and host specific factors contribute to viral evasion and present important impediments to vaccine development. Both, innate and adaptive immune responses are of major importance for the control of HCV infection. However, HCV has evolved ways of evading the host's immune response in order to establish persistent infection. For example, HCV inhibits intracellular interferon (IFN) signaling pathways, impairs the activation of dendritic cells, CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses, induces a state of T-cell exhaustion and selects escape variants with mutations CD8<sup>+</sup> T cell epitopes<sup>[63]</sup>. An effective vaccine will need to produce strong and broadly cross-reactive CD4<sup>+</sup>, CD8<sup>+</sup> T cell and neutralizing antibody (NAb) responses to be successful in preventing or clearing HCV. Vaccines in clinical trials now include recombinant proteins, synthetic peptides, virosome based vaccines, tarmogens, modified vaccinia Ankara based vaccines, and DNA based vaccines. Several pre-clinical vaccine strategies are also under development and include recombinant adenoviral vaccines, virus-like particles, and synthetic peptide



vaccines. Moreover, vaccines may also be used in the future in combination with the recent direct acting antiviral (DAA) drugs enabling IFN-free treatment regimens<sup>[63]</sup>. Indeed understanding the immune mechanisms, particularly HCV-specific cell mediated immune response, of patients who have successfully cleared the infection is essential to design and develop a vaccine<sup>[64]</sup>.

Because there is no vaccine and no post-exposure prophylaxis for HCV, the focus of primary prevention efforts should be safer blood supply in the developing world, safe injection practices in health care and other settings, and decreasing the number of people who initiate injection drug abuse<sup>[6]</sup>. People with known HCV infection should be counseled regarding ways to reduce the risk of transmitting HCV to others, and means of minimizing their risk for HCV-related complications. As part of secondary prevention efforts, HCV-infected people should be referred for medical evaluation and antiviral treatment consideration, and programs ensuring access to these services should be in place<sup>[6]</sup>.

Health education is also essential to reduce the HCV burden, and specific programs should be provided to increase public awareness on transmission and prevention of infection<sup>[62]</sup>.

## TREATMENT

HCV is a potentially curable disease<sup>[65]</sup> with a good percentage of treated patients getting a sustained virologic response (SVR) defined as undetectable serum HCV RNA 24 wk after the end of therapy (and now at 12 wk after the end of therapy<sup>[8]</sup>). Although the available standard of care (SOC) therapy has led to significant improvements in treatment response rates, less than 50% of HCV-infected persons are aware of their diagnosis<sup>[66]</sup>, and among them, only 1%-30% receive treatment. The true rate-limiting factor in achieving better outcomes may turn out to be access to diagnosis and treatment<sup>[66]</sup>.

Multiple barriers may impede the delivery of HCV therapy<sup>[67]</sup>. To increase cure rates, the psychological (psychiatric illness, attitudes and coping skills), lifestyle (alcohol consumption, diet, and exercise), social (income, education, social class, poverty), and other different barriers to treatment adherence and completion must be identified and overcome<sup>[68]</sup>.

For adults, standard IFN has been approved for the treatment of HCV since 1991, Ribavirin (RBV) since 1998 and pegylated-IFN (peg-IFN) since 2002. Nine years had passed before the American Food and Drug Administration (FDA) approved a new drug to be added to the existing SOC, the DAA oral protease inhibitors, boceprevir and telaprevir<sup>[69]</sup>. New drugs under development include other protease inhibitors, the NS5B polymerase and NS5A inhibitors<sup>[70]</sup>. In the coming years, the number of the new drugs will multiply exponentially and pharmaceutical companies have begun to combine them in triple and quadruple regimens (with and without peg-IFN)<sup>[69]</sup>.

In children 3-17 years old, treatment with peg-IFN $\alpha$ -2a or b plus RBV for 24-48 wk is the SOC therapy<sup>[71,72]</sup>, whereas the recently approved DAA still need evaluation in children.

In the United States and most European countries, the current first line therapy for infection with genotype-1 is a combination of peg-IFN alpha plus RBV plus either boceprevir or telaprevir. High SVR rates can be achieved even in those with evidence of fibrosis and cirrhosis, but response is poor in prior null responders, especially those with cirrhosis<sup>[73]</sup>.

Preliminary data from investigational studies suggest the potential for cure rates of 80%-90% in genotype-1 infection using combinations of DAAs and RBV without peg-IFN, but larger studies will be needed to confirm these results across a wider range of populations<sup>[74-76]</sup>.

Quadruple therapy with pegylated-IFN combines BI201335, a protease inhibitor, and BI207127, a non-nucleoside NS5B polymerase inhibitor, with peg-IFN and RBV<sup>[77]</sup>. The only quadruple peg-IFN-free study is the Gilead Sciences all-oral quad regimen<sup>[78]</sup>. It is a phase II study for genotype 1, treatment-naïve patients who are not cirrhotic. It combines GS-5885, GS-9451, tegobuvir, and RBV for 24 wk. The triple peg-IFN-free therapy combines mericitabine, a nucleoside NS5B polymerase inhibitor; danoprevir, a protease inhibitor; ritonavir, a booster for danoprevir; and RBV or placebo<sup>[79]</sup>.

SVR varies considerably from 26%-80% depending on age, duration of infection, viral load, viral genotype, adiposity, hepatic fibrosis iron scores, aminotransferase elevation, compliance with therapy, SNPs of IL-28B gene locus. It was found that a single IL28B genotype SNP rs12979860 determination predicts treatment response in patients with chronic hepatitis C Genotype 1 virus<sup>[80,81]</sup>. IL-28B has been also reported to play a role in spontaneous clearance of HCV genotype 4 in Egypt/North Africa<sup>[82]</sup>. Regarding the HCV genotypes 2 and 3, the polymorphisms rs12979860 and rs8099917 showed significant associations. However, the strength of this association was almost three times lower than for genotypes 1 and 4<sup>[83]</sup>. In addition regarding genotype 2, it was found that the Asian population was solely responsible for this association in rs8099917<sup>[84]</sup>. The generally reduced association for patients with HCV genotypes 2/3 could be related to the high rate of SVR present in these IFN-sensitive genotypes<sup>[85]</sup>.

SNPs of IL-28B gene received considerable interest also for their association with spontaneous clearance of HCV among vertically-infected children<sup>[86]</sup>.

SNPs of IL-28B as well as IL-10 are good predictors of response to IFN/RBV therapy in HCV genotype 4 infected Egyptian children<sup>[87]</sup>.

The combination of serum level of IFN-gamma inducible protein and SNPs of IL-28B can identify patients with acute HCV who are most likely to undergo spontaneous clearance and those in need of early antiviral therapy<sup>[88]</sup>.

SNPs of osteopontin gene were also reported as pre-



dictors for the efficacy of IFN therapy in chronic HCV Egyptian patients with genotype 4<sup>[89]</sup>.

## CONCLUSION

HCV infection is an increasing health and economic burden in adults as well as children, in both developing and developed countries. The natural history and histopathology of HCV-related liver disease in children are still conflicting and variable. Prevention of infection depends on screening of blood with the most sensitive tests, avoiding nosocomial infections, and avoiding injection drug abuse and unprotected sex in adolescents; as well as education. Development of a vaccine preventing HCV infection is of thorough public health importance. The SOC therapy is peg-IFN plus RBV. The SVR is variable (26%-80%) and depends on several viral and host factors. Eradication of HCV in a child (if possible) is cost-effective as it may prevent cirrhosis and HCC; and can have major family, public health and global benefits.

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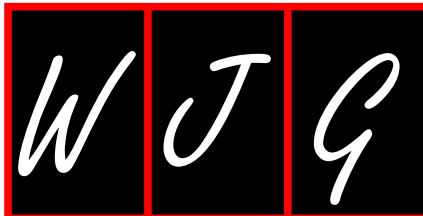


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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Direct effects of hepatitis C virus on the lymphoid cells

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## Abstract

It has been reported that the direct binding of hepatitis C virus (HCV) and/or the replication of HCV in the extrahepatic organs and, especially, lymphoid cells, might affect the pathogenesis of extrahepatic diseases with HCV infection. More than one decade ago, several reports described the existence of HCV-RNA in peripheral blood mononuclear cells. Moreover, many reports describing the existence of HCV in B lymphocytes and B cell lymphoma have been published. In addition to B lymphocytes, it was reported that HCV replication could be detected in T lymphocytes and T cell lines. Among the extrahepatic diseases with HCV infection, mixed cryoglobulinemia-related diseases and autoimmune-related diseases are important for understanding the immunopathogenesis of HCV persistent infection. Moreover, HCV persistent infection can cause malignant lymphoma. The biological significance of lymphotropic HCV has not yet become clear. However, several candidates have been considered for a long time. One is that lymphotropic HCV is an HCV reservoir that might contribute to the recurrence of HCV infection and difficult-to-treat disease status. The other important issue is the carcinogenesis of the lymphoid cells and disturbances of the immune responses. Therefore, the extrahepatic

diseases might be induced by direct interaction between HCV and lymphoid cells. In this article, we summarize various studies showing the direct effect of HCV on lymphoid cells and discuss the biological significance of lymphotropic HCV.

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**Key words:** Hepatitis C virus; Lymphotropism; T cell; B cell; Immunology

**Core tip:** In this article, we summarize various studies showing the direct effect of hepatitis C virus (HCV) on lymphoid cells and discuss the biological significance of lymphotropic HCV.

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## INTRODUCTION

An estimated 130-170 million people are infected with hepatitis C virus (HCV) worldwide<sup>[1]</sup>. Around 75% of the patients with acute HCV infection undergo chronic HCV infection and are subsequently at risk of progressing to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. Persistent infection of HCV involves not only the liver but also various extra-hepatic organs<sup>[3-7]</sup>. HCV can infect hepatocytes, lymphoid cells, and probably other cells through CD81 and receptor candidates<sup>[8]</sup>. Moreover, the expression of microRNA (miR)-122 facilitates efficient replication of HCV in nonhepatic cells<sup>[9]</sup>. These reports indicated that the direct binding of HCV and/or the replication of HCV in the extrahepatic organs, especially lymphoid cells, might affect the pathogenesis of

extrahepatic diseases with HCV infection. Among the extrahepatic diseases with HCV infection, mixed cryoglobulinemia (MC)-related diseases and autoimmune-related diseases are important for understanding the immunopathogenesis of HCV persistent infection<sup>[10-13]</sup>. Moreover, HCV persistent infection could cause malignant lymphoma<sup>[4]</sup>. The status of a disease might depend on the direct interaction between HCV and lymphoid cells<sup>[6,14-17]</sup>. The biological significance of lymphotropic HCV has not yet become clear. However, several candidates have been considered for a long time. One is that lymphotropic HCV is an HCV reservoir that might contribute to the recurrence of HCV infection and difficult-to-treat disease status<sup>[18-23]</sup>. The other important issue is the carcinogenesis of the lymphoid cells and disturbances of the immune responses<sup>[8,14,24-28]</sup>. Previously, Sung *et al*<sup>[29]</sup> reported a lymphotropic HCV strain that was isolated from B cell lymphoma. This lymphotropic HCV strain can infect and replicate in established B cell lines and primary B lymphocytes<sup>[29]</sup>. Moreover, we reported that T cell lines and primary naïve T lymphocytes were infected with this HCV strain<sup>[8,25,26]</sup>. In these studies, we demonstrated that lymphotropic HCV had various effects, especially on T cell development and proliferation. Therefore, understanding of the direct effects of HCV on the lymphoid cells is needed to clarify the immunopathogenesis of HCV persistent infection. In this report, we summarize various studies showing the direct effect of HCV on lymphoid cells and discuss the biological significance of lymphotropic HCV.

## ROLE OF VIRUS RESERVOIR

### *HCV infection in peripheral blood mononucleated cells*

More than one decade ago, several reports described the existence of HCV-RNA in peripheral blood mononucleated cells (PBMCs)<sup>[30,31]</sup>. The detection rate of HCV-RNA in PBMCs was increased if the patients were infected with human immunodeficiency virus (HIV) and HCV<sup>[31]</sup>. This phenomenon indicated that immune-suppressive circumstances and/or HIV antigen might enhance the replication activity of HCV in lymphoid cells<sup>[32]</sup>. HIV-1 accessory protein transactivator of transcription (TAT) can activate HCV replication by upregulating IP10 production. Moreover, it was reported that continuous release of HCV by PBMCs was detected in HCV-infected patients, especially in HIV co-infected patients<sup>[18]</sup>. The detection of HCV-RNA in the PBMCs from HIV-HCV co-infected patients could contribute to the recurrence of HCV viremia after pegylated-interferon and ribavirin treatment. It was reported that the presence of positive/negative strand HCV RNA at the end of treatment is associated with relapse among HCV-HIV co-infected patients<sup>[33]</sup>. In addition to HCV-HIV co-infected patients, a low level of HCV replication could be detected in peripheral lymphoid cells from HCV mono-infected patients after antiviral treatment<sup>[20,23]</sup>. Moreover, it was reported that

HCV persisting at low levels long after therapy-induced resolution of chronic hepatitis C could remain infectious<sup>[20]</sup>. This continuous viral presence could result in the persistence of humoral and cellular immunity for many years after treatment and could present a risk of infection reactivation.

### *Responsible lymphocyte subsets as a viral reservoir*

It has been reported that HCV replication could be detected in various kinds of lymphoid cells. Many reports describing the existence of HCV in B lymphocytes and B cell lymphoma have been published<sup>[5,29,34]</sup>. Recently, one group reported that CD19<sup>+</sup> B lymphocytes had significantly higher viral loads than CD14<sup>+</sup> monocytes<sup>[35]</sup>. Among B lymphocytes, CD27<sup>+</sup> memory B lymphocytes were more resistant to apoptosis than CD27<sup>-</sup> B lymphocytes. CD27<sup>+</sup> B lymphocytes might be a candidate subset of the HCV reservoir in chronic hepatitis C (CH-C)<sup>[36]</sup>. In addition to B lymphocytes, it was reported that HCV replication could be detected in T lymphocytes and T cell lines<sup>[20,37,38]</sup>. We also reported that a lymphotropic HCV strain could infect T cell lines and primary human naïve CD4<sup>+</sup> T lymphocytes<sup>[8,25,26]</sup>. HCV infects hepatocytes, lymphoid cells, and probably other cells through CD81 and several candidate receptors. The expression of CD81 could be detected in B cells, T cells, and monocytes, indicating that these types of cells are potential targets of HCV infection. Recently, one group reported that HCV infection of human T lymphocytes is mediated by CD5<sup>[39]</sup>. In contrast to T lymphocytes, hepatocytes do not express CD5. Therefore, the mechanism of HCV lymphotropism might be different from that of HCV hepatotropism. Moreover, the other candidate receptors were analyzed using HCV-prone and resistant T cell lines, PBMCs, primary T cells, Huh7.5 cells and HepG2 cells<sup>[40]</sup>. CD5 and CD81 expression coincided with lymphotropism and that of occludin with the permissiveness of T cell lines, but probably not primary T lymphocytes<sup>[40]</sup>.

In addition to B and T lymphocytes, it has been reported that HCV can infect monocytes, especially CD14<sup>+</sup>CD16<sup>+</sup> monocytes, but not CD14<sup>+</sup>CD16<sup>-</sup> monocytes<sup>[41]</sup>. The detection of HCV-RNA in monocytes was reported in HCV-HIV co-infected patients and HCV-monoinfected patients<sup>[19]</sup>. HIV might facilitate the infection/replication of HCV in human macrophages<sup>[42]</sup>. One group reported the frequent compartmentalization of HCV in circulating CD19<sup>+</sup> B lymphocytes and CD14<sup>+</sup> monocytes<sup>[43]</sup>. Moreover, it was reported that immature and mature dendritic cells are susceptible to HCV genotype 1 infection, supporting at least HCV RNA replication *in vitro*<sup>[44]</sup>. Another group reported that replicative-strand HCV-RNA was detected in peripheral blood dendritic cells<sup>[28]</sup>. Although other lymphoid cells might be susceptible to HCV infection<sup>[45,46]</sup>, these reports suggested that B and T lymphocytes, monocytes, and dendritic cells could be reservoirs for HCV.

## DIRECT EFFECT OF HCV ON THE CARCINOGENESIS OF LYMPHOID CELLS

Many reports have focused on the relevance of HCV infection and B-cell lymphoma, especially non-Hodgkin lymphoma (NHL)<sup>[4,47]</sup>. Compared to the high association between HCV infection and HCC, epidemiologic reports on the relationship between HCV and NHL show a moderate risk for the development of lymphoma. However, no association between HCV and NHL was also reported in low HCV prevalence countries<sup>[48,49]</sup>. Different hypotheses have been suggested to explain the difference in the HCV-NHL prevalence: (1) Geographic differences in the HCV genotype distribution might contribute to differences in the HCV-NHL prevalence; (2) The duration of persistent infection of HCV might influence the carcinogenesis of lymphoid cells; and (3) Studies in low prevalence countries might not have included enough patients to detect the association. However, meta-analyses indicated a significant association between HCV and B-NHL<sup>[48,50]</sup>.

Many groups reported the mechanisms of lymphomagenesis. However, we have to understand that HCV-infected patients with MC are at a higher risk of developing HCV-NHL<sup>[51]</sup>. MC could be an intermediary step in the development of NHL. Although, different theories have been proposed to explain the mechanism of HCV-induced lymphomagenesis, we can classify most of the theories into two categories. One of them is direct HCV binding with B lymphocytes. The external stimulation of lymphocyte receptors (CD19, CD21, CD81, B-cell receptor) by HCV antigen might induce a proliferation signal<sup>[52]</sup>. The HCV-core protein induces the production of interleukin (IL) 6 in CD14<sup>+</sup> cells *via* Toll like receptor 2 and leads to increased B cell proliferation<sup>[53]</sup>. In addition to the classical cytokine proliferation signal, the down regulation of miR-26b, an miRNA known to have tumor-suppressive properties, was found in splenic marginal zone lymphoma with HCV persistent infection<sup>[54]</sup>. This theory was supported by the phenomenon of lymphoma remission when the HCV antigens are removed by treatment. In addition to the proliferation signal, HCV-E2 CD81 on B cells triggers the enhanced expression of activation-induced cytidine deaminase (AID), which could contribute to enhancing the mutation frequency<sup>[14]</sup>. The other category of lymphomagenesis mechanism is HCV infection and/or replication in B lymphocytes. It has been reported that the replication of HCV in B lymphocytes could induce error-prone DNA polymerase zeta, polymerase iota, and AID, which contribute to enhancing the mutation frequency<sup>[14]</sup>. Moreover, the cellular DNA damage and mutation were mediated by nitric oxide and reactive oxygen species<sup>[55,56]</sup>. In addition to *in vitro* study, interferon regulatory factor-1-null mice with inducible and persistent expression of HCV structural protein showed a high incidence of lymphoma and lymphoproliferative diseases<sup>[57]</sup>. In this mouse model, the overexpression of apoptotic related genes and aberrant cytokine

production were detected in the first step of carcinogenesis. Another group also reported that the expression of HCV-core protein could increase the incidence of lymphoma in transgenic mice<sup>[58]</sup>. Moreover, it has been reported that persistent expression of the full genome of HCV in B cells induces the spontaneous development of B-cell lymphoma *in vivo*<sup>[59]</sup>. HCV transgenic mice that expressed the full HCV genome in B cells showed a 25% incidence of diffuse, large B-cell non-Hodgkin lymphomas. Although the relationship between HCV persistent infection and lymphomagenesis could become clarified by various epidemiological studies, the mechanism of lymphomagenesis still needs to be considered carefully.

## DIRECT EFFECT OF HCV ON THE IMMUNE EVASION

Many studies have described a failure of the innate and cellular immune response, including type 1 helper T cells (Th1) hypo-responsiveness, cytotoxic T lymphocytes (CTL) exhaustion, excessive function of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells, failure of dendritic cell function, occurs in HCV persistent infection<sup>[60-69]</sup>. Among the numerous mechanisms, the lymphoid cells, *via* direct binding and/or infection in B cells, T cells, NK cells and DCs *etc.*, should be considered, especially in HCV persistent infection<sup>[8,25-28,70-73]</sup>. In our previous study, we used SB-cell lines that continuously produce infectious HCV virions in culture. The virus particles produced from the culture had a buoyant density of 1.13-1.15 g/mL in sucrose and could infect primary human PBMCs and an established B-cell line *in vitro*<sup>[29]</sup>. This lymphotropic HCV strain was useful to investigate the biological significance of HCV replication in lymphoid cells. In this *in vitro* system, HCV could infect and transiently replicate in T cells and HCV replication suppressed the interferon (IFN)- $\gamma$ /STAT-1/T-bet signaling due to the reduction of STAT-1 and inhibition of its activation<sup>[26]</sup>. Moreover, HCV replication in T cells suppressed cellular proliferation and enhanced susceptibility to Fas signaling by inhibiting CD44v6 signaling and expression<sup>[25]</sup>. In addition to cell lines, we used primary T lymphocytes to analyze the biological meaning of lymphotropic HCV<sup>[8]</sup>. Another group reported that HCV core protein modulates the transcription of *IL-2* promoter in T lymphocytes by activating the nuclear factor of activated T lymphocyte pathway<sup>[74,75]</sup>. Moreover, the expression of HCV core protein could induce Ca<sup>2+</sup> oscillations that regulate both the efficacy and information content of Ca<sup>2+</sup> signals<sup>[74]</sup>. In addition to HCV replication in T cells, Yao *et al*<sup>[76]</sup> reported that the direct binding of HCV core to gC1qR on CD4<sup>+</sup> and CD8<sup>+</sup> T cells leads to impaired activation of Lck and Akt. We could also detect a relationship between HCV core protein and immune suppression in HCV persistent infection<sup>[77]</sup>. Double filtration plasmapheresis for CH-C patients could reduce the amounts of HCV core proteins in the peripheral blood and on the surface of T lymphocytes<sup>[77]</sup>. Moreover, it has been reported that the



engagement of gC1qR on DCs by HCV core limits the induction of Th1 responses and may contribute to viral persistence. Another group reported that NK cell-derived cytokines secreted in the presence of HCV cc showed a diminished antiviral effect that correlated with a reduction of IFN- $\gamma$ <sup>[72]</sup>. DCs play essential roles in the triggering of primary antiviral immune reactions. DCs are the most potent activators of CD4 T cells for supporting Th1 differentiation, which is important for the cellular immune response. Several reports described that persistent HCV infection is associated with an allostimulatory defect of monocyte-derived DC<sup>[67,70]</sup>. These reports supported that HIV/HCV co-infected patients were difficult-to-control in comparison with HCV mono-infected patients, since lymphotropic HCV is frequently detected in HIV/HCV co-infected patients<sup>[78]</sup>. Co-infection with HCV and HIV is associated with increased HCV replication and a more rapid progression to severe liver disease, including the development of cirrhosis and HCC.

## DIRECT EFFECT OF HCV ON IMMUNE STIMULATION

We need to focus not only on the suppression of the immune system but also on the stimulation of the immune system, since the prevalence of cryoglobuline-related and autoimmune-related diseases is much higher than in healthy subjects<sup>[10,79]</sup>. HCV core protein activates interleukin-2 gene transcription through the nuclear factors of activated T cells pathway<sup>[75,80]</sup>. IL-2 has a role in T cell proliferation. Recently, we reported that lymphotropic HCV and high frequency of Th17 cells were detected in CH-C patients with pyoderma gangrenosum-like lesions<sup>[16]</sup>. In that report, the eradication of HCV could improve the immunological status and pyoderma gangrenosum-like lesions. A study regarding the relationship between lymphotropic HCV and autoimmune diseases is ongoing in our laboratory. Another group reported that HCV-core induced STAT3 activation might play a role in the alteration of inflammatory responses in human monocytes<sup>[81]</sup>. Moreover, HCV infection of macrophage/monocytes *in vitro* might be associated with the induction of cytokines tumor growth factor- $\alpha$  and IL8. In addition to T lymphocytes and monocytes, Machida *et al*<sup>[17,27]</sup> reported that HCV could induce immunoglobulin hypermutation in B lymphocytes. These reports together suggest that HCV could stimulate an unfavorable immune response. As for the understanding of autoimmune diseases, HCV persistent infection might be one of the representative models of viral-induced autoimmune diseases.

## CONCLUSION

Although various reports have described the direct effects of HCV on lymphoid cells, few have addressed whether the disturbance of the immune system induced by the direct binding and/or infection of HCV on lymphoid cells might coordinately influence the pathogenesis of

HCV persistent infection. In this article, we summarized various reports indicating the direct effects of HCV on lymphoid cells. In addition to the direct effect of HCV, the indirect effects of HCV on lymphoid cells could influence the pathogenesis of HCV persistent infection. Therefore, we must treat a vast array of data to clarify the real pathogenesis of HCV persistent infection. Recently, the technologies of deep sequencing, immunoassays with increased numbers of multicolor flow cytometry analyses, and chimera mice with human lymphocytes have been developed. These technologies, together with previous data, might be able to clarify the direct effects of HCV on lymphoid cells.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# An insight into the diagnosis and pathogenesis of hepatitis C virus infection

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## Abstract

This review focuses on research findings in the area of diagnosis and pathogenesis of hepatitis C virus (HCV) infection over the last few decades. The information based on published literature provides an update on these two aspects of HCV. HCV infection, previously called blood transmitted non-A, non-B infection, is prevalent globally and poses a serious public health problem worldwide. The diagnosis of HCV infection has evolved from serodetection of non-specific and low avidity anti-HCV antibodies to detection of viral nucleic acid in serum using the polymerase chain reaction (PCR) technique. Current PCR assays detect viral nucleic acid with high accuracy and the exact copy number of viral particles. Moreover, multiplex assays using real-time PCR are available for identification of HCV-genotypes and their isotypes. In contrast to previous methods, the newly developed assays are not only fast and eco-

nomic, but also resolve the problem of the window period as well as differentiate present from past infection. HCV is a non-cytopathic virus, thus, its pathogenesis is regulated by host immunity and metabolic changes including oxidative stress, insulin resistance and hepatic steatosis. Both innate and adaptive immunity play an important role in HCV pathogenesis. Cytotoxic lymphocytes demonstrate crucial activity during viral eradication or viral persistence and are influenced by viral proteins, HCV-quasispecies and several metabolic factors regulating liver metabolism. HCV pathogenesis is a very complex phenomenon and requires further study to determine the other factors involved.

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**Key words:** Hepatitis C virus; Diagnosis; Pathogenesis; Immunity; Steatosis

**Core tip:** This article focuses on the diagnosis and pathogenesis of hepatitis C virus infection. Both of these aspects are important in order to eradicate this endemic virus and to prevent serious liver diseases.

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## INTRODUCTION

Hepatitis C virus (HCV) was first characterized by Choo *et al*<sup>[1]</sup> and Kuo *et al*<sup>[2]</sup> in 1989. It was soon identified as the main causative agent of the disease previously known as post transfusion non-A, non-B hepatitis virus infec-



tion. HCV has been found to be an important cause of liver disease and remains a major public health problem worldwide. According to the World Health Organization, nearly 3% of the world population has been infected with HCV. Therefore, more than 170 million people are chronic carriers of HCV and at high risk of developing liver cirrhosis and/or hepatocellular carcinoma (HCC). Three to 4% of chronically infected individuals develop fatal HCC. Currently, HCC caused by HCV infection is considered an indication for liver transplantation<sup>[3-5]</sup>.

HCV was the leading cause of post-transfusion and community-acquired non-A, non-B hepatitis until characterization of the virus in 1989 and the introduction of blood screening in 1990. The initiation of blood screening for HCV has markedly reduced its incidence. However, it still remains a significant problem in intravenous drug abusers. HCV infection is the most common cause of liver transplantation in adults. HCV and HIV-1 frequently co-infect humans and it has been estimated that as many as 18% of HIV-infected persons are also infected with HCV<sup>[4]</sup>.

HCV is an enveloped RNA virus and belongs to the genus Hepacivirus of the family Flaviviridae. The HCV genome consists of 9.6-kb single-stranded RNA of positive polarity and a single open reading frame of 9033-9099 nucleotides flanked by a conserved 5' and 3' noncoding region (NCR) at the ends. Its genome codes for a long polyprotein of approximately 3000 amino acids<sup>[6]</sup> which is processed co-translationally and post-translationally to yield structural proteins (core, envelope E1, and E2) and non-structural (NS) proteins (NS1/p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)<sup>[7]</sup>. The envelope proteins (E1 and E2) are the outer surface proteins of the viral particles and play important roles in virus entry into the host cell. NS5B is a variable region of the HCV genome and codes for an RNA-dependent RNA polymerase (RdRp).

RNA polymerase lacks proof reading activity and this may alter the detection, sensitivity to interferon anti-viral activity and pathogenicity of the virus (Figure 1)<sup>[8]</sup>.

Like several other viruses, the RNA virus has a high degree of heterogeneity<sup>[5]</sup> that varies 30%-35% among different genotypes. Based on previous studies, six major genotypes and more than 120 subtypes of HCV have been characterized to date<sup>[9]</sup>. These HCV genotypes have distinct geographic distributions, with genotype 1 and 2 frequently found worldwide<sup>[10]</sup>. In India, genotype 3 is reported to be the most prevalent, followed by genotype 1<sup>[11,12]</sup>. Different HCV genotypes have important epidemiological implications. Despite nucleotide sequence divergence between genotypes, they remain quite similar in their transmission pattern, persistence and disease development<sup>[13]</sup>. Although genetic variation is attributed to several factors, two major theories *i.e.*, the Darwinian and Neutral evolution theories are thought to be the prominent theories in causing genetic diversity in HCV<sup>[13]</sup>. The nucleotide sequence variability is distributed throughout the viral genome. Regions encoding envelope proteins

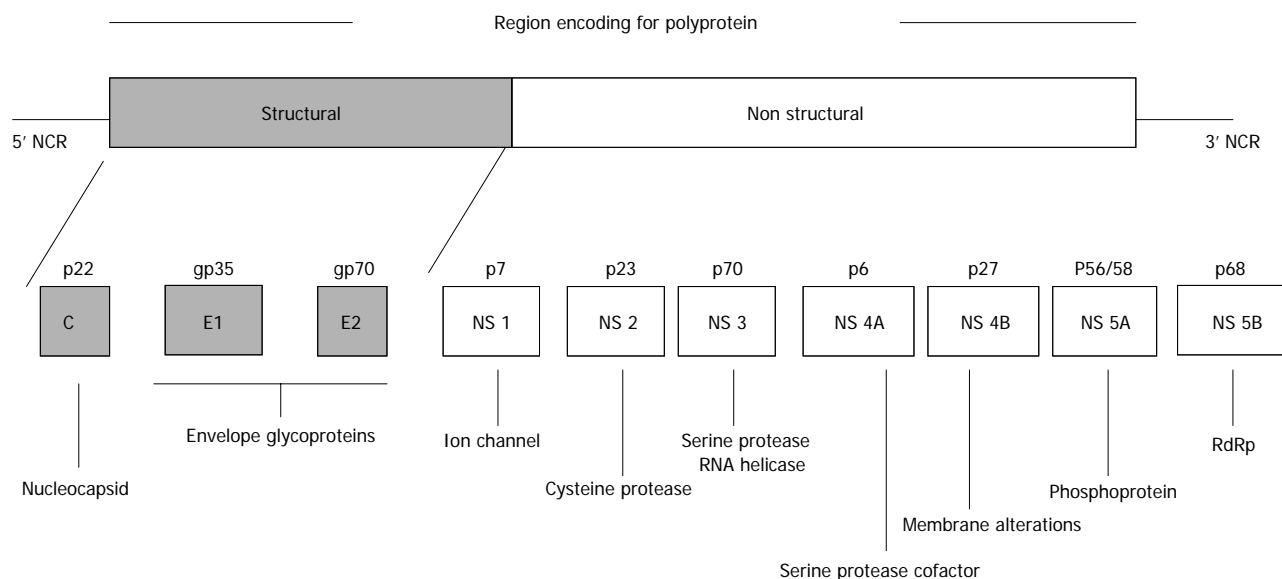
(E1, E2) and NS-1 are the most variable, whereas the 5' NCR is the most conserved region.

HCV patients show a poor response to antiviral therapy based on the combination of pegylated interferon (IFN)- $\alpha$  and ribavirin. Only 40%-50% of patients infected with HCV genotype-1 and 80% of those infected with genotype-2 or 3 achieve a sustained virological response (SVR) with this regimen<sup>[14]</sup>. The recent use of direct acting anti-viral (DAA) molecules, which are active on HCV during treatment, has led to a substantial improvement in SVR rates in HCV genotype-1 infected patients. However, this may lead to the selection of resistant virus if DAA molecules are used alone<sup>[15]</sup>. Moreover, there is a high relapse rate of HCV infection after discontinuation of therapy. Recently, host genetic factors including human leukocyte antigen (HLA) and cytokine genes have been implicated in HCV infection or persistence<sup>[16]</sup>. Genetic polymorphism of cytokine genes including IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-10, IL-20 and SNPs in the promoter region of osteopontin gene, have been found to be crucial in determining the therapeutic outcome of HCV infection<sup>[17]</sup>. Therefore, every effort is being made to understand the pathogenesis of HCV infection to create a therapeutic model for an effective treatment against HCV. Although recent reports describe the development of *in vitro* replication systems leading to the production of infectious viral particles<sup>[18,19]</sup>, there is currently no cell culture model suitable for synthesizing vaccines based on killed or attenuated virus. All efforts have been focused on sub-unit vaccines, composed of one or several antigens, either in the form of recombinant proteins, synthetic peptides or vectored vaccines. The earliest vaccine developed for HCV was that by the Chiron group<sup>[20]</sup>. However, very little progress was noted in this direction in subsequent years.

This article reviews the major aspects of HCV infection including the diagnosis and pathogenesis of HCV infection. Both of these aspects have a strong association with therapy, thus, newer means of accurate diagnosis and a better understanding of HCV infection pathogenesis may allow the development of a therapeutic model. This article attempts to update readers regarding the information available on these two aspects to date.

## DIAGNOSIS OF HCV INFECTION

During HCV infection, every attempt is made to diagnose and differentiate acute from chronic hepatitis C infection. Acute HCV infection is typically mild. It is often not diagnosed, and the infection may be recognized only when it becomes chronic<sup>[21]</sup>. The diagnostic tests used, including the presence of anti-HCV antibodies in serum, cannot differentiate between acute and chronic HCV infection because anti-HCV IgM, used as marker of acute infection, is variable in acute infectious disease and is also detected at high rates in patients with chronic HCV infection<sup>[22,23]</sup>. The diagnostic procedures for hepatitis C virus infection used in laboratories are based on the detection

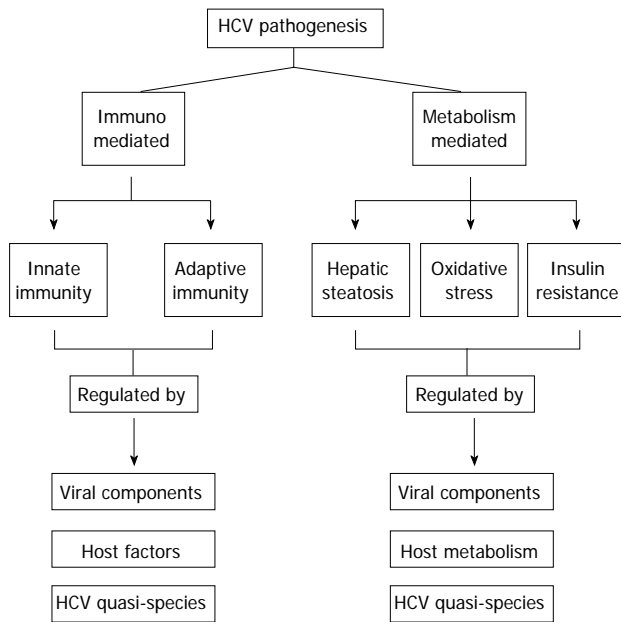


**Figure 1** Proteins encoded by the hepatitis C virus genome. Genome organization of hepatitis C virus showing the structure of the viral genome, including the long open reading frame encoding structural and nonstructural proteins, and 5' and 3' non-coding regions (NCRs). [Source: Monica A *et al.* *Expert Rev Mol Med* 2003; 5].

of anti-HCV antibodies against recombinant HCV proteins using enzyme immunoassay (EIA) and chemiluminescence immunoassay. Non-structural and recombinant antigens are used in these assays. Four different generations of anti-HCV test kits have been developed to date. The first generation EIA detected antibodies against the nonstructural proteins (NS4) with recombinant antigen c100-3. Subsequently, the second generation assay was developed and this included antigens from the core region (c22-3), the NS3 region (c33c) and a part of c100-3 (5-1-1) from the NS4 region. The third-generation EIA included an additional antigen from the NS5 region and a reconfiguration of the core and NS3 antigens. However, all these anti-HCV assays had the disadvantages of giving high false positive results and a lack of sensitivity to detect antibodies during the window period. In addition, these antibody-based assays could not distinguish between acute, past and chronic infections. This was followed by the development of supplementary tests involving the recombinant immunoblot assay (RIBA) which was commercialized. This assay contained recombinant antigen (c33c, NS5) and synthetic peptides (5-1-1, c100 and c22). Similarly, a few other commercial assays, known as third generation immunoassays incorporated HCV antigens from the core region, E2 hypervariable region, NS3 region, NS4A, NS4B and NS5A region. All these recombinant immunoblot assays were used as supplementary tests to the anti-HCV assays. Similar to EIA, the RIBA had the disadvantages of difficulty in performance and a high percentage of indeterminate results. Therefore, these are no longer used in diagnostic laboratories. Recently, fourth generation anti-HCV assays incorporating additional nonstructural proteins are being used as screening tests<sup>[24]</sup>. These kits for anti-HCV detection target different HCV antigens and detect more than five primary antibodies to ensure the specificity and sensitivity

of the detection kit.

Anti-C22c and anti-C33c may be the first HCV antibodies to appear during the acute phase of the disease, which is defined by elevated alanine aminotransferase (ALT) levels and/or clinical symptoms<sup>[25]</sup>. Anti-NS5 appears somewhat later, while anti-C100-3 is the last antibody to be detected in acute self-limited HCV infection. The diagnosis and differentiation of acute from chronic HCV infection poses another problem. Patients chronically infected with one HCV-genotype develop acute hepatitis on infection with another genotype. Multiple episodes of acute hepatitis were observed in polytransfused thalassemic children reinfected with different HCV genotypes<sup>[26,27]</sup>. Therefore, discrimination between acute and chronic infection in the same patient is sometimes very difficult. HCV RNA in the serum or liver appears to be the earliest detectable marker of acute HCV infection, preceding the appearance of anti-HCV by several weeks<sup>[25]</sup>. HCV viremia may persist despite the normalization of serum ALT levels. Thus, the use of ALT levels in the diagnosis of HCV is not helpful. However, HCV RNA in serum usually lasts for fewer than 4 mo in patients with acute self-limited HCV infection. The average time from transfusion to sero-conversion is approximately 11 to 12 wk with EIA-1 (Enzyme immunoassay-1) and 7 to 8 wk with EIA-2 (Enzyme immunoassay-2). Now attempts are being made to develop EIA assays to differentiate HCV sub-types<sup>[28]</sup>. Patients with post-transfusion chronic non-A, non-B hepatitis develop anti-HCV antibodies in the majority of cases. Anti-HCV antibodies are not neutralizing, especially with HCV envelope proteins E1 and E2<sup>[29]</sup>. High levels of anti-C100-3 were correlated with high titers of circulating HCV in chimpanzees<sup>[30]</sup>. Therefore, the development and persistence of diagnostic antibodies to HCV seem to reflect concomitant virus replication and consequently a high



**Figure 2** Regulation of hepatitis C virus pathogenesis by host immunity and metabolic factors. HCV: Hepatitis C virus.

potential for infectivity.

HCV RNA is frequently detected in patients with chronic hepatitis C and in patients carrying anti-HCV antibodies. A study carried out in Hong Kong demonstrated that 83% of anti-HCV positive patients were viremic when HCV RNA was determined using polymerase chain reaction (PCR) with two different sets of primers for noncoding regions<sup>[27]</sup>. Similarly, in another study, 98 of 100 patients with chronic non-A, non-B liver disease were positive for antibodies by EIA-2, but all 100 patients were positive for HCV RNA by PCR. With the currently available EIA systems, chronic HCV infection can readily be identified in most patients. Measurement of HCV RNA by PCR does not substantially increase the numbers of patients found to have chronic HCV infection<sup>[31]</sup>. Following the introduction and wider use of real-time PCR, it is now easier to diagnose and monitor the progress of HCV viremia in a very short time period<sup>[32]</sup>. In addition, the use of multiplex PCR by real time is another advancement in the detection of possible hepatitis viral co-infections in single attempt analysis<sup>[33]</sup>.

Based on published information regarding various aspects of HCV infection including the currently available diagnostic assays and therapeutic regimens, the American Association for the Study of Liver Diseases and Centers for Disease Control and Prevention, United States have approved a document as “practice guidelines” for use in the diagnosis and treatment of HCV infection. This is an important document and describes details of the guidelines to be followed for laboratory diagnosis of acute/chronic HCV infection<sup>[34]</sup>.

## PATHOGENESIS OF HCV INFECTION

HCV is a non-cytopathic virus<sup>[35]</sup> that enters the liver cell

and undergoes replication simultaneously causing cell necrosis by several mechanisms including immune-mediated cytolysis in addition to various other phenomena such as hepatic steatosis, oxidative stress and insulin resistance. The proteins/peptides encoded by different sub-genomic regions of the HCV genome and their quasispecies influence the above mechanism, and thus, have a significant role in HCV pathogenesis and disease causation. A brief description of HCV pathogenesis in the light of these factors is given in the following section (Figure 2).

### Viral entry

HCV is a blood-transmitted virus that reaches the liver *via* circulation. The entry of HCV isolates requires at least 4 host-derived factors including scavenger receptor class B type I, Occludin, Claudin-I (CLDN1) and CD81. In addition, CLDN6 and CLDN9 have been shown to substitute for CLDN1 as HCV entry factors in human non-liver cells<sup>[36]</sup>. The CD81 molecule on host cell surfaces acts as a viral receptor, which binds with the viral particle and facilitates its entry in the liver cell<sup>[37,38]</sup>. CD81 is expressed on the surface of almost all nucleated cells and complexes with a variety of other cell-surface receptors such as CD19 and CD21 on B cells, and sends a costimulatory signal to the cells<sup>[39]</sup>. The viral envelop protein, E2, binds to the major extracellular loop of CD8<sup>[40]</sup>. HCV shows multi-site binding and can also bind to several other molecules such as the receptor for low-density lipoprotein, the dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and its liver counterpart<sup>[41,42]</sup>. E2 is the most variable viral protein, and therefore, its interactions with CD81 have been reported to be strain-specific<sup>[43]</sup>. It has two hyper variable regions, HVR-1 and HVR-2 which undergo frequent mutations, possibly due to virus-neutralizing antibodies and HCV-specific cytolytic T lymphocytes (CTLs). HCV also has a high mutation rate due to the lack of proofreading ability of its RNA-dependent RNA polymerase. Therefore, HCV exists in several distinct, but closely related virus species within an infected individual. These species are called HCV quasispecies.

## HOST IMMUNITY

### Innate immunity

Innate immunity presents a first line defense for the control of HCV infection as it does for several other viral infections. During HCV infection, cells produce Type 1 IFN which prepares and induces the cells to resist infection, check viral replication, promote adaptive immunity and activate natural killer (NK) cells, DCs and Kupffer cells *etc.* Once inside the cell, the innate immunity *vs* HCV is triggered through host recognition of viral macromolecular motifs, known as pathogen-associated molecular patterns (PAMPs), as non-self by cellular pathogen recognition receptors. These receptors includes toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs)<sup>[44]</sup>. RIG-I binds PAMP on

HCV-RNA and activates interferon regulatory factor-3 (IRF-3) for expression of IFN- $\alpha/\beta$  and anti-viral/interferon stimulated genes (ISGs)<sup>[45]</sup>. The secreted IFN and cytokines then activate NKs, DCs and Kupffer cells *etc.* These cells also play a significant role in mounting T/B cell-based immunity<sup>[46]</sup>. The PAMP region lies on the 3' untranslated region (UTR) of HCV and induces RIG-1 signaling<sup>[47]</sup> that results in a RIG-1 interaction with IFN- $\beta$  promoter stimulator (IPS-1) which causes activation of IRF-3 and nuclear factor  $\kappa$ B (NF $\kappa$ B).

HCV can effectively evade innate immunity resulting in persistent viral infection. This occurs because HCV has evolved to counteract the RIG-1 pathway<sup>[48]</sup> and thus evade the immune challenge. This phenomenon is the reason for chronicity in the majority of HCV infected patients. For this, the non-structural proteins of HCV *i.e.*, NS3 and NS4A form a complex which activates the NS protease domain to target cleavage of IPS-1. After cleavage, IPS-1 can no longer signal downstream to activate IRF-3 and NF $\kappa$ B and the infected cells no longer produce IFN- $\beta$  or express ISGs<sup>[49]</sup>.

NK cells, a major arm of innate immunity, play an important role in eradication of HCV. The liver is enriched in NK cells that are usually activated in an early phase of HCV infection. The activated NK cells recruit virus-specific T cells and induce antiviral immunity in the liver. They also eliminate virus-infected hepatocytes directly by cytolytic mechanisms and indirectly by secreting cytokines including IFN- $\gamma$  and TNF- $\alpha$ . These cytokines induce an antiviral state in host cells. Surprisingly, HCV has evolved multiple strategies to counter the host's NK cell response. It is interesting that activated NK cells contribute toward liver injury, while inactive or compromised NK cells permit the virus to continue invasion<sup>[50]</sup>.

### Adaptive immunity

After entry and replication of the virus inside liver cells, the viral molecules are transported to the endoplasmic reticulum and associate with major histocompatibility complex (MHC) molecules, which are finally transported to the cell surface. These molecules on the cell surface are recognized by T cells for their immune action. The majority of CTLs are CD8<sup>+</sup> and recognize antigens presented on MHC class I molecules. Approximately 10% of CTLs are CD4<sup>+</sup> which recognize antigens presented on MHC II molecules. These CTLs eliminate cells infected with virus. However, HCV is reported to have evolved mechanisms to avoid recognition by CTLs. They either reduce the expression of MHC molecules or prevent the viral peptide from presentation at the cell surface. Thus, CTLs play a major role in viral eradication<sup>[51]</sup> and immunopathogenesis of HCV infection<sup>[52]</sup>.

In another pathway of the disease mechanism, the destruction of HCV-infected hepatocytes release HCV fragments that are taken up by myeloid DCs. These DCs migrate to the draining lymph nodes and express HCV antigens on HLA class II molecules. Subsequently, they increase expression of costimulatory molecules (CD80,

CD86) which interact with and activate antigen-specific helper T (Th) cells<sup>[53]</sup>. These activated Th cells promote the maturation of DCs and increase the expression of CD40 ligand and TNF- $\alpha$ . The mature DCs induce T-cell activation by overexpression of their surface molecules. They also enhance antigen presentation capacity *via* HLA-I and production of cytokines that stimulate T-cell activation. IL-12 has been shown to play an important role in stimulating IFN- $\gamma$  production from activated T cells<sup>[54,55]</sup>, and thus, induces development of the type 1 (Th1) immune response characteristic of CTL activation. The effector CTLs release perforin, granzyme, and TNF- $\alpha$ , or express Fas ligand, and initiate a direct attack on HCV-infected hepatocytes<sup>[56,57]</sup>.

The hepatocytes infected with HCV and DCs produce Type I IFNs which suppress viral replication by inducing enzymes such as 2'-5' oligoadenylate synthetase (OAS) and RNA-dependent protein kinase (PKR) in hepatocytes<sup>[58]</sup>. The plasmacytoid DC recognizes HCV-related markers through TLR-7, which interacts with single-stranded RNA<sup>[59]</sup>. The TLR-signaling up-regulates PDC-triggering receptor expressed on myeloid cells (PDC-TREM) which induce further production of IFN- $\alpha$ <sup>[60]</sup>. Activated OAS destroys viral RNAs, whereas PKR inhibits forming polysomes of viral mRNA<sup>[58]</sup>. When HCV-specific CTL responses are not strong enough to eradicate the virus this leads to persistent infection<sup>[61]</sup>.

Successful clearance of HCV during acute HCV infection depends on the rise, vigor and persistence of the Th1 immune response<sup>[62,63]</sup>. Patients who developed a strong Th1 response showed efficient viral clearance and a self-limited disease course. In contrast, those who lacked IL-12 and IFN- $\gamma$  production invariably developed chronic persistence of the virus. The majority of patients fail to control the infection and develop a chronic infection with a variable degree of hepatitis and viremia<sup>[64,65]</sup>. Experimental studies have also demonstrated that HCV components induce an antigen processing mechanism and IFN-stimulated genes in infected livers<sup>[66-68]</sup>. Impaired function of DCs, as antigen-presenting cells in inducing immunity, may be responsible for the impaired immune responses. Various studies have reported that viral proteins including HCV core, E1, and NS3 inhibit DC maturation<sup>[69,70]</sup>. HCV infects DCs through the binding of HCV E2 protein and thereby suppress DC function in promoting an antiviral effect<sup>[41,71]</sup>.

CTLs activated by viral proteins, not only kill virus-infected cells, but also contribute to virus control through a noncytolytic mechanism by secreting cytokines, *e.g.*, IFN- $\gamma$ , IFN- $\alpha/\beta$  and TNF- $\alpha$ . These cytokines induce an antiviral state in host cells. This also renders uninfected cells resistant to infection and prevents viral replication. The progression of the majority of infected persons to chronic infection suggests inability of the antiviral immunity to contain this infection. There may be several reasons for this failure, including the emergence of escape variants as a result of a high rate of virus mutations, decreased production of antiviral cytokines or "stunning"



of HCV-specific CTLs, a compromised cytolytic potential of the CTLs and antagonistic peptides<sup>[72]</sup>.

It is important to note here that the HCV genome in a single host is a dynamic population of different, but closely related genomes, designated quasispecies. The generation of quasispecies is usually ascribed to high variation in hyper variable region-1 (HVR-1) during viral replication<sup>[73]</sup>. In acute resolving hepatitis, HVR-1 shows very little variation, as compared to that in chronic hepatitis<sup>[74]</sup>. HVR-1 induces anti-HCV neutralizing antibodies<sup>[75,76]</sup> and HVR-1 specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>[77,78]</sup>. Using the responding host cellular immune response differentially, HVR-1 favors viral escape<sup>[79,80]</sup>. HVR-1 variations result from the action of a continuous immune-driven positive selection<sup>[81,82]</sup>. Thus, HVR-1 complexity helps in the virus adaptive strategy to escape the immune onset. HCV clearance is associated with a vigorous HCV specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell response in the acute phase of infection. In contrast, viral persistence is associated with a weak and dysfunctional virus specific T cell response<sup>[79-83]</sup>. T cell failure and HCV immune evasion have been explained in several reports<sup>[84-86]</sup>.

### Role of T regulatory cells in adaptive immunity

Recent studies have suggested a possible role for different regulatory T cell populations in HCV persistence. These studies showed a higher frequency of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in the blood and CD4<sup>+</sup>FoxP3<sup>+</sup> T cells in the liver of chronically HCV infected patients<sup>[87-89]</sup>. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells suppress HCV specific CD8<sup>+</sup> T cell and CD4<sup>+</sup> T cell proliferation as well as CD8<sup>+</sup> T cell IFN- $\gamma$  secretion<sup>[87,90-92]</sup>. After HCV antigen stimulation, Treg cells secrete IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) which suppress virus specific T cell responses<sup>[91-93]</sup>. CD4<sup>+</sup>CD25<sup>+</sup> Treg cells obtained from chronically HCV infected patients demonstrated greater suppressive activity against HCV specific CD8<sup>+</sup> T cells compared to Treg cells isolated from acute HCV infected patients. However, the suppressive effect observed in patients who successfully cleared the virus was still significant<sup>[90]</sup>. Another study showed that the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells and their suppressive capacity against virus specific T cell responses were as high in HCV recovered chimpanzees as those in persistently HCV infected chimpanzees<sup>[94]</sup>. This observation requires further in-depth studies to explore the actual suppressive effect of Treg cells during HCV infection. Induction of Treg cells by HCV antigens was first demonstrated by the response of CD4<sup>+</sup> T cell to HCV core protein. HCV-specific IL-10 secreting T cells were detected in the blood of chronic HCV infected persons<sup>[95]</sup>. Regulatory CD8<sup>+</sup> T cells may play an important role in chronic HCV infection. HCV-specific CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells from the blood of chronically infected patients suppress HCV-specific T cell responses *via* TGF- $\beta$  secretion. The blockade of TGF- $\beta$  markedly enhanced HCV specific IFN- $\gamma$  secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>[96]</sup>.

Few other studies have shown that chronic HCV in-

fection results in exhaustion or impairment of HCV-specific CD8<sup>+</sup> T cells. During chronic HCV infection, CD8<sup>+</sup> T cells fail to proliferate or secrete antiviral cytokines including IFN- $\gamma$ . This phenomenon is promoted by a lack of CD4<sup>+</sup> T cells and the expression of immunomodulatory cytokines such as IL-10<sup>[97]</sup>. The major cause of HCV-specific CD8<sup>+</sup> T cell impairment is ascribed to the expression of inhibitory receptors such as Programmed death-1, lymphocyte-activation gene-3 (a protein related to CD4), CTLA-4 (a member of the CD28 receptor family), T-cell immunoglobulin mucin-3 and 2B4 on HCV-specific CD8<sup>+</sup> T cells in blood and liver<sup>[98]</sup>. Expression of these inhibitory receptors is associated with low levels of CD127 expression and impaired proliferation and differentiation of T cells. Thus, different mechanisms contribute to the dysfunction of HCV-specific CD8<sup>+</sup> T cells in chronic HCV infection.

In addition to cytotoxic T lymphocytes, humoral immune response against viral and cellular components during HCV infection is also present. Patients positive for HCV RNA and/or anti-HCV antibodies have type I anti-liver kidney microsome antibodies, which also recognize cytochrome P450 (CYP) 2D6. The patient's liver is infiltrated with auto reactive mononuclear cells, which recognize CYP2D6. It is interesting that the viral core protein residues 178-187 bear sequence homology with human cytochrome P450 (CYP2A6 and CYP2A7) residues 8-17<sup>[96]</sup>. Although HCV is a hepatotropic virus and infects hepatocytes, viral genome and its replicative intermediates are frequently present in peripheral blood mononuclear cells and lymphoid tissues of chronically infected persons. The viral glycoprotein E2 has been implicated in the oligoclonal expansion of several lymphoma cells<sup>[99]</sup>. The most common rheumatic and cutaneomucous symptoms in HCV-infected patients include fatigue, arthralgia, paraesthesia, myalgia, pruritus, and the sicca syndrome<sup>[100]</sup>.

## ROLE OF VIRAL PROTEINS AND GENOTYPES

The role of structural and non-structural components of the HCV virion has been explained by variation in their interactions with metabolites affecting pathogenic pathways leading to liver damage. HCV-core protein has a prominent role in all these interactions as compared to envelope and non-structural proteins. Moreover, when the mechanism of this interaction was studied in relation to various HCV genotypes, it was observed that different genotypes behave differently to regulate all these pathogenic pathways.

The role of NS5A and E2 region was found be important. NS5A has a role in viral replication, inactivating PKR<sup>[101-104]</sup>, blocking the apoptotic pathway, binding of growth factor receptor-bound protein 2<sup>[105,106]</sup> and induction of anti-inflammatory interleukin secretion<sup>[107,108]</sup>. Similarly, E2 protein inhibits PKR<sup>[109,110]</sup>. The region of NS5A which interacts with PKR, shows clustering of amino acid changes during IFN treatment and plays an

important role in the evasion mechanism<sup>[111]</sup>. Furthermore, this association varies with genotype and thus, alters their sensitivity to IFN treatment. NS5A remains under strong immune selection, has T- and B-cell epitopes and possibly, in combination with individuals' HLA, selects immune cells to produce sensitivity/resistance to IFN therapy<sup>[112]</sup>. The functional activity of NS5A towards immune selection is clearly governed by the HCV-genotypes and varies accordingly. The response of genotype 2 and 3 to IFN treatment may be due to individuals recognizing the NS5A protein immunologically<sup>[113]</sup>.

Binding of HCV E2 protein to DCs induces their maturation. Several HCV viral proteins, including core, NS3, NS5A and NS5B proteins, have been shown to inhibit DC functions<sup>[69]</sup>. Consequently, the functions of both CD4<sup>+</sup> Th cells and CD8<sup>+</sup> CTLs are impaired in chronic HCV patients. This has been suggested to be one of the mechanisms that HCV utilizes to weaken host immune responses and spread the infection. Indeed, many clinical studies have shown that in chronic HCV patients, not only the functions of DCs are impaired<sup>[113,114]</sup>, the functions of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are also impaired<sup>[115]</sup>. A similar inductive effect of E2 protein was also reported in other cell types, including T cells, B cells<sup>[116]</sup>, hepatocytes<sup>[117]</sup> and hepatic stellate cells<sup>[118]</sup>.

The role of HCV genotypes in the progression of liver disease is one of the most controversial areas of HCV research. In patients with chronic HCV, infection with genotype-1b is reportedly associated with a more severe liver disease and a more aggressive course than the infection with other HCV genotypes. Similarly, it was found that HCV genotype-1b was significantly more prevalent among patients with liver cirrhosis and those with decompensated liver disease requiring liver transplantation than among those with chronic active hepatitis C<sup>[119-121]</sup>. Although this is indirect evidence, it suggests an association between HCV genotype-1b and the development of these complications. HCV genotype-1b is a marker for more severe HCV associated liver disease, because it reflects a longer time of infection than a mere aggressive form of hepatitis C.

## METABOLIC CONDITIONS AFFECTING HCV PATHOGENESIS

In addition to immune mediated HCV pathogenesis, there are several other clinical and metabolic conditions that have a strong association with HCV pathogenesis. These include HCV-induced insulin resistance, oxidative stress and hepatic steatosis. The following is a brief description of the conditions affecting HCV pathogenesis:

### HCV-induced insulin resistance

HCV infection influences overall metabolism leading to increased steatosis, fibrosis, inflammation, apoptosis and insulin resistance (IR)<sup>[122,123]</sup> during the course of the disease. The resulting IR shows a modulating impact on liver pathogenesis by HCV infection<sup>[124]</sup>. IR increases the

*de novo* lipogenesis *i.e.*, fatty acid (FA) synthesis *via* over-expression and maturation of SREBP-1c. This in turn, increases the activities of lipogenic enzymes including Acetyl CoA carboxylase and FA synthase. At the same time, intermediates of triglyceride biosynthesis also activate inhibitors of insulin signaling. For example, activation of protein kinase C- $\epsilon$  by phosphorylating insulin receptor substrate, and thus inhibiting phosphatidylinositol-3,4,5-triphosphate<sup>[125]</sup>, inhibits Akt translocation by ceramides *etc.*<sup>[126]</sup>. HCV-core protein, either directly or *via* increased secretion of TNF- $\alpha$ , causes IR<sup>[127,128]</sup>. The HCV core can activate inhibitors of insulin signaling including mammalian target of rapamycin<sup>[129]</sup> and suppressor of cytokine signaling (SOCS)-3 and C-Jun N-terminal kinase (JNK)<sup>[130,131]</sup>. The activation of JNK by HCV core may follow a direct or indirect proinflammatory cytokine-mediated mechanism.

### HCV-associated oxidative stress

Oxidative stress is reported to be an important part of HCV-induced liver damage. Previous studies investigated the role of different molecular components of HCV structure in modulating oxidative stress during HCV infection. HCV-core protein present within the outer membrane of mitochondria induces oxidation of glutathione and promotes Ca<sup>2+</sup> uptake into mitochondria. Clément *et al.*<sup>[96]</sup> explained the molecular mechanism and demonstrated that following glutathione oxidation, there is increased reactive oxygen species (ROS) production by mitochondrial electron transport complex I and III. The HCV non-structural protein, NS5A, promotes ROS production in the membrane of endoplasmic reticulum (ER) by activating the release of Ca<sup>2+</sup> from ER, thereby inducing oxidative stress<sup>[97]</sup>. NS3 protein induces ROS production by activation of NADPH oxidase<sup>[97]</sup>. Increased ROS production and consequent oxidative stress is evident by the presence of markers of increased oxidative stress in the blood. Levels of 8-hydroxy deoxyguanosine and 4-hydroxy-2-nonenol are increased in HCV infection<sup>[132,133]</sup>. Similarly, few studies have shown reduced levels of glutathione during HCV infection. Another study showed that the serum level of thioredoxin, a marker of oxidative stress, was significantly reduced in HCV infection<sup>[134-136]</sup>.

The presence of oxidative stress has been noted in different types of hepatitis including hepatitis B. However, there is a marked increase in oxidative stress (OS) in HCV infection<sup>[132]</sup>. Several studies have shown that structural components of HCV induce effective OS<sup>[132]</sup>. HCV-core and non-structural components, NS3 and NS5A proteins, directly induce OS<sup>[137-139]</sup>. Core protein is involved in OS generation *via* oxidation of mitochondrial glutathione and uptake of Ca<sup>2+</sup> into mitochondria<sup>[139,140]</sup> thus, changing the permeability of its membrane<sup>[141]</sup>. Electron transport complex I increases production of ROS and redistributes cytochrome from mitochondria to the cytosolic fraction<sup>[93]</sup>. NS5A is associated with the ER membrane<sup>[142]</sup> and activates signal transducer transcription and NF $\kappa$ B<sup>[107]</sup>. These activations lead to inflammation,

immune response and apoptosis<sup>[143]</sup>. Similarly, NS3 triggers ROS by activating NADPH oxidase 2 in mononuclear and polymorphonuclear phagocytes<sup>[144]</sup> which increase apoptosis of hepatocytes<sup>[144]</sup>. All these reports conclude that the structural and non-structural components of HCV induce a significant increase in OS that results in liver damage during HCV infection.

### HCV-induced steatosis

HCV infection is reported to have a strong association with hepatic steatosis. There are several other factors also responsible for steatosis, which include alcohol consumption, obesity, and diabetes<sup>[145-147]</sup>. Studies on steatosis in relation to hepatotropic viruses demonstrated that HCV infection directly causes steatosis in some patients<sup>[148]</sup>. Studies in experimental animals have shown that HCV-core protein promotes liver steatosis<sup>[149,150]</sup>. Furthermore, when steatosis was studied in relation to HCV-genotypes, it was noted that although steatosis is induced by all HCV-genotypes, it appears more prominent and frequent with HCV-genotype 3 infection<sup>[151-153]</sup>. In patients carrying genotype-3 infection, there was a good correlation between the level of steatosis and HCV replication<sup>[153,154]</sup> and the presence of HCV-core in the liver. In addition, steatosis resolves in patient with genotype-3 when treated successfully with anti-viral therapy as compared to those with non-genotype-3 who remain steatotic<sup>[155,156]</sup>. Steatosis reappears with relapse of infection<sup>[155]</sup>. This clearly demonstrates that some HCV-genotypes have more steatogenic potential. Subsequent studies<sup>[157]</sup> indicated that genotype-3 interferes with very low-density lipoprotein (VLDL) secretion. Core protein, which promotes lipid accumulation in hepatocytes<sup>[158,159]</sup>, was more efficient from genotype-3 compared to core protein from genotype-1.

All these reports concluded that HCV causes steatosis in three different ways: (1) Impaired secretion of lipids from hepatocytes; (2) Increased *de novo* synthesis of free fatty acids (FFAs); and (3) Impaired FA degradation. The first aspect of HCV-induced steatosis was proposed due to the impaired secretion of VLDL. To substantiate this, reports from different studies demonstrated a decreased level of apolipoprotein B and cholesterol in chronic HCV infected patients<sup>[159,160]</sup>. These low levels pointed to HCV disturbing the assembly and secretion of VLDL from the liver<sup>[161]</sup>. Another important aspect in this relationship was increased *de novo* synthesis of FFAs in the presence of HCV infection. In this context, it is suggested that HCV upregulated the sterol regulatory element binding protein-1c (SREBP-1c) signaling pathway<sup>[158]</sup> with NS2 and NS4B proteins inducing SREBP at the transcriptional level<sup>[162,163]</sup>. SREBP was also induced by expression of HCV core protein. Studies in chimpanzees infected with HCV also demonstrated that HCV increased the activity of lipogenic enzymes such as ATP citrate lyase<sup>[164]</sup>. HCV-core, in particular, activates and helps in cellular lipid synthesis<sup>[164]</sup>, possibly *via* its binding with retinoid receptor.

HCV-induced steatosis is also due to impaired FA

degradation by HCV. Expression of HCV-core protein is reported to reduce the expression of peroxisome proliferation activated receptor- $\alpha$  (PPAR $\alpha$ ), a nuclear receptor involved in FA degradation and down-regulation of mitochondria  $\beta$ -oxidation<sup>[165]</sup>. Genotype-3 shows significant down-regulation of PPAR $\alpha$  as compared to genotype-1<sup>[166,167]</sup>. HCV-core protein also down-regulates PPAR $\alpha$  and therefore, is more effective when from genotype-3 as compared to genotype-1. The core protein from genotype-3 also down-regulated the PPAR $\gamma$  and up-regulated SOCS-7 in human hepatoma cells<sup>[167]</sup>. These data clearly show that HCV-core protein may modulate the expression of various genes responsible for FA degradation *via* down-regulation of PPARs.

## CONCLUSION

HCV infection, previously known as blood borne non-A, non-B infection, is a serious public health problem worldwide. The diagnosis of HCV is based on the detection of anti-HCV antibodies and/or viral nucleic acid in serum. Studies over the last few years have developed assays not only for the accurate serodiagnosis of infection, but also identification of HCV serotypes. The pathogenesis of HCV infection is quite complex and regulated by host immunity as well as several metabolic activities influencing liver function. Whereas both innate and adaptive immunity are involved in the pathogenic action of HCV, the cytotoxic lymphocytes are crucial in deciding the eradication or persistence of viral particles. Moreover, the persistence of HCV infection is also affected by viral proteins, HCV isotypes and liver metabolism. In order to understand HCV pathogenesis further investigations are needed.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Scotomas in molecular virology and epidemiology of hepatitis C virus

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## Abstract

In the 1970s, scientists learned of a new pathogen causing non-A, non-B hepatitis. Classical approaches were used to isolate and characterize this new pathogen, but it could be transmitted experimentally only to chimpanzees and progress was slow until the pathogen was identified as hepatitis C virus (HCV) in 1989. Since then, research and treatment of HCV have expanded with the development of modern biological medicine: HCV genome organization and polyprotein processing were delineated in 1993; the first three-dimensional structure of HCV nonstructural protein (NS3 serine protease) was revealed in 1996; an infectious clone of HCV complementary DNA was first constructed in 1997; interferon and ribavirin combination therapy was established in 1998 and the therapeutic strategy gradually optimized; the HCV replicon system was produced in 1999; functional HCV pseudotyped viral particles were described in 2003; and recombinant infectious HCV in tissue culture was produced successfully in 2005. Recently, tremendous advances in HCV receptor discovery, understanding the HCV lifecycle, decryption of the HCV genome and proteins, as well as new anti-HCV compounds have been reported. Because HCV is difficult

to isolate and culture, researchers have had to avail themselves to the best of modern biomedical technology; some of the major achievements in HCV research have not only advanced the understanding of HCV but also promoted knowledge of virology and cellular physiology. In this review, we summarize the advancements and remaining scotomas in the molecular virology and epidemiology of HCV.

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**Key words:** Hepatitis C virus; Hepatitis C virus lifecycle; Molecular virology; Hepatitis C virus models; Epidemiology

**Core tip:** The review summarizes the advancements, as well as remaining scotomas, in the molecular virology of hepatitis C virus (HCV). We emphasize the contributions of HuH-7 hepatocellular carcinoma cell line to development of the HCV replicon, cell culture-derived HCV, and HCV pseudoparticles. In addition, we reiterate the importance of epidemiological issues because accurate assessment of HCV-related disease burden has been overlooked. This review provides a history of the fight against HCV, which has required scientists to avail themselves to the best of modern biomedical technology, which in turn has enriched our knowledge of virology and cellular physiology.

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## INTRODUCTION

The hepatitis C virus (HCV) is an enveloped, single-

stranded, positive-sense RNA virus, classified as a *Hepacivirus* within the *Flaviviridae* family<sup>[1-3]</sup>. The 9.6-kb RNA genome contains one long open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR)<sup>[4-6]</sup>. The single ORF encodes an approximately 3000 amino acid (aa) polyprotein that undergoes co- and post-translational cleavage by host and viral proteases to yield 10 viral proteins, not including the F protein<sup>[7,8]</sup>. The structural proteins, nucleic acid-binding nucleocapsid core protein and envelope proteins (E1 and E2/P7) are encoded by 25% of the N-terminal portion of the genome<sup>[9]</sup>. The remaining 75% of the genome encodes the non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B<sup>[9]</sup>.

Humans are the primary reservoir of HCV<sup>[10]</sup>. HCV transmission occurs primarily through exposure to infected blood and the majority of individuals with persistent infection develop chronic hepatitis, which can progress to cirrhosis or hepatocellular carcinoma<sup>[11-13]</sup>. Different from other viruses, such as influenza A viruses and human immunodeficiency viruses, HCV is difficult to isolate and culture<sup>[14-16]</sup>. Since HCV was identified in 1989<sup>[1]</sup>, basic research on HCV has been being hindered by the absence of reliable, reproducible, and efficient culture systems<sup>[11]</sup>. Recently, tremendous advances in understanding the HCV replicon<sup>[15,16]</sup>, the pseudo-typed HCV viral particle<sup>[17]</sup>, cell based culture systems<sup>[18,19]</sup>, receptors<sup>[20-24]</sup>, life cycle<sup>[25,26]</sup>, structural biology and HCV therapy strategy<sup>[27-29]</sup> have been gained. However, several scotomas in the molecular virology and epidemiology of HCV remain to be elucidated. This review summarizes the advancements and remaining scotomas in the molecular virology and epidemiology of HCV.

## MAJOR PROGRESS IN FIGHTING HCV

Since HCV was identified in 1989<sup>[1]</sup>, virological research has led to a great deal of progress in the pathogenesis, diagnosis, treatment, control and prevention of the disease<sup>[11,30]</sup>. Since virus elimination is the ultimate goal of viral disease therapy, here we emphasize two major recent achievements in hepatitis C treatment. The first achievement was the development of direct-acting antiviral (DAA) agents, which are inhibitors of the HCV protease<sup>[31-37]</sup>. Although peginterferon and ribavirin remain vital components of therapy, the emergence of DAA agents has led to an unprecedented improvement in sustained virologic response rates to approximately 94%<sup>[30,38]</sup>. This is indicative of two milestones in virology: a therapy with the highest documented antiviral effects and optimism that the virus could be eliminated by medications. The second achievement is the identification of several single-nucleotide polymorphisms associated with spontaneous and treatment-induced clearance of HCV infection<sup>[39,40]</sup>. This discovery is also a milestone because only the rare single nucleotide polymorphisms of rs12980275 and rs8099917, near the interleukin28B gene, have any reported biological effect<sup>[39,40]</sup>.

## CURRENT HCV MODELS

The *in vitro* and *in vivo* models for HCV have evolved significantly since the discovery of the virus. With any virus, cell-based culture systems and animal models are the essential tools for virological study, vaccine development, and antiviral drug discovery. Many viruses, such as the influenza virus, are easy to isolate and culture in cell lines<sup>[41,42]</sup>; however, HCV is difficult to isolate and propagate<sup>[15,16,18,19]</sup>. Before HCV was identified, many virologists had attempted to isolate and culture the pathogen of non-A, non-B hepatitis using traditional cell-based approaches<sup>[43]</sup>. After struggling for decades, it was determined that HCV could only survive in human or chimpanzee fetal liver cells and hepatocytes or human peripheral blood mononuclear cells<sup>[44-49]</sup>. These cells are inconvenient to obtain and have a finite lifespan in culture, so even though these early studies showed that HCV was selective with a narrow host range<sup>[50,51]</sup>, these methods made little contribution to HCV research (Table 1).

Defeated by classical approaches to isolate HCV, virologists were forced to reproduce the HCV lifecycle using split models, which included the HCV genome RNA replication model (HCV replicon)<sup>[15,16]</sup>, HCV structural proteins model (virus-like particle, VLP)<sup>[52]</sup> and HCV pseudotyped viral particles model<sup>[17]</sup> (Table 1). In 1999, Bartenschlager's group in Germany established a HCV replicon system<sup>[15]</sup>, followed soon after in 2000 by Rice's group in the United States<sup>[16]</sup>. These models simulated the structure of the subgenomic selectable HCV replicons composed of the HCV 5'-UTR, the gene encoding the neomycin phosphotransferase or firefly luciferase, the encephalomyocarditis virus internal ribosome entry site, the region encoding HCV NS2-5B or NS3-5B, the authentic 3'-UTR, and the 12-16 5'-terminal codons of the core<sup>[15,16]</sup>. The replicon could replicate autonomously in hepatic cell cultures (*e.g.*, HuH7 cell line)<sup>[15,16]</sup>, leading to a series of experiments that examined the function of 5'- and 3'-UTR and NS3 to NS5B in HCV genome replication, described the viral life cycle, and led to the development of antiviral drugs<sup>[27]</sup>. The HCV replicon was able to replicate itself within the cell; however, it was not capable of producing infectious viruses<sup>[27]</sup>. Furthermore, this replicon was not able to reproduce in HuH-7 cells with high efficiency and for an extended period of time<sup>[50]</sup>. Virologists attempted to improve the replication efficiency and modify the robust HCV replicon using several methods, including adaptive mutation hunting, to reduce the non-HCV genome and increase the HCV genome composition, by attempting to replace various wild HCV strains of different genotypes<sup>[27,50,53]</sup>.

Pseudotyped viral particles are commonly known as lentiviral vectors. These vectors are composed primarily of three viral elements; the gag-pol, which forms the viral structure, recognizes the viral genome and is responsible for the genome lifecycle; the viral mimic genome, which provides the genome elements that will be recognized

Table 1 Summary of *in vitro* and *in vivo* models for hepatitis C virus

<i>In vitro</i> and <i>in vivo</i> models	Established year	Advantages	Deficiencies
<i>In vitro</i>			
Cultivation of HCV	1993-1999	Achieved cultivation of HCV in human foetal liver cells, human hepatocytes or PBMC. Illustrated HCV is quite species selective and has a narrow range of hosts	Requires specific cellular factors to support viral lifecycle. Primary human and chimpanzee hepatocytes or highly differentiated cells dependent. Most of them have yielded limited success. Poor reproducibility and low levels of HCV replication
HCV replicon	1995-2000	Provided a cell-based model for the study on HCV genome replication	
HCV VLP	1998-1999	Rare evidence to support that HCV structural proteins core, E1, and E2 could form VLP	
HCVpp	2003	Provided a convenient and feasible tool for studies on viral entry, HCV receptor, neutralizing antibody, etc.	
HCVcc	2005	A break through in production of infectious hepatitis C virus in tissue culture	
<i>In vivo</i>			
Chimpanzee	1979	The only recognized animal model for HCV study, played a critical role in HCV discovery and play an essential role in defining the natural history of HCV	Chimpanzees differ from humans in their course of infection, that chronic carriers do not develop cirrhosis or fibrosis, limited availability, cost performance, and public resistance
Tree shrew	1998	Might be a succedaneum for chimpanzees	Persistent HCV infection could not be established and only 25% of infected animals developed transient or intermittent viremia. Germ line was not available to a small animal model
Chimeric human liver mouse	2001	Exhibited prolonged infection with high viral titers following inoculation with HCV isolated from human serum. HCV can be transmitted horizontally. Drug evaluation	Since the mice were immunodeficient, they were not appropriate models to study HCV pathogenesis
Genetically humanized mouse	2011	Represents the first immunocompetent mice model for HCV study. Allows for the studies of HCV coreceptor biology <i>in vivo</i>	Operation is difficult

HCVpp: Hepatitis C virus (HCV) pseudotyped viral particles; VLP: Virus like particle; HCVcc: Cell culture derived HCV.

by gag-pol and ensures complete viral RNA metabolism; and the envelope proteins, which are presented onto the artificial viral particle<sup>[54-58]</sup>. Additionally, a lentiviral vector contains a reporter gene inserted into the artificial viral genome. Although the lentiviral vector was used widely in gene transduction, presenting an HCV envelope protein functionally in this viral particle was not considered. Virologists tried to generate the HCV VLP<sup>[52]</sup>, because classic virological experience told us that VLP of a certain virus could be produced by cloning and expressing virus structural proteins, and Liang's group at the National Institutes of Health (Bethesda, MD) was successful in establishing the HCV-like particles using a baculovirus expression vector system<sup>[52]</sup>. In 2003, French virologist Bartosch *et al*<sup>[17]</sup> produced HCV pseudoparticles (HCVpp) using viral elements derived from murine leukemia virus. The HCVpp system led to advanced studies that identified a neutralizing antibody against HCV<sup>[59]</sup>, explored HCV receptors and described the structure and function of the HCV envelope proteins<sup>[60-63]</sup>. In 2005, the Japanese virologist Wakita obtained a genotype 2a HCV strain (JFH-1) from a Japanese patient with a rare case of fulminant hepatitis C<sup>[18]</sup>. Based on the experience and methods accumulated in studying the HCV replicon, Wakita and his group rescued HCV in the HuH7 cell line, which was designated as HCVcc, for cell-culture-derived HCV<sup>[18]</sup>. HuH-7 cells infected with cloned

and *in vitro* transcribed JFH-1 genomes produced viruses that were capable of infecting naïve HuH-7 cells<sup>[18]</sup>. In addition, the virus particles could be neutralized with a monoclonal antibody against the viral glycoprotein E2<sup>[18]</sup>. The study was the first *in vitro* experiment that showed the complete lifecycle of HCV. More importantly, virus obtained from the cell cultures was highly infectious in chimpanzees and immunodeficient mice with partial human livers<sup>[64]</sup>. As early as the 1970s, it was known that the etiological agent responsible for non-A and non-B hepatitis could be transmitted to chimpanzees<sup>[65]</sup>, and chimpanzees were subsequently recognized as the only animal model of HCV<sup>[66]</sup> (Table 1). Chimpanzees played a critical role in defining the natural history of HCV<sup>[66]</sup> and since they are closely related to humans, any study of chimpanzees could reflect more closely what happens in humans than other animal models. However, chimpanzees that are chronic carriers of HCV do not develop cirrhosis or fibrosis<sup>[66,67]</sup>, which are the most important consequences of HCV infection in humans. Because chimpanzee studies are expensive and restricted by ethical responsibilities<sup>[67]</sup>, scientists diverted their attention to other small animal models, such as the tree shrew and a chimeric human liver mouse. Xie *et al*<sup>[68]</sup> demonstrated that *Tupaia* could be infected by HCV when severely immunosuppressed; however, persistent



HCV infection could not be established and only 25% of infected animals developed transient or intermittent viremia<sup>[51]</sup>. By genetically manipulating the urokinase-type plasminogen activator transgenic mouse, Mercer *et al*<sup>[69]</sup> transplanted normal human hepatocytes into severe combined immunodeficient mice carrying a plasminogen activator transgene. The chimeric mice exhibited prolonged infection with high viral titers following inoculation with HCV isolated from human serum<sup>[69]</sup>. Since the mice were immunodeficient, they were not appropriate models for investigation of HCV pathogenesis, although they were useful in assessing the activity of antiviral drugs, as well as neutralizing antibodies<sup>[51]</sup> (Table 1).

Mouse models of HCV provided little information about the human hepatocellular factors required for HCV entry. Thus, Ploss *et al*<sup>[24]</sup> introduced human CD81, scavenger receptor type B class 1, claudin 1, and OCLN genes into mice using a recombinant adenovirus expression system. They found that mice expressing these human factors were sufficient for HCV infection<sup>[24]</sup>. This system allowed for the investigation of HCV co-receptor biology *in vivo* and evaluation of passive immunization strategies and, therefore, represented the first immunocompetent small animal model for HCV<sup>[70]</sup> (Table 1).

## SCOTOMAS IN MOLECULAR VIROLOGY

Although much progress has been made in all aspects of HCV research in the last few decades, we are still far from achieving the ultimate goal of complete HCV control and prevention. Thus, a better understanding of the HCV life cycle is essential to optimize the antiviral strategy. As mentioned above, the major challenges to HCV research are that HCV is difficult to isolate and culture, and no vaccine is available<sup>[14-16,71]</sup>. However, rather than summarizing the many achievements in HCV research, we have chosen to enumerate the scotomas in HCV molecular virology.

### Structural biology of the HCV particle

Since HCV was first proposed to be a distinct infectious pathogen, virologists of that era attempted to visualize this enigmatic microbe using electron microscopy<sup>[43]</sup>. Different from other hepatitis viruses, including hepatitis A virus, hepatitis B virus and hepatitis E virus and the other viruses within the *Flaviviridae* family<sup>[43]</sup>, no clear electron microscope image of HCV was reported until Chisari's and Rice's groups provided high-resolution images of highly enriched cell culture-derived HCV (HCVcc) particles in 2010 and 2013, respectively<sup>[72,73]</sup>. The reason for the difficulty in observing the crude HCV particle in HCV-harboring tissue remains unclear. The viral titer should not be an issue since viral copies in blood samples produced by HCV RNA are  $> 10^6/\text{mL}$ <sup>[74]</sup>. Serum-derived HCV particles are associated with the lipoprotein components apolipoprotein A-I (apoA-I), apoB-48, apoB-100, apoC-I and apoE<sup>[75]</sup>. The interaction between virus particles and serum lipoproteins suggests that HCV may form

hybrid lipoviral particles<sup>[75]</sup> that facilitate virus entry into hepatocytes and protect the virus from the host immune response. In addition, the lipoprotein components might affect the morphological observation of crude HCV particles by electron microscopy. Our current knowledge of HCV morphology indicates that HCV particles are 40-80 nm in diameter, pleiomorphic, lack obvious symmetry or surface features and contain electron-dense cores<sup>[72,73]</sup>. The lack of details describing the overall architecture of HCV limits the ability of molecular biologists to study HCV structural biology and topology.

### Molecular virology of HCV structural proteins

A quarter of the N-terminal region of the HCV polyprotein encodes the core structural protein and glycoproteins E1 and E2, which are believed to be incorporated into the HCV particle<sup>[17]</sup>. Based on general virological knowledge, the core protein should be a major component of the viral capsid, responsible for viral genome RNA recognition, binding and packaging<sup>[27]</sup>. Glycoproteins E1 and E2 located in the viral surface are also called envelope proteins; E1 and E2 are responsible for receptor recognition, receptor binding, endocytosis and membrane fusion<sup>[27]</sup>. The mature core protein contains a positively charged N-terminal RNA binding domain and a C-terminal domain that consists of two amphipathic helices and a palmitoylated cysteine residue to facilitate peripheral membrane binding<sup>[27]</sup>. Antibodies against core proteins are important for HCV serological detection<sup>[43]</sup>. Previous studies demonstrated that the core protein is involved in many pathogenic processes<sup>[43]</sup>. Furthermore, the core protein induces hepatocellular carcinoma in transgenic mice<sup>[76]</sup> and is a potent inhibitor of RNA silencing-based antiviral response<sup>[77]</sup>. However, the basic function of the core protein in capsid formation remains unknown. Although the region between amino acids 82 and 102 contains a tryptophan-rich sequence involved in homotypic core proteins interaction<sup>[78]</sup>, the HCV core particle had not been successfully produced. *In vitro* nucleocapsid reconstitution experiments using the 1-124 or 1-179 core segments and structured RNA molecules have yielded irregular particles larger than those reported by the limited electron microscopy observations<sup>[79]</sup>. Scientists in China at Xiamen University successfully generated human papillomavirus VLP<sup>[80]</sup>, hepatitis E virus VLP<sup>[81]</sup> and hepatitis B virus core capsids but failed to produce the HCV capsid (personal communication). In addition, the crystal structure of the HCV core protein is not yet available (Table 2).

E1 and E2 are type I transmembrane glycoproteins assumed to be class II fusion proteins, with N-terminal ectodomains of 160 and 334 amino acids, respectively, and a short C-terminal transmembrane domain of approximately 30 amino acids<sup>[27,43]</sup>. Studies of HCVpp have indicated that 14 amino acids from the HCV core and 12 amino acids from the E1 C-terminus are required for E1 and E2 function<sup>[62]</sup>. The hemagglutinin and neuraminidase of influenza A viruses matches each other in a

Table 2 Summary of the properties of hepatitis C virus structural proteins

	Core	E1	E2	p7
Genome location	342-914	915-1490	1491-2579	2580-2769
Translation processing site		Rough ER		
Amino acid composition	191	192	363	63
Molecular weight (kDa)	21-23	33-35	70-72	7
Glycosylation	No	Yes	Yes	No
Cleavage		ER signal peptidase and SPP		
Crystal structure		Not available		
Functional unit	Dimer	Heterodimer?		Revealed Hexamer
Common function	Viral particle formation. Core, E1 and E2, together with p7 and NS2, are required for virus assembly (assembly module)			
Unique function	Capsid protein, viral particle formation, viral genome recognizing and packaging. Interacts with cLDs in early viral particle formation process. Counters host antiviral factors and involves pathogenesis	Envelope glycoproteins, interact with SRB1, CD81, CLDN1, OCLN, <i>etc.</i> to trigger viral entry. Promote fusion with the endosomal membrane. Counter host immune response <i>via</i> hypervariable regions		Viroporin. Has key roles in organizing the virus assembly complex. p7-NS2 complex interacts with the NS3-4A enzyme to retrieve core protein from cLDs to form viral particle
Major scotomas	How do the core form the viral capsid? The signals and processes that mediate RNA packaging are largely unknown. What impeded us to resolve the structure of the viral glycoproteins? What is the real process in HCV entry? How are these receptors and co-receptors temporally and spatially used to ensure the early infection processes?			

Start co-ordinates based on H77 (accession number, NC\_004102). SRB1: Scavenger receptor class B member 1; CD81: Tetraspanin CD81; CLDN1: The tight junction protein claudin 1; OCLN: The tight junction protein occludin; cLDs: Cytosolic lipid droplets; ER: Endoplasmic reticulum; SPP: Signal peptidase and signal peptide peptidase; HCV: Hepatitis C virus. The molecular weights of E1 and E2 refer to the glycosylated forms.

relative slack manner regardless of gene homology<sup>[54-58]</sup>, while the matching pattern of HCV E1 and E2 is relatively strict. We separated E1 and E2 of HCV genotypes 1a, 1b, and 2a into two individual expression plasmids and replaced the transmembrane domains of 1b and 2a E1 and E2 with that of genotype 1a. The complementation features of E1 and E2, as well as the contributions of both the ecto- and transmembrane domains to the formation of the E1E2 complex, were evaluated using the HCVpp system<sup>[63]</sup>. We found that 1aE2 could not only complement its native 1aE1 but also 1bE1; in genotype 1b, glycoprotein complex formation is dependent primarily on the overall biological characteristics of the intact native E1 and E2; in genotype 2a, although the interaction of intact native E1 and E2 is critical for the formation of the glycoprotein complex, the ectodomain made a greater contribution than did the transmembrane domain<sup>[63]</sup>. This study suggested that E1 and E2 formed a functional envelope protein complex dependent on E1 and E2 expression<sup>[63]</sup>. E1 and E2 are assembled as non-covalent heterodimers<sup>[82,83]</sup>, although the number of E1 molecules necessary to aggregate with E2 for biological function has not been elucidated. We highlight this scotoma because the envelope proteins of many viruses do not function simply in a 1:1 ratio<sup>[54-58]</sup> and viral proteins are not translated in equal numbers<sup>[43]</sup>. A lack of understanding of this point will impede receptor discovery, regardless of how many receptors and co-receptors are identified<sup>[75]</sup>. The crystal structure of the dengue virus glycoprotein, which is another member of the *Flaviviridae* family, was revealed in 2004<sup>[84]</sup>. By contrast, virologists failed to produce the HCV E1 or E2 crystal, which limits our understanding of the biological characteristics of HCV envelope proteins. Since one of the most important biological functions of HCV envelope proteins is

membrane fusion, E1 and E2 are assumed to be class II fusion proteins<sup>[83]</sup>. This assumption has been challenged by recent studies suggesting that HCV and pestiviruses share an uncharacterized mechanism of membrane fusion<sup>[85]</sup>. These contradictory issues are common in HCV research and await further advances in our understanding of HCV virology (Table 2).

Molecular virology of non-structural HCV proteins

HCV proteins can be categorized into an assembly module (from core to NS2) and a replication module (from NS3 to NS5B) on the basis of viral essential functions<sup>[27,75]</sup>. Details on the function of structural and non-structural HCV proteins are lacking due to the limitations of *in vitro* models. There is also some controversy on topics such as whether the assembly module is necessary for viral particle formation or whether p7 is a structural or non-structural protein. Although the HCV replicon system provided solid evidence that non-structural proteins activate HCV RNA replication *in vitro*<sup>[15,16]</sup>, some unresolved issues remain. These include why it is not possible to turn this system into a fully competent HCV cell culture model or why all replicons, except for the genotype 2a JFH-1 clone, contain cell-culture-adaptive mutations that when introduced back into viral genome, render it non-infectious in chimpanzees<sup>[67]</sup>.

The p7 polypeptide is a small, 63-aa intrinsic membrane protein with a double-membrane-spanning topology in which its N- and C-terminal ends face the ER lumen<sup>[27]</sup>. Recent data indicate that p7 can mediate membrane ion permeability and form hexamers<sup>[86,87]</sup>. The three-dimensional structure of a hexameric p7 channel revealed a highly tilted, flower-shaped protein architecture with six protruding petals oriented toward the ER lumen<sup>[86,87]</sup>. These structural and membrane-permeability

Table 3 Summary of the properties of hepatitis C virus non-structural proteins

	NS2	NS3	NS4A	NS4B	NS5A	NS5B
Genome location	2769-3419	3420-5312	5313-5474	5475-6257	6258-7601	7602-9378
Translation processing site			Rough ER			
Amino acid composition	810-1026	1027-1657	1658-1711	1712-1972	1973-2420	2421-3012
Molecular weight (kDa)	21-23	70	8	27	56-58	65-68
Cleavage						
Crystal structure	C-terminal (aa904-1026) was solved					
Functional unit	Homodimer	Monomer or oligomer	Monomer	Not available	Revealed Homodimer	Revealed Monomer
Common function			Replication module			
Unique function	A metal-dependent proteinase, many functions dependent on the interaction with P7 and NS3. Participation in proteolytic cleavage at the NS2-NS3 junction of the polypeptide. Both the TMDs and protease domain of NS2 are required for the production of virus particles	The DAA targeting protein, NS3 was anchored in ER membrane by cofactor NS4A. NS3-4A complex has serine-type protease activity and NTPase/RNA helicase activities. Nonspecific cleavage of two critical interferon induction proteins: MAVS and TRIF	The central portion of NS4A, residues 21-32, intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site. The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication as well as assembly	A master organizer of replication complex formation. NTPase activity? RNA binding?	Produced as multiple phospho-variants. RNA-binding phosphoprotein involved in RNA replication. Phosphorylation of a specific serine residue within the C-terminus by CK II $\alpha$ is essential for virus assembly. The interaction of NS5A with the cLD-bound core protein is the key steps in HCV assembly	RNA-dependent RNA polymerase
Major scotomas	How HCV particles are organized? What is the accurate duty of each nonstructural protein in viral lifecycle? How do the nonstructural proteins utilize host cellular factors for its own survival? Why					

Start co-ordinates based on H77 (accession number, NC\_004102). Aa: Amino acid; TMD: Transmembrane domain; CK II: Casein kinase II; cLD: Cytoplasmic lipid droplet; LDL: Low-density lipoprotein; VLDL: Very-low-density lipoprotein; MAVS: Mitochondrial antiviral signaling protein; TRIF: TIR-domain-containing adaptor inducing interferon; HCV: Hepatitis C virus.

properties suggest that p7 belongs to the viroporin family and could play an important role in viral particle release and maturation<sup>[86,87]</sup>. However, the role of p7 in calcium and ion metabolism is unknown. Furthermore, HCVpp with or without p7 showed no changes in viral particle formation and pp infectivity<sup>[62,63]</sup>. A study of the closely related GB virus B, which infects tamarins and has an analogous but larger protein, p13, showed that p13 is processed into two components p6 and p7, and that p6 was dispensable while p7 was essential for infectivity<sup>[88]</sup>.

NS2 is a metal-dependent proteinase, whose functions are dependent on the interaction with p7 and NS3<sup>[27]</sup>. Although the NS2 protease is dispensable for RNA replication, NS2 participates in proteolytic cleavage at the NS2-NS3 junction of the polypeptide<sup>[27]</sup>. The transmembrane and protease domains of NS2 are required for infectious virus assembly<sup>[89]</sup>. Why NS2 is critical for viral particle formation remains unknown and the interactions between NS2 and other structural and non-structural viral proteins to form an unknown viral particle formation network should be explored (Table 3).

NS3 is a 70-kDa multifunctional protein anchored by the cofactor NS4A<sup>[27,89]</sup>. NS2/NS3 junction cleavage is essential to liberate fully functional NS3 protein<sup>[27,89]</sup>. NS3-4A is a non-covalent complex, with a serine protease located in the N-terminus (aa 1-180) and an NTPase/RNA helicase in the C-terminus (aa 181-631)<sup>[27,89]</sup>. The substrate specificity of NS3-4A is low and causes non-specific cleavage of host proteins; *e.g.*, mitochondrial antiviral-signaling (MAVS) and TIR domain-containing adaptor inducing interferon  $\beta$  (TRIF), and thus might impact host IFN response<sup>[90]</sup>. The central portion of NS4A, residues 21-32, intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site<sup>[27]</sup>. The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication, as well as assembly<sup>[27]</sup>. The DAAs telaprevir and boceprevir<sup>[31-37]</sup> are inhibitors targeting the NS3-4A protease that displayed promising effects in clinical trials, indicating that the NS3-4A protease is critical for viral life cycle (Table 3).

NS4B is a poorly characterized hydrophobic 27-kDa protein<sup>[27]</sup> comprised of a 66-aa N-terminal portion, a 120-aa central portion, and a 70-aa C-terminal portion<sup>[91]</sup>. Four transmembrane-spanning regions were predicted in the central portion, while the N-terminal portion plays an important role in assembly of a functional replication com-



plex<sup>[27,91]</sup>. Einav *et al*<sup>[92,93]</sup> and Thompson *et al*<sup>[94]</sup> demonstrated that NS4B harbors NTPase activity and has a role in viral assembly.

NS5A is a 447-aa membrane-associated protein that plays an important role in modulating HCV RNA replication and particle formation<sup>[91]</sup>. NS5A can be detected in basally phosphorylated and hyper-phosphorylated forms with molecular weights of 56- and 58-kDa, respectively<sup>[95,96]</sup>. NS5A is comprised of four domains: a N-terminal membrane anchor and three domains separated by two low complexity sequences<sup>[27,91]</sup>. The three separated domains are domain 1, aa 36-213; domain 2, aa 250-342 and domain 3, aa 356-447. Domains 1 and 2 are involved in RNA replication and domain 1 is involved in cellular lipid drop binding, domain 3 is essential for viral assembly and is involved in interaction with the core protein accumulated in cellular lipid drops<sup>[27,91]</sup>. Although studies showed that NS5A is critical for HCV RNA replication, deletions in D2 and D3 are tolerated in RNA replication<sup>[97]</sup>, and viable replicons and viruses harboring GFP insertions displayed no change on HCV RNA replication<sup>[98]</sup>. Phosphorylation of a specific serine residue within C-terminal by casein kinase II  $\alpha$  is essential for virus assembly<sup>[99]</sup>. The interaction of NS5A with the cytosolic lipid droplets-bound core protein is a key step in HCV assembly<sup>[97,100,101]</sup>.

NS5B is an RNA-dependent RNA polymerase (RdRp). Its crystal structure was revealed in 1999<sup>[102]</sup>, the active site is highly conserved and located in the palm subdomain<sup>[91]</sup>. The low substrate specificity allows for the incorporation of ribavirin into nascent RNA. Thus, ribavirin remains a perfect RNA analog in HCV therapy<sup>[103]</sup>. Although recombinant NS5B is available and its crystal structure is known<sup>[104,105]</sup>, its role in HCV RNA replication remains unclear (Table 3).

### 5'-non-translated regions and 3' non-translated regions

Viral non-translated regions (NTRs) and non-coding regions, harbor important biological functions, involving viral genome reorganization, replication, translation initiation, and viral assembly<sup>[106]</sup>. The HCV 5'NTR contains 341 bp (H77 strain, NCBI Reference Sequence: NC\_004102.1)<sup>[107]</sup>. The predicted secondary structure of the HCV 5'NTR consists of four domains (domains I-IV, numbered from 5' to 3'), and the largest domain III was further categorized into sub-domains a-f<sup>[107]</sup>. The major functional unit in the HCV 5'NTR is an IRES, which includes three domains (II-IV)<sup>[106-109]</sup>. Initiation of protein synthesis in host cells utilized by HCV is different from mRNA translation in eukaryotes because HCV initiates viral protein synthesis *via* its IRES, which is known as internal translation initiation. This process is a cap-independent mechanism of recruiting, positioning and activating the host cellular protein synthesis machinery driven by the HCV IRES<sup>[106-109]</sup>, which is relatively weak in directing protein translation compared to the IRESs of other viruses, and may contribute to an insufficient host immune response<sup>[110]</sup>. Although structural and biochemical stud-

ies of the IRES found in HCV have provided the most detailed information thus far regarding the mechanism of IRES driven translation, unresolved issues remain. For example, it is unknown whether the HCV IRES acts as one determining factor for hepatotropism or how the HCV 5'NTR interacts with the 3'NTR to support HCV RNA replication and polyprotein translation. Additionally, the biological impact of the NTR to each hepatitis virus remains unclear since the HCV NTRs have a different structure compared to those of hepatitis A and E viruses. Finally, the interaction of miR-122 with the HCV 5'NTR to facilitate replication of viral RNA remains to be fully elucidated<sup>[111]</sup>.

The 3' terminal of any genome is technically difficult to identify and the available complete sequence of the HCV 3'NTR is unusual. The 3' UTR is divided into three structurally distinct domains from 5' to 3', an upstream variable region of about 40 nucleotides, a long poly (U)-poly (U/UC) tract and a 98-nucleotide (3' X) sequence that forms three stem-loop structures<sup>[43,106]</sup>. The long poly (U)-poly (U/UC) tract was a major obstacle to obtaining an HCV genomic clone because no known DNA polymerase could amplify this region and the fidelity of a reverse transcriptase in this region was suspect. The function of the 3' UTR remains to be determined<sup>[43,106]</sup>. It may play an important role in minus intermediate RNA and genome RNA synthesis during HCV RNA replication<sup>[43,106]</sup> since variable region deletions of RNA replicons could replicate, albeit at a much lower level<sup>[112]</sup>. However, deletion of either the poly (U/UC) or the 3' X was not viable, which suggested that the poly (U/UC) and 3'X regions are critical for HCV RNA replication<sup>[107]</sup>.

### HuH7 cell line

HCV researchers should be familiar with the human hepatocellular carcinoma cell line HuH-7, also known as Huh7 or HuH7. This cell line is critical because the HCV replicon, HCVcc, and HCVpp are all dependent on this cell line or its derivatives, indicating that it harbors all critical factors for HCV replication, assembly, budding and entry<sup>[43,113-115]</sup>. HuH-7 is a well-differentiated hepatocyte-derived hepatocellular carcinoma cell line that originated from a liver tumor in a 57-year-old Japanese male in 1982 (<http://huh7.com/>). It was established by scientists at Okayama University of Japan in the 1980s (<http://cellbank.nibio.go.jp/legacy/celldata/jcrb0403.htm>). HuH-7 remains the only hepatocellular carcinoma cell line that can fully support the HCV life cycle. Improvements in hepatocellular carcinoma cell line isolation could provide more effective HCV-supporting cell lines; alternatively the advances in induced pluripotent stem cells could result in a breakthrough in HCV culture and isolation.

## SCOTOMAS IN EPIDEMIOLOGY OF HCV

HCV carries a large disease burden in some countries and is the second most studied virus. Most HCV infections are subclinical with a long and insidious disease



**Table 4 Epidemiological features of hepatitis C virus infection**

Epidemiological index	Current consensus
Source of infection	Chronic HCV carriers
Route of transmission	HCV transmission occurs primarily through exposure to infected blood. Past: Receiving infected blood or organ transplantation, from accidental exposure to infected blood, and sexual transmission in persons with high risk behaviours. Present: HCV is usually spread by sharing infected needles with a chronic HCV carrier, and some people acquire the infection through nonparenteral means that have not been fully defined.
Susceptible population	General population
Incubation period	Average 6-10 wk
Prevalence and incidence	3% of the world's population have HCV
Rate of chronic infection	Up to more than 80%
Outcome of chronic infection	10%-20% of chronic HCV carriers may develop into cirrhosis and liver failure. 1%-5% of chronic HCV carriers are associated with the development of hepatocellular carcinoma
Molecular epidemiology	HCV is classified into eleven major genotypes (designated as 1-11), many subtypes (designated a, b, c, etc.), and about 100 different strains (numbered 1, 2, 3, etc.) based on the genomic sequence heterogeneity. Genotypes 1-3 have a worldwide distribution. Types 1a and 1b are the most common, accounting for about 60% of global infections. Type 2 is less frequently represented than type 1. Type 3 is endemic in southeast Asia and is variably distributed in different countries. Genotype 4 is principally found in the Middle East, Egypt, and central Africa. Type 5 is almost exclusively found in South Africa, and genotypes 6-11 are distributed in Asia.
Stability	HCV is inactivated by exposure to lipid solvents or detergents, heating at 60 °C for 10 h or 100 °C for 2 min in aqueous solution, formaldehyde (1:2000) at 37 °C for 72 h, $\beta$ -propiolactone and UV irradiation.
Vaccine	Not available

HCV: Hepatitis C virus.

course. However, epidemiological surveillance of HCV is relatively weak compared to some acute respiratory transmission diseases ([http://www.who.int/influenza/surveillance\\_monitoring/en/](http://www.who.int/influenza/surveillance_monitoring/en/)).

Chronic HCV carriers are the only reservoir of HCV since chimpanzees could be infected with HCV only experimentally<sup>[43,106]</sup>. HCV transmission occurs primarily through exposure to HCV-infected blood<sup>[43,106]</sup>. Blood transfusion, solid organ transplantation from an infected donor, and unsafe medical practices were the major transmission routes before HCV was identified in 1989<sup>[43,106]</sup>. Beginning in the early 1990s, strict screening of blood donors and precise control over the blood supply were implemented by national governments<sup>[43,106,116]</sup>. The majority of HCV infections are now limited to specific subpopulations, such as intravenous drug users and patients with certain hemopathies<sup>[117]</sup>. Although unsafe medical practices, occupational exposure to infected blood, maternal-fetal transmission, sex with an infected person and high-risk sexual practices are believed to be HCV transmission routes, the rate of acquisition of infection by these routes is low<sup>[118]</sup>. The average incubation period is 6-10 weeks and most virologists and hepatologists consider that up to 80% of HCV infected persons do not eliminate HCV spontaneously<sup>[43,106]</sup>. Cirrhosis and liver failure develop in 10%-20% of chronic HCV carriers; 1%-5% of chronic HCV carriers develop hepatocellular carcinoma<sup>[43,106]</sup> (Table 4).

The World Health Organization estimates that up to 3% of the global population is infected with HCV (<http://www.who.int/csr/disease/hepatitis/Hepc.pdf>), and the peak disease burden is expected around 2020<sup>[119]</sup>. However, these estimations lack sufficient evidence. Firstly, it is hard to track the origin of the data since most authors citing this statistic used inaccurate citations.

Secondly, the HCV serological detection kit has undergone at least three iterations<sup>[117]</sup>. The first test developed in 1990 detected antibody to a single epitope within the core protein by enzyme linked immunosorbent assay and provided data on 170 million HCV carriers, even though it was plagued by poor sensitivity<sup>[43]</sup>. Third-generation enzyme immunoassays included antibodies against multiple antigens, which increased the sensitivity significantly<sup>[43]</sup>, although no large-scale serological investigations have been performed. In China, a nationwide HCV serological survey performed in 2006 showed the prevalence of anti-HCV antibodies to be < 0.5% among more than 80000 Chinese subjects<sup>[116]</sup>. Furthermore, the rates of HCV were much lower than those of hepatitis B among clinical inpatient and outpatient populations, which was significantly different from a Japanese population<sup>[119,120]</sup>. Epidemiology is important because it will provide basic knowledge of disease and inaccurate epidemiological data will lead to inaccuracies in our knowledge of the disease burden, natural history and therapeutic efficacy.

The number of people that will become chronic carriers after HCV infection remains unknown. Scientists believe that as many as 40%-80% of HCV infections will develop into chronic infections<sup>[121-123]</sup> (Table 3). While these estimates are also likely inaccurate, how and when people are infected must be determined to ascertain a more precise figure. One recent cross-sectional study performed in intravenous drug users challenged the current assumptions regarding the rate of chronic infection; in that study as many as 77.8% of individuals cleared HCV infection without the need for anti-viral therapy<sup>[117]</sup>.

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## Liver function impairment in liver transplantation and after extended hepatectomy

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### Abstract

Extended hepatectomy, or liver transplantation of reduced-size graft, can lead to a pattern of clinical manifestations, namely "post-hepatectomy liver failure" and "small-for-size syndrome" respectively, that can range from mild cholestasis to irreversible organ non-function and death of the patient. Many mechanisms are involved in their occurrence but in the recent past, high portal blood flow through a relatively small liver vascular bed has taken a central role. Therefore, several techniques of inflow modulation have been attempted in cases of portal hyperperfusion first in liver transplantation, such as portocaval shunt, mesocaval shunt, splenorenal shunt, splenectomy or ligation of the splenic artery. However, high portal flow is not the only factor responsible, and before major liver resections, preoperative assessment of the residual liver function is necessary. Techniques such as portal vein embolization or portal vein ligation can be adopted to increase the future liver volume, preventing post-hepatectomy liver failure. More recently, a new surgical procedure, that combines *in situ* splitting of the liver

and portal vein ligation, has gradually come to light, inducing remarkable hypertrophy of the healthy liver in just a few days. Further studies are needed to confirm this hypothesis and overcome one of the biggest issues in the field of liver surgery.

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**Key words:** Small-for-size syndrome; Liver transplantation; Extended hepatectomy; Liver failure; Cirrhosis

**Core tip:** In this review we focus on the small-for-size syndrome and post-hepatectomy liver failure, the most feared complications of liver surgery, fundamentally similar in pathogenesis and clinical manifestations, occurring when the residual liver is not large enough to accommodate the markedly increased portal vein blood flow. Our aim is to simplify a concept, which has been a major concern in hepatic surgery for some time. Many efforts have been and are being made to overcome such an important problem in this field.

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### INTRODUCTION

The liver is a unique organ, capable of regeneration and functional recovery after parenchymal injury. When the volume is too small to satisfy the metabolic demand, the liver loses this peculiar ability, resulting in delayed synthetic dysfunction with poor bile production, coagulopathy, prolonged cholestasis and intractable ascites, which can lead to septic complications and high mortality. The

term “small-for-size syndrome” (SFSS) was first<sup>[1]</sup> coined in liver transplantation as a consequence of size mismatch between graft and recipient, an event occurring especially in the setting of living donor liver transplantation (LDLT) or split liver transplantation<sup>[2]</sup>, where the use of partial grafts has gained worldwide acceptance to overcome the shortage of cadaveric organs. However, the same concept can also be applied to the field of liver resection, where patients with marginally resectable tumors are at high risk of developing post-hepatectomy liver failure (PHLF)<sup>[3]</sup>, a clinical manifestation comparable to the SFSS.

## DEFINITION

There is not full consensus about the definition of SFSS. It was introduced in 1996 by Emond *et al*<sup>[1]</sup> and regarded the clinical manifestation following transplantation of small grafts in LDLT. The term SFSS on the basis of personal working experience, and no threshold values of liver function tests, was suggested. In 2005, Dahm *et al*<sup>[4]</sup> proposed a more precise definition. These authors described SFSS after liver transplantation as the presence of two of the following criteria recorded on three consecutive postoperative days: serum bilirubin > 100  $\mu\text{mol/L}$  (6 mg/dL), international normalized ratio (INR) > 2 and presence of encephalopathy grade III or IV. The small-for-size syndrome usually occurs during the first postoperative week and is diagnosed after the exclusion of other causes such as technical complications (*e.g.*, arterial or portal occlusion, outflow congestion, bile leak) and/or rejection or infections (*e.g.*, cholangitis, sepsis).

The same concept is applicable to the field of hepatic surgery, where extended resections can lead to the development of PHLF. Many different definitions of PHLF have been proposed in the literature<sup>[5-7]</sup>. In trying to propose a more standardized definition, in 2011, Rahbari *et al*<sup>[7]</sup> suggested a simple and easily applicable definition of PHLF as a “postoperative acquired deterioration in the ability of the liver to maintain its synthetic, excretory and detoxifying functions, which are characterized by an increased INR and concomitant hyperbilirubinemia on or after postoperative day 5”. They differentiated severity in three grades (A, B, C), according to whether changes in clinical management of the patient or invasive treatments are required. It is of interest that even if SFSS and PHLF can be viewed as the same manifestation of liver function impairment, the two terms and their relative definitions are currently separated. It would probably be of interest to join the two definitions into a single one, but at present no suggestions, regarding this topic, are present in the literature.

## PATHOPHYSIOLOGY

The magnitude of the effect of increased portal flow after hepatectomy on the development of PHLF, though recognized, is currently not yet well established and most

of the studies regarding this topic come from the transplantation experience.

High portal blood venous flow (PVF) has gained a central role in the pathogenesis of SFSS. Under normal physiological conditions, portal vein blood flow accounts for 75% of total hepatic inflow, or 90 mL/min per 100 g of liver tissue, while the hepatic artery contributes for 20%-25%<sup>[8]</sup>. The portal vein lacks intrinsic auto-regulation. Hence, after extended hepatectomy or transplantation of small grafts, the remnant liver is subjected to the portal flow destined to a whole liver, through a reduced micro-vascular bed<sup>[9]</sup>. Such a substantial increase of PVF and shear-stress on sinusoidal lining cells is inversely related to graft size. In > 75% partial hepatectomy, PVF increases by more than twice the baseline values, resulting in PHLF, with high morbidity and mortality<sup>[10]</sup>. Although shear-stress is considered to be a necessary stimulus for hepatic regeneration<sup>[11]</sup>, excessive forces can be detrimental to both the function and survival of the reduced-size organ: the result is damage of sinusoidal spaces with release of inflammatory cytokines, responsible for progressive hepatocyte necrosis<sup>[12]</sup>. Pathological findings include hepatocyte ballooning, tremendous mitochondrial swelling, irregular large gaps between sinusoidal lining cells, and collapse of the space of Disse<sup>[13]</sup>.

Although portal vein pressure (PVP) is considered a reliable predictor of graft failure<sup>[14]</sup>, the latter and PVF do not run parallel to each other; furthermore, the lack of correlation between graft weight/recipient body weight ratio (GRWR) and PVP has been investigated<sup>[15]</sup>.

Blood flow regulation, which allows a steady rate of hepatic perfusion, depends not only on the classical arterial intrinsic regulation but also on an inverse relationship between portal and hepatic arterial flow, also known as hepatic arterial buffer response (HABR)<sup>[16]</sup>. When the portal blood flow increases, this leads to an elevated wash-out of adenosine levels in the space of Mall, contracting the hepatic artery<sup>[17]</sup>. Adenosine is unlikely to be the sole vascular regulator and other vaso-active compounds may contribute to HABR<sup>[18]</sup>. The consequences of such a diminished arterial blood flow manifest in the peripheral circulation as a centrilobular microvesicular steatosis or infarcts, or, in severely affected cases, as ischemic cholangitis in the hilum<sup>[19]</sup>. Hence, the clinical manifestations can range from mild cholestasis to liver failure. However, the optimal rate needed to sustain liver regeneration and function, without damage to the liver, is still not known and further experimental studies on animal models are needed.

## PREOPERATIVE PREDICTION

Hepatectomy remains the first curative option for neoplasms of the liver. The mortality rate after major liver resections, *i.e.*, the removal of three or more Couinaud segments, ranges from 3% to 7% in non-injured liver parenchyma and increases up to 32% in patients with cirrhosis<sup>[20]</sup>. Thus, the extent of parenchymal resection

**Table 1 Predictive factors of small-for-size syndrome and post-hepatectomy liver failure**

Liver volume	Liver function	Patient-related	Other
FLR/TLV	CHILD-PUGH	CALI	Cholestasis
GRWR or GV/SLV	HVPG	Age > 65 yr <sup>[37]</sup>	Liver stiffness <sup>[31]</sup>
	ICG	Male sex <sup>[37]</sup>	Donor factors <sup>[44]</sup>
	MEGX <sup>[30]</sup>	Diabetes mellitus <sup>[37]</sup>	

FLR: Future liver remnant; TLV: Total liver volume; GRWR: Graft weight-recipient body weight ratio; GV: Graft volume; SLV: Standard liver volume; HVPG: Hepatic vein pressure gradient; ICG: Indocyanine green clearance; MEGX: Monoethylglycinexylidide; CALI: Chemotherapy-induced liver injury.

is an essential parameter in establishing both the operability of each patient and the risk of PHLF and this, to date, is still a subject of debate, probably due to different methods of measurement, variability in the segment volumetric distribution and degree of underlying disease.

The 3D volumetric computed tomography reconstruction allows preoperative calculation of the liver volume, even of the single segments, and, more important, of the future liver remnant (FLR). With a normal function, FLR should range between 20% and 30% of total liver volume, whereas smaller volumes are correlated with increase of liver failure and infections<sup>[21,22]</sup>. Care must be taken when an underlying liver disease pre-exists. In “injured” livers, (steatosis, cholestasis, fibrosis, cirrhosis or chemotherapy) the FLR should be greater than 30%-40%<sup>[23]</sup>. Therefore, an accurate preoperative assessment of liver function is needed.

In patients with cirrhosis, the Child-Pugh score and the hepatic vein pressure gradient are the two most important restrictive criteria in selecting candidates for surgery<sup>[24,25]</sup> even if they do not provide precise assessment of liver resectability<sup>[26]</sup>. Metabolic tests based on the detoxifying properties of the liver have the advantage of providing a more reliable estimation of the hepatic function, and they are based on quantitative measures. Indocyanine green clearance is the most popular test<sup>[27]</sup>, especially in Eastern countries, where it constitutes the pillar of preoperative algorithms for liver resection<sup>[28,29]</sup>. Other quantitative tests, such as the monoethylglycinexylidide<sup>[30]</sup> test, have led to good prediction of PHLF, but they have gained less popularity and are not routinely used. A simple and non-invasive method of measurement of liver stiffness (Fibroscan<sup>®</sup>) has recently been gaining broad consensus for predicting PHLF in selected patients<sup>[31]</sup>, but further studies are needed to establish its potential role in patient selection for surgery.

Chemotherapy-induced liver injury is common in patients that received chemotherapy for colorectal liver metastases, and the two typical patterns are sinusoidal injury (sinusoidal obstruction syndrome) in oxaliplatin-based regimens, and steatohepatitis (CASH), associated with irinotecan treatment<sup>[32]</sup>. More than 6 cycles of oxaliplatin need a longer time interval before major hepatectomy, even though accountability for PHLF still remains a matter of debate<sup>[33]</sup>, whereas irinotecan is associated with

an increased risk of peri-operative mortality after hepatectomy<sup>[34]</sup>. Biopsy of the liver before surgery might be helpful to assess the grade of steatosis or the histological features of CASH, thus defining more precise windows between drug administration and surgery.

Cholestasis impairs liver regeneration, and levels of bilirubin above 2.9 mg/dL are related to a higher rate of liver failure after major hepatectomy<sup>[35]</sup>. Nevertheless, the use of preoperative biliary drainage is still controversial, except for acute cholangitis or small FLR that are candidates for portal vein embolization<sup>[36]</sup>, in which case biliary drainage is highly recommended. Besides such patient-related factors, others, like age > 65 years, male sex and diabetes mellitus, are related to a high risk of PHLF<sup>[37]</sup>. Obesity is not per se a major predictor of liver failure<sup>[38]</sup>.

In the setting of transplantation, liver volume assessment is represented by the GRWR or graft volume/standard liver volume ratio (GV/SLV): in LDLT safe thresholds are at least 0.8% of GRWR or 30%-40% of GV/SLV<sup>[2,39,40]</sup>, with greater values in patients affected by portal hypertension or advanced chronic liver disease. There are reports on the successful use of smaller grafts<sup>[41]</sup>, but in association with some intraoperative inflow modulations: a case report of a left lobe LDLT as low as 0.34% of GRWR underwent splenectomy and did not develop post-operative SFSS<sup>[42]</sup>. In liver transplantation, size is not always the sole factor responsible for graft post-transplant liver function<sup>[43]</sup>, because graft quality is likewise important in order to avoid liver dysfunction or other complications. Aside from basic requirements for donor livers, the following donor factors have a negative impact on graft prognosis: age > 50 years, prolonged intensive care unit stay > 5 d, hypernatremia, prolonged cardiac/respiratory arrest and long ischemia times, administration of high dosage of vasopressors, severe systemic sepsis, steatosis > 30%, anatomic variations in vascular structure and, obviously, abnormal liver function, particularly with elevated serum bilirubin and gamma glutamyltransferase<sup>[44]</sup>.

Prediction of SFSS and PHLF is feasible and is based on the calculation of liver volume up to the assessment of liver function. Evaluation of patient status can help to find the best candidate for surgery. In the field of liver transplantation, donor characteristics also have to be taken into account, defining which grafts are at higher risk of developing SFSS than others. A list of the above mentioned factors is shown in Table 1.

## ATTENUATING SFSS IN LIVER TRANSPLANTATION

In the presence of high portal blood flow and/or small grafts (GRWR < 0.8%), several different technical flow manipulations can be performed to overcome graft hyperperfusion and reduce PVF, although there is no full consensus about their indications: portocaval shunt, mesocaval shunt, splenorenal shunt, splenectomy or ligation of the splenic artery. Boillot *et al*<sup>[45]</sup> reported the first



successful mesocaval shunt with downstream ligation of the superior mesenteric vein in a left lobe transplantation (GRWR of 0.61%), based on previous experimental studies on pigs.

Hemi-portocaval shunt, *i.e.*, anastomosis between the left or the right portal branch and the inferior vena cava in a permanent fashion, is advocated by Troisi *et al.*<sup>[10]</sup> whenever the PVF at reperfusion exceeds three-four times the one recorded in the donor. None of the patients undergoing such a graft inflow modulation developed SFSS, with significant decrease of portal vein flow.

The effects of splenic flow diversion have been investigated in the presence of portal hypertension (PVP > 20 mmHg)<sup>[15]</sup> and/or of portal hyperperfusion (PVF > 250 mL/min per gram)<sup>[46]</sup>. However, when PVF exceeds 500 mL/min per gram, portosystemic shunt cannot be avoided.

Both splenic artery ligation (SAL) and splenectomy can be performed and are comparable in terms of outcome and overall survival<sup>[47]</sup>, although for the latter, septic complications must always be taken into account. Splenectomy is considered superior to SAL for the purpose of increasing white balance and platelet count after LDLT, which is not achieved by SAL alone.

Splenic artery embolization represents a valid alternative to achieve portal decompression<sup>[48]</sup>. Furthermore, a linear correlation between PVF and graft-to-recipient spleen size ratio has been found, thus including the spleen size as a likely predictor of post-transplant portal hyperperfusion and SFSS<sup>[49]</sup>.

Techniques of graft inflow modulation account for a certain risk of steal phenomenon<sup>[50]</sup>: portal vein thrombosis, encephalopathy, septic complications or hampered liver regeneration are described as principal side effects. It remains an open question whether and when portosystemic shunts should be removed<sup>[51]</sup>, since hypoperfusion, as well as hyperperfusion, can also be detrimental for liver function.

According to the definition of Dahm *et al.*<sup>[4]</sup>, who stated that SFSS should be considered as a distinct entity, outflow obstruction per se should be excluded as a possible trigger, as it may reduce the hepatic function. However, one of the most discussed topics concerns the reconstruction of the middle hepatic vein (MHV) in right lobe grafts, since congestion of anterior segments (V-VIII) may lead to graft dysfunction<sup>[52]</sup>. A graft with inclusion of the MHV has been demonstrated to be technically and physiologically superior, but the use of this technique should be limited to selected cases in LDLT due to an increased risk for donor safety<sup>[53]</sup>. For MHV reconstruction, several transplant centers use various types of vascular grafts, with a predilection for large caliber autologous vessels (*i.e.*, the superficial femoral vein), or also cryopreserved venous or arterial grafts<sup>[54]</sup>.

future metabolic demand, a number of strategies can be adopted to increase the liver volume, preventing post-hepatectomy liver failure. Portal vein embolization (PVE) has become the most standardized procedure due to its safety and feasibility: it consists in the occlusion of portal flow ipsilateral to the lesion, inducing hypertrophy in the contralateral lobe. Makuuchi *et al.*<sup>[55]</sup> first used this technique in 1982 to extend the limits of hepatic resection, thus increasing the number of cases suitable for curative surgery: in this early report, 14 patients underwent pre-operative PVE followed by major liver resection 6-41 d after embolization, with no occurrence of postoperative liver failure. After almost 30 years, the indications of PVE are still very poorly standardized: many authors indicate a residual liver volume less than 30% of total liver volume or up to 40% in injured livers as the critical threshold<sup>[56,57]</sup>. Surgery is usually performed 2-8 wk after PVE, with future liver remnant volume increased by 10%-46%. From 70% to 100% of patients who underwent PVE, hemi-hepatectomy or extended hepatectomy could be performed. Following resection, the perioperative morbidity and mortality was less than 15% and 0%-7%, respectively<sup>[58-60]</sup>.

Portal vein ligation (PVL) represents a good alternative, although there are no controlled studies clearly showing the superiority of PVE *vs* PVL. Portal vein ligation requires laparotomy and, furthermore, the volume gain is often limited due to formation of collaterals between the two different lobes<sup>[20]</sup>. PVL is not considered such a standardized and safe procedure as PVE, but patients who are candidates for 2-stage hepatectomy can benefit from this technique<sup>[61,62]</sup>, recently adopted in a new surgical approach aimed at enhancing and accelerating the regeneration of the remnant liver<sup>[63]</sup>. In 2009, Schnitzbauer *et al.*<sup>[63]</sup> reported on a case series of 25 marginally resectable patients with massive involvement of the right lobe by neoplastic nodules, on which an innovative 2-step technique was carried out. In the first step, right portal vein ligation and *in situ* splitting of the liver on the right side of the falciform ligament was performed; in the second step, after a median time interval of 9 d, extended hepatectomy (right trisectionectomy) was completed. The observed median increase in volume of the left lobe was 74%, but morbidity and mortality were significant (68% and 12%, respectively). Thereafter, the so-called advanced liver partition and PVL for staged hepatectomy, also known by the acronym ALPPS<sup>[64]</sup>, has spread to many centers worldwide: the obtained median increase in volume ranges from 74% up to 87%, with surgery usually performed 5-30 d after the first step. However, mortality rates of 13%-22% are still reported<sup>[65-68]</sup>. Although the procedure is innovative and attractive, these latter figures make it imperative to increase the number of patients treated with this strategy to better define its feasibility and limits<sup>[69]</sup>.

In addition to the above, more studies are needed to understand the exact mechanisms of hepatic regeneration, also through biopsy of the remnant liver before and after hepatectomy, and measurements of portal flow

## ATTENUATING PHLF IN EXTENDED HEPATECTOMY

If the remnant liver volume is not sufficient to meet the

and pressure should be provided. In fact, although the preserved functional capacity of the hypertrophied remnant liver could be established with functional tests (*e.g.*, indocyanine green clearance) and through the uptake of  $^{99m}\text{Tc}$  dimethyl iminodiacetic acid<sup>[64]</sup>, excessive portal flow represents one of the main problems, determining a possible discrepancy between the relevant increase in volume and the amount of actually functioning parenchyma. de Santibañes *et al*<sup>[70]</sup>, in 2012, claimed that the diseased right hemi-liver, left in place, acts as an auxiliary liver to assist the future liver remnant for the first and critical week after resection, but in true auxiliary transplantation, both the portal and arterial flows to two hemi-livers are maintained. Thus, contrary to auxiliary transplantation, in which the growth and functional recovery may progress harmonically with a real portal flow modulation, this phenomenon is not certain after extended hepatectomy with a small residual parenchyma. In other words, how can this “beneficial” re-direction of the entire portal flow to a “small-for-size” remnant liver comply with established principles of portal flow modulation in small-for-size transplantation? Research in animal models clearly shows that a portocaval shunt has a positive effect in attenuating liver injury after extensive hepatectomy, suggesting that a slower regeneration following reduction of portal flow may be more advisable than faster regeneration associated with temporary portal hyperflow<sup>[71,72]</sup>. In this view, more insights on the mechanisms and features of liver regeneration are needed to better understand the potential benefit of portal flow modulation to prevent postoperative liver failure<sup>[64]</sup>.

## PHARMACOLOGICAL INTERVENTIONS

Many drugs have been demonstrated to be effective in attenuating SFSS after living donor liver transplantation of small grafts, but most of them have been tested only in animal models<sup>[73,74]</sup>, whereas clinical trials on human beings are still lacking. Furthermore, pharmacological portal flow modulation has been investigated: shear-stress attenuation has been achieved by somatostatin<sup>[75]</sup>, through down-regulation of the endothelin-1 (sinusoidal vasoconstrictor) and up-regulation of heme-oxygenase-1 (vasodilator and antioxidant). Nitric oxide pathway activation seems to be protective against ischemia-reperfusion injury both in liver resection and liver transplantation<sup>[76]</sup>. Therapeutic agents promoting liver regeneration, such as serotonin, are still a matter of debate for their controversial role<sup>[77]</sup>. Recently, autologous bone marrow stem cells have been used to increase liver regeneration prior to major liver resection. In particular, an enhanced parenchymal growth after portal vein embolization through the portal injection of CD133<sup>+</sup> cells (in the non-embolized hepatic lobe) has been demonstrated, with a subsequent improvement of outcome after surgery<sup>[78]</sup>. Even though the specific effect of CD133<sup>+</sup> cells is not completely understood<sup>[79]</sup>, this approach is intriguing due to the possibility of combination with other techniques

favoring post-transplant or post-hepatectomy liver function recovery, such as procedures of portal flow modulation.

## CONCLUSION

Post-hepatectomy liver failure and small-for-size liver syndrome can be viewed as two sides of the same coin, since both of them can lead to an identical pattern of clinical manifestations, that is cholestasis, impairment of coagulation and development of ascites, and that can range up to irreversible organ non-function and death of the patient. Safe thresholds of remnant liver volume differ between liver transplantation and after extended hepatectomy, probably due to graft denervation, immunosuppressive therapy and severity of ischemia-reperfusion injury. However, preoperative assessment of liver function and size is crucial, while intraoperative recording of hemodynamic changes, before and after hepatectomy or liver transplantation, should be mandatory in order to perform inflow modulation, if necessary. Other strategies, which include pharmacological perioperative protection of the liver and stem cell injection, are being explored, but further studies are needed before they can be applied in the clinical field.

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## Exocrine pancreatic insufficiency in adults: A shared position statement of the Italian association for the study of the pancreas

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sensus was reached. The final draft of the manuscript was then sent to the AISP Council for approval and/or modification. All concerned parties approved the final version of the manuscript in June 2013.

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**Key words:** Exocrine pancreatic insufficiency; Chronic pancreatitis; Gastric surgery; Pancreatic surgery; Pancreatic neoplasms; Risk factors; Clinical studies

**Core tip:** Pancreatic exocrine insufficiency represents a condition related to pancreatic and extrapancreatic disease. We have reviewed the evidence related to the pathophysiological aspects of exocrine pancreatic diseases and we have also reported the recommendations for treating this condition in the most common pancreatic and extrapancreatic diseases. Pancreatin minimicrospheres is a drug which is cost-effective according to a survey of Polish patients, but studies demonstrating its cost-efficacy in Italy are necessary.

### Abstract

This is a medical position statement developed by the Exocrine Pancreatic Insufficiency collaborative group which is a part of the Italian Association for the Study of the Pancreas (AISP). We covered the main diseases associated with exocrine pancreatic insufficiency (EPI) which are of common interest to internists/gastroenterologists, oncologists and surgeons, fully aware that EPI may also occur together with many other diseases, but less frequently. A preliminary manuscript based on an extended literature search (Medline/PubMed, Cochrane Library and Google Scholar) of published reports was prepared, and key recommendations were proposed. The evidence was discussed at a dedicated meeting in Bologna during the National Meeting of the Association in October 2012. Each of the proposed recommendations and algorithms was discussed and an initial con-

Pezzilli R, Andriulli A, Bassi C, Balzano G, Cantore M, Delle Fave G, Falconi M, Frulloni L; the Exocrine Pancreatic Insufficiency collaborative (EPIc) Group. Exocrine pancreatic insufficiency in adults: A shared position statement of the Italian association for the study of the pancreas. *World J Gastroenterol* 2013; 19(44): 7930-7946 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i44/7930.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.7930>

### INTRODUCTION

This is a medical position statement developed by the Exocrine Pancreatic Insufficiency collaborative group

which is a part of the Italian Association for the Study of the Pancreas (AISP). We covered the main diseases associated with exocrine pancreatic insufficiency (EPI) of common interest to internists/gastroenterologists, oncologists and surgeons, fully aware that EPI may occur in many other diseases, but less frequently (Giardia and HIV infections, lymphoma, Whipple's disease, amyloidosis).

## LITERATURE SEARCH METHODS

A preliminary manuscript based on an extended literature search (Medline/PubMed, Cochrane Library and Google Scholar) of published reports was prepared, and key recommendations were proposed. A MESH term "EPI" was used for the search on Medline/PubMed and key words (exocrine pancreatic insufficiency) were used for both Cochrane Library and Google Scholar. A total of 1465 manuscript were retrieved on Medline/PubMed, 64 on Cochrane Library and 1234 on Google Scholar. After deduplication only 282 papers regarding the specific aims of the study were selected and 151 were utilized. The evidence and recommendations were discussed at a dedicated meeting in Bologna during the National Meeting of the Association in October 2012 in which was present 130 participants. Each of the proposed recommendations and algorithms was discussed and an initial consensus was reached. The final draft of the manuscript was then sent to the AISP Council for approval and/or modifications. All concerned parties approved the final version of the manuscript in June 2013.

## PHYSIOLOGY OF PANCREATIC DIGESTION OF NUTRIENTS

The pancreatic secretion is a clear fluid liquid, 97% of which is water and electrolytes<sup>[1]</sup>, and 3% proteins. In turn, these are made up of proteins (3%) mainly represented by proteases (80%), amylase (7%), lipase (4%) and nucleases (1%)<sup>[2]</sup>. The normal absorption of nutrients involves a complex mixture of digestive enzymes and bile salts, and an intact intestinal mucosa to enable the uptake of these hydrophobic complexes. Under normal condition, all major pancreatic enzymes act simultaneously with postprandial chyme decrease during duodenal-ileal transit<sup>[3]</sup>; the rate of intraluminal degradation differs widely among the major enzymes due to their different stability regarding inactivating mechanisms<sup>[4]</sup>. Pancreatic amylase is a very stable enzyme, probably because of its high resistance to enzymatic proteolysis<sup>[5]</sup>; the majority of that released into the duodenum reaches the terminal ileum in an active form<sup>[4,6,7]</sup> whereas approximately 60% of the protease activities released into the duodenum are delivered to the mid-jejunum, and only between 20% and 30% reach the terminal ileum<sup>[4]</sup>. As regards lipolytic enzymes, lipase is most susceptible to inactivation during small intestinal transit. In the absence of triglycerides, a large proportion of lipase activity is also lost between the duodenum and the jejunum, and only small quantities

**Table 1 Causes of pancreatic insufficiency**

### Chronic pancreatitis

Primary pancreatic insufficiency
Agenesis of the pancreas
Congenital pancreatic hypoplasia
Shwachman-Diamond syndrome
Johanson-Blizzard syndrome
Adult pancreatic lipomatosis or atrophy
Isolated lipase or colipase deficiency
Pancreatic resection
Pancreatic cancers
Secondary pancreatic insufficiency
Mucosal small bowel disease: Decreased cholecystokinin release
Somatostatinoma or exogenous somatostatin analog intake: Decreased pancreatic secretion
Gastrinoma: Intraluminal destruction of enzymes
Surgery and Billroth II anastomosis: Poor mixing or decreased hormone release, disturbance of innervations
Periampullary tumors (pancreatic duct obstruction)

Modified from reference 155.

are delivered to the terminal ileum<sup>[4,5]</sup>. After ingestion, dietary lipids are initially emulsified in the stomach and then hydrolyzed by the action of gastric and pancreatic lipase and colipase; hydrolyzed lipids are then aggregated into micelles or liposomes with the addition of bile salts in the duodenum and jejunum, the micelles are absorbed across the intact intestinal villi by both active and passive processes and, finally, packaged into chylomicrons within intestinal epithelial cells and transported to the circulatory system via the lymphatic system<sup>[8]</sup>.

## MECHANISMS OF EXOCRINE PANCREATIC INSUFFICIENCY

Exocrine pancreatic insufficiency results from a progressive loss of acinar pancreatic cells which leads to the secretion of an insufficient amount of digestive enzymes into the duodenum. As indicated in Table 1, chronic pancreatitis is the most well-known cause of EPI<sup>[9]</sup> but also several other conditions, such as partial or total surgical resection of the gland, loss of function of pancreatic tissue or obstruction of the main pancreatic duct as well as diabetes, celiac disease, inflammatory bowel diseases, and gastrectomy should also be considered. Maldigestion results when exocrine (mainly lipase and trypsin) pancreatic function is reduced by more than 90%; other pancreatic and extra-pancreatic causes of maldigestion are reported in Table 2<sup>[10]</sup>.

## CLINICAL MANIFESTATION AND ASSESSMENT OF EXOCRINE PANCREATIC INSUFFICIENCY

### Patient complaints

Patients with steatorrhea typically report an increase in daily bowel movements, with fatty, bulky stools which are

**Table 2 Pathogenesis of maldigestion**

Mechanism	Explanation
Decreased pancreatic production	Lack of functional tissue or decreased endogenous neurohormonal stimulation
Decrease in delivery	Pancreatic duct obstruction
Decreased activation	Low duodenal pH
Premature enzymatic degradation	Decreased contact time due to increased motility, impaired interaction with chyme and biliary salts, and intestinal bacterial overgrowth

difficult to flush away. This occurs mainly after high fat-containing meals and is sometimes not a daily symptom. As steatorrhea occurs after meals, it typically happens 2 to 3 times a day in individuals with a normal lipid-content diet. Weight loss and anorexia may also develop over time due to malnutrition.

### Physical examination

Chronic malabsorption results in weight loss, such as temporal scalloping, interosseous wasting, and lack of subcutaneous fat. Nail leukonychia due to hypoalbuminemia may be present in the late stages of chronic malabsorption. Signs of liposoluble vitamin lack may appear; ecchymoses due to clotting abnormalities in the case of vitamin K deficiency, ataxia and peripheral neuropathy resembling Friedreich ataxia due to vitamin E deficiency, abnormalities of night blindness and xerophthalmia (dry corneas) due to vitamin A deficiency; contraction or muscle spasms, osteomalacia and osteoporosis may also occur due to hypocalcemia. Examination of the stool is an important tool for recognizing steatorrhea.

### Investigations

Exocrine pancreatic function is currently diagnosed using two groups of tests, usually referred to as direct and indirect (or tubeless) tests; the principal tests are reported in Table 3. The most sensitive test is a direct test based on aspiration of the pancreatic contents during secretin or secretin-cholecystokinin/cerulein administration<sup>[11]</sup>; this test is only available in a few centers, it is invasive and is not indicated in clinical practice. Other tests currently available in clinical practice are indirect tests. At present, fecal elastase-1 determination is the most diffuse test for screening pancreatic exocrine insufficiency<sup>[12]</sup>, usually using a monoclonal test<sup>[13]</sup>. This test does not require the withdrawal of enzyme supplementation therapy and is based on analysis of a single stool sample. Concentrations of elastase-1 less than 200 µg/g in feces are compatible with exocrine pancreatic insufficiency and less than 100 µg/g are indicative of severe pancreatic insufficiency<sup>[14]</sup>. The <sup>14</sup>C-triolein breath test and the cholesteryl-[1-<sup>13</sup>C] octanoate breath test have been used for assessing fat malabsorption<sup>[15]</sup>; the D-xylose test (normal serum D-xylose concentration greater than 1.33 mmol/L 1 h after an oral dose of D-xylose) for exploring the malabsorption of carbohydrates, and fecal chymotrypsin excretion (normal > 6 U/g) for evaluating the malabsorption of proteins<sup>[12]</sup>.

Two other tests, not presently available commercially, are the N-benzoyl-L-tyrosyl-p-aminobenzoic acid (PABA) test and the pancreolauryl test which are based on the recovery of an ingested dose of PABA and fluorescein dilaurate from the urine<sup>[16]</sup>. In clinical trials, objective confirmation of excess fecal fat may be undertaken, and the following methods are usually used<sup>[9]</sup>: Sudan staining of random homogenized stool, steatocrit and quantitative fat analysis. Sudan staining evaluates the number and size of fat globules per high-power field (hpf), and the test results are scored as normal ( $\leq 20$ /hpf, 1 to 4 micrometers in size), moderately increased ( $> 20$ /hpf, 1 to 8 µm in size) and definitely increased ( $> 20$ /hpf, 6 to 75 µm in size)<sup>[17]</sup>. Compared to chemical fat analysis, Sudan staining has a sensitivity of 94% and a specificity of 95% for diagnosing abnormal fecal fat excretion<sup>[18]</sup>. Steatocrit is a quantitative measurement of fat and is expressed as a proportion of an entire centrifuged homogenized stool sample<sup>[19]</sup>. A spot acid steatocrit level (normal < 10%) has been reported as having a sensitivity of 100% and a specificity of 95% when compared to 72-h quantitative fat analysis<sup>[20]</sup>. The best reported method is the 72-h fat chemical analysis using the van de Kamer method. The patients need to keep a food diary to ensure that adequate dietary fat (100 g/d) is consumed during the test; the normal output is less than 7 g of fat per 24-h period<sup>[21]</sup>. Coefficient of fat absorption (CFA) should be used to better quantify the steatorrhea; it is calculated using the following equation:  $CFA (\%) = 100 [(mean\ fat\ intake - mean\ stool\ fat)/mean\ fat\ intake]$ <sup>[22]</sup>; in healthy subjects, the CFA is usually greater than 80%<sup>[23]</sup>.

A new assessment for pancreatic malabsorption which takes into consideration some serum parameters reflecting nutritional status (magnesium < 2.05 mg/dL, reduced serum levels of prealbumin, albumin, retinol binding protein, ferritin, and hemoglobin) has recently been reported<sup>[24]</sup>, but it requires further validation<sup>[25]</sup>.

Finally, bioelectrical impedance has been proposed for assessing nutritional status in patients with pancreatic cancer<sup>[26]</sup>. This method is based on the different conductive and resistive properties of the various body tissues; it is not invasive, it is inexpensive and it can be performed at the bedside. In brief, fixed low-voltage and high-frequency alternating current introduced into the body is conducted through the fluid compartment of the fat-free mass and it is able to measure both body resistance and capacitance. Capacitance causes the current to lag behind the voltage, creating a phase shift; this shift is quantified geometrically as the angular transformation of the capacitance: resistance ratio, also called phase angle.

### Pancreatic enzyme replacement therapy

In order to avoid maldigestion and ameliorate the nutritional status of patients with EPI, the cornerstone of treatment is pancreatic enzyme replacement therapy (PERT). Available formulations contain pancreatic enzymes encapsulated in microgranules or minimicrospheres with a pH sensitive coating in order to either



**Table 3** Indirect diagnostic tests for evaluating pancreatic exocrine insufficiency

Test	In favour	Against
CFA	Gold standard	72 h stool collection; 100 g standard diet; no simultaneous PERT; not pancreas specific
Acid steatocrit	Linear correlation with CFA also in a single sample; Good as screening	High fat diet needed; 24-72 h stool collection is ideal
Fecal elastase 1	Single stool sample; PERT can be continued	Poor sensitivity in mild EPI, watery stools and small bowel disease
<sup>13</sup> C-mixed triglyceride breath test	Simple; Also for mild forms of EPI and therapy assessment	Requires further validation
Fecal chymotrypsin	Good for compliance control; Single small stool sample	Sensitivity low for clinical practice (chymotrypsin is variably inactivated during intestinal transit); not for mild EPI; watery stools decrease enzyme activity; PERT must be discontinued
Secretin-enhanced magnetic resonance cholangiopancreatography	Morphological and semi-quantitative functional changes	Requires further validation
Nutritional status (magnesium < 2.05 mg/dL, ↓ prealbumin, ↓albumin, ↓retinol binding protein, ↓ferritin, ↓hemoglobin)	Simple	Requires further validation

CFA: Coefficient of fat absorption; PERT: Pancreatic enzyme supplementation therapy; EPI: Pancreatic exocrine insufficiency.

prevent the release and the subsequent inactivation of enzymes by gastric acidity or to release the enzymes into the intestinal lumen where the pH is higher and optimal for the digestion and absorption of food. Currently, the Italian guidelines also suggest minimicrospheres to be the ideal pancreatin formulation<sup>[9]</sup>.

The initial recommended dose of pancreatic extract which should be given is 40000-50000 units of lipase per meal and 25000 U per snack, and this dose should be progressively increased until the steatorrhea is totally or sufficiently reduced<sup>[27,28]</sup>; this dosage should be maintained over time.

### Dietary and drug recommendation

Food intake should be distributed between three main meals per day, and two or three snacks. The pancreatic extracts should be ingested during the meals.

Even if a diet which is low in fat reduces steatorrhea and improves maldigestion, it restricts caloric intake and is not a good option.

Medium-chain triglycerides (MCTs) have not been shown to be effective in patients suffering from chronic pancreatitis with EPI. Moreover, their poor palatability and high cost reduce patient compliance. Evidence exists that MCTs also require enzyme supplements for proper digestion and absorption<sup>[29]</sup>. They should be used only in patients with persistence of symptoms or weight loss despite adequate enzyme supplementation<sup>[30]</sup>. Medium-chain triglycerides have been proposed in PERT non-responders as an “ultima ratio”. The quantity of energy administered by MCTs is limited (ca 8.3 kcal/g) and the dose must be increased slowly in order to achieve intestinal adaptation, even when using enteral nutrition<sup>[31]</sup>. However, trials have shown no advantage between a normal balanced diet and MCT-enriched preparations<sup>[29,32,33]</sup>.

A diet rich in fiber content is contraindicated because the fibrous material will interfere with proteolytic and amylolytic enzyme activity; lipolytic activity is most af-

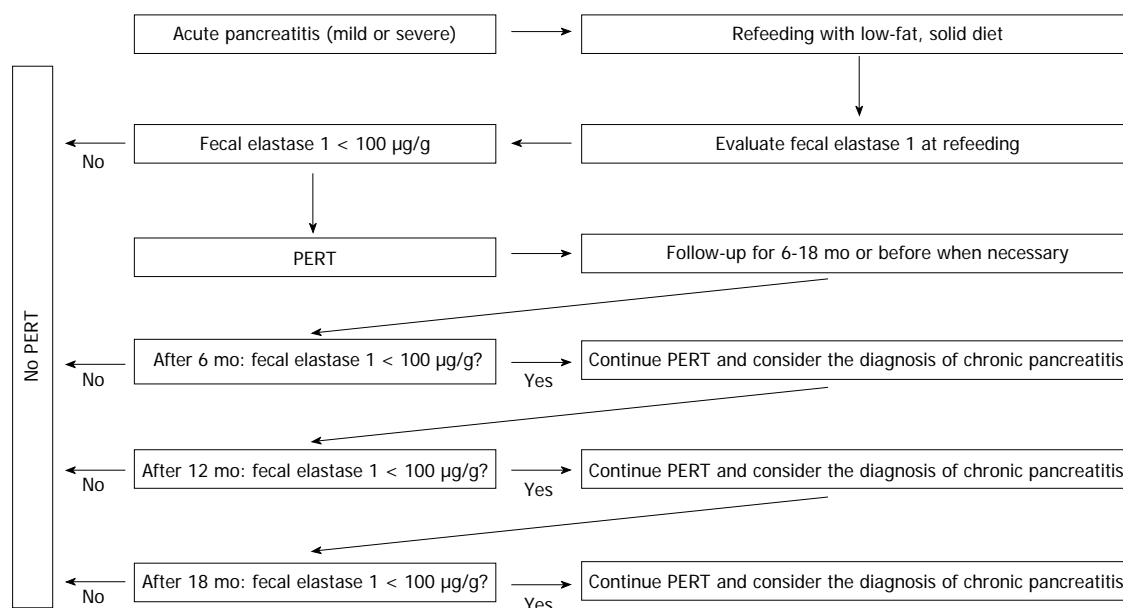
fected<sup>[30,34]</sup>, whereas enzymes contained in gastroprotected minimicrospheres can be assumed also with food having a pH less than 5.5. Acid-suppressing agents should be utilized only in patients who continue to experience symptoms of maldigestion despite the adequate administration of PERT<sup>[35]</sup>.

### Goal of the treatment

Steatorrhea in severe pancreatic insufficiency is very difficult to resolve completely, and only a 60%-70% reduction is usually achieved using PERT<sup>[36]</sup>. This may be due the fact that there are numerous interactions between pancreatic maldigestion, intestinal ecology and intestinal inflammation; consequently, to the methods of achieving optimal management of pancreatic maldigestion need to be fully re-evaluated, considering not only the correction of pancreatic insufficiency using PERT and, the best duodenal pH to allow for the optimal efficacy of these extracts, but also the decontamination of the intestinal lumen, the supplementation of bile acids and, probably, the use of probiotics to attenuate intestinal inflammation in chronic pancreatitis patients<sup>[37]</sup>. Fat soluble vitamins and micronutrients, such as zinc and selenium, should be routinely assessed and administered whenever necessary<sup>[38]</sup>.

### Warnings regarding PERT

An appropriate clinical response to PERT does not allow predicting a normal nutritional status in patients with chronic pancreatitis. Up to 2/3 of patients with an apparently good clinical response have some residual nutritional deficiency<sup>[39]</sup>. Crushing, chewing or holding the pancreatic extract capsules in the mouth may cause local irritation. The fine powder of the pancreatic enzymes may also be irritating to the nasal mucosa and the respiratory tract and can precipitate an asthma attack. Extremely high doses of pancreatic extracts have been associated with hyperuricemia and hyperuricosuria<sup>[40]</sup>. Submucosal



**Figure 1** Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients hospitalized for acute pancreatitis. PERT: Pancreatic enzyme replacement therapy.

strictures in the proximal colons of children with cystic fibrosis have been reported (“fibrosing colonopathy”), and it is now recommended that not more than 10000 units of lipase per kg of body weight per day be given to children<sup>[41]</sup>; to our knowledge, this complication has been never reported in adults<sup>[42]</sup>.

## RECOMMENDATION FOR SPECIFIC DISEASES

### Acute pancreatitis

In Italy, there are approximately 20000 admissions per year for acute pancreatitis (AP)<sup>[43]</sup>. Acute pancreatitis is an inflammatory disease most commonly caused by gallstones or alcohol abuse, and is associated with significant morbidity and mortality<sup>[44]</sup>. Pathological values of fecal elastase-1 have been found in 12.0% of patients with AP (9.3% with mild and 2.7% with severe pancreatitis). Pathological fecal elastase-1 was not significantly related to sex, age or day of refeeding. Finally, only 4.0% of patients may have severe EPI (*i.e.*, fecal elastase-1 concentrations less than 100 µg/g). Thus, in selected cases (approximately 800 Italian AP patients per year), there is the need for enzyme supplementation during refeeding if the elastase-1 fecal determination is clearly abnormal<sup>[45]</sup>. The suggestion is that these patients be monitored for EPI for at least 6-18 mo and treated with oral pancreatic enzymes at a dosage of 40000-50000 U per meal and 25000 U per snack unless otherwise indicated<sup>[27]</sup> (Figure 1).

### Chronic pancreatitis

The greatest benefit of PERT is in the patients who excrete more than 15 g of fecal fat per day or have weight loss<sup>[46,47]</sup>. However, German and Spanish guidelines treat patients with a daily fecal fat output < 15 g in the pres-

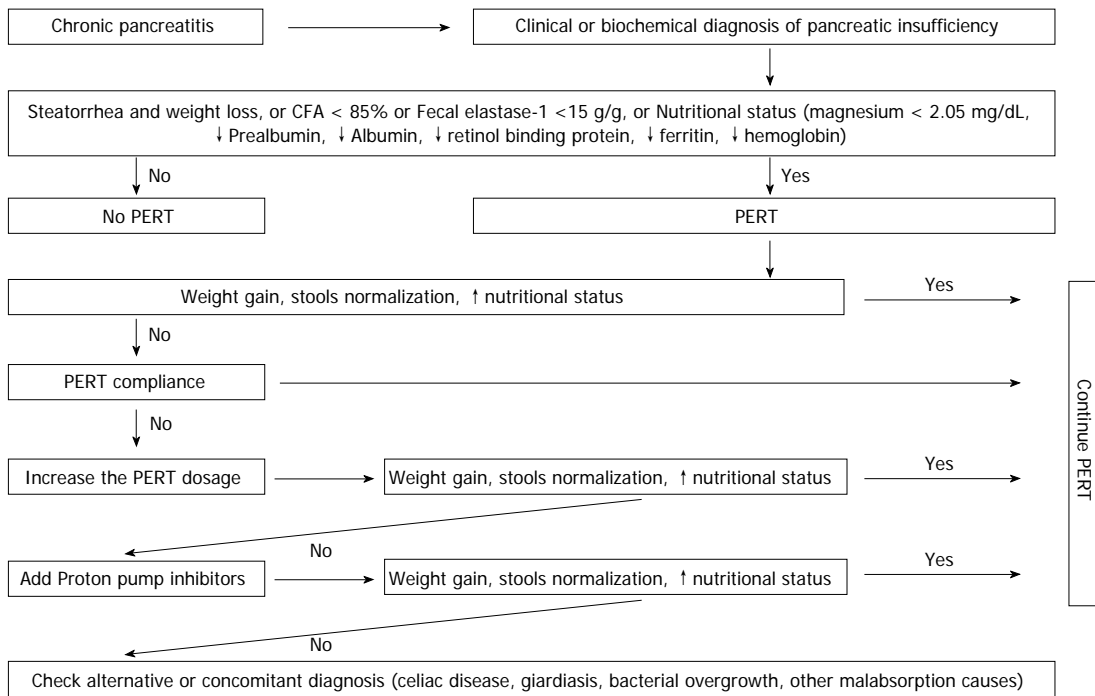
ence of symptoms of malabsorption (weight loss, osteopenia, loss of muscular mass)<sup>[9,31,48,49]</sup>. Alcohol should also be avoided to prevent additional impairment of the pancreatic exocrine function<sup>[50]</sup>.

The initial dose of pancreatic enzymes should be 40000 units as a starting dose for a meal and 20000 units for a snack<sup>[9,31,48,51]</sup>.

Increasing doses of PERT are recommended in non-responder patients<sup>[9,48,51]</sup>. Furthermore, acid suppression is also suggested to ensure optimal enzymatic delivery into the duodenum, despite the lack of clinical trials<sup>[52]</sup>. Moreover, as reported by Domínguez-Muñoz *et al*<sup>[53]</sup>, gastric acid inhibition avoids bile acid precipitation and allows lipase release in the proximal gut. It has been shown that patients with EPI respond properly to PERT if bicarbonate secretion is preserved and/or gastric secretion reduced. Calcium and magnesium-containing antacids should be avoided as they produce soaps, precipitate with glycine conjugated bile salts in the intestine and worsen steatorrhea<sup>[54]</sup>.

Lack of patient compliance may be a cause of treatment failure and can be discovered by measuring fecal chymotrypsin<sup>[55]</sup>. If chymotrypsin activity in the stool is low, the patient should be educated to take supplements during or just after meals<sup>[9,56]</sup>. Intestinal bacterial overgrowth, found in up to 40% of the patients with chronic pancreatitis<sup>[57,58]</sup>, intestinal giardiasis or other intestinal malabsorption disorders, should be ruled out in non-responder patients.

Parameters to be used for the assessment of therapy include clinical improvement/normalization of nutritional parameters and clinical symptoms<sup>[9,24]</sup>. In non-responder patients, laboratory methods for assessing fat absorption (CFA, C-13 mixed triglyceride breath test) may be used. Fat soluble vitamin deficiency should be



**Figure 2 Algorithm for monitoring and treating exocrine pancreatic insufficiency.** Algorithm for monitoring and treating exocrine pancreatic insufficiency summarized from Italian<sup>[9]</sup>, German<sup>[47]</sup> and Spanish<sup>[31]</sup> guidelines, and a synopsis of the guidelines<sup>[51]</sup>. CFA: Coefficient of fat absorption; FE1: Fecal elastase-1; PERT: Pancreatic enzymes replacement therapy; PPI: Proton pump inhibitor.

corrected parenterally<sup>[9]</sup>.

Before starting PERT, evaluation of the fasting glucose levels and quantification of the malabsorption is suggested, if possible. Moreover, determining glucose fasting levels during the first 1-2 wk of treatment is also suggested<sup>[51]</sup>. An algorithm for PERT in chronic pancreatitis patients is summarized in Figure 2.

### Unresectable pancreatic ductal adenocarcinoma

The prevalence of EPI is high but of moderate degree in the majority of cases; it has been reported that 65% of pancreatic cancer patients have fat malabsorption, and 50% protein malabsorption<sup>[59,60]</sup>. The causes of the EPI are mainly related to the obstruction of and/or the loss of the pancreatic parenchyma<sup>[61]</sup>. Thus, the most important predictors of the onset of EPI malabsorption in pancreatic cancer patients are the site of the tumor in the pancreatic head, the tumor replacing at least 90% of the normal pancreatic tissue and main duct obstruction<sup>[62-64]</sup>.

Even if the most widely accepted prognostic factors in unresectable pancreatic carcinoma are the presence of metastases and the value of CA 19-9 at presentation<sup>[65,66]</sup>, the prognostic factor “weight loss” has received particular attention from the “Eastern Cooperative Oncology Group” study<sup>[66]</sup> and in this study the weight loss ranged from 30% in patients with non-Hodgkin’s lymphoma to 87% in patients with gastric cancer; patients with pancreatic cancer showed weight loss in 65% of cases and it correlates with worsening of the performance status even if this factor was not a negative prognostic factor for survival<sup>[66]</sup>. In contrast, a more recent retrospective study found a direct relationship between the percentage

of weight loss and the risk of death, with a value greater than 7 times the expected value when the decrease exceeded 10%<sup>[67]</sup> and these data were confirmed by a retrospective study regarding 58 patients with unresectable pancreatic carcinomas showing that a phase angle of less than 5 degrees was a negative prognostic factor<sup>[26]</sup> and by a prospective non-randomized study enrolling 194 patients with unresectable advanced pancreatic cancer showing that a value of fecal elastase-1 less than 20 µg/g was a negative prognostic factor for survival. Of interest, a value of fecal elastase-1 of less than 20 µg/g and extremely severe pancreatic insufficiency were found more frequently in the group of patients with tumors in the head of the pancreas<sup>[68]</sup>.

The main question is whether replacement therapy with pancreatic enzymes and nutritional therapy have a positive impact on the quality of life and survival in patients with advanced pancreatic cancer. Pancreatic enzyme replacement therapy can partially prevent weight loss in patients with unresectable tumors of the pancreatic head, at least in the period before biliary endoprosthesis placement<sup>[69]</sup>. Two different phase II studies have shown that, in patients with advanced pancreatic cancer, having a weight loss of more than 5% in the previous 4 wk and a body mass index of less than 19, parenteral nutrition improved all nutritional parameters, as evaluated by the bioelectrical impedance without, however, reaching normality<sup>[70,71]</sup>.

The algorithm for monitoring EPI and malnutrition in unresectable pancreatic ductal adenocarcinoma patients is reported in Figure 3. Of course, appropriate amounts of pancreatic extracts should be administered during each

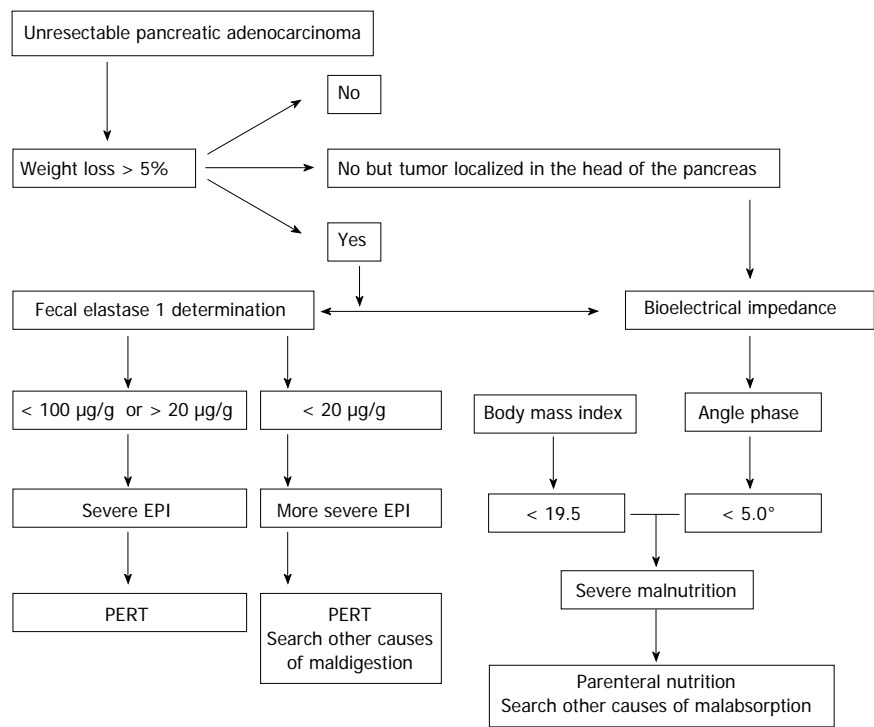


Figure 3 Algorithm for monitoring and treating exocrine pancreatic insufficiency and malnutrition in unresectable pancreatic ductal adenocarcinoma patients. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzymes replacement therapy.

Table 4 Fecal elastase 1 concentrations in type 1 and type 2 diabetes mellitus n (%)						
Ref.	Type 1 DM			Type 2 DM		
	Overall	FE-1 (100-200 µg/g)	FE-1 (< 100 µg/g)	Overall	FE-1 (100-200 µg/g)	FE-1 (< 100 µg/g)
Hardt <i>et al</i> <sup>[80]</sup>	322	73 (23)	92 (28)	697	108 (15)	138 (20)
Vesterhus <i>et al</i> <sup>[81]</sup>	140	10 (7)	16 (11)	63	2 (3)	6 (9)
Larger <i>et al</i> <sup>[82]</sup>	195	28 (14)	38 (19)	472	35 (7)	50 (10)
Icks <i>et al</i> <sup>[83]</sup>	112	22 (20)	29 (26)			
Cavalot <i>et al</i> <sup>[84]</sup>	37	17 (46)	4 (11)			
Rathmann <i>et al</i> <sup>[85]</sup>				544	100 (18)	65 (12)
Nunes <i>et al</i> <sup>[86]</sup>				42	6 (14)	9 (21)
Yilmaztepe <i>et al</i> <sup>[87]</sup>				32	9 (28)	1 (3)
Overall	837	158 (18.9)	188 (22.5)	1933	275 (14.2)	283 (14.6)

DM: Diabetes mellitus; FE-1: Fecal elastase-1.

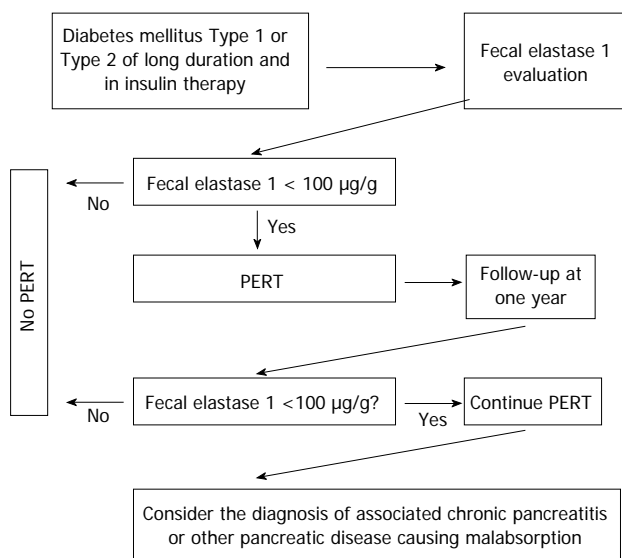
meal (40000-50000 U of lipase) and per snack (25000 U).

Diabetes mellitus

Exocrine pancreatic insufficiency was demonstrated in approximately 50% of patients with insulin-dependent diabetes, and in 30%-50% of those with non insulin-dependent diabetes<sup>[72-78]</sup>. Nine prospective reports evaluated EPI by means of fecal elastase-1 estimation in patients with either type-1 or type-2 diabetes<sup>[79-87]</sup> (Table 4) and included 2770 diabetic patients, 837 of them (30%) with type-1, and the remaining 1933 with type-2 diabetes mellitus (DM). Overall, fecal elastase-1 concentrations were abnormal (*i.e.*, < 200 µg/g) in 904 patients (32.6%) and the impairment was mild (*i.e.*, fecal elastase-1 > 100 but < 200 µg/g) in 439 patients (15.8%, overall); severe EPI (< 100 µg/g) was documented in 465 (16.8%). Of the 904 diabetics with abnormal fecal elastase-1, exocrine

impairment was mild in 48.9%, and severe in 51.4%. The prevalence of EPI differed slightly between type 1 and type 2 DM. Abnormal (< 200 µg/g) fecal elastase-1 concentrations were found in 346 (41.3%) of 837 type 1 diabetic patients, and in 558 (28.9%) of 1933 type 2 diabetic patients, a 12.4% difference in prevalence rates. More patients with type 1 DM (188 of 837, 22.5%) had signs of severe EPI, as compared to the 14.3% rate (277 of 1933) in type 2 DM. Exocrine pancreatic insufficiency is usually only of a mild to a moderate degree, and will not lead to clinically overt steatorrhea in the majority of diabetics. Thus, the clinical relevance of EPI and the role of functional tests in these patients are questionable. However, patients with DM frequently suffer from a wide range of abdominal complaints which contribute to impairment of the quality of life<sup>[88]</sup>. Although data are controversial, at least some of these symptoms may be attributable in





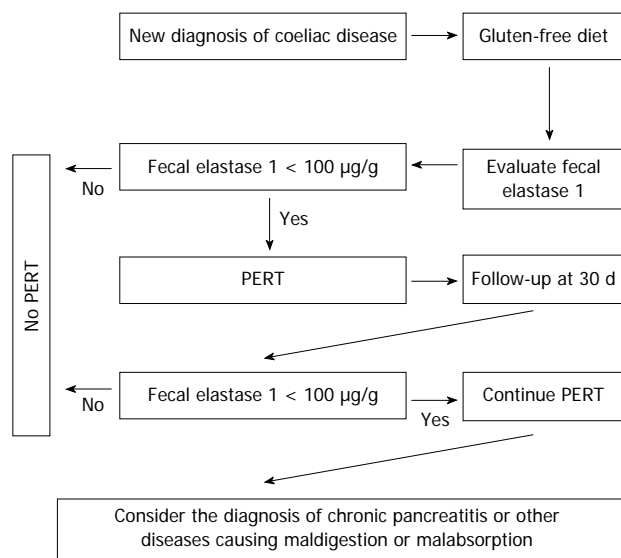
**Figure 4** Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients with diabetes mellitus. PERT: Pancreatic enzymes replacement therapy.

part to EPI (mild to moderate) and might respond to enzyme treatment<sup>[81,89-94]</sup>. Thus, pancreatic tests should be part of the diagnostic work-up in patients with symptoms and do not respond to simple therapeutic measures. As reported in Figure 4, patients with fecal elastase-1 < 100 µg/g should be given pancreatic enzymes in adequate daily doses (40000-50000 U of lipase) administered during meals. Treatment improves symptoms significantly, the supply of soluble fat vitamins is normalized, and the risk of osteoporosis is reduced. Enzyme replacement therapy might have an impact on glucose metabolism since it can reduce the insulin requirement and contribute to improved control of the glucose metabolism, but the evidence is contradictory<sup>[93,94]</sup> as improvement of glucose metabolism was not seen in all studies<sup>[95,96]</sup>.

### Celiac disease

The prevalence of adult celiac disease (CD) in the general population is reported to be 1%-2%<sup>[96-99]</sup>; diarrhea remains a common presenting symptom<sup>[100]</sup> and it is usually attributed to continued gluten ingestion; however, other causes of chronic diarrhea in patients who are compliant with their gluten-free diet exist, and one of them is exocrine pancreatic insufficiency. Using a secretin test, it has been found a mild reduction in the pancreatic secretion of bicarbonates and pancreatic enzymes (especially lipase) in untreated celiac patients these alterations revert to normal after going on a gluten-free diet; mild pancreatic insufficiency is present in about 40% of untreated CD patients and severe pancreatic insufficiency in 10%<sup>[101,102]</sup>. More recently, other authors using tubeless test, such as fecal chymotrypsin or elastase 1 determination and the C mixed-triglyceride breath test, confirmed that pancreatic insufficiency in untreated CD patients in percentages ranging from 11.4% to 56.2%<sup>[103-107]</sup>.

It has also been suggested exocrine pancreatic func-



**Figure 5** Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients with celiac disease. PERT: Pancreatic enzymes replacement therapy.

tion impairment may be related to the degree of mucosal villous atrophy and that the level of fecal elastase may improve once the mucosa has recovered after an appropriate gluten-free diet<sup>[104,108]</sup>. In addition, it seems that pancreatic insufficiency does not depend on nutritional status<sup>[105]</sup>. Regarding the use of PERT in these patients, the data come from a double blind randomized study carried out on children showing that pancreatic enzyme therapy is certainly useful in the first 30 d after the diagnosis of CD<sup>[106]</sup>. In fact, after 30 d of a gluten-free diet associated with pancreatic extracts, body weight significantly increases with respect to patients treated with only a gluten-free diet. Similar results were obtained in a longitudinal study<sup>[109]</sup>. The conclusion is that pancreatic enzyme therapy is certainly useful in the first 30 d after the diagnosis of CD and that enzyme supplementation may possibly be discontinued as symptoms improve and fecal elastase-1 concentrations normalize. The dosage of pancreatic extracts should be 40000-50000 U per meal and 25000 U per snack. In CD patients who continue to experience clinical steatorrhea despite being on a gluten-free diet, a search for possible exocrine pancreatic insufficiency must be carried out<sup>[110]</sup>. In addition, we should bear in mind that, in adult CD patients have a risk developing chronic pancreatitis more than 3 times as compared to general population and there is also an increased need for PERT<sup>[111]</sup>. An algorithm for PERT in CD is reported in Figure 5.

### Inflammatory bowel diseases

**Crohn's disease:** About 35% of the patients with Crohn's disease have an impaired exocrine pancreatic function<sup>[112]</sup> and no relationship are present between exocrine pancreatic insufficiency and age or nutritional status. Interestingly, patients having steatorrhea had a defect of lipase output ranging from 10% to 67% and, especially in this

latter group of patients, the use of PERT was hypothesized. In patients with Crohn's disease, enzyme activities were not correlated to the duration of disease or to the extent or localization of a previous bowel resection<sup>[113]</sup>. The lowest enzyme values were found in patients with the most extensive bowel involvement, and they were significantly lower than in patients with disease confined to the terminal ileum. Thus, the factors related to the impaired pancreatic function in Crohn's disease seem to be disease activity, and the localization and extent of the disease. Finally, patients with Crohn's disease may have an autoimmune involvement of the pancreatic gland and those having positive serum pancreatic autoantibodies may also have impaired exocrine pancreatic function more frequently<sup>[114]</sup>. However, we have no evidence that PERT can be utilized in patients with Crohn's disease and exocrine pancreatic insufficiency to improve the maldigestion present in these patients.

**Ulcerative colitis:** Pancreatic exocrine insufficiency, assessed using a secretin-cerulein test, may be present in about 40%-50% of patients with ulcerative colitis<sup>[112,115,116]</sup> especially during a active phase of the disease and the majority of patients with pancreatic insufficiency had active disease with loose stools; thus, the reduced fecal elastase-1 concentration could have been due to dilution of the enzyme and not to pancreatic involvement. In addition, in those patients who were also studied during the remission phase of the disease and had a solid stool, the fecal elastase-1 concentration became normal<sup>[112]</sup>. More recently, the possibility of autoimmune pancreatitis associated with ulcerative colitis has been reported. Thus, it is possible that only a small number of ulcerative colitis patients having severe pancreatitis insufficiency due to autoimmune pancreatitis may benefit from PERT.

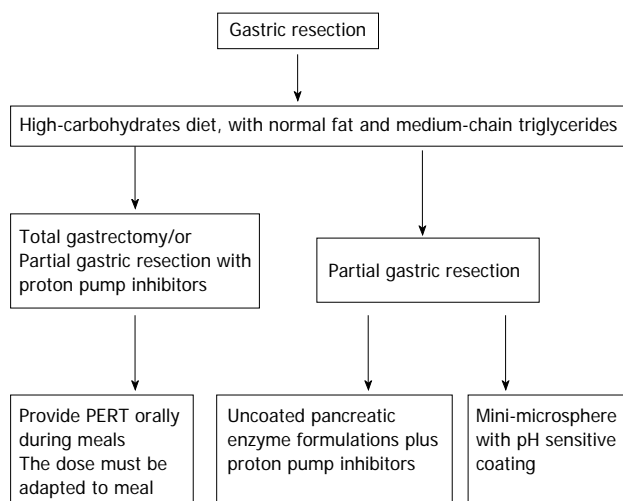
### Gastric surgery

Exocrine pancreatic insufficiency is a common clinical problem after gastric surgery<sup>[117]</sup>. The side effects of gastric resections, in particular total gastrectomy, include diarrhea, anorexia, weight loss and EPI that are responsible for a global status of malnutrition, malabsorption and maldigestion<sup>[118]</sup>. Malnutrition is considered one of the major complications after gastric surgery for gastric cancer<sup>[119]</sup> and EPI contributes to the pathogenesis of global malnutrition. After gastric surgery, EPI can result from various causes, such as a deficient trituration of nutrients, altered gastric emptying, alteration of pancreatic denervation and post-cibal asynchrony<sup>[120,121]</sup>.

Any surgical procedure, such as total or subtotal gastrectomy, total or subtotal pancreatectomy, with or without duodenal resection (*e.g.*, in the context of a Whipple procedure), causing distortion in the anatomic-physiology of digestion can be responsible for EPI<sup>[122-127]</sup>. Several events can be considered as being responsible for EPI after gastrectomy. Alterations of gastric relaxation due to the absence of nervous gastric reflexes; the absence of nervous gastric stimulation responsible for pancreatic

secretion caused by the lack of fundus relaxation and the reduction of exocrine pancreatic secretion due to the absence of cholecystokinin after intestinal resection. Rapid gastric emptying and asynchrony between gastric emptying and biliopancreatic secretion due to new tracts of various reconstructions, bacterial overgrowth after gastrectomy, extensive denervation of the pancreas due to lymph node dissection and truncal vagotomy are the most frequent alterations involved in the pathogenesis of EPI<sup>[123,128,129]</sup>. The latter has been shown to cause mild to moderate EPI by itself<sup>[130,131]</sup>. In 1996, Friess *et al.*<sup>[123]</sup> demonstrated that 100% of patients develop severe primary EPI three mo after a total gastrectomy. Chymotrypsin and trypsin were the most severely deficient enzymes after gastric surgery, with a decreased production of up to 91% three mo after surgery. Low levels of gastrin and postprandial pancreatic polypeptides, and high levels of cholecystokinin were also reported<sup>[123]</sup>. Exocrine pancreatic insufficiency is reported in both total and partial gastrectomy; Büchler *et al.*<sup>[127]</sup> demonstrated that the pancreolauryl test was pathological in 47%-64% of patients after Billroth-I surgery and in 64%-70% after Billroth-II surgery. On the contrary, Heptner *et al.*<sup>[124]</sup> reported EPI after gastric resection in only 30% of patients, even if the pancreolauryl test was abnormal in 90% of these patients. Armbricht *et al.*<sup>[132]</sup> conducted a double-blind, crossover study of 15 patients who underwent surgery for gastric cancer (total gastrectomy) and compared PERT with a placebo. The authors concluded that PERT reduced massive steatorrhea and improved stool consistency after total gastrectomy. Nevertheless, Bragelmann and co-workers reported an overall improvement in abdominal symptoms, fecal frequency and fecal consistency when following 52 institutionalized patients with a fecal fat output greater than or equal to 14 g/d after gastric resection for cancer, but no differences were found regarding body mass index, bowel habits or fat malabsorption<sup>[133]</sup>. Interestingly, Huddy and coworkers found that EPI contributes to postoperative morbidity after an esophagectomy, and that these patients can benefit from PERT<sup>[134]</sup>.

The main goal of the therapy in patients suffering from EPI is to reverse all the secondary events caused by enzyme deficiency (Figure 6). Therapeutic efficacy is closely connected with two important aspects: time and dosage of the pancreatic enzymes administered, and dietary changes<sup>[135]</sup>. Nutritional changes should include a high carbohydrate diet, with normal fat and medium-chain triglycerides<sup>[136]</sup>. It is also recommended that a personalized diet be created after major gastrointestinal surgery in order to prevent weight loss, anorexia, inflammation and changes in homeostasis<sup>[53]</sup>. Following a total gastrectomy and in patients receiving therapy with proton pump inhibitors (PPIs) for some reason, unprotected pancreatin powder is preferred<sup>[120]</sup>. In addition to dietary changes it is mandatory to resort to PERT, orally ingested, during meals. The dose must be adapted to the meal and should not be less than 40000-50000 U of lipase per meal<sup>[135]</sup>. For partial gastric resection, patients receiving



**Figure 6** Algorithm for treating exocrine pancreatic insufficiency in patients who undergo gastric resection. PERT: Pancreatic enzymes replacement therapy.

uncoated pancreatic enzyme formulations should also require simultaneously administered proton pump inhibitors<sup>[53]</sup> since lipase is irreversibly deactivated by gastric acid. However, the administration of PPIs can improve fat digestion even in patients who do not benefit from PERT<sup>[53]</sup>. Several authors agree with the need for liposoluble vitamin supplementation, especially for patients with severe EPI<sup>[135]</sup>.

### Pancreatic resections

Suggestions on this topic are based on a consensus reached by the experts and are not fully based on data coming from literature. Partial or total pancreatectomy (TP) is frequently associated with EPI. In this setting, PERT is essential for maintaining adequate digestion. In a TP, the removal of the entire pancreatic parenchyma produces inevitable exocrine failure while, in a partial pancreatectomy, the severity of EPI depends on both the underlying disease, the preoperative pancreatic function, and the extent and type of the resection. Most importantly, any pancreatic neoplasm can be associated with chronic obstructive pancreatitis (focal or extended) which might affect pancreatic function/secretion, leading to EPI before any type of resection.

### Extent and type of the resection

**Pancreaticoduodenectomy:** Anatomical changes secondary to reconstruction after a pancreaticoduodenectomy (PD) lead to important physiological alterations which frequently correlate with the severity of postoperative EPI. A PD (either Whipple or pylorus-preserving) is associated with several and complex patho-physiological events such as: (1) disturbance of gastric fundus relaxation caused by the disappearance of antro-fundic and duodeno-fundic reflexes; (2) the absence of neurally stimulated pancreatic excretion caused by the lack of fundus relaxation; (3) the reduction of cholecystokinin-mediated stimulation of pancreatic secretion secondary

to duodenal resection; (4) large and hard to digest nutrient particles reaching the jejunal lumen due to resection of the distal stomach (Whipple procedure); (5) reduction in exocrine pancreatic secretion due to pancreatic head resection; and (6) asynchrony between the gastric emptying of nutrients and bilio-pancreatic secretion as a result of anatomical reconstruction<sup>[122,128,137,138]</sup>.

For these reasons, every patient who is candidate for a PD should be considered at increased risk for EPI regardless of the underlying disease<sup>[128,139]</sup>. Therefore, it has been suggested that, after PD, PERT be given to all patients with pancreatic cancer, especially those with impending adjuvant therapy. Furthermore, it should be considered that pancreatic cancer is often associated with obstructive chronic pancreatitis, a preoperative risk factor for the development of EPI by itself<sup>[128,139]</sup>. The development of pancreatic insufficiency after PD could also be related to the different techniques used for the pancreatic anastomosis<sup>[117,140,141]</sup>.

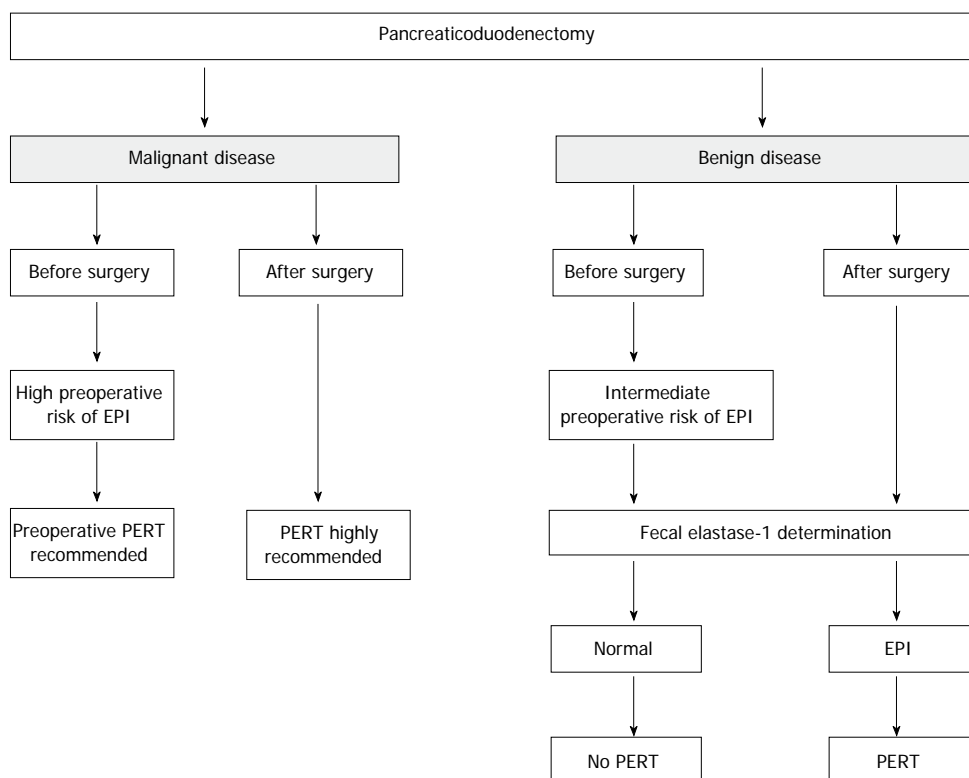
**Distal pancreatectomy:** Distal pancreatectomy (DP) is the procedure of choice for treating lesions affecting the body-tail of the gland. A DP may affect pancreatic exocrine function depending on the amount of normal tissue removed<sup>[142,143]</sup>. Based on these data, permanent postoperative EPI, as a result of parenchymal loss from pancreatic resection, was not observed and these conclusions have been subsequently confirmed<sup>[139]</sup>.

### Atypical resections: Middle pancreatectomy and enucleation

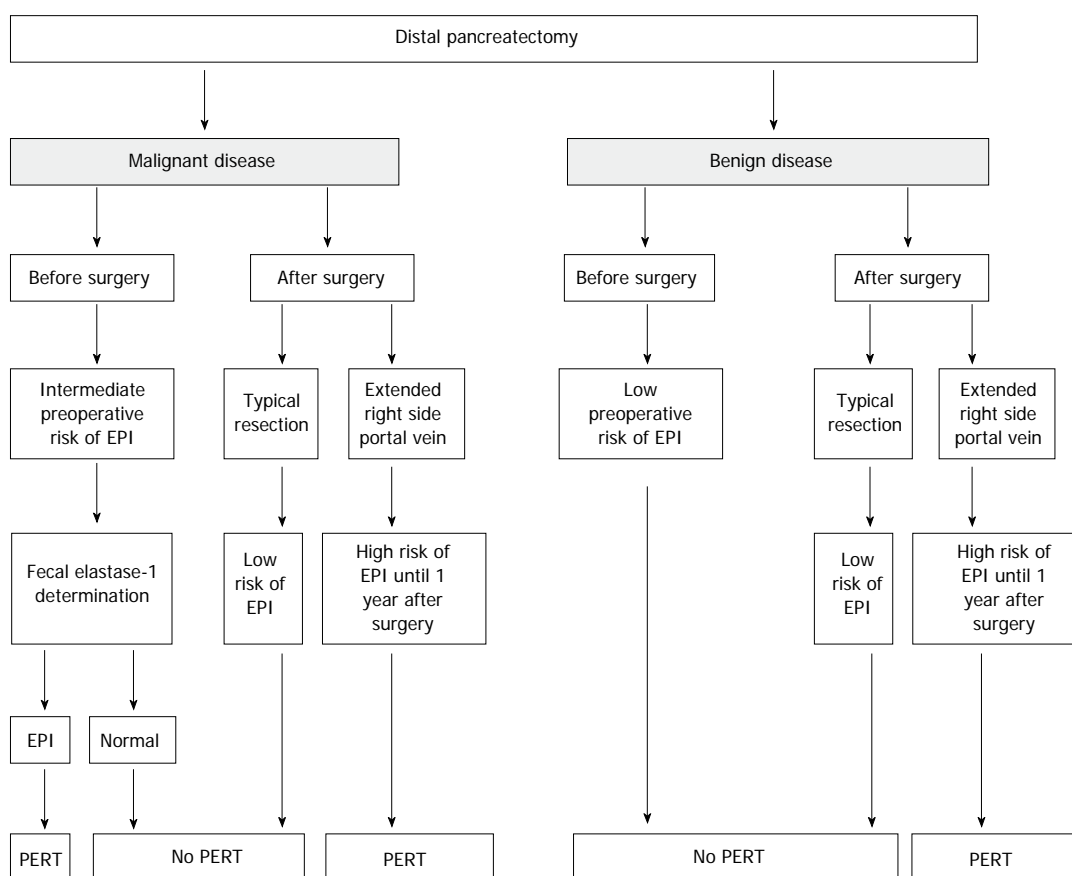
Atypical resections are usually performed for benign or borderline pancreatic tumors, such as small (< 2 cm) pancreatic neuroendocrine tumors, cystic papillary tumors, low grade intraductal papillary mucinous neoplasms and serous cystic adenomas. Enucleation is usually performed for tumors smaller than 2 cm located in any part of the pancreas but sufficiently far from the main pancreatic duct. A MP is indicated for neoplasm in the neck of the pancreas which could not be safely enucleated<sup>[139,144-146]</sup>. Using the <sup>13</sup>C-mixed triglyceride breath test (normal test > 5%), it has been found an EPI rate of 5% after a median follow up of 71 mo<sup>[147]</sup>. In addition, Crippa *et al.*<sup>[148]</sup>, after a median follow-up of 54 mo, observed a rate of clinical EPI of 5% in a cohort of 100 patients who had undergone MP for benign or borderline tumors. The authors compared this result with an EPI rate of 15.6% in patients who underwent an extended left pancreatectomy (at the right side of the superior mesenteric vein), the alternative surgical procedure to MP for lesions located in the pancreatic neck. These data are consistent with those of others<sup>[149,150]</sup>.

### Treatment of EPI in pancreatic resection

It has been reported that in patients undergoing a pylorus-preserving pancreaticoduodenectomy for pancreatic neoplasia, gastro-protected microspheres were less effective than those in patients who had undergone a classic Whipple technique<sup>[151]</sup> probably because microspheres are retained in the stomach. One of the few randomized

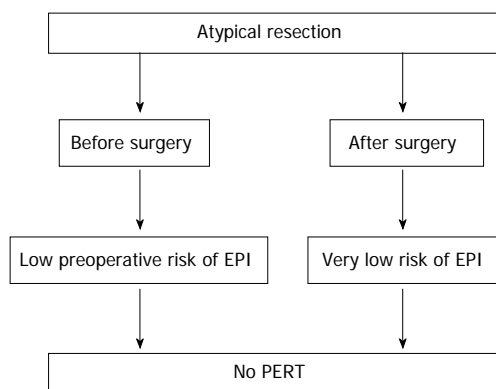


**Figure 7** Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients who receive pancreaticoduodenectomy. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzyme replacement therapy.



**Figure 8** Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients who undergo distal pancreatectomy. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzymes replacement therapy.





**Figure 9** Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients who undergo atypical resection of the pancreas. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzymes replacement therapy.

studies explaining the efficacy of pancreatic extracts for the control of malabsorption was carried out on a small group of patients with chronic pancreatitis who had undergone a pancreatic resection with longitudinal pancreaticojejunostomy<sup>[152]</sup> and showed that treatment with pancreatic extracts ameliorated not only the nitrogen balance but also the fat and protein absorption. Another randomized controlled double-blinded crossover study explored the comparative efficacy of two pancreatin preparations of gastroprotected microspheres with different doses in pancreatectomized patients having chronic pancreatitis<sup>[153]</sup>. All patients were stabilized before enrollment in the study with a standard dose of pancreatic extracts. After this stabilization period, 56% of the patients still had a fecal fat excretion greater than 7 g/d, and 38% greater than 15 g/d. The results demonstrated that there was a significant relationship between fecal fat excretion, fecal volume and evacuation frequency but there was no relationship between fecal fat excretion, and abdominal pain or malabsorption. Both the pancreatin standard dose and the elevated dose demonstrated equal efficacy; in pancreatectomized patients, high dose pancreatic extracts significantly reduced the number of capsules needed per day with a better compliance to substitutive therapy. From the clinical point of view, pancreatic enzyme replacement therapy needs to be routinely considered and based on pragmatic clinical evaluation of the patient<sup>[22,38,63]</sup>. The suggested algorithms for PERT in patients undergoing surgery, according to the type of pancreatic resection, are reported in Figures 7-9, taking into consideration that the dosage should be no less than 40000-50000 U of lipase per meal and 25000 per snack.

## CONCLUSION

We should point out that there is a paucity of information regarding some areas of managing EPI there is a lack of good quality of literature. Finally, studies on the economic aspects of this treatment with the different formulations commercially available are also necessary; it has been calculated that the treatment of chronic pan-

creatitis-related EPI with pancreatin minimicrospheres is cost-effective according to a survey of Polish patients but it is necessary that these results also be evaluated for the Italian National Health System<sup>[154]</sup>.

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## MicroRNAs as tools to predict glucocorticoid response in inflammatory bowel diseases

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expression of certain miRNA networks in the pathogenesis of autoimmune and inflammatory diseases, such as IBD. There is a great interest in the identification of the role of miRNAs in the modulation of pharmacological response; however, the association between miRNA and GC response in patients with IBD has not yet been evaluated in a prospective clinical study. The identification of miRNAs differently expressed as a consequence of GC treatment in comparison to diagnosis, represents an important innovative approach that could be translated into clinical practice. In this review we highlight the altered regulation of proteins involved in GC molecular mechanism by miRNAs, and their potential role as molecular markers useful for predicting in advance GC response.

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**Key words:** Glucocorticoids; Inflammatory bowel diseases; MicroRNA; Molecular markers; Pharmacogenomics

### Abstract

In spite of the introduction in therapy of highly effective biological agents, glucocorticoids (GCs) are still employed to induce remission in moderate to severe inflammatory bowel diseases (IBD), but considerable inter-individual differences in their efficacy and side effects have been reported. The effectiveness of these drugs is indeed very variable and side effects, particularly severe in pediatric patients, are common and often unpredictable: the understanding of the complex gene regulation mediated by GCs could shed light on the causes of this variability. In this context, microRNAs (miRNAs) represent a new and promising field of research. miRNAs are small non-coding RNA molecules that suppress gene expression at post-transcriptional level, and are fine-tuning regulators of diverse biological processes, including the development and function of the immune system, apoptosis, metabolism and inflammation. Emerging data have implicated the deregulated

**Core tip:** Studies on microRNAs (miRNAs) and pharmacogenomics represent a promising investigation topic that could increase the understanding of the pharmacology of steroids in inflammatory bowel diseases (IBDs) and possibly in other diseases. A number of studies have shown that glucocorticoids (GCs) can modify the expression profiles of different miRNAs, however, the obtained results have been highly variable, and to date it is not possible to recognize a specific miRNA pattern regulated by GCs. Moreover, existing studies employed techniques based on the use of reverse transcription quantitative polymerase chain reaction and microarrays, through the analysis and quantification of already known miRNAs. Using next generation sequencing technologies, it could be possible to detect novel, still unrecognised miRNAs, and identify new miRNA isoforms (iso-miRs) as well. This innovative approach could be a valuable tool for a better understanding of the role

of miRNAs to predict steroid response in IBDs. In the future, the increased availability and the reduced costs of RNA profiling should enable the clinicians to stratify patients on specific miRNA biomarkers before starting GC treatment.

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## INTRODUCTION

To date, a curative pharmacological therapy for inflammatory bowel diseases (IBD) does not exist and the therapeutic approach is mainly aimed at controlling inflammation, with drugs capable of inducing and maintaining remission. Despite the introduction in therapy of highly effective biological agents, in IBD patients with moderate to severe disease glucocorticoids (GCs) are effective in inducing remission and are still considered the standard for treatment<sup>[1]</sup>. In spite of the large clinical use, the benefits of these agents are often narrowed by high inter-individual variability. Given the high incidence of suboptimal response, associated with a significant number of side effects, the identification of subjects that are most likely to respond poorly to these agents is extremely important. However, the mechanisms of this variability are scarcely understood and there is presently no means to predict the response in advance<sup>[2-5]</sup>; in this context, microRNAs (miRNAs) represent a new and promising field of research.

miRNAs are small (18-24 nucleotides) non-coding RNAs, which bind the 3'UTRs and the coding exons of their target genes and inhibit gene expression<sup>[6]</sup> either by messenger RNA (mRNA) cleavage (most common in plants) or by translational repression (most common in metazoan)<sup>[7,8]</sup>. According to the miRNA database miRBase, 1872 precursors and 2578 human mature miRNA sequences have been published (<http://www.mirbase.org><sup>[9,10]</sup>) and we are only on the verge of understanding their physiological impact on gene regulation. A single miRNA can regulate a multitude of mRNAs (approximately 200), and each mRNA can be regulated by multiple miRNAs<sup>[11,12]</sup>; overall, it is predicted that protein production for at least 20% of all human genes is regulated by miRNAs<sup>[13,14]</sup>.

By affecting gene regulation, miRNAs are likely to be implicated in the control of diverse biological processes, such as cellular proliferation and apoptosis<sup>[10,15-17]</sup>, stem cell differentiation<sup>[15,18-20]</sup>, and organ development and morphogenesis<sup>[21,22]</sup>; in addition a strong association between miRNA expression dysregulation and induction of cancer has been shown<sup>[23-26]</sup>. Moreover, miRNAs have important regulatory roles in the innate and adaptive immune system<sup>[27-29]</sup>, and characteristic miRNA expression

profiles have been demonstrated even in IBD<sup>[30-33]</sup>.

There is a lot of interest in identifying the role of miRNAs in the modulation of drug response<sup>[34]</sup>, but studies about this topic are still very limited, and the possible correlation between miRNAs expression and variability on GC response in IBD patients has not yet been examined. A better knowledge of miRNAs role could lead to their use as biomarkers for IBD, and consequently, to the development of new strategies for therapy personalization in these diseases.

This review tries to highlight the altered regulation of proteins involved in GC molecular mechanism by miRNAs in different diseases and *in vitro* models, and their potential role as molecular markers useful for predicting in advance GC response.

## GLUCOCORTICOIDS IN INFLAMMATORY BOWEL DISEASES

GCs are effective inhibitors of cytokine secretion and T-cell activation, and are consequently largely employed in different inflammatory conditions, including IBD. Despite the introduction of novel therapies, these agents are still currently used for induction of remission in moderate to severe IBDs, however, a wide variability in response to these agents is evident and, in these diseases, GC resistance or dependence is particularly frequent. Among the adult IBD population, a prospective analysis has described the 1-year outcome in patients with Crohn's disease (CD) treated with a first oral prednisone course (40-60 mg/d) and tapering to a maintenance dose of 10-15 mg/d<sup>[35]</sup>. Prolonged steroid response was obtained in 44% of patients, 36% of subjects were steroid dependent while 20% of subjects did not respond and were steroid resistant; a high frequency of surgery was reported within 1 mo after steroid treatment. Similar results have been obtained in a retrospective American study: immediate outcomes for CD and ulcerative colitis (UC), respectively, were complete remission in 58% and 54% of cases, partial remission in 26% and 30%, resistance in 16% of patients<sup>[36]</sup>. In paediatric IBD patients, clinical reports have shown that up to 90% of subjects has a rapid improvement of symptoms when prednisone treatment is given; however, after 1 year, only 55% of patients were still in remission and were considered steroid responsive. In around 38% of patients, steroid therapy could not be discontinued as patients experienced an increase of disease activity when the dose was reduced (steroid dependent)<sup>[37]</sup>.

Demographic and/or clinical markers<sup>[36,38,39]</sup> have been evaluated and related with this variability in GC response, but results have not been consistently replicated. Genetic and epigenetic markers are likely to complement clinical and demographic predictors: phenotypes resulting from genetic changes and regulation can markedly influence drug pharmacokinetics or alter drug efficacy and/or toxicity profiles. The identification of genetic biomarkers that can be useful for classifying the disease and help to improve therapy is paramount.



## MOLECULAR MECHANISM OF GC ACTION

The effects of GCs are mediated by the glucocorticoid receptor (GR)- $\alpha$ , a member of the nuclear receptor superfamily of ligand-dependent transcription factors<sup>[40,41]</sup>. The human GR gene is encoded on chromosome 5q31.3 and consists of nine coding exons<sup>[42]</sup>. Alternative splicing of exon 9 generates two receptor isoforms, GR- $\alpha$  and GR- $\beta$ <sup>[43-46]</sup>. GR- $\beta$  is not able to bind GCs, resides constitutively in the nucleus of cells, has a longer half-life than GR- $\alpha$ , and does not transactivate GC-inducible reporter genes<sup>[47]</sup>. It has been suggested<sup>[48,49]</sup> that cell specific expression and function of GR isoforms may explain the tissue and individual selective actions of GCs.

The function of GR is conditioned by chaperone and co-chaperone proteins that form a molecular heterocomplex with the GR itself<sup>[50,51]</sup>, required for proper ligand binding, receptor activation and transcription: abnormalities in proteins that make up the heterocomplex may contribute to altered GC responsiveness<sup>[52,53]</sup>. Several studies have demonstrated differences in the heterocomplex gene expression profiles in steroid resistant in comparison with responder patients, but it is not clear if this different expression is the cause of the variability in response or the consequence of GC treatment<sup>[54-59]</sup>. After GC binding and dissociation from heterocomplex proteins, the GR translocates into the nucleus; translocation is mediated by specific nuclear transport factors that belong to the importin  $\beta$  family of nuclear transporters, and in particular by importin 13<sup>[60]</sup>. The activated receptor then binds as homodimer two palindromic DNA-binding sites, the so-called glucocorticoid responsive elements (GREs), localized in the promoter region of target genes<sup>[61-63]</sup>. As a consequence of DNA binding, GCs can induce trans-activation and trans-repression processes: binding to positive GREs leads to activation of the transcription of anti-inflammatory [*e.g.*, interleukin 10 (IL-10), Annexin 1] as well as of regulator proteins involved in metabolic processes (*e.g.*, enzymes of gluconeogenesis)<sup>[64-66]</sup>. The second mechanism of GC action is trans-repression<sup>[67]</sup>, which leads to a reduced expression of immune-regulatory and proinflammatory proteins such as cytokines [IL-1, IL-2, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )] and prostaglandins<sup>[68]</sup>, and is believed to be responsible for the majority of beneficial anti-inflammatory effects.

Steroid hormones can regulate gene expression post-transcriptionally, by destabilizing mRNAs<sup>[69]</sup>. In addition, these hormones can induce rapid non genomic effects within the cytoplasm; for example, they induce the release of Src kinase from the GR heterocomplex, resulting in lipocortin activation and inhibition of arachidonic acid release<sup>[70,71]</sup>, and alter cytoplasmic ion content<sup>[72,73]</sup>.

## miRNAS AND GC RESPONSE

### miRNA regulation by GCs

It has been demonstrated that activation of GR by GCs

might induce or repress specific miRNAs in various target genes. The majority of studies have evaluated the effect of GCs on miRNA expression levels in tumor leukemic cells, during GC induced apoptosis<sup>[74]</sup>.

Rainer *et al.*<sup>[75]</sup> have correlated miRNA levels with expression data of their host genes in cell lines and clinical samples of children with acute lymphoblastic leukemia (ALL) undergoing systemic GC monotherapy. At least 5 miRNAs were significantly regulated by GC therapy. Importantly, the miR-15/16 cluster, which induces cell cycle arrest, was up-regulated by GCs in a subset of ALL patients and cell lines, consistent with the known apoptotic effect of GCs in immature lymphoblasts. Indeed, overexpression of miR-15b/16 increased GC sensitivity in leukemia cell lines whereas silencing miR-15b/16 with inhibitors decreased GC sensitivity *in vitro*.

Another study in a T-cell lymphoma cell line has shown that GC treatment repressed the expression of the miRNA cluster miR-17-92, which results in elevated protein expression of Bim, a proapoptotic member of the B-cell lymphoma-2 family (Bcl-2). Overexpression of miRNA cluster miR-17-92 decreased Bim induction, and attenuated GC mediated apoptosis, while cluster knockdown increased Bim induction and GC mediated apoptosis<sup>[76]</sup>. These findings suggest a novel mechanism that could contribute to the induction of lymphocyte apoptosis by GCs.

Harada *et al.*<sup>[77]</sup> demonstrated that in the leukemic cell line RS4; 11 dexamethasone down-regulated miRNA levels; miR17HG was rapidly down-regulated, and chromatin immunoprecipitation demonstrated that the promoter is a target of GC transcriptional repression; in particular, the miR-17-92 cluster was identified as a prime target for dexamethasone induced repression. In the sensitive leukemia cell line SUP-B15, but not in the resistant line REH, dexamethasone reduced the expression of the miR-17 family and concomitantly increased its target protein Bim. Up-regulation or inhibition of miR-17 resulted in a decrease and increase, respectively in Bim protein levels and in dexamethasone induced cytotoxicity. Down-regulation of miR-17 levels was observed in *ex vivo* patients' leukemia cells that underwent dexamethasone induced apoptosis<sup>[77]</sup>.

Another recent study<sup>[78]</sup>, by genome wide miRNA microarray on diagnostic bone marrow samples of ALL pediatric patients treated with GCs, identified a reduced expression of miR-355 as the most significant miRNA abnormality associated with poor outcome. Moreover, the authors demonstrated that exogenous expression of miR-355 in ALL cells increases sensitization to prednisolone-induced apoptosis. MAPK1 was identified as a target of miR-355, and the MEK/ERK inhibitor treatment increased GC induced cytotoxicity through the activation of Bim.

Smith *et al.*<sup>[79]</sup> have demonstrated that miRNAs are repressed during GC induced apoptosis of primary rat thymocytes, and further demonstrated the repression

of the miRNA processing enzymes Dicer, Drosha and DGCR8/Pasha. Silencing of Dicer expression in two human leukemic lines significantly enhanced GC induced apoptosis, while overexpression of the GC-repressed miR-17-92 polycistron reduced apoptosis.

Among the few studies that have considered the effect of GCs on miRNA expression in non tumor cells, Ledderhose *et al.*<sup>[80]</sup> in native and CD3/CD28 stimulated cells from healthy volunteers, demonstrated that miR-24 is expressed in human T cells, and expression is increased 1.7 fold upon stimulation. Hydrocortisone significantly enhanced by 3 fold the miRNA induction<sup>[80]</sup>.

In human corneal fibroblast treated for 16 h with dexamethasone, genome microarray and microRNA analyses were used to evaluate gene and miRNA expression. In response to treatment with the steroid, 261 genes were up-regulated and 123 were down-regulated more than three-fold. Several miRNAs, including miR-16, miR-21 and miR-29C were up-regulated, whereas miR-100 was down-regulated by the steroid, suggesting a posttranscriptional control of gene expression through miRNAs<sup>[81]</sup>.

Studies of the miRNAs profile on mucosal biopsies of patients with eosinophilic esophagitis, before and after successful treatment with GCs were conducted by Lu and collaborators<sup>[82]</sup>; of the 377 miRNA sequences examined, 32 miRNAs were significantly up-regulated and 4 down-regulated in the biopsies obtained before treatment compared to samples obtained after GC therapy. miR-214 was the most up-regulated (150 fold) and miR-146b-5b, 146a, 145, 142-3p and 21 were up-regulated by at least 10 fold.

Williams *et al.*<sup>[83]</sup>, using a highly sensitive reverse transcription-polymerase chain reaction, measured 277 miRNAs in airway biopsies obtained from normal subjects and mild asthmatic patients before and after one month twice daily treatment with inhaled budesonide. No significant difference in miRNA expression was evident in the airway biopsies of normal and asthmatic subjects, and, despite improved lung function, no change in miRNAs expression was evident after one month budesonide treatment. However, a specific miRNA expression profile was observed in different cell types (alveolar epithelial cells, airway smooth muscle cells, alveolar macrophages, lung fibroblasts).

Finally, in a recent study<sup>[84]</sup>, activated human CD4<sup>+</sup> T cells from healthy donors were exposed *in vitro* to 1  $\mu$ mol/L of methylprednisolone and changes in miRNA and mRNA expression profiles were analyzed by microarrays; a number of steroid responsive genes and miRNAs were identified. Further studies with qPCR, flow cytometry and ELISA, demonstrated that methylprednisolone increased the expression of miR-98 and suppressed the levels of predicted targets, including the pro-inflammatory cytokine IL-13 and three TNF receptors FAS, FASL, and TNF receptor superfamily member 1B (TNFRSF1B); these data suggest that methylprednisolone acts through miR-98 to inhibit specific pro-inflammatory targets<sup>[84]</sup>.

### GR as miRNA target

The role of miRNAs in the regulation of the GR has been examined, indeed, computational studies showed that the 3' UTR of the GR is predicted to contain numerous seed regions recognized by a variety of miRNAs<sup>[85]</sup>.

Using a combination of *in silico* prediction of miRNA binding sites, miRNA overexpression studies and mutagenesis of the GR 3'UTR, Vreugdenhil and collaborators<sup>[86]</sup> found that miR-18 and miR-124a bind GR mRNA and decrease GR activity in neuronal tissues. These miRNAs were tested for their ability to alter the translational activity of GR and reduce GR protein levels in cell cultures *in vitro*; miR-18 and miR-124a overexpression reduced GR protein levels and impaired the activation of the GR responsive gene glucocorticoid-induced leucine zipper (GILZ). In addition these authors have demonstrated by miRNA reporter assay that miR-124a is able to bind to the predicted seed region in the GR 3' UTR.

Ledderose *et al.*<sup>[80]</sup> have investigated the role of miR-124 in the regulation of GR expression; these authors have studied the influence of the GR isoforms (the active isoform  $\alpha$ , and the dominant negative non-ligand-binding isoform  $\beta$ ) on GC effects in human T-cells, and found that, in patients with critical illness-related corticosteroid insufficiency, miR-124 specifically down-regulated GR- $\alpha$ : a slight increase of miR-124 and a reduction of GR- $\alpha$  was observed in patient T-cells compared to healthy controls. The authors suggested a novel miR-124-mediated mechanism in the down-regulation of GR- $\alpha$  in patients with critical illness-related corticosteroid insufficiency, that could explain, at least in part, GC resistance in this disease.

Tessel *et al.*<sup>[87]</sup> have identified and characterized miR-130b as an important down-regulator of GR in GC-resistant multiple myeloma cell line: the overexpression of this miRNA was also associated with a decreased regulation of the downstream GC controlled gene GILZ, suggesting this mechanism as one of the possible causes of resistance to GCs.

### miRNA involved in IBD

The pathophysiology of IBD is not yet clear, and genetic, epigenetic, infectious and immunological factors seem to play a role. It has been suggested that the gastrointestinal inflammation is the result of an altered activation of the immune system to a luminal factor, such as intestinal flora, in genetically predisposed subjects.

Among the many biological processes regulated by miRNAs, it is now accepted that these small non coding RNAs contribute to the maintenance of immunological homeostasis at mucosal sites<sup>[88,89]</sup>. The role of miRNAs in the pathogenesis of IBD has been thoroughly considered (see recent reviews<sup>[32,90,91]</sup>), and it has been suggested that these small non coding RNAs represent an important player in the complex interactions which results in IBD clinical features. Of particular interest is the observation that miRNA expression changes during tissue progres-

sion from normal to inflamed and varies according to the type and evolutionary stage of IBD<sup>[92]</sup>. Indeed, a number of studies have identified a specific differential expression of miRNAs in IBD and unique miRNA expression profiles for the different subtypes of IBDs, both in human tissues collected by colonoscopic biopsies and in peripheral blood samples, have been demonstrated<sup>[32,90,91]</sup>.

It has been argued that genetic polymorphisms in miRNAs, as well as in miRNA target genes can affect their regulatory function and, consequently, the expression level of their target mRNAs. Most studies have described an association between SNPs in miRNA genes and human cancers<sup>[93-98]</sup>, and only recently the association between mRNA related SNPs and the risk of IBD has been examined<sup>[99]</sup>. Bioinformatic approaches have been used to analyze the association between diseases-linked SNPs, miRNAs and mRNAs: SNP data derived from genome wide association studies that were correlated with miRNA, revealed a CD phenocode comprising rs11209026, rs7807268, rs254215, rs2542151 in miR-125, rs11805303 in miR-519, and rs6908425 in miR-181<sup>[30]</sup>. Of interest, miR-181, miR-519 and miR-119 could target mRNAs encoded by genes involved in the importin pathway, whereas miR-181 and miR-125 are potential regulators of components of inflammasome pathway. Both importin and inflammasome are involved also in GC molecular mechanism: importin is a nuclear transport protein responsible for the translocation of the complex GR-GC into the nucleus<sup>[2]</sup>, and variants in inflammasome gene have been correlated with steroid resistance in pediatric IBD patients<sup>[100]</sup>.

An association between rs3746444 in miR-499 and UC susceptibility has been observed in 170 Japanese patients: this SNP may alter the function or expression of miR-499, altering the regulation of target mRNAs related to inflammatory immune responses, and influencing the pathophysiological features of UC<sup>[101]</sup>. Of particular interest is the observation that the rs3746444 AG genotype was associated also with steroid dependence and refractory phenotype, whereas the rs3746444 AA genotype was inversely related to hospitalization time, steroid dependence, and refractory phenotype. In addition, the rs11614913 TT genotype held a significantly higher risk of refractory phenotype.

## CONCLUSION

There is a lot of interest in identifying the role of miRNAs in the modulation of drug response, but studies about this topic are still very limited, and the possible correlation between miRNAs expression and variability in GC response in IBD patients has not yet been extensively examined. Studies about miRNAs and pharmacogenomics may represent a promising investigation topic that could increase the understanding of the pharmacology of steroids in IBDs and possibly in other diseases.

A number of studies have shown that GCs can modify the expression profile of different miRNAs, however,

the obtained results have been highly variable. The differences observed can possibly be ascribed to the different tissues or cell lines analysed or different experimental protocols, and to date it is not possible to recognize a specific miRNA pattern regulated by GCs.

miRNA regulation by GCs in IBDs has never been analyzed in clinical prospective studies, in which patients are followed from diagnosis and throughout steroid therapy: the identification of miRNAs differently expressed as a consequence of GC treatment in comparison to diagnosis, could be an important innovative approach. This type of study design will reduce to the minimum the effect of confounding factors and results should be easier to translate into clinical practice.

Moreover, existing studies employ techniques based on the use of reverse transcription quantitative PCR and microarrays, based on the analysis and quantification of already known miRNAs. Using next generation sequencing technologies it should be possible to detect novel, still unrecognised miRNAs, and identify new miRNA isoforms (iso-miRs) as well.

In the future, the increased availability and the reduced costs of RNA profiling should enable the clinicians to stratify patients on specific miRNA biomarkers before starting GC treatment. This will allow the personalization of therapy, avoiding a treatment doomed to failure, increasing efficacy and reducing toxicity.

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## Anti-angiogenic therapies for metastatic colorectal cancer: Current and future perspectives

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**Core tip:** Metastatic colorectal cancer is a very aggressive disease. However, recently developed chemotherapeutic protocols and targeted drugs have emerged as a valuable tool for treating this set of patients. Our manuscript brings the readers current trends and future perspectives in this field.

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### Abstract

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in both men and women in the United States, with about 142820 new cases and 50830 deaths expected in 2013. Metastatic disease (mCRC) remains a challenge for oncologists worldwide due to its potential comorbidities. Recently, chemotherapy regimens containing 5-fluorouracil, leucovorin, oxaliplatin and irinotecan combinations are a standard of care in the metastatic disease. Currently, biological therapies involving vascular endothelial growth factor and epidermal growth factor receptor pathways, such as bevacizumab and cetuximab, have emerged as good option for improving mCRC patient survival. Now, aflibercept plus standard chemotherapy has also been approved in second line regimen for mCRC patients. Our review will discuss novel biological drugs and their indications for mCRC patients and will bring future perspectives in this regard.

### INTRODUCTION

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in both men and women in the United States, with about 142820 new cases and 50830 deaths expected in 2013<sup>[1]</sup>. In Europe, CRC represents the second most common cancer and leading cause of cancer death, in both genders combined<sup>[2]</sup>. Consequently, CRC is considered a prominent global health problem.

Usually, early CRC has no symptoms, and this is why screening is so important. Moreover, almost all symptoms (*i.e.*, change in bowel habits, general abdominal discomfort, weight loss with no apparent cause, constant tiredness) are not well specific. Consequently, CRC might be diagnosed when a patient has symptoms or as a result of a screening program<sup>[3]</sup>. Colonoscopy is the main diagnostic tool for primary screening due to its great benefit on either flexible sigmoidoscopy or guaiac fecal occult



blood test<sup>[4]</sup>.

The 1- and 5-year relative survival rates for patients with CRC are respectively 83.2% and 64.3%, considering all stages. Additionally, ten years after diagnosis, survival continues to decline to 57.6%<sup>[3]</sup>. The most important problem remains disease relapse following surgery since, commonly, it is the cause of death in these patients<sup>[3]</sup>. This fact becomes relevant when we observe that when CRC are detected at a localized stage, the 5-year relative survival rate is 90.1% and, after disease involves adjacent organs or lymph nodes, the 5-year survival rate falls to 69.2%. Moreover, when cancer has spread to distant organs, the 5-year survival rate is 11.7%<sup>[5]</sup>.

Many patients have metastatic disease (mCRC) initially not suitable for resection<sup>[6]</sup>. The majority of patients with mCRC cannot be cured, and the goals of chemotherapy for them are to prolong survival, improve quality of life and provide palliation, when applicable<sup>[7]</sup>. Over the past years, the outcome of these patients has been improved, with median survival reaching almost 24 mo<sup>[6,8]</sup>.

The liver is the most common site of hematogenous metastasis in CRC, and its appearance is a frequent event for patients with CRC and remains a major cause of cancer-related death<sup>[9]</sup>. Approximately 25% of patients present synchronous liver metastasis at time of diagnosis, and another 25% of patients will develop liver metastases during the course of their disease, usually within a 2-year period after initial surgical treatment of their primary tumor<sup>[10]</sup>. The only potentially curative treatment for patients with liver metastasis is surgical resection, which results in a 5-year survival rate of 36%<sup>[11]</sup>. Nevertheless, 70% of these patients will suffer a relapse after resection of their hepatic metastasis, with the majority in the first 2 years after surgery and the remaining continuing to occur up to 10 years<sup>[12]</sup>.

Over the past years, the development and incorporation of agents that target angiogenesis in clinical practice have led to improvements in the treatment of mCRC, with benefits in progression-free survival (PSF) and overall survival (OS) in these patients<sup>[13]</sup>. This paper aims to review the impact of known and new anti-angiogenic therapies for mCRC, especially those which target vascular endothelial growth factor (VEGF) pathways.

### Angiogenesis and CRC-molecular mechanisms

Blood vessel formation comprises two main types: vasculogenesis and angiogenesis. During early embryonic development, vasculogenesis is the process responsible for the formation of the primary vasculature of the body, which consists in the formation of blood vessels from endothelial cell progenitors (*i.e.*, hemangioblasts)<sup>[14]</sup>. On the other hand, angiogenesis is a complex and highly regulated biological process that refers to the formation of new vascular segments. During this process, the combination of sprouting, splitting, and remodeling of the existing vessels occurs<sup>[15]</sup>. Physiologically angiogenesis occurs under tight regulation by a wide range of pro-angiogenic inducers, such as growth factors, chemokines,

angiogenic enzymes, endothelial-specific receptors, and adhesion molecules as well as various antiangiogenic factors including angiostatin, endostatin, thrombospondin, canstatin, and pigment epithelium-derived factor<sup>[16]</sup>. As blood vessels are needed to supply nutrients and oxygen to tissues, angiogenesis plays an essential role in normal growth and development. Nevertheless, imbalances between the angiogenic mediators and inhibitors may result in the development of pathologies, as cancer<sup>[17]</sup>.

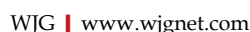
In order to continue grow and metastasize, tumors need to continually acquire an adequate blood supply, which is accomplished by inducing angiogenesis<sup>[18]</sup>. Since Folkman recognized, in the early 1970s, the therapeutic potential for the inhibition of angiogenesis process in cancer, angiogenesis has been largely studied<sup>[19]</sup>.

Figure 1 shows the main angiogenic mechanisms related to VEGF pathways. The VEGF family, which plays a critical role in tumor angiogenesis<sup>[20]</sup>, includes six members: VEGF-A, -B, -C, -D, -E and placental growth factor (PlGF)<sup>[21]</sup>. VEGF-A, also known as VEGF, is the most important member and the major physiologic and pathologic mediator of angiogenic mechanism<sup>[20]</sup>. The *VEGF-A* gene, located on chromosome 6 (6p21.3), undergoes alternative splicing to yield mature isoforms of 121, 145, 165, 183, 189, and 206 amino acids<sup>[22-24]</sup>. *In vivo*, only three isoforms have been related to angiogenesis, VEGF<sub>121</sub>, VEGF<sub>145</sub> and VEGF<sub>165</sub>. The latter has been demonstrated to be a predominant isoform secreted by malignant and benign cells<sup>[25]</sup>. VEGF signals, mainly through VEGF receptor 2 (VEGFR-2) which is tightly expressed by endothelial cells, are involved in angiogenesis. VEGF binds to VEGF receptor 1 (VEGFR-1), with approximately 10 times the affinity of VEGFR-2 binding. However, its signal-transducing properties are extremely weak<sup>[26]</sup>.

Most solid tumors present hypoxic regions as they grow and, thus, outweigh their blood supply. The results of the cellular adaptation to hypoxic microenvironment are aggressive disease, resistance to therapy, and decreased patient survival<sup>[27]</sup>. The transcription factor hypoxia-inducible factor-1 (HIF-1 or HIF) is the most important regulator of the hypoxic response, which up-regulates expression of proteins involved in the regulation of several aspects of tumor biology, such as oxygen transport, iron metabolism, glycolysis, glucose transport, cell survival and proliferation, angiogenesis, invasion and metastasis<sup>[28,29]</sup>. VEGF is one of several proangiogenic factors directly activated by HIF-1 and acts to promote new blood vessel formation and thereby provide the re-establishment of oxygen and nutrient supply<sup>[27]</sup>.

Paracrine mechanisms generated through VEGF production by tumor cells may also influence angiogenesis pathways. However, those cells cannot adequately respond to VEGF directly since they do not have enough cell surface VEGF receptors for that purpose. In contrast, endothelial cells recruited during angiogenesis express numerous VEGF receptors, but produce little or no detectable VEGF ligand. In this context, the amount of





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ways but also by other pathways including Notch, angiopoietins and integrins<sup>[20]</sup>. The Notch pathway, an intercellular signaling pathway, influences many biological processes, including cell fate determination, cellular differentiation, proliferation, survival and apoptosis<sup>[37,38]</sup>. There are four Notch cell-surface receptors (Notch-1, -2, -3 and -4) and five Notch membrane-anchored ligands [Jagged-1, Jagged-2, Delta-like (Dll)-1, -3, and -4], expressed by various cell types. Both ligand and receptor are transmembrane proteins with large extracellular domains that consist of epidermal growth factor (EGF)-like repeats. Notch is synthesized as a precursor protein that is processed in the Golgi before being transported to the cell surface, where it resides as a heterodimer. Interaction of Notch receptors with Notch ligands, between two bordering cells, initiates a series of successive proteolysis cleavages. The first cleavage, mediated by ADAM-family metalloproteases such as ADAM10 or tumor necrosis factor  $\alpha$ -converting enzyme (TACE), generates a substrate for cleavage by the gamma-secretase complex. This cleavage leads to the release of Notch intracellular domain (NICD) from the cell membrane. This protein fragment, then, translocates into the nucleus and operates as a cofactor to regulate transcription of Notch target genes<sup>[39]</sup>. The induction of Dll4-Notch signaling acts as a mechanism intended to prevent excessive angiogenesis and to control the development of new blood vessels<sup>[40]</sup>.

Vascular endothelial cells express Notch 1 and Notch

4 receptors and the Jagged-1, Dll1, and Dll4 ligands. Among these, Dll4 is expressed exclusively by endothelial cells<sup>[20]</sup>. Dll4 is usually induced by VEGF as a negative-feedback regulator of vascular growth. In contrast to VEGF blockade, which results in a loss of many tumor vessels and an apparent normalization of the remaining vessels of the tumor, DLL4 blockade results in a striking increase in these vessels. Paradoxically, this increased vascularity is associated with decreased tumor growth, even for tumors that are highly resistant to blockade of VEGF<sup>[41]</sup>. Since VEGF induces Dll4 and Dll4 induces vascular quiescence and differentiation, and down-regulates VEGFR-2<sup>[42]</sup>, it is obvious that the balance of these two pathways may be important to the development and outcomes of therapeutic acting in these pathways<sup>[43]</sup>.

Recently, the angiopoietins have emerged as important regulators of angiogenesis<sup>[16]</sup>. The human angiopoietin family comprises Ang-1, -2 and -3, all of which act as ligands for endothelial cell-specific tyrosine kinase receptor Tie2, expressed principally on the vascular endothelial cells<sup>[44-46]</sup>.

Ang-1, which is predominantly expressed in perivascular cells such as pericytes, vascular smooth muscle cells, fibroblasts and tumor cells, binds to Tie2 receptor as an antagonist. Upon binding of Ang-1, Tie-2 receptor autophosphorylates, leading to stimulation of various intracellular signaling pathways which promote endothelial cell survival and the maintenance of an endothelial barrier and a quiescent vasculature. Mural cells, such as vascular smooth muscle cells and pericytes, constantly produce Ang-1 under physiological conditions, and maintain vascular stabilization and maturation<sup>[47]</sup>. On the other hand, Ang-2 produced by the endothelium, acts as an antagonist for Tie2 by competing with Ang-1<sup>[45]</sup>. It induces vascular destabilization and vessel proliferation. VEGF and angiopoietins have complementary roles in angiogenesis. In the presence of VEGF, Ang-2 stimulates tumor angiogenesis by promoting vessel destabilization, whereas in the absence of VEGF, Ang-2 promotes endothelial cell death and vessel regression<sup>[48]</sup>.

Blockade of Tie-2 pathway has been more difficult than blockade of the VEGF pathway, due to the complexity of agonistic and antagonistic ligands for the same receptor. Moreover, it has been a challenge to find and design effective and specific drugs against Tie-2 or angiopoietins<sup>[20]</sup>.

Fibroblast growth factor (FGF)/FGF receptor (FGFR) signaling is involved in multiple cellular processes, such as proliferation, anti-apoptosis, drug resistance, and angiogenesis<sup>[49]</sup>. FGFs are heparin-binding growth factors that are part of a family that comprises 23 members (FGF-1 to -23), of which only 18 are functional ligands for FGFR in humans. The members of the FGFR family (FGFR-1 to -4) share a common domain architecture consisting of extracellular immunoglobulin-like domains and a cytoplasmic tyrosine kinase domain<sup>[50]</sup>. Although FGF1 and FGF2 are among the first discovered molecules that contribute to angiogenesis, some mem-

bers of the VEGF ligand family and VEGFR are now accepted to play a main role in driving embryonic vascularization, angiogenesis and lymphangiogenesis<sup>[51]</sup>. Nevertheless, both FGFs and VEGF cooperate to promote angiogenesis. FGF-2 induces the expression of VEGF in vascular endothelial cells, while the blockade of VEGF reduces the expression of endogenous FGF-2, suggesting a positive feedback mechanism. Furthermore, inhibition of FGFR-1 and FGFR-2 activity can reduce tumor vascularization as well as VEGF expression. Therefore, promotion of angiogenesis by FGFs may be dependent of crosstalk between FGF-VEGF signaling pathways<sup>[52]</sup>.

EGF signaling is initiated by the binding of EGF family members to the extracellular domain of erythroblastic leukemia viral oncogene homologue (ErbB) receptors. The ErbB receptor tyrosine kinase family comprises 4 members, namely, EGF receptor (EGFR)/ERBB1/HER1, ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4<sup>[53]</sup>. The major contributors of these receptors are a complex signaling cascade that modulates growth, signaling, differentiation, adhesion, migration and survival of cancer cells<sup>[54]</sup>.

The EGF family members bind to the ErbB receptors and are classified into 3 groups based on their receptor affinities: in the first group, EGF, transforming growth factor- $\alpha$ , amphiregulin (AR), and epigen (EPG), specifically bind to EGFR; in the second group, betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR), which exhibit dual specificity, bind to both EGFR and ErbB4; and the third group, which includes neuregulins (NRGs), forms two subgroups on the basis of their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4)<sup>[53,55]</sup>. On binding, ErbBs form homo or heterodimers and initiate multiple pathways involving effectors including rat sarcoma viral oncogene homologue (RAS)/mitogen-activated protein kinase, phosphatidylinositol 3-kinase-AKT, mammalian target of rapamycin, signal transducer and activator of transcription, SRC tyrosine kinase, phospholipase C- $\gamma$ 1/protein kinase C (PKC) and p27. The activation of these pathways plays a relevant role in several aspects of development and tissue homeostasis<sup>[54]</sup>. Increased EGFR signaling is particularly common in several cancers, including CRC, through one or more of the family members<sup>[56]</sup>. EGFR and its family members, due to their vast role in the progression of cancer, have emerged as attractive candidates for anti-cancer therapy.

## TREATMENT OF mCRC

Nowadays, there are many therapeutic strategies approved by the Food and Drug Administration (FDA) for the management of mCRC: 5-fluorouracil (5-FU), leucovorin (LV), irinotecan, capecitabine, oxaliplatin, regorafenib, ziv-aflibercept, and the monoclonal antibodies bevacizumab, cetuximab, and panitumumab. Of these drugs, only few have FDA-approved indications for use as monotherapies and reveal activity as a single agent

**Table 1 Clinical trials and main anti-angiogenic drugs in metastatic disease**

Clinical trial	Phase	Line	Regimen	Median PFS (mo)	Median OS (mo)	ORR (%)
Aflibercept VELOUR NCT00561470 <sup>[87]</sup>	III	2 <sup>nd</sup>	FOLFIRI + aflibercept <i>vs</i> FOLFIRI + placebo	6.90 <i>vs</i> 4.67 HR = 0.758, <i>P</i> = 0.0001	13.50 <i>vs</i> 12.06 HR = 0.817, <i>P</i> = 0.0032	19.8 <i>vs</i> 11.1 <i>P</i> = 0.001
AFFIRM NCT00851084 <sup>[86]</sup>	II	1 <sup>st</sup>	mFOLFOX6 + aflibercept <i>vs</i> mFOLFOX6	8.48 <i>vs</i> 8.77		49.1 <i>vs</i> 45.9
Brivanib NCT00640471 <sup>[90]</sup>	III	3 <sup>rd</sup>	Cetuximab + brivanib <i>vs</i> cetuximab + placebo	5.0 <i>vs</i> 3.4 HR = 0.72, <i>P</i> < 0.001	8.8 <i>vs</i> 8.1 HR = 0.88, <i>P</i> = 0.12	13.6 <i>vs</i> 7.2 <i>P</i> = 0.004
Regorafenib CORRECT NCT01103323 <sup>[99]</sup>	III	2 <sup>nd</sup>	Regorafenib <i>vs</i> placebo	1.9 <i>vs</i> 1.7 HR = 0.49, <i>P</i> < 0.000001	6.4 <i>vs</i> 5.0 HR = 0.77, <i>P</i> = 0.0052	
Sorafenib RESPECT NCT00865709 <sup>[107]</sup>	II	1 <sup>st</sup>	mFOLFOX6 + sorafenib <i>vs</i> mFOLFOX6 + placebo	9.1 <i>vs</i> 8.7 HR = 0.88, <i>P</i> = 0.46	17.6 <i>vs</i> 18.1 HR = 1.13, <i>P</i> = 0.51	
Sunitinib NCT00668863 NCT00457691 <sup>[108]</sup>	II III	1 <sup>st</sup> 1 <sup>st</sup>	FOLFIRI + sunitinib <i>vs</i> FOLFIRI + placebo	7.8 <i>vs</i> 8.4 HR = 1.095, <i>P</i> = 0.807	20.3 <i>vs</i> 19.8 HR = 1.171, <i>P</i> = 0.916	32 <i>vs</i> 34 <i>P</i> = 0.683
Valatanib CONFIRM1 NCT00056459 <sup>[110]</sup>	III	1 <sup>st</sup>	FOLFOX4 + valatanib <i>vs</i> FOLFOX4 + placebo	7.7 <i>vs</i> 7.6 HR = 0.88, <i>P</i> = 0.118	21.4 <i>vs</i> 20.5 HR = 1.08, <i>P</i> = 0.260	<i>P</i> > 0.05
CONFIRM 2 NCT00056446 <sup>[111]</sup>	III	2 <sup>nd</sup>	FOLFOX4 + valatanib <i>vs</i> FOLFOX4 + placebo	5.6 <i>vs</i> 4.2 HR = 0.83, <i>P</i> = 0.013	13.1 <i>vs</i> 11.9 HR = 1.00, <i>P</i> = 0.957	

mCRC: Metastatic colorectal cancer; PFS: Progression-free-survival; OS: Overall survival; ORR: Overall response rate; FOLFIRI: 5-fluorouracil + leucovorin + irinotecan; mFOLFOX6: 5-fluorouracil + leucovorin + oxaliplatin; HR: Harzard ratio.

against CRC, including fluoropyrimidines (5-FU and capecitabine), irinotecan, cetuximab, and panitumumab.

The combination chemotherapy is the only standard for first-line treatment of mCRC. Regardless of which regimen is used, outcome may be maximized in patients who receive, alone or in combination, 5-FU, LV, irinotecan, and oxaliplatin sometime during the course of treatment. These chemotherapy regimens have been extensively studied in phase II and III trials, both as first- and second-line therapies<sup>[57,58]</sup>. Tables 1 and 2 summarizes current and future trials on mCRC anti-angiogenic therapies.

### Antiangiogenic drugs

Bevacizumab is a humanized monoclonal antibody that binds and inactivates VEGF, preventing angiogenesis and, hence, tumor growth and proliferation. Bevacizumab contains human framework regions and the complementarity-determining regions of a murine antibody that inhibits all active isoforms of VEGF<sup>[59]</sup>. Currently, bevacizumab is the only agent specifically targeting the VEGF pathway for the treatment of CRC<sup>[60]</sup>.

Over the past decades, many trials have investigated bevacizumab in mCRC. It has been studied with different active chemotherapy and biological agents, as well as in multiple treatment setting, sequencing and duration<sup>[61]</sup>.

The phase II trial conducted by Kabbinavar *et al*<sup>[62]</sup> compared two doses of bevacizumab plus 5-FU/LV (low-dose bevacizumab: 5 mg/kg every 2 wk; high-dose bevacizumab: 10 mg/kg every 2 wk) with 5-FU/LV alone in 104 patients untreated. Compared with the 5-FU/LV control arm, treatment with bevacizumab (at both dose

levels) plus 5-FU/LV resulted in a higher response rate (RR) (control arm: 17%; low-dose arm: 40%; high-dose arm: 24%), longer median time to disease progression (control arm: 5.2 mo; low-dose arm: 9.0 mo; high-dose arm: 7.2 mo), and longer median survival (control arm: 13.8 mo; low-dose arm: 21.5 mo; high-dose arm: 16.1 mo). Based on these results, in the most subsequent phase III trials on mCRC the 5 mg/kg bevacizumab dosing is chosen<sup>[61]</sup>.

The phase III AVF 2107 trial (NCT00109070) randomized 813 patients to receive IFL plus either bevacizumab 5 mg/kg (*n* = 402) or placebo (*n* = 411), every 2 wk. The addition of bevacizumab compared with IFL alone provided significantly clinical and statistical improvement in median OS (20.3 mo *vs* 15.6 mo; HR = 0.66, *P* < 0.001), PFS (10.6 mo *vs* 6.2 mo, HR = 0.54, *P* < 0.001) and overall response rate (ORR) (44.8% *vs* 34.8%, *P* = 0.004)<sup>[13]</sup>.

In the NO16966 phase III trial (NCT00069095), with 2 × 2 factorial design, 1401 patients with mCRC were randomized to receive FOLFOX or XELOX and then bevacizumab or placebo. Median PFS was significantly increased when bevacizumab was added (9.4 mo in bevacizumab group *vs* 8.0 mo in placebo group; HR = 0.83, *P* = 0.0023). Median OS was 21.3 mo in the bevacizumab group and 19.9 mo in the placebo group (HR = 0.89, *P* = 0.077), and RR was similar in both arms. A planned subset analysis demonstrated significant improvement of PFS with bevacizumab in the XELOX subgroup (*P* = 0.0026), as opposed when FOLFOX4 (*P* = 0.187) was added. Safety results showed that grade 3 or higher adverse events were slightly higher in the bevacizumab

**Table 2** Current clinical trials considering anti-angiogenic therapies for colorectal cancer

	Trial	Phase	Line	Therapy/arms	Status of trial
Bevacizumab	NCT01321957	II	1 <sup>st</sup>	FOLFOX + bevacizumab <i>vs</i> FOLFOX + bevacizumab + irinotecan	Currently recruiting participants
	NCT00819780	II	1 <sup>st</sup>	Panitumumab + mFOLFOX6 <i>vs</i> bevacizumab + mFOLFOX6	Ongoing, but not recruiting participants
	NCT01531595	II	1 <sup>st</sup>	3 cycles of XELOX + bevacizumab alternating with 3 cycles of XELIRI + bevacizumab	Currently recruiting participants
	NCT01067053	II	1 <sup>st</sup>	Bevacizumab + capecitabine + oxaliplatin - 6 cycles; after the first 6 cycles of treatment, continuing only with bevacizumab and capecitabine	Ongoing, but not recruiting participants
	NCT01765582	II	1 <sup>st</sup>	FOLFOXIRI + bevacizumab <i>vs</i> Sequential FOLFOXIRI + bevacizumab <i>vs</i> FOLFOX + bevacizumab	Currently recruiting participants
	NCT01006369	II	-	FOLFOX6 + bevacizumab + hydroxychloroquine <i>vs</i> XELOX + bevacizumab + hydroxychloroquine	Currently recruiting participants
	NCT01417494	II	1 <sup>st</sup>	Chemotherapy (FOLFIRI, FOLFOX, LV5FU2) + bevacizumab <i>vs</i> Chemotherapy (FOLFIRI, FOLFOX, LV5FU2)	Currently recruiting participants
	NCT01532804	II	2 <sup>nd</sup>	FOLFOX6 + bevacizumab (day 1 = day 15, 12 cycles) <i>vs</i> Raltitrexed + oxaliplatin + bevacizumab ( day 1 = day 21, 8 cycles)	Currently recruiting participants
	NCT00952029	II / III	1 <sup>st</sup>	FOLFIRI + bevacizumab and during the chemotherapy-free interval maintenance with bevacizumab <i>vs</i> FOLFIRI + bevacizumab and during the chemotherapy-free interval NO maintenance	Currently recruiting participants
Cetuximab	NCT01640405	III	1 <sup>st</sup>	mFOLFOX6 + bevacizumab <i>vs</i> FOLFIRI + bevacizumab	Currently recruiting participants
	NCT00444678	II	-	Cetuximab + capecitabine + oxaliplatin	Ongoing, but not recruiting participants
	NCT01251536	II	1 <sup>st</sup>	Cetuximab (standard dose: 250 mg/m <sup>2</sup> weekly) <i>vs</i> Cetuximab (dose escalation: days 22 and 29-350 mg/m <sup>2</sup> , from day 36 onwards - 500 mg/m <sup>2</sup> weekly)	Currently recruiting participants
	NCT01718808	II	1 <sup>st</sup>	Cetuximab + capecitabine <i>vs</i> Cetuximab	Currently recruiting participants
	NCT01867697	II	1 <sup>st</sup>	Cetuximab (biweekly) + FOLFIRI (continuously) <i>vs</i> Cetuximab (biweekly) + alternating FOLFIRI and mFOLFOX6	Currently recruiting participants
	NCT00640081	II	1 <sup>st</sup>	Intermittent chemotherapy plus intermittent cetuximab treatment (12 wk), plus cetuximab followed by a period off all therapy; reintroduction of the same chemotherapy and cetuximab regimen (12 wk after initial progression off treatment) <i>vs</i> Intermittent chemotherapy plus continuous cetuximab treatment (12 wk), plus cetuximab followed by a period of withdrawal of the chemotherapy, but continued weekly cetuximab monotherapy with reintroduction of the same chemotherapy regimen to the cetuximab (12 wk after initial progression off chemotherapy treatment)	Ongoing, but not recruiting participants
	NCT00479752	II	1 <sup>st</sup>	FOLFOX4 + cetuximab (initial dose: 400 mg/m <sup>2</sup> in week 1, followed by weekly doses of 250 mg/m <sup>2</sup> ) <i>vs</i> FOLFOX4 + cetuximab (500 mg/m <sup>2</sup> every 2 wk)	Ongoing, but not recruiting participants
	NCT00482222	III	1 <sup>st</sup>	Oxaliplatin/fluoropyrimidine <i>vs</i> oxaliplatin/fluoropyrimidine + cetuximab pre and post surgery	Currently recruiting participants
	NCT00433927	III	1 <sup>st</sup>	FOLFIRI + cetuximab <i>vs</i> FOLFIRI + bevacizumab	Ongoing, but not recruiting participants
Panitumumab	NCT01228734	III	1 <sup>st</sup>	Cetuximab + FOLFOX4 <i>vs</i> FOLFOX4	Ongoing, but not recruiting participants
	NCT01030042	III	2 <sup>nd</sup>	FOLFOX4 followed, after progression, by irinotecan + cetuximab <i>vs</i> Cetuximab + irinotecan	Currently recruiting participants
	NCT00885885	II	-	Panitumumab + FOLFOX4 <i>vs</i> Panitumumab + FOLFOX4	Ongoing, but not recruiting participants
	NCT01215539	II	1 <sup>st</sup>	Panitumumab + capecitabine + oxaliplatin	Currently recruiting participants
	NCT01126112	II	1 <sup>st</sup>	Panitumumab (6 mg/kg every 2 wk )	Ongoing, but not recruiting participants
	NCT00819780	II	1 <sup>st</sup>	Panitumumab + mFOLFOX 6 <i>vs</i> bevacizumab + mFOLFOX 6	Ongoing, but not recruiting participants
	NCT01328171	II	1 <sup>st</sup>	FOLFOXIRI + panitumumab <i>vs</i> FOLFOXIRI	Currently recruiting participants
	NCT01508000	II	1 <sup>st</sup>	mFOLFOX6 (6 cycles after and before surgery) + surgery <i>vs</i> mFOLFOX6 + bevacizumab (6 cycles after and before surgery) + surgery <i>vs</i> mFOLFOX6 + panitumumab (6 cycles after and before surgery) + surgery	Not yet open for participant recruitment
	NCT01814501	II	2 <sup>nd</sup>	5-FU + irinotecan + panitumumab	Currently recruiting participants
	NCT00940316	II	2 <sup>nd</sup>	Erlotinib + panitumumab + irinotecan (treatment repeats every 2 wk) <i>vs</i> Erlotinib + panitumumab (treatment repeats every 2 wk) <i>vs</i> Erlotinib + panitumumab	Currently recruiting participants
	NCT00364013	III	1 <sup>st</sup>	FOLFOX + panitumumab <i>vs</i> FOLFOX	Ongoing, but not recruiting participants



	NCT01910610	III	1 <sup>st</sup>	FOLFIRI + cetuximab, followed by oxaliplatin-based chemotherapy + bevacizumab <i>vs</i> OPTIMOX + bevacizumab, followed by irinotecan-based chemotherapy + bevacizumab, followed by an anti-EGFR agent (cetuximab +/- irinotecan or panitumumab) with or without irinotecan	Not yet open for participant recruitment
Aflibercept	NCT01669720	II	2 <sup>nd</sup>	Aflibercept <i>iv</i> (4 mg/kg every 2 wk)	Currently recruiting participants
	NCT01652196	II	1 <sup>st</sup>	Aflibercept <i>iv</i> + mFOLFOX 6 <i>iv</i> (days 1 and 15; repeats every 28 d)	Currently recruiting participants
	NCT01802684	II	1 <sup>st</sup>	Induction therapy (sequence #1) Regimen: Aflibercept + mFOLFOX7 - 6 cycles (3 mo) Maintenance after induction (sequence #2) First phase (sequence #2A); Regimen: Aflibercept + fluoropyrimidine (simplified LV5FU2 or capecitabine) - 6 cycles (3 mo) Second phase (sequence #2B); Regimen: Aflibercept +/- fluoropyrimidine (simplified LV5FU2 or capecitabine) - until PD or limiting toxicity Reintroduction (sequence #3); Regimen: Aflibercept + mFOLFOX7 - 6 cycles (3 mo) Maintenance after reintroduction (sequence #4); Regimen: Aflibercept + fluoropyrimidine - until PD or limiting toxicity	Not yet open for participant recruitment
	NCT01882868	II	2 <sup>nd</sup>	Aflibercept <i>iv</i> + FOLFIRI Aflibercept + FOLFIRI (every 2 wk)	Currently recruiting participants
	NCT01889680	II	1 <sup>st</sup>	mFOLFOX6 + aflibercept (every 14 d for 6 cycles) plus 5-FU/LV (every 14 d) <i>vs</i> mFOLFOX6 + aflibercept (every 14 d for 6 cycles) plus 5-FU/LV + aflibercept (every 14 d)	Not yet open for participant recruitment
	NCT01646554	II / III	1 <sup>st</sup>	mFOLFOX6 and SURGERY 6 cycles before and 6 cycles after surgery consisting in: Hour 0: Oxaliplatin 85 mg/m <sup>2</sup> <i>iv</i> 2-h infusion; Hour 0: Folinic acid 400 mg/m <sup>2</sup> (DL form) or 200 mg/m <sup>2</sup> (L form) <i>iv</i> 2-h infusion; Hour 2: 5-FU 400 mg/m <sup>2</sup> <i>iv</i> bolus over 2-4 min; Hour 2: 5-FU 2400 mg/m <sup>2</sup> given as a continuous infusion over 46 h; On day 1 of a 14 d cycle <i>vs</i> mFOLFOX6 + aflibercept and surgery; 6 cycles before and 6 cycles after surgery consisting in: Hour 0: Aflibercept 4 mg/kg intravenous infusion 1-h; Hour 1: Oxaliplatin 85 mg/m <sup>2</sup> 2-h infusion; Hour 1: Folinic acid 400 mg/m <sup>2</sup> (DL form) or 200 mg/m <sup>2</sup> (L form) 2-h infusion; Hour 3: 5-FU bolus 400 mg/m <sup>2</sup> <i>iv</i> bolus over 2-4 min; Hour 3: 5-FU 2400 mg/m <sup>2</sup> given as a continuous infusion over 46 h; Day 1 of a 14 day cycle	Not yet open for participant recruitment
	NCT01661270	III	2 <sup>nd</sup>	Aflibercept <i>iv</i> (day 1 of each cycle, every 2 wk) + FOLFIRI <i>vs</i> Placebo <i>iv</i> (day 1 of each cycle, every 2 wk) + FOLFIRI	Currently recruiting participants
	NCT01571284	III	2 <sup>nd</sup>	Aflibercept IV (every 2 wk) + FOLFIRI	Currently recruiting participants
	NCT01670721	III	2 <sup>nd</sup>	Aflibercept IV (on day 1) + FOLFIRI administered as follows: dI-leucovorin infusion over 2 h on day 1; Irinotecan: infusion over 90-min infusion, on day 1, followed by bolus 5-FU and 5-FU continuous infusion over 46 h or as individualized by physician's clinical judgment; Treatment cycle to be administered every 2 wk	Currently recruiting participants
Brivanib	NCT01367275	II	2 <sup>nd</sup>	Brivanib (800 mg orally daily days 1-14) + Irinotecan <i>iv</i> (180 mg/m <sup>2</sup> on day 1)	Ongoing, but not recruiting participants
Cediranib	NCT00588900	II	2 <sup>nd</sup>	Irinotecan <i>iv</i> (days 1 and 8) + Cediranib oral (days 1-21)	The recruitment status of this study is unknown because the information has not been verified recently
Ramucirumab	NCT01111604	II	2 <sup>nd</sup>	mFOLFOX-6 <i>vs</i> mFOLFOX-6 + ramucirumab (8 mg/kg <i>iv</i> infusion, administered every 2 wk) <i>vs</i> mFOLFOX-6 + icrucumab (15 mg/kg <i>iv</i> infusion, administered every 2 wk)	Ongoing, but not recruiting participants
	NCT01183780	III	2 <sup>nd</sup>	FOLFIRI + ramucirumab (8 mg/kg administered intravenously every 2 wk) <i>vs</i> FOLFIRI + placebo	Currently recruiting participants
Regorafenib	NCT01298570	II	2 <sup>nd</sup>	Regorafenib (160 mg, <i>po</i> , daily, per 7 day cycle) + FOLFIRI (day 1 and day 15 of each 28 d cycle) <i>vs</i> Placebo (oral administration, days 4-10 and days 18-24 of 28 day cycle +) + FOLFIRI (day 1 and day 15 of each 28 d cycle)	Currently recruiting participants
	NCT01289821	II	1 <sup>st</sup>	Day 1 and day 15 of each cycle: 85 mg/m <sup>2</sup> oxaliplatin + folinic acid (either 400 mg/m <sup>2</sup> D/L-folinic acid or 200 mg/m <sup>2</sup> L-folinic acid), <i>iv</i> + 400 mg/m <sup>2</sup> 5 FU <i>iv</i> + 2400 mg/m <sup>2</sup> 5 <i>iv</i> ; Days 4 to 10 and days 18 to 24: regorafenib 160 mg (four 40 mg tablets)	Ongoing, but not recruiting participants
	NCT01875380	II	1 <sup>st</sup>	Regorafenib (orally, 160 mg per day for 3 wk, followed by 1 wk of rest)	Not yet open for participant recruitment
	NCT01103323	III	2 <sup>nd</sup>	Regorafenib (160 mg per oral once daily for 3 wk on 1 wk off of every 4 wk cycle) <i>vs</i> Placebo (per oral once daily for 3 wk on 1 wk off of every 4 wk cycle)	Ongoing, but not recruiting participants
	NCT01584830	III	2 <sup>nd</sup>	Regorafenib [3 wk on/1 wk off (160 mg <i>od po</i> )] Placebo [3 wk on/1 wk off (160 mg <i>od po</i> )]	Ongoing, but not recruiting participants
	NCT01853319	III	2 <sup>nd</sup>	Regorafenib (160 mg per oral every day for 3 wk of every 4 wk cycle)	Not yet open for participant recruitment
	NCT01538680	III	2 <sup>nd</sup>	Regorafenib (160 mg <i>po</i> every day for 3 wk on, 1 wk off)	Expanded access is currently available for this treatment

Semaxanib	NCT00021281	III	1 <sup>st</sup>	Semaxanib <i>iv</i> (on days 1, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36 and 39) + irinotecan <i>iv</i> , leucovorin calcium <i>iv</i> , fluorouracil <i>iv</i> (on days 1, 8, 15, and 22) (every 6 wk) <i>vs</i> Irinotecan <i>iv</i> , leucovorin calcium <i>iv</i> , fluorouracil <i>iv</i> (on days 1, 8, 15, and 22) (every 6 wk) <i>vs</i> Semaxanib <i>iv</i> (on days 1, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36 and 39) + irinotecan <i>iv</i> (days 1, 15 and 29) + leucovorin calcium <i>iv</i> , fluorouracil <i>iv</i> (on days 1, 2, 15, 16, 29 and 30) <i>vs</i> Irinotecan <i>iv</i> , leucovorin calcium <i>iv</i> , fluorouracil <i>iv</i> (on days 1, 8, 15, and 22) (every 6 wk)	The recruitment status of this study is unknown because the information has not been verified recently
Sorafenib	NCT01715441 NEXIRI 2	II	2 <sup>nd</sup>	Irinotecan 180 mg/m <sup>2</sup> <i>iv</i> with cross over to irinotecan and sorafenib combination at progression <i>vs</i> Sorafenib 400 mg twice daily with cross over to irinotecan and sorafenib combination at progression <i>vs</i> Irinotecan 120 mg/m <sup>2</sup> <i>iv</i> at cycle 1, 150 mg/m <sup>2</sup> at cycle 2 and 180 mg/m <sup>2</sup> at cycle 3 + sorafenib 400 mg twice daily from cycle 1	Currently recruiting participants
	NCT01471353	II	2 <sup>nd</sup>	Sorafenib 200-400 mg <i>po</i> twice daily on days 1-21 (dose escalation scheme) + capecitabine 1000 mg/m <sup>2</sup> <i>po</i> twice daily on days 1-14 repeated every 21 d	Currently recruiting participants
	NCT00826540	II	2 <sup>nd</sup>	Sorafenib twice daily on days 1-5 and 8-12 + bevacizumab <i>iv</i> on day 1	Ongoing, but not recruiting participants
	NCT00839111	II	2 <sup>nd</sup>	Sorafenib (400 mg twice daily from day 3 to day 14, day 17-28) + FOLFIRI	The recruitment status of this study is unknown because the information has not been verified recently
	NCT01290926	II	2 <sup>nd</sup>	Sorafenib (200 mg in the morning, 400 mg in the evening) + capecitabine (850 mg/m <sup>2</sup> twice daily)	The recruitment status of this study is unknown because the information has not been verified recently
	NCT00326495	II	2 <sup>nd</sup>	Oral sorafenib (400 mg by twice daily) plus cetuximab (400 mg/m <sup>2</sup> , week 1; 250 mg/m <sup>2</sup> <i>iv</i> , weekly)	Currently recruiting participants
Sunitinib	NCT00936832	II	1 <sup>st</sup>	FOLFIRI (on days 1, 15, and 29) + oral sunitinib (on days 1-28). (repeats every 6 wk)	The recruitment status of this study is unknown because the information has not been verified recently

Research on July 25, 2013 (<http://clinicaltrials.gov>). 5-FU: 5-fluorouracil; FOLFIRI: 5-fluorouracil + leucovorin + irinotecan; mFOLFOX6: 5-fluorouracil + leucovorin + oxaliplatin; HR: Hazard ratio.

group (30% *vs* 21%)<sup>[63]</sup>.

In the phase III MAX study, 471 patients with previously untreated and unresectable mCRC were randomly assigned to the following arms: capecitabine alone, capecitabine plus bevacizumab, or capecitabine, bevacizumab, and mitomycin. Median PFS was 5.7 mo for the capecitabine arm, 8.5 mo for the capecitabine-bevacizumab arm, and 8.4 mo for the capecitabine-bevacizumab-mitomycin arm. Thus, there was statistical improvement in PFS between the capecitabine arm and the other two arms (capecitabine *vs* capecitabine-bevacizumab: HR = 0.63,  $P < 0.001$ ; capecitabine *vs* capecitabine-bevacizumab-mitomycin: HR = 0.59,  $P < 0.001$ )<sup>[64]</sup>. Based on these results, in United States and Europe, bevacizumab in association with standard chemotherapy has been approved for first-line treatment of KRAS-mutant mCRC or for second-line treatment of KRAS-wild type patients previously treated with anti-EGFR drugs.

Despite those interesting benefits reported in previous trials, researchers and clinicians should be knowledgeable about toxicities, such as for hypertension and bleeding.

### Anti-EGFR agents

Cetuximab and panitumumab are two EGFR inhibitors currently indicated as monotherapy in patients with wild-type KRAS tumors as a first or second-line treatment<sup>[65]</sup>. Only cetuximab is indicated in combination with irinotecan, and has been approved for use in first-line in Europe

as mono-therapy or in combination with chemotherapy<sup>[66]</sup>.

Cetuximab is a recombinant human-murine chimeric IgG1 monoclonal antibody that binds to the extracellular region of the EGFR with high specificity and with higher affinity than EGF on normal and tumor cells<sup>[67]</sup>.

A phase II clinical trial conducted by Tabernero *et al*<sup>[68]</sup> assessed 43 patients who received cetuximab and FOLF-FOX4 as first-line chemotherapy. RR was 72%; median PFS was 12.3 mo and median OS was 30 mo. Cetuximab did not increase the characteristic toxicity of FOLFOX4 and was collectively well tolerated. The most commonly reported grade 3 or higher adverse events were diarrhea, neutropenia, and paresthesia.

The OPUS study, also a phase II trial (NCT00125034), included 337 patients who were randomized to receive FOLFOX4 with cetuximab ( $n = 169$ ) or alone ( $n = 168$ ) in first-line chemotherapy<sup>[69]</sup>. In 93% of measured KRAS patient samples, 57% were KRAS-wild type. Patients whose tumors were KRAS-wild type who received cetuximab plus FOLFOX4 had a 2.6-fold increased odds ratio of response (ORR: 57% *vs* 34%, OR = 2.551,  $P = 0.0027$ ) and a 43% decrease in the risk of disease progression (median PFS 8.3 mo *vs* 7.2 mo, HR = 0.567,  $P = 0.0064$ ) compared with those who received FOLFOX4 alone. Also, median OS was improved by the addition of cetuximab to FOLFOX4 for patients in that group (22.8 mo *vs* 18.5 mo, HR = 0.855,  $P = 0.39$ ). On the other hand, patients whose tumors carried KRAS mutations who

received cetuximab plus FOLFOX4 had a decreased odd of response (34% *vs* 53%, OR = 0.459,  $P = 0.0290$ ) and a higher risk of disease progression (median PFS 5.5 mo *vs* 8.6 mo, HR = 1.720,  $P = 0.0153$ ) compared with those who received FOLFOX4 alone<sup>[70]</sup>.

In the phase III CRYSTAL study (NCT00154102), 1198 patients who received cetuximab plus FOLFIRI ( $n = 599$ ) or FOLFIRI alone ( $n = 599$ ) were included. The addition of cetuximab to chemotherapy significantly reduced the risk of progression by 15% (8.9 mo *vs* 8.0 mo, HR = 0.85,  $P = 0.048$ ) and improved ORR (46.9% *vs* 38.7%, OR = 1.40,  $P = 0.048$ ). On the other hand, no significant difference in median OS between the two treatment groups was observed (19.9 mo *vs* 18.6 mo, HR = 0.93,  $P = 0.31$ )<sup>[71]</sup>. In that study, *KRAS* and *BRAF* mutations were detected in 37% and 6% of patients, respectively. The addition of cetuximab to FOLFIRI in patients with wild-type *KRAS* resulted in significant improvement in median OS (23.5 mo *vs* 20.0 mo, HR = 0.796,  $P = 0.0093$ ), median PSF (9.9 mo *vs* 8.4 mo, HR = 0.696,  $P = 0.0012$ ), and RR (57.3% *vs* 39.7%, OR = 2.069,  $P < 0.001$ ) compared with FOLFIRI alone. These results showed the role of *KRAS* mutation status as a powerful predictive biomarker for the efficacy of cetuximab plus FOLFIRI. Concerning grade 3 or 4 adverse events, they were more common with use of regimen with cetuximab and included skin reactions, infusion reactions and diarrhea<sup>[72]</sup>.

In the phase III study NORDIC V II (NCT00145314), 571 patients with mCRC were randomized to one of the following three arms: continuous FLOX alone or with cetuximab or intermittent FLOX with continuous weekly cetuximab. No differences were found in RR, median PFS or OS in patients receiving cetuximab, either in *KRAS*-mutant or -wild-type<sup>[73]</sup>.

In the phase III trial MRC COIN, 1630 patients were randomized to receive oxaliplatin-based chemotherapy (FOLFOX or XELOX) with or without cetuximab. The determination of *KRAS* mutation was performed in 1316 (81%) patients and it was identified in 729 (55%) patients<sup>[74]</sup>. Patients with wild-type *KRAS* tumors showed no improvements in median OS for cetuximab combined with chemotherapy when compared with chemotherapy alone (17.0 mo *vs* 17.9 mo, HR = 1.038,  $P = 0.68$ ) or PFS (8.6 mo *vs* 8.6 mo, HR = 0.96,  $P = 0.60$ ); however, there was an increase in ORR (57% *vs* 64%,  $P = 0.049$ ). Furthermore, there was a potential benefit with improvement in PFS for wild-type *KRAS* patients who received cetuximab plus infused 5-FU (HR = 0.72,  $P = 0.037$ ) but not cetuximab plus capecitabine (HR = 1.02, 95%CI: 0.82-1.26,  $P = 0.88$ )<sup>[74]</sup>.

Based on those trials, cetuximab in addition with standard chemotherapy has been approved in United States and Europe for wild-type *KRAS* mCRC patients in first-line regimen. It is important to monitor toxicity profile such as skin rash, diarrhea, nausea and mucositis in order to provide a good tolerability for patients. Regular medical visits before each cycle and support medication could

help address this concern.

Panitumumab is a recombinant human IgG2k monoclonal antibody that binds EGFR and prevents receptor dimerization, tyrosine autophosphorylation of EGFR, and the activation of downstream signaling molecules<sup>[75]</sup>.

The phase III trial PRIME (NCT00364013) included 1183 patients without prior chemotherapy for mCRC, who were randomly assigned to receive FOLFOX4 with or without panitumumab therapy. In the wild-type *KRAS* subgroup, panitumumab plus FOLFOX4 produced a significantly improved median PFS compared with FOLFOX4 alone (9.6 mo *vs* 8.0 mo, respectively; HR = 0.80,  $P = 0.02$ ). Nevertheless, a non-significant increase in median OS was found for panitumumab plus FOLFOX4 versus FOLFOX4 alone (23.9 mo *vs* 19.7 mo, respectively, HR = 0.83,  $P = 0.072$ ). In the mutant *KRAS* subgroup PFS was significantly reduced in the panitumumab plus FOLFOX4 arm when compared with the FOLFOX4 alone arm (HR = 1.29,  $P = 0.02$ ), and median OS was 15.5 mo *vs* 19.3 mo, respectively (HR = 1.24,  $P = 0.068$ )<sup>[76]</sup>.

As a conclusion, the use of cetuximab or panitumumab for wild-type *KRAS* mCRC patients will depend on the patient fitness, toxicity profile and drug wiliness in each circumstance. Both drugs are safe and prove to improve OS in the metastatic setting.

### Double monoclonal antibody therapy

The efficacy of bevacizumab and anti-EGFR agents in first-line treatment of mCRC encouraged two clinical trials of double monoclonal antibody therapy<sup>[77]</sup>.

In the phase III PACCE (NCT00115765) study, a total of 1053 patients were randomized to receive first-line chemotherapy [oxaliplatin/5-FU/LV ( $n = 823$  patients) or irinotecan/5-FU/LV ( $n = 230$  patients)] and bevacizumab with or without panitumumab. The study was discontinued early after a planned interim analysis showed reduced PFS and increased toxicity in the panitumumab arm. In the final analysis, median PFS (10.0 mo *vs* 11.4 mo for the panitumumab and control arms, respectively, HR = 1.27) and OS (19.4 mo *vs* 24.5 mo for the panitumumab and control arms, respectively) were shorter in the panitumumab arm in the entire study cohort as well as in the subset with wild-type *KRAS*. Grade 3/4 adverse events in the oxaliplatin (panitumumab *vs* control) cohort included skin toxicity (36% *vs* 1%), diarrhea (24% *vs* 13%), infections (19% *vs* 10%), and pulmonary embolism (6% *vs* 4%)<sup>[78]</sup>.

Similarly, in the phase III CAIRO2 trial, 755 patients with previously untreated mCRC were randomly assigned to receive capecitabine, oxaliplatin, and bevacizumab (CB regimen,  $n = 378$  patients) or the same regimen plus weekly cetuximab (CBC regimen,  $n = 377$  patients). The addition of cetuximab to XELOX plus bevacizumab resulted in shorter PFS in the entire study cohort (10.7 mo in the CB group *vs* 9.4 mo in the CBC group,  $P = 0.01$ ) and in the wild-type *KRAS* subset compared with XELOX plus bevacizumab. No difference in OS (20.3 mo in the CB group *vs* 19.4 mo in the CBC group,  $P = 0.16$ )

or ORR (50.0% in the CB group *vs* 52.7% in the CBC group,  $P = 0.49$ ) was verified between treatment arms. Patients treated with cetuximab who had tumors bearing a mutated *KRAS* gene had significantly decreased PFS as compared with cetuximab-treated patients with wild-type *KRAS* tumors (8.1 mo *vs* 10.5 mo,  $P = 0.04$ ) or patients with mutated *KRAS* tumors in the CB group (8.1 mo *vs* 12.5 mo,  $P = 0.003$ ). Grade 3 or 4 adverse events were more frequent in the CBC group, which were attributed to cetuximab-related adverse cutaneous effects<sup>[79]</sup>.

On the basis of these studies, double monoclonal antibody therapy with bevacizumab and an anti-EGFR agent is not recommended<sup>[77]</sup>.

### Management of liver metastasis

In order to determine the treatment strategy for hepatic metastases of CRC, it is important to verify the presence of one of three situations: metastases are readily resectable; metastatic disease is initially considered to be unresectable, principally due to location; or liver metastases are unlikely ever to become resectable<sup>[80]</sup>. Surgical resection undoubtedly remains the gold standard for the treatment of resectable colorectal liver metastases because it improves patient's prognosis if the metastases are resectable. When surgery is not indicated for hepatic metastases, chemotherapy is the first-choice treatment. In cases where surgical resection becomes possible and chemotherapy is effective, the long-term prognosis may be good<sup>[81]</sup>.

For patients with initially resectable disease, with good prognostic factors, one approach is immediate surgical resection and another is perioperative chemotherapy such as FOLFOX4<sup>[82,83]</sup>. Today, chemotherapy before surgery, even in patients with resectable metastases, can increase the complete resection rate, facilitate limited hepatectomies, improve postoperative recovery, treat micrometastases, provide a test of chemoresponsiveness, identify aggressive disease, spare ineffective therapy and prolong relapse-free survival<sup>[80]</sup>.

In potentially resectable colorectal liver metastases, neoadjuvant chemotherapy, infused 5-FU/LV, in combination with either irinotecan or oxaliplatin, as well as triple cytotoxic drug therapy, *e.g.*, FOLFOXIRI, and more recent combination chemotherapy regimens with targeted agents cetuximab and bevacizumab, should be considered to enhance the chance of cure of patient with initially unresectable liver metastases<sup>[80,82]</sup>.

In liver metastases that are unlikely to ever become resectable, palliative chemotherapy based on FOLFOX4/XELOX, FOLFIRI, with or without biological therapies, should be considered. In this setting, the possibility of doing a resection should not be excluded<sup>[82]</sup>.

showed in Tables 1 and 2. Further down, we will discuss the main trials in each field.

### Aflibercept

Aflibercept (Ziv-aflibercept, VEGF-Trap) is a recombinant VEGFR-antibody protein generated by the fusion of second immunoglobulin (Ig) domain of the VEGFR-1 and the third Ig domain of the VEGFR2 to the Fc domain of human IgG1<sup>[84]</sup>. In contrast to bevacizumab, which only binds to VEGF-A and forms multimeric complexes, aflibercept traps the different isoforms of VEGF-A, with approximately 1000-fold higher affinity than bevacizumab. In addition, aflibercept binds to VEGF-B and PlGF<sup>[85]</sup>. This VEGF-Trap effectively suppresses tumor growth and vascularization *in vivo*, resulting in stunted and almost completely avascular tumors<sup>[84]</sup>.

To investigate the potential role of aflibercept in the first-line treatment of mCRC with chemotherapy, the phase II AFFIRM trial (NCT00851084) recruited 236 patients who had never received therapy for mCRC or angiogenesis inhibitors. A total of 117 patients received mFOLFOX6 alone and 119 received mFOLFOX6 plus aflibercept (4 mg/kg *iv* every 2 wk). This study showed similar efficacy of FOLFOX plus aflibercept *vs* FOLFOX alone with respect to ORR (49.1% *vs* 45.9%, respectively) and median PFS (8.48 mo *vs* 8.77 mo, respectively)<sup>[86]</sup>.

The purpose of the phase III randomized, placebo-controlled clinical trial VELOUR (NCT00561470) was to investigate the efficacy and safety of aflibercept plus FOLFIRI in the second-line treatment of mCRC after oxaliplatin failure. 614 participants were randomly assigned to receive aflibercept (4 mg/kg intravenously; 612 patients) or placebo (614 patients) every 2 wk in combination with FOLFIRI. Median OS was 13.50 mo for aflibercept and 12.06 mo for placebo ( $HR = 0.817$ ,  $P = 0.0032$ ). Adding aflibercept to FOLFIRI also increased PFS relative to placebo plus FOLFIRI ( $HR = 0.758$ ,  $P = 0.0001$ ), with median PFS times of 6.90 mo *vs* 4.67 mo, respectively. The ORR in the aflibercept group was 19.8% compared with 11.1% in the placebo group ( $P = 0.001$ ). Grade 3/4 adverse events with an at least 2% higher incidence with aflibercept versus placebo were diarrhea, asthenia/fatigue, stomatitis/ulceration, infections, hypertension, gastrointestinal/abdominal pain, neutropenia/neutropenic complications and proteinuria<sup>[87]</sup>. Approximately one third of study participants had previously been treated with bevacizumab (187 in the placebo and 186 in the aflibercept group). Aflibercept produced a consistent trend towards prolonged OS ( $P = 0.7231$ ) and PFS ( $P = 0.6954$ ), regardless of prior use of bevacizumab. The incidence of adverse events in the aflibercept arm was similar in patients with prior bevacizumab (100%) to those without (98.9%), with a similar incidence of grade 3/4 events (82.5% and 83.9%, respectively). Results of this subgroup analysis showed that the addition of aflibercept to FOLFIRI leads to a consistent trend of increased OS and PFS, regardless of prior bevacizumab use<sup>[88]</sup>.

## TARGET THERAPIES-OTHERS

Others drugs are also under investigation or have been recently approved for the use in the metastatic setting, as



### Brivanib

Brivanib alaninate (BMS582664) is an oral, potent selective inhibitor of both the FGF and VEGF family of receptors<sup>[89]</sup>. Besides its antiangiogenic activity from blocking VEGFR-2 and -3, its ability to disrupt FGF receptors (FGFRs) -1, -2 and -3 has been suggested to circumvent primary and/or acquired resistance to VEGF blockade, and block FGF-dependent tumor proliferation<sup>[90]</sup>. In pre-clinical studies using *in vivo* tumor xenograft models of CRC resistant to bevacizumab, the strong antiangiogenic effects and antitumor activity of brivanib<sup>[91]</sup> were established. Phase I studies evaluated brivanib in combination with cetuximab in advanced gastrointestinal malignancies, including CRC, and demonstrated good tolerability and some evidence of clinical activity<sup>[92,93]</sup>.

A phase III study (NCT00640471) was carried out to evaluate combined use of brivanib and cetuximab without chemotherapy in third-line therapy for mCRC. A total of 750 patients were randomly assigned to treatment: 376 on brivanib plus cetuximab arm and 374 on placebo plus cetuximab arm. Patients included in this trial had wild-type *K-RAS*, had received prior fluoropyrimidine, and had been treated with irinotecan and oxaliplatin. Despite positive effects on PFS (5.0 mo in brivanib arm and 3.4 mo in placebo arm-HR = 0.72,  $P < 0.001$ ) and objective response, cetuximab plus brivanib increased toxicity and did not significantly improve OS in patients with metastatic, chemotherapy-refractory, wild-type *K-RAS* colorectal cancer (8.8 mo in brivanib arm and 8.1 mo in placebo arm-HR = 0.88,  $P = 0.12$ ). A total of 51 patients in brivanib arm and 27 patients in placebo arm had complete or partial response, yielding ORR of 13.6% and 7.2% for brivanib and placebo arms, respectively. The difference in ORR was statistically significant, supporting the brivanib plus cetuximab combination ( $P = 0.004$ ). The median duration of response was 5.8 mo in brivanib arm and 5.4 mo in placebo arm ( $P = 0.04$ ). Incidence of grade 3 or higher adverse events was 78% in brivanib arm and 53% in placebo arm, particularly fatigue, hypertension, rash, abdominal pain, diarrhea, dehydration, and anorexia. Hematologic adverse events were uncommon in both arms<sup>[90]</sup>.

### Cediranib

Cediranib (AZD2171) is a highly potent and selective inhibitor of the three VEGFRs and has a half-life suitable for once-daily oral dosing<sup>[94]</sup>. Cediranib is currently in phase III development for the first-line treatment of mCRC. The clinical development program includes two global phase II / III studies (HORIZON II and HORIZON III) in the first-line treatment setting, and a phase II study in second-line treatment.

HORIZON II (NCT00399035) is a randomized phase II / III trial aimed to compare chemotherapy (FOLFOX or XELOX) with cediranib or placebo as first-line therapy in patients with mCRC. In this study, cediranib plus chemotherapy significantly improved PFS (HR = 0.84) but not OS (HR = 0.94) or ORR, compared

with placebo plus chemotherapy.

HORIZON III (NCT00384176) incorporated a phase II / III study design. An end-of-phase-II analysis of efficacy and safety was undertaken to determine whether the study should continue into the phase III part. In this study, a randomized comparison of mFOLFOX6 in combination with cediranib versus mFOLFOX6 in combination with bevacizumab as first-line chemotherapy was made in patients with mCRC.

### Ramucirumab

Ramucirumab (IMC-1121B) is a fully humanized IgG1 monoclonal antibody that binds with high affinity to the extracellular VEGF-binding domain of VEGFR-2. Ramucirumab binds to a VEGFR-2 epitope involved in ligand binding and blocks VEGF ligands from binding this site and activating the receptor<sup>[95]</sup>. The inhibition of VEGF-stimulated VEGFR-2 activation provides ramucirumab significant antitumor activity in a range of malignancies in animal models as a single agent or in combination with other therapeutics<sup>[96]</sup>.

Several studies assessing ramucirumab in mCRC are currently underway (Table 2), without reported results. In a phase II study (NCT00862784) participants were treated with ramucirumab (8 mg/kg infusions every 2 wk) in combination with mFOLFOX6 as first-line therapy. In another phase II study (NCT01111604), patients with disease progression on an irinotecan-based, first-line chemotherapy regimen (FOLFIRI or CAPIRI) received mFOLFOX-6 alone or in combination with ramucirumab (8 mg/kg infusions every 2 wk). The phase II study NCT01079780 evaluated the combination of ramucirumab, cetuximab, and irinotecan versus cetuximab and irinotecan in patients with mCRC and progression following a bevacizumab-based regimen. A phase III study (NCT01183780) evaluates the role of ramucirumab, in combination with FOLFIRI chemotherapy, in patients with progression following first-line combination therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine. Soon, ramucirumab may show its place in the current clinical practice scenario.

### Regorafenib

Regorafenib (BAY 73-4506) is an oral multikinase inhibitor that blocks the activity of multiple protein kinases, including kinases involved in the regulation of tumor angiogenesis (VEGFR-1, -2, and -3, and angiopoietin-1 receptor), oncogenesis (KIT, RET, RAF1, BRAF, and BRAFV600E), and the tumor microenvironment (PDGFR and FGFR)<sup>[97]</sup>. Preclinical studies (both *in vitro* and *in vivo*) showed a broad spectrum of antitumor activity of Regorafenib as a result of its ability to block several angiogenic, stromal and oncogenic kinases<sup>[98]</sup>.

The phase III trial CORRECT (NCT01103323) investigated the use of regorafenib in 760 patients who had received all locally approved standard therapies and had progressed during or within 3 mo after the last standard therapy. Patients were randomized in a 2:1 ratio to

receive regorafenib (160 mg orally daily for 3 out of 4 wk;  $n = 500$ ) versus placebo (3 wk on and 1 wk off;  $n = 253$ ), respectively. Randomization was based on pre-allocated block sizes and patients were stratified by previous treatment with VEGF-targeting drugs, time from diagnosis of metastatic disease ( $\geq 18$  or  $< 18$  mo), and geographical region. This study reported an increase in OS for regorafenib-treated patients against best supportive care, after progression on standard therapy (6.4 mo *vs* 5.0 mo, respectively, HR = 0.77, one-sided  $P = 0.0052$ ). Also, median PFS was 1.9 mo *vs* 1.7 mo when compared with placebo (HR = 0.49, one-sided  $P < 0.000001$ ). After the interim analysis, the study was unblinded and patients were allowed to cross over to the regorafenib arm. Treatment-related adverse events occurred in 93% of patients in the regorafenib arm and in 61% of those in the placebo arm. The most common grade 3 or higher side effects related to regorafenib were hand-foot skin reaction (17%), fatigue (10%), diarrhoea (7%), hypertension (7%), and rash or desquamation (6%)<sup>[99]</sup>. Based on the CORRECT study, regorafenib received approval from the FDA in October 2012 for the treatment of chemorefractory mCRC patients. However, we believe that this drug should be provided only in a specific context due to the modest results reported on OS benefit and pharmaco-economic evaluation.

### Semaxanib

Semaxanib (SU5416) is a potent, specific and competitive (with respect to ATP) inhibitor of the tyrosine kinase activity of Flk-1/KDR. Semaxanib was shown to inhibit VEGF-dependent mitogenesis of human endothelial cells, without inhibiting the growth of a variety of tumor cells *in vitro*<sup>[100,101]</sup>.

A clinical phase III study (NCT00004252) studied the combination of 5-FU/LV with semaxanib or alone, as a first-line therapy for mCRC patients. Although the study had already been completed, its results are not yet known.

### Sorafenib

Sorafenib is an oral multikinase inhibitor with anti-proliferative and anti-angiogenic effects. It inhibits the activity of the serine/threonine kinases c-Raf and B-Raf; the mitogen-activated protein kinases MEK and ERK; VEG; PDGFR; the cytokine receptor c-KIT; the receptor tyrosine kinases Flt-3 and RET; and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway<sup>[102]</sup>. *In vivo* and *in vitro* studies showed that sorafenib inhibits tumor growth and disrupts tumor microvasculature through antiproliferative, antiangiogenic, and/or proapoptotic effects<sup>[103]</sup>.

In the double-blind placebo-controlled phase II study RESPECT (NCT00865709), the addition of sorafenib to mFOLFOX6 was evaluated. 198 patients were randomized to receive sorafenib (400 mg *bid*) ( $n = 97$ ) or placebo ( $n = 101$ ), combined with mFOLFOX6 every 14 d. Median PFS was 9.1 mo for the sorafenib arm and 8.7 mo for the placebo arm (HR = 0.88,  $P = 0.46$ ). Similar

results were observed in the subgroup analyses: in patients with wild-type *KRAS*, the median PFS was 9.5 mo *vs* 9.2 mo, respectively (HR = 0.84), with corresponding medians of 7.8 mo *vs* 7.6 mo, respectively, in the mutant *KRAS* subgroup (HR = 0.96). In patients with wild-type *BRAF*, the median PFS was 9.2 mo *vs* 9.0 mo, respectively (HR = 0.91), and the median PFS for patients with mutant *BRAF* was 8.6 mo *vs* 7.3 mo, respectively (HR = 0.89). There was no difference between treatment arms for median OS (17.6 mo in the sorafenib arm *vs* 18.1 mo in the placebo arm-HR = 1.13,  $P = 0.51$ ). In patients with wild-type *KRAS*, median overall survival was 19.9 mo *vs* 16.8 mo, respectively (HR = 0.89), and 17.0 mo *vs* 19.4 mo, respectively, in patients with mutant *KRAS* (HR = 1.29). In patients with wild-type *BRAF*, median overall survival was 18.8 mo *vs* 18.3 mo, respectively (HR = 1.09), and 13.9 mo *vs* 11.9 mo, respectively, in patients with mutant *BRAF* (HR = 0.46). The most common grade 3/4 adverse events in the sorafenib and placebo arms were neutropenia (48% *vs* 22%), peripheral neuropathy (16% *vs* 21%), and grade 3 hand-foot skin reaction (20% *vs* 0%). Treatment discontinuation because of adverse events was 9% and 6%, respectively. Generally, dose intensity (duration and cumulative doses) was lower in the sorafenib arm than in the placebo arm. This study did not detect a PFS benefit with the addition of sorafenib to first-line FOLFOX6 for mCRC, and *KRAS* and *BRAF* status did not seem to impact treatment outcomes. These results do not support further development of sorafenib in combination with mFOLFOX6 in molecularly unselected patients with mCRC<sup>[104]</sup>.

The clinical phase II study FOSCO (NCT00889343) studied the combination of FOLFOX6 or FOLFIRI with sorafenib or alone, as a second-line therapy in mCRC patients. Although the study had already been completed, its results are not yet known.

### Sunitinib

Sunitinib malate (SUTENT) is an oral, multitargeted tyrosine kinase inhibitor that selectively inhibits the VEGFR and PDGFR family members, as well as stem-cell factor receptor (KIT), glial cell line-derived neurotrophic factor receptor (rearranged during transfection; RET), colonystimulating factor receptor (CSF-1R), and FMS-like tyrosine kinase-3<sup>[105-107]</sup>.

In a phase III trial (NCT00457691), 768 patients with mCRC were randomly assigned to receive intravenous FOLFIRI (every 2 wk) plus sunitinib (37.5 mg/d, 4 wk on, 2 wk off) ( $n = 386$ ) or placebo ( $n = 382$ ). Median PFS was 7.8 mo in the sunitinib plus FOLFIRI arm and 8.4 mo in the placebo plus FOLFIRI arm (HR = 1.095;  $P = 0.807$ ), indicating a lack of superiority for sunitinib plus FOLFIRI. Median OS was 20.3 mo in the sunitinib arm and 19.8 mo in the placebo arm (HR = 1.171, one-sided stratified Log-rank  $P = 0.916$ ). In addition, the ORR in the sunitinib arm failed to be significantly better than that in the placebo arm (32% *vs* 34%;  $P = 0.683$ ). The study failed to demonstrate superiority for FOLFIRI plus sunitinib.

tinib. Sunitinib plus FOLFIRI was associated with more grade  $\geq 3$  adverse events and laboratory abnormalities when compared to FOLFIRI plus placebo [neutropenia (68% *vs* 30%), diarrhea (16% *vs* 8%), thrombocytopenia (11% *vs* 1%), anemia, stomatitis, fatigue, hand-foot syndrome and febrile neutropenia)]. In addition, more deaths as a result of toxicity (12 *vs* 4) and significantly more dose delays, dose reductions and treatment discontinuations occurred in the sunitinib arm<sup>[108]</sup>.

A phase II, open-label, single-arm study (NCT00668863) investigated oral sunitinib (37.5 mg/d 4 wk on, 2 wk off) combined with intravenous FOLFIRI (every 2 wk) for the first-line treatment of Japanese patients with unresectable or metastatic CRC. Median PFS was 6.7 mo by independent review and 7.2 mo by investigator assessment. ORR was 36.6% by independent review and 42.3% by investigator assessment. There was a high incidence of adverse events such as neutropenia (97.2%), leukopenia (97.2%); thrombocytopenia (84.5%), diarrhea (78.9%), nausea (78.9%), decreased appetite (74.6%) and fatigue (66.2%). Furthermore, almost 20% of patients discontinued study treatment permanently, due to adverse events and over 90% of required temporary interruptions of study treatment to perform treatment for related toxicities. The study was closed early when the concurrent phase III study of first-line sunitinib plus FOLFIRI in non-Japanese patients with mCRC was stopped due to futility, as discussed previously.

### Vatalanib

Vatalanib (PTK 787/ZK 222584; PTK/ZK) is a potent, orally active angiogenesis inhibitor that interferes with the kinase activity of all three VEGF receptors, acting as a competitive inhibitor at the ATP-binding site of the receptor kinase. This inhibition is reversible, highly selective for VEGFRs and translates to growth inhibition in a variety of different experimental tumor models. Although tumor regression did not occur, an attenuation of tumor growth was observed<sup>[109]</sup>.

In the clinical phase III trial CONFIRM1 (NCT00056459), 1168 patients with untreated mCRC were randomly assigned 1:1 to receive FOLFOX4 plus vatalanib or placebo. This study showed that the addition of vatalanib to FOLFOX4 did not improve PFS (7.7 mo in vatalanib arm and 7.6 mo in placebo arm: HR = 0.88,  $P = 0.118$ ) or OS (21.4 mo in vatalanib arm and 20.5 mo in placebo arm: HR = 1.08,  $P = 0.260$ ) and no statistically significant differences between the two treatment groups were observed in ORR (42% in vatalanib arm and 46% in placebo arm). Furthermore, vatalanib increased toxicity and more patients withdrew from treatment because of events other than disease progression in the vatalanib arm. Incidence of adverse event was 85.3% in vatalanib group and 77.5% in placebo group, particularly neutropenia, hypertension, and diarrhea. Concerning grade 3 or higher adverse events, the most notable differences were noted for hypertension (23.0% *vs* 6.8%, respectively), diarrhea (15.4% *vs* 11.1%, respectively), dizziness (7.4%

*vs* 2.3%, respectively), and pulmonary embolism (5.7% *vs* 1.7%, respectively)<sup>[110]</sup>.

The CONFIRM 2 (NCT00056446) was a phase III trial aimed to compare treatment with vatalanib plus FOLFOX4 versus placebo plus FOLFOX4 in patients with previously treated mCRC, whose disease had recurred or progressed during or within 6 mo of treatment with irinotecan in combination with a fluoropyrimidine. The median OS was 13.1 and 11.9 mo (HR = 1.00,  $P = 0.957$ ). Median PFS was longer with vatalanib than with placebo (5.6 and 4.2 mo, respectively; HR = 0.83,  $P = 0.013$ ). Treatment-related adverse events occurred in 81.4% patients in vatalanib arm and in 71% of those in placebo arm. The most common grade 3 or higher side effects related to vatalanib were neutropenia, hypertension, diarrhea, fatigue and nausea<sup>[111]</sup>.

## CONCLUSION

Nowadays, mCRC treatment remains a challenge for oncologists worldwide. Over last three decades, mCRC treatment has come from fluoropyrimidine based chemotherapy to the addition of innovative chemotherapies regimen combination, such as FOLFOX, FOLFIRI, XELOX, XELIRI, 5-FU + LV, and innovative biologic therapies, such as bevacizumab, cetuximab and panitumumab. More recently, Afibercept was approved for combination with standard chemotherapy in second line regimens for mCRC patients. Therefore, many options are now available with a powerful capacity to improve survival for metastatic patients. Thus, we should be aware for those previous mentioned innovative opportunities to fit them for each patient according to the adequate indication and tolerability. Also, pharmaco-economic studies are warranted to provide useful tools for public health entities, which might allow better clinical decisions, especially when willing those advances in research.

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## Alcoholism and liver disease in Mexico: Genetic and environmental factors

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### Abstract

Alcoholism and cirrhosis, which are two of the most serious health problems worldwide, have a broad spectrum of clinical outcomes. Both diseases are influenced by genetic susceptibility and cultural traits that differ globally but are specific for each population. In contrast to other regions around the world, Mexicans present the highest drinking score and a high mortality rate for alcoholic liver disease with an intermediate category level of per capita alcohol consumption. Mexico has a unique history of alcohol consumption that is linked to profound anthropological and social aspects. The Mexican population has an admixture genome inherited from different races, Caucasian, Amerindian and

African, with a heterogeneous distribution within the country. Thus, genes related to alcohol addiction, such as dopamine receptor D2 in the brain, or liver alcohol-metabolizing enzymes, such as alcohol dehydrogenase class I polypeptide B, cytochrome P450 2E1 and aldehyde dehydrogenase class 2, may vary from one individual to another. Furthermore, they may be inherited as risk or non-risk haplogroups that confer susceptibility or resistance either to alcohol addiction or abusive alcohol consumption and possibly liver disease. Thus, in this era of genomics, personalized medicine will benefit patients if it is directed according to individual or population-based data. Additional association studies will be required to establish novel strategies for the prevention, care and treatment of liver disease in Mexico and worldwide.

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**Key words:** Alcohol; Genes; Alcoholism; Alcohol dependence; Alcohol addiction; Alcohol abuse; Alcoholic liver cirrhosis; Anthropology

**Core tip:** Alcoholism and liver disease are leading global health problems. However, the severity and outcome of liver disease appear to vary between individuals and populations. In the present review, we analyze the general scope of alcohol consumption and its relationship with the pattern of drinking score in different countries. We focus on the development of alcoholism in Mexico, which has a strong historical background, and emphasize the need to understand the genetic and environmental factors affecting each population or geographical region of the world.

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## INTRODUCTION

The human history of alcohol consumption has been documented for several thousand years<sup>[1]</sup>. Alcohol was undoubtedly the result of a fortuitous coincidence that occurred when fruits, grains and flower stalks were fermented for a long time. People may have begun to experience pleasure and happiness after tasting alcoholic beverages<sup>[1,2]</sup>.

Alcoholic beverages are obtained from different sources, depending on the region of the world. Traditionally, must and wines are produced from grapes of the Middle East and Europe, whisky is made from various grains and sake is obtained from rice in Asia. In Mexico, “pulque” was introduced first, followed by tequila, which are made from the maguey and agave plants, respectively<sup>[3,4]</sup>.

Historically, alcohol-based beverages have served as a source of needed nutrients and have been widely used for their medicinal, antiseptic and analgesic properties. However, during the last century, alcohol abuse has increased in several countries, thereby augmenting the rate of accidents and liver diseases. The range of liver diseases secondary to alcohol consumption is extensive, including acute alcoholic hepatitis, alcoholic liver disease (ALD), cirrhosis and hepatocellular carcinoma<sup>[5]</sup>. Different factors may affect the development of alcoholic liver damage, including the dose, duration and type of alcohol consumption, drinking patterns, gender and ethnicity<sup>[1,6-8]</sup>. Other associated risk factors include obesity, iron overload, concomitant viral hepatitis infection<sup>[1,7,9]</sup> and genetic factors<sup>[7,8]</sup>. Nonetheless, the degree of the association among alcohol consumption, morbidity and mortality due to ALD varies among individuals and populations worldwide. Alcohol consumption and ALD are linked to specific genetic and environmental factors that are prevalent in each population. However, which factors and how they are involved in both alcohol addiction and the adaptation of hepatic genes capable of metabolizing large amounts of ethanol without developing liver disease are challenging questions.

In this comprehensive review, we revisit the information on the worldwide consumption of alcohol and patterns of drinking associated with liver disease, emphasizing the history of alcoholism in Mexico and the differences in the genetic and environmental backgrounds with respect to alcoholism and liver disease among the different countries, with a focus on the genetic factors involved in alcohol dependence and alcohol abuse as well as liver-metabolizing enzymes.

## WORLDWIDE ALCOHOL CONSUMPTION

The World Health Organization (WHO) published the total adult per capita alcohol consumption (liters of pure alcohol consumption/year) by distinct geographical

regions of the world<sup>[10]</sup>. Three primary categories, high (10-12 L and > 12 L), intermediate (7.5-9.99 L and 5-7.49 L) and low (2.5-4.99 L and < 2.5 L), were created to compare alcohol consumption among different countries.

The countries with the highest alcohol consumption are located primarily in Europe (Czech Republic, United Kingdom, Ireland, Germany, France, Portugal and the Russian Federation) but also in other regions, such as South Korea, Australia, Nigeria, Uganda and Argentina. The intermediate category includes countries located in the Americas, such as the United States, Canada, Mexico, Chile, Brazil and Colombia, a few African countries, such as Cameroon, South Africa, Namibia and Botswana, and Norway in Europe. The low alcohol consumption category includes several countries within the Eastern Mediterranean region and Asia, generally representing those countries where religious beliefs prohibit alcohol consumption.

However, there have been different trends in the last 50 years regarding alcohol consumption in countries worldwide. Although several countries have increased alcohol consumption, others have decreased alcohol consumption (Figure 1). Furthermore, since 2008, the WHO has been in the process of drafting a global strategy to reduce the harmful use of alcohol<sup>[11]</sup>. These observations led us to analyze the effectiveness of these strategies to avoid or decrease alcohol consumption and to improve the understanding of the biological and social events involved in the drinking habits of alcohol in Mexico compared with other regions of the world.

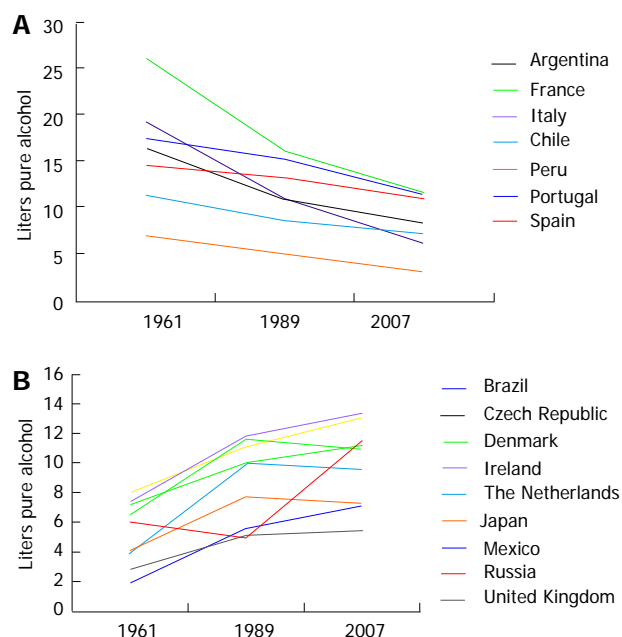
## MORTALITY DUE TO ALD

To address these concerns, we examined the mortality related to ALD within several countries. Interestingly, there is a discrepancy between mortality related to ALD and the per-capita alcohol consumption<sup>[12]</sup>; *e.g.*, Mexico is one of the countries with the highest mortality rate due to ALD but is not included among the countries with the highest alcohol consumption<sup>[12]</sup>. However, global comparisons among different populations are limited because not all countries report mortality related to ALD<sup>[12]</sup>.

## PATTERN OF DRINKING SCORE

The pattern of drinking score is a composite scale that ranges from 1 to 5 and focuses primarily on the degree of risk associated with how the alcohol is consumed rather than the amount of alcohol consumed. To build this scale, the following indicators are used: quantity of alcohol consumed by occasion, festive drinking, proportion of drinking events that involve becoming drunk, proportion of drinkers who drink daily, drinking with meals and drinking in public places<sup>[13]</sup>.

Unlike alcohol consumption, which is measured by the amount of pure alcohol per capita/year, the pattern of drinking score is closely related to ALD. For example, the countries with the highest pattern of drinking score



**Figure 1** Trends of per-capita alcohol consumption in different countries. A: Countries that decreased consumption per capita since 1961 to 2007; B: Countries that increased consumption per capita since 1961 to 2007.

are Kazakhstan, Mexico, the Russian Federation, South Africa and Ukraine, and the countries with a lower-risk pattern of drinking are Portugal, Spain, France, Italy and Germany<sup>[13]</sup>.

Taken together, alcohol consumption indicators, mortality rates and pattern of drinking scores, which all may contribute to ALD, are heterogeneous worldwide<sup>[12,13]</sup>. Thus, because ALD is a multifactorial problem, researchers should consider the anthropological and historical aspects prevalent among the different societies.

## ALCOHOL CONSUMPTION IN MEXICO

### Early history of alcohol consumption

To understand the interaction between the evolutionary and genetic changes associated with specific environments, it is necessary to know when and how these events occurred among the different populations. In the case of Mexico, the establishment of a sedentary lifestyle required approximately 5000 years<sup>[14,15]</sup>. During this period, the Mesoamericans began the domestication of the well-known staple foods of Mexico, such as maize (*Zea mays* L.), beans (*Phaseolus* spp), squash and pumpkin (*Cucurbita* spp) and chili (*Capsicum* spp). This process was accompanied by the discovery and consumption of fermented alcoholic beverages made from a number of endemic agave plants (*Agave* spp). The origin of alcoholic beverages, as described by the Aztecs, was a mythical love story between two deities, “Mayahuel” and “Quetzalcóatl” (Figure 2)<sup>[16-18]</sup>.

The core of the mature agave plant produces a honey water, or “aguamiel”, rich in amino acids and proteins<sup>[19]</sup>, which once fermented, produces the traditional alcoholic beverage. The Nahuas in their native language named the



**Figure 2** The tale of Mayahuel. The ancient gods gathered in the heavens understood that their people got bored eating only maize and chili; thus, they sent Quetzalcóatl, the god of the winds, to bring the young and beautiful goddess Mayahuel, granddaughter of a “tintzimitl”, a star who attempted to prevent the sun from rising. Quetzalcóatl and Mayahuel fell in love and together promised that they would give their people a magic plant to recover their happiness. Meanwhile, the evil grandmother noticed that Mayahuel had disappeared; thus, she and other “tintzimitl” went down to the earth to find her. Mayahuel and Quetzalcóatl were hidden in the form of a tree with two arms (branches); one arm was Mayahuel, and the other was Quetzalcóatl. When the grandmother found them, she cut Mayahuel’s arm into many small pieces, but not Quetzalcóatl’s, who was then transformed into a human again. Nothing could be done for Mayahuel, so Quetzalcóatl buried the leftover pieces in the ground and wept for his loss deeply. Finally, from these parts, the maguey was born. The mature maguey “cries” the honey-water, or “aguamiel”, that emerges from the center of the plant, representing the tears of Quetzalcóatl. “Octli polihuhqui” is the fermented nectar of the maguey that brings happiness. Thus, Mayahuel in the Nahuatl language stands for all that surrounds the maguey<sup>[16,17]</sup>. Mayahuel may be considered a dual deity. On the one hand, she represented a woman with many breasts who nourished many children, the 400 rabbits (“Centzon Totochtin”); thus, she was associated with the earth and fertility. On the other hand, she was associated with drunkenness and adultery. This mythological symbol had such great influence that one day of the month was devoted to the rabbit, and those born on that specific date were destined to either be a drunk or commit adultery<sup>[17,18]</sup>.

former “iztac octli” and the latter “octli polihuhqui”<sup>[20]</sup>. However, when the Spaniards arrived on the continent, “octli polihuhqui” was phonetically derived as the term “pulque”<sup>[20,21]</sup>.

The “octli” but not “octli polihuhqui” served as nourishment for the elderly and sick and for women after childbirth<sup>[22]</sup>. The “octli” and perhaps the “octli polihuhqui” were given to all family members, including babies and children, in public ceremonies<sup>[17]</sup>. The “octli polihuhqui” was also used for medicinal purposes as an antidepressant or as an anesthetic before human sacrifice<sup>[16,18]</sup>.

Additionally, the early Mexicans were familiar with the effects of the abuse of “octli polihuhqui”; thus, excessive drinking was strictly prohibited by law primarily during the religious holidays, and a death penalty was implemented<sup>[17]</sup>. The Aztec rulers often declared that the abuse of “octli polihuhqui” was the source and beginning of all evil and all ruin<sup>[17]</sup>. Unfortunately, these laws were not reinforced after the 15<sup>th</sup> century, granting a tolerance of the abusive consumption of alcoholic beverages during the colonial period<sup>[4,23]</sup>.

The rich history of the consumption of “pulque” by the Mexicans over many centuries is an essential compo-



Figure 3 Main types of alcoholic beverages consumed by geographical region in Mexico.

ment of the framework that is required to understand the relationship among the Mexican genome, alcoholism and liver disease at present.

### **Alcohol consumption in Mexico at present**

Mexico is one of the leading countries with a high mortality rate due to liver diseases in the world<sup>[10]</sup>. The National Health Secretariat reported an average of 25000 cases of cirrhosis per year from 2000 to 2010<sup>[24]</sup>. The primary etiologies of cirrhosis are alcohol, followed by hepatitis C infection and non-alcoholic steatohepatitis<sup>[25-27]</sup>.

For Mexico, the WHO reported that the amount of alcohol consumed is 8.4 L of pure alcohol per capita among individuals older than 15 years of age, which corresponds to an intermediate category as previously described<sup>[10]</sup>. However, if this parameter is applied only to drinkers, alcohol consumption increases to 27.1 L, which is similar to what had been reported in countries with the highest levels of alcohol consumption per capita<sup>[10]</sup>.

However, the pattern of drinking score shows a better scope of alcoholism among the Mexican population. By examining the amount of alcohol consumed by occasion, we observed that alcohol consumption occurs primarily during the weekends<sup>[28]</sup>, unlike in Europe where they drink wine almost daily, at lunch or dinner.

Hepatologists may advise their patients not to drink any alcoholic beverage to maintain a healthy liver. However, a large proportion of adults around the world drink alcoholic beverages<sup>[1,5,6]</sup>. Thus, the recommendation to avoid liver damage is that the amount of alcohol consumed should be equal or less than 2 drinks per occasion (20-40 g ethanol), not more than 4 drinks per day and not more than 10 to 12 drinks per week, allowing the liver to rest at least 1 or 2 d<sup>[1,5,6]</sup>. Furthermore, it has been suggested that the number of alcoholic drinks should be less in women than in men because women have a higher risk for developing ALD<sup>[1,5,6]</sup>.

However, each weekend, approximately 30 million Mexicans have been estimated to consume more than five drinks per occasion (more than 80 g of ethanol),

with another 10 million consuming at least one alcoholic drink daily. However, alcohol abuse has been detected in 5 million people with a strong dependence on alcohol<sup>[28]</sup>.

The average Mexican begins consuming alcohol before the age of 18 years perhaps because of a strong cultural influence. Studies conducted in the western region of Mexico have shown that 61.4% of the 12- to 17-year-old have already begun to drink alcohol<sup>[27]</sup>. The primary types of alcoholic beverages consumed in Mexico are beer, tequila and “pulque”, and other distilled beverages are consumed in a lower proportion<sup>[27-30]</sup>. However, the distribution of alcoholic beverage preferences is heterogeneous. Thus, in central Mexico, “pulque” is preferred, in contrast to tequila in the west or beer in the northern and southern parts of the country (Figure 3). These preferences are associated with the historical cultural background of each region and may be related to the mortality caused by cirrhosis. The mortality rate in central Mexico is greater than 30/100000, followed by the north at less than 10/100000 and the west at less than 5/100000<sup>[30,31]</sup>.

In western Mexico, young people begin to drink beer either during the weekend or at any social or religious event, such as weddings, coming-of-age parties and christenings. After the initiation of alcohol use, the number of beers consumed per occasion over the weekend ranges from 4 to 6 (80-100 g); this number gradually increases to 20-24 beers/355 mL each (300-360 g of alcohol)/occasion per person over a period of approximately 10 years. The second stage involves the combination of beer with tequila or any other distilled beverage during a period of 8 to 10 years. During this stage, the amount of alcohol consumed ranges from 380 to 640 g daily. In the third stage, alcohol dependence is severe, and patients may or may not present with cirrhosis. By this time, they drink an average of 510 g of alcohol per day (450-720 g)<sup>[26,32-34]</sup>.

The time between the initiation of alcohol use and the diagnosis of cirrhosis is 23 to 30 years<sup>[26,35]</sup>. However, we have identified two distinct age peaks of clinical cirrhosis. In the first group, patients are young, approximately 30 years old, and a plausible genetic predisposition to liver cirrhosis has been proposed to be involved. In the second group, the average age is approximately 45 years<sup>[33]</sup>. Compared to other countries, Mexico, according to our findings, may have the youngest people with alcoholic cirrhosis in the world. Apparently, the Apo E2<sup>[33]</sup> and FABP2<sup>[36]</sup> gene polymorphisms may be involved in the early onset of ALD among the Mexican population.

### **Clinical profile of Mexican patients with ALD**

The majority of patients with ALD seek medical attention in the advanced stages of the disease with a Child-Pugh score of C and multiple complications, such as encephalopathy, variceal bleeding, infections and ascites<sup>[25,27,35]</sup>. These clinical characteristics are present in the two primary age groups of patients with alcoholic cirrhosis<sup>[33]</sup>. Furthermore, the patients with alcoholic cirrhosis continue to drink high amounts of alcohol after diagnosis



and may die earlier in life due to clinical complications<sup>[25]</sup>. This observation may be one of the foremost reasons why hepatocellular carcinoma is rare in Mexico compared with other regions of the world<sup>[37,38]</sup>, in conjunction with other environmental factors<sup>[39]</sup>.

ALD has been associated with nutritional deficiencies and malnutrition worldwide<sup>[40]</sup>. However, preliminary data from a reference center in western Mexico have shown that obesity is also present. Among 90 patients, 17% of the alcoholic cirrhotic patients were malnourished, whereas overweight and obesity were detected in 33% of the patients, with another 50% of normal weight<sup>[35]</sup>. These data are consistent with the fact that Mexico has the highest prevalence of obesity<sup>[41]</sup>, thus adding a new risk factor for liver disease. Furthermore, in this group of patients, 34% of the patients had drug additions, which is an increasing social and health problem<sup>[35]</sup>.

Thus, the combination of alcoholism, obesity, drugs and, in several cases, viral hepatitis B or C, leads us to explore specific strategies for treatments and prevention programs to detect cirrhosis at early stages of the disease.

## GENETICS OF ALCOHOL DEPENDENCE OR ALCOHOL ABUSE

In recent decades, researchers have been using various strategies to identify genes that may be associated with alcohol dependence or alcohol abuse. Studies based on candidate genes<sup>[42-45]</sup> or linkage disequilibrium were followed by the advances in genotyping that have resulted in the widespread use of genome-wide association studies<sup>[46,47]</sup>. Previous studies in families, twins and adoption studies have shown that approximately 40%-60% of the variance in the risk for developing alcoholism can be explained by genetic factors<sup>[43-47]</sup>. However, the interactions between genes and several environmental factors have led experts in the field to identify at least two types of alcoholism: (1) a more severe, more genetic and early-onset type of alcoholism; and (2) a less severe, more environmental and late-onset type of alcoholism<sup>[48-52]</sup>.

Regarding the role of genetic factors in the susceptibility to alcohol dependence and alcohol abuse, research has primarily aimed to study the expression of brain and liver genes. For example, the major brain genes that modulate the neuroadaptive mechanism that translates alcohol stimuli into pleasure, anxiety or cravings are opioid receptor mu 1<sup>[53-55]</sup>, catechol-O-methyltransferase<sup>[56]</sup>,  $\gamma$ -aminobutyric acid receptor A<sup>[57,58]</sup>, 5-hydroxytryptamine (serotonin) receptor adenylate cyclase-coupled<sup>[59,60]</sup>, cholinergic receptor muscarinic 2<sup>[61,62]</sup>, vesicular monoamine transporter 2<sup>[63-65]</sup> and dopamine receptor D2<sup>[48,66-68]</sup>.

In the liver, several alcohol dehydrogenase (ADH) enzymes, primarily alcohol dehydrogenase class I polypeptide B (ADH1B)<sup>[52]</sup>, cytochrome P450 2E1 (CYP2E1)<sup>[69]</sup> and aldehyde dehydrogenase class 2 (ALDH2)<sup>[52,70]</sup>, and other minor ADHs, such as ADH1C<sup>[71]</sup> and ADH4<sup>[72]</sup>, have been related to alcohol metabolism and alcoholism. The three major enzyme genes express variants with different

catalytic activities ( $V_{\max}$ ) and Michaelis constants ( $K_m$ ); thus, their ability to metabolize substrates is variable.

The combination of the allelic profile of these brain and liver genes may affect the risk of or protection against alcohol dependence or alcohol abuse as well as the amount of alcohol metabolized in the liver and the susceptibility to liver damage. Variances in the distribution of these gene polymorphisms may mark phenotypic differences among populations for the aforementioned features. Hence, for this review, the biological functions of dopamine receptor D2 (DRD2), ADH1B, CYP2E1 and ALDH2 are briefly described, and their global allelic frequencies are compared, including those reported for the Mexican population.

### DRD2

Alcohol has a stimulatory effect on the dopaminergic neurons of the ventral tegmental area. Dopamine is captured by DRD2 in these neurons in the nucleus accumbens, causing a pleasant effect that is integrated into the mesolimbic system<sup>[48,67,68]</sup>.

The DRD2 *Taq I A1* polymorphism consists of a T/C nucleotide substitution (rs1800497) that alters the *Taq I* restriction site located 10541 bp downstream of the termination codon. Several studies have investigated the association of this gene polymorphism with alcohol dependence. *Taq I A1* allele carriers reportedly have lower amounts of DRD2 receptors than the *Taq I A2* carriers<sup>[73]</sup>. Thus, *A1* allele patients require higher amounts of alcohol to achieve the desired effect<sup>[66-68,74-76]</sup>. In additional studies, the association between the *A1* allele *Taq I* and alcohol use disorders has been corroborated in some but not in others. However, in several meta-analyses, a significant association between Caucasian *A1* allele carriers and alcohol addiction has been found<sup>[48,77]</sup>.

The allelic distribution of DRD2 displays a wide range of frequencies worldwide<sup>[74-79]</sup>, but the highest prevalence of the *A1* allele is found among the Amerindian Pima (83%) and Mayas (71%) from Mexico<sup>[80,81]</sup> (Figure 4).

### ADH1B

The *ADH1B* gene has a polymorphic site, resulting in the Arg47His substitution (rs1229984). The *A2* allele (ADH1B His47) confers a 100-fold higher catalytic activity to the ADH1B enzyme than the *A1* allele (ADH1B Arg47). The *A2* allele carriers have a higher ethanol oxidation capacity than the *A1* carriers. However, the *A2* carriers have a higher acetaldehyde production that leads to an alcohol-flushing response that has been considered to be protective.

The protective effect of the *A2* allele against alcohol dependence is well known in the East Asian population<sup>[82]</sup>. A study conducted in a cohort of pregnant women from England demonstrated that the *A2* carriers consumed less alcohol before pregnancy, had less incidents of binge drinking during pregnancy and were abstainers during the first trimester of gestation<sup>[83]</sup>. However, al-



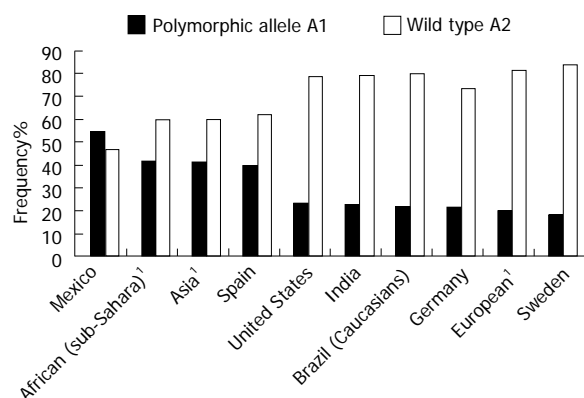


Figure 4 Frequency of Taq I A dopamine receptor D2 polymorphisms in different countries. <sup>1</sup>HapMap.

though this allele apparently reduces the risk of alcohol dependence, it may confer a substantial risk of chronic liver disease, especially among heavy drinkers<sup>[84]</sup>.

In contrast, *A1* allele carriers do not present with the rapid production of acetaldehyde, which eliminates the alcohol-flushing response. A study conducted in a large cohort of individuals from Copenhagen revealed that *A1* allele homozygotes drank more alcohol and had a higher risk for developing alcohol dependence<sup>[85]</sup>. The *A1* allele has also been associated with alcohol dependence in several other studies<sup>[86-88]</sup>.

### CYP2E1

The *CYP2E1* gene, which encodes the enzyme that oxidizes ethanol in the microsomal oxidation system, is essential in the pathophysiology of ALD. Alcohol consumption induces the expression of *CYP2E1*, which is directly involved in the primary oxidation pathway of this substrate, displacing the enzymatic activity of *ADH1B* ( $K_m$ : 8-10 mmol/L for *CYP2E1* vs 0.2-2.0 mmol/L for *ADH1B*)<sup>[69,89]</sup>.

The *C2* allele, which is less common, has a C/T transition at nucleotide position -1019 within the 5' terminal regulatory region. The *C2* allele is associated with a 100-fold higher transcription activity, higher protein concentration and increased enzyme activity, which lead to a faster rate of alcohol oxidation<sup>[90,91]</sup>.

The *C2* allele carriers have been demonstrated to consume excessive amounts of alcohol, which may be caused by the high transcriptional activity of *CYP2E1*. *C2* allele carriers metabolize ethanol (alcohol) to acetaldehyde at a higher rate. Acetaldehyde is a highly toxic and mutagenic metabolite that increases oxidative stress by producing reactive oxygen species and lipid peroxides, such as 4-hydroxy-2,3-nonenal, 4-hydroxy-2,3-alkenals and malondialdehyde<sup>[92]</sup>. An association between an increased risk for ALD and alcoholic cirrhosis has been reported among the carriers of the *C2* allele<sup>[93-95]</sup>, several of whom belong to the mestizo population of western Mexico<sup>[96]</sup>.

### ALDH2

The *ALDH2* gene encodes the primary mitochondrial

isoform enzyme that oxidizes acetaldehyde to acetate in the liver<sup>[70,97]</sup>. The C/G transition in exon 12 of *ALDH2* causes an amino acid substitution of glutamic acid for lysine at position 487 (*ALDH2* Glu487Lys, rs671). The *A2* allele (*ALDH2* Lys487) has little or null enzymatic activity. This deficiency leads to the accumulation of acetaldehyde and consequently provokes a flushing response, which discourages alcohol drinking<sup>[97]</sup>. Because flushing is an undesirable symptom, it confers relative protection against abusive alcohol consumption. An association between *A2* allele carriers and a lower risk for alcohol dependence and reduced alcohol use has been reported<sup>[98]</sup>.

With regard to the distribution of the polymorphisms of the liver alcohol-metabolizing genes, all present contrasting frequencies among different population groups (Figure 5A-C). Among Asians, the *ADH1B* gene displays the highest frequency of the *A2* protective allele, with 78% in Japan and 69% in China. In contrast, the lowest frequency for the *A2* allele was detected in Germany and Mexico, at 4% and 3%, respectively (Figure 5A)<sup>[91,99-105]</sup>, wherein the frequency of the *A1* allele associated with alcohol dependence was much higher.

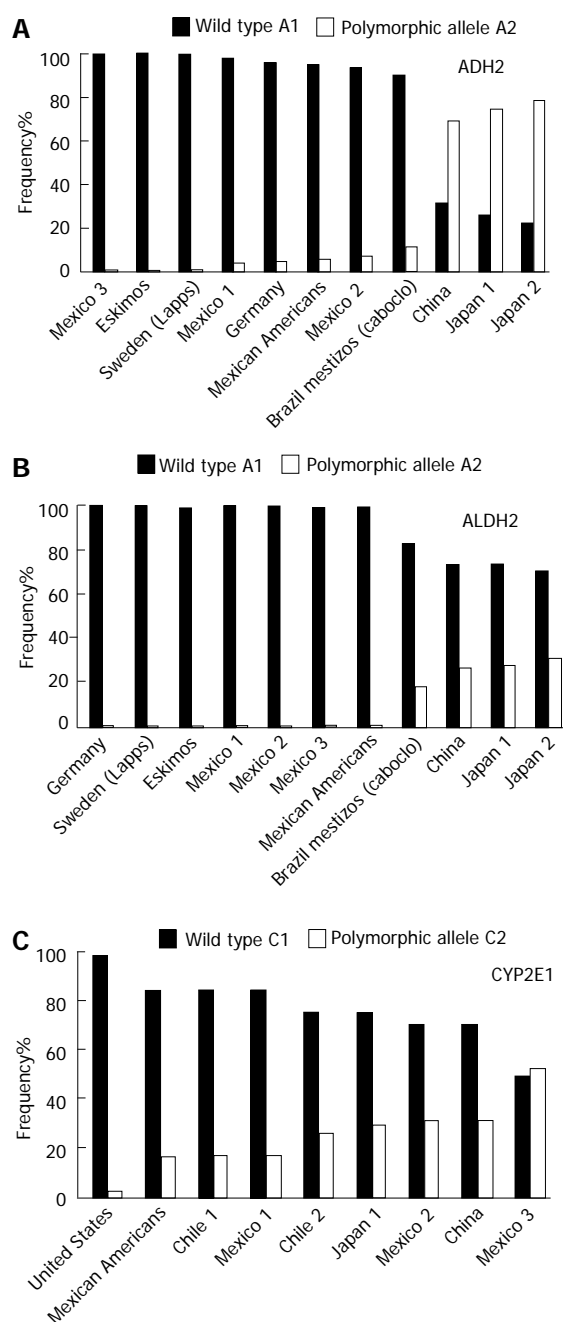
The *C2* allele for the *CYP2E1* gene has a frequency of approximately 30% in Japan and China and 2% in the United States. In Chile and Mexico, the frequency is 16% among the mestizo population (Figure 5B)<sup>[91,93,94,105-108]</sup>. Interestingly, among the Amerindians of western Mexico, such as the "Huichol" people, this gene polymorphism shows a prevalence of 50%, which is the highest rate reported to date<sup>[91]</sup>.

The highest frequency of the *A2* allele for the *ALDH* gene has been reported in China (29%) and Japan (26%). In Germany, Sweden and Mexico, its frequency is extremely low or absent<sup>[91,99-105]</sup>, which could explain, to some extent, the high amount of alcohol consumption that has been reported in those countries.

We could speculate that the selective evolution of both the brain and liver genes was not necessarily directed only by the exposure to alcohol. For example, liver cytochrome genes metabolize a large variety of xenobiotics, whereas those expressed in the brain fulfill the addiction criteria. However, an alternative point of view is to consider these genes as part of a general survival mechanism. Thus, the basic biological necessities of life, such as food (sugars, e.g., glucose) or sexual reproduction, are ensured and rewarded by pleasure; however, these necessities may not be driven by pleasure exclusively.

## CONCLUSION

At the end of the last century, we began to understand how liver genes are involved in the metabolism of ethanol and how cerebral genes are related to addictions. Additional genetic studies, including genome-wide association studies, will corroborate the association of specific alleles with alcoholism and ALD. The next step may be a personalized medicine strategy for the prevention, diagnosis and treatment of liver diseases. However, as aforementioned in this review, genes and environmental



**Figure 5** Frequency of polymorphisms of enzymes that metabolize alcohol. A: Alcohol dehydrogenase 2 (ADH2); B: Aldehyde dehydrogenase class 2 (ALDH2); C: Cytochrome P450 2E1 (CYP2E1) in different countries. A-B: Mexico 1: Western Mexico; Mexico 2: Native from Mexico "Otomi"; Mexico 3: Native from Mexico "Huicholes"; Japan 1: Rural country; Japan 2: Mestizos from Japan; C: Mexico 1: Western Mexico; Mexico 2: North an central Mexico; Mexico 3: Native from Mexico "Huicholes"; Chile 1: Mestizos; Chile 2: Native from Chile "Mapuches".

factors are involved in the development of ALD, which requires an in-depth analysis of the different populations. Therefore, the data found in several regions of the world may not correlate to populations from different geographic areas.

Mexicans are an admixture population that has inherited specific alleles from different races, predominantly Caucasian, Amerindian and African<sup>[109,110]</sup>. Based on the

current data on allelic frequencies in different countries, the Mexican population has a particular genetic profile that may explain the epidemiological and clinical manifestations of alcohol-related liver diseases. Thus, the expectation that the different allelic variants of the aforementioned genes (*DRD2*, *ADH2*, *CYP2E1* and *ALDH2*) will express themselves individually is plausible. However, considering these alleles as a haplogroup may generate risk or non-risk phenotypes related to liver disease, as well as to proneness towards or resistance against the high intake of alcohol. Haplogroups that could confer a non-risk phenotype for alcoholism and liver damage could be *DRD2*\*A2, *ADH2*\*A2, *CYP2E1*\*C1, *ALDH2*\*A2 and *DRD2*\*A2, *ADH2*\*A1, *CYP2E1*\*C1, *ALDH2*\*A2 because they are related to non-addiction plus flushing by the accumulation of acetaldehyde, exhibiting a protective effect. The haplogroups that could confer risk phenotypes for alcoholism and liver damage could be *DRD2*\*A1, *ADH2*\*A1, *CYP2E1*\*C1, *ALDH2*\*A1 and *DRD2*\*A1, *ADH2*\*A1, *CYP2E1*\*C2, *ALDH2*\*A1 because these are related to addiction plus the efficient metabolism of alcohol but exposure to acetaldehyde. This observation may explain why some patients who consume heavy amounts of alcohol per day (> 80 g/d) for more than 20 years do not have liver damage, whereas others with a less than or equal to consumption level and less exposure suffer liver damage and even die from cirrhosis or its complications<sup>[1,6,7]</sup>. However, additional studies are required to demonstrate the association between these hypothetical allelic profiles and the clinical outcomes of alcohol-dependent patients in Mexico and worldwide.

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## Management of post-hepatectomy complications

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### Abstract

Hepatic resection had an impressive growth over time. It has been widely performed for the treatment of various liver diseases, such as malignant tumors, benign tumors, calculi in the intrahepatic ducts, hydatid disease, and abscesses. Management of hepatic resection is challenging. Despite technical advances and high experience of liver resection of specialized centers, it is still burdened by relatively high rates of postoperative morbidity and mortality. Especially, complex resections are being increasingly performed in high risk and older patient population. Operation on the liver is especially challenging because of its unique anatomic architecture and because of its vital functions. Common post-hepatectomy complications include venous catheter-related infection, pleural effusion, incisional infection, pulmonary atelectasis or infection, ascites, subphrenic infection, urinary tract infection, intraperitoneal hemorrhage, gastrointestinal tract bleeding, biliary tract hemorrhage, coagulation disorders, bile leakage, and liver failure. These problems are closely related to sur-

gical manipulations, anesthesia, preoperative evaluation and preparation, and postoperative observation and management. The safety profile of hepatectomy probably can be improved if the surgeons and medical staff involved have comprehensive knowledge of the expected complications and expertise in their management. This review article focuses on the major post-operative issues after hepatic resection and presents the current management.

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**Key words:** Hepatectomy; Postoperative complication; Management

**Core tip:** Despite technical advances and high experience of liver resection of specialized centers, it is still burdened by relatively high rates of postoperative morbidity and mortality. Common post-hepatectomy complications include fever, hemorrhage, bile leakage, liver failure, pleural effusion, and subphrenic infection. The aim of this study was to summary the causes for post-hepatectomy complications and to discuss the prevention and treatment trick for postoperative complications.

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### INTRODUCTION

The era of hepatic surgery began with a left lateral hepatic lobectomy performed successfully by Langenbuch in Germany in 1887. Since then, hepatectomy has been widely performed for the treatment of various liver diseases, such as malignant tumors, benign tumors, calculi in the intrahepatic ducts, hydatid disease, and abscesses.

Operation on the liver is especially challenging because of its unique anatomic architecture and because of its vital functions. Despite technical advances and high experience of liver resection of specialized centers, it is still burdened by relatively high rates of postoperative morbidity (4.09%-47.7%) and mortality (0.24%-9.7%)<sup>[1-16]</sup> (Table 1). Common post-hepatectomy complications include fever, hemorrhage, bile leakage, liver failure, pleural effusion, and subphrenic infection, which we will discuss.

## POSTOPERATIVE FEVER AND INFECTIONS

### *Venous catheter-related infection*

Deep-vein catheterization is routinely performed for hepatic surgery, and venous catheter-related infection is the most common cause of fever after hepatectomy. This source of fever should be considered if the fever cannot be attributed to some other cause. If it cannot, the catheter should be immediately removed and its tip cultured, so that appropriate antibacterial therapy can be instituted promptly<sup>[17-21]</sup>.

### *Pleural effusion*

Reactive pleural effusion may occur after hepatectomy and usually is the result of diaphragmatic injury, obstruction of thoracic venous or lymphatic systems, or surgical manipulation on the hepatic coronary ligament (usually causing a subphrenic fluid collection). The pleural effusion, which most often occurs in the right chest, can cause fever even though it is aseptic. X-ray and ultrasound examinations should be performed promptly in febrile patients in order to determine whether a pleural effusion has developed. If only a small effusion is present it may spontaneously resolve, and if the patient has no significant symptoms or signs, no special treatment will be needed; otherwise, thoracic puncture and drainage of the effusion should be carried out<sup>[22-24]</sup>.

### *Incisional infection*

Incisional infection usually occurs within 1 wk after operation. Swelling and exudation at the incision site, or in the case of severe infection, dehiscence of the wound may be seen. If infection is found, the sutures and necrotic tissue should be removed and adequate drainage established. Antibiotics may be prescribed to help control the infection. If wound dehiscence is present, tension sutures may be placed<sup>[25-27]</sup>. Albumin may be administered intravenously in order to help relieve intra-abdominal pressure if present<sup>[28]</sup>.

### *Pulmonary atelectasis or infection*

Postoperative atelectasis or pulmonary infection most commonly presents 3-5 d after the operation. Symptoms and signs may include chest tightness, shortness of breath, and cyanosis. Surgical trauma, prolonged bed rest, and limited coughing because of incisional pain are the major factors predisposing to pulmonary atelectasis or infection. The findings of hypoxemia, determined by blood-gas anal-

ysis, and abnormalities seen on chest X-ray films, will assist in making the diagnosis. If the pulmonary infection progresses to pneumonia, the patient may have fever, cough, and pulmonary rales; increased bronchovascular shadows and pulmonary consolidation may be seen on chest X-ray films. Analgesic drugs may be given to relieve patient's pain and to facilitate deep breathing; bronchial lavage may be performed for relief of airway obstruction; and antibiotics may be prescribed after sputum culture and testing bacteria for drug sensitivity<sup>[28-30]</sup>.

### *Ascites*

Ascites is common in hepatectomy patients who have associated liver malfunction or cirrhosis. Ascitic fluid may drain from the incision site or the drainage tube<sup>[31]</sup>. Accumulation of much ascites may result in imbalance of water and electrolytes. Paracentesis for treatment of the ascites usually is not recommended; administration of diuretics and albumin is preferred. However, if the ascites is suspected of being infected and the source of fever, diagnostic paracentesis under ultrasonic guidance should be performed<sup>[32-35]</sup>.

### *Subphrenic infection*

Subphrenic infection is a severe complication of hepatectomy, usually resulting from incomplete or premature removal of a subphrenic fluid collection or a bile leak. Fever, tenderness in the upper abdomen, and abdominal muscle tension are the major manifestations of subphrenic infection. Septicopyemia or septicemia may develop if infection is severe, which may occur with pleural effusion or pulmonary atelectasis<sup>[36]</sup>. Thorough drainage of the fluid, in addition to anti-inflammatory therapy, is critical in the treatment. Ultrasonic guidance may be useful in the aspiration of subphrenic fluid collections or in the evacuation of abscesses. Open operation may be needed if the infection is severe<sup>[37]</sup>.

### *Urinary tract infection*

Fever, back pain and bladder irritation are the common symptoms of upper urinary tract infection. In contrast, fever is not common in lower urinary tract infection, which is usually manifested by dysuria and urinary frequency and urgency. Treatment of the urinary infection includes anti-inflammatory medications, oral hydration, and medications to relieve cystospasm and symptoms of bladder irritation.

## POSTOPERATIVE HEMORRHAGE

### *Intraperitoneal hemorrhage*

The incidence of intraperitoneal hemorrhage ranges from 4.2% to 10%<sup>[6,38-41]</sup>. Three common reasons for intraperitoneal hemorrhage are: (1) bleeding from the surfaces of the residual liver, which may be a consequence of arterial branch truncation or congestion of the hepatic vein due to stenosis or ligation; (2) incomplete intraoperative hemostasis, which sometimes is due to inappropriate



**Table 1** Summary of studies investigating the post-hepatectomy mortality and morbidity

Ref.	Journal	Date of publication	Country of study	NO. of Patients studied	Disease's diagnosis	Mortality and morbidity of hepatectomy
Savage <i>et al</i> <sup>[1]</sup>	<i>Ann Surg</i>	December 1991	United States	300	Liver trauma or liver tumors	The operative mortality was 19% (1962-1979) or 9.7% (1980-1988), and the overall complication rate was 12.3%
Wu <i>et al</i> <sup>[2]</sup>	<i>Zhonghua Waike Zazhi</i>	May 2002	China	1762	Liver cancer	The total mortality was 0.40%, and the total complication rate was 4.09%
Ishikawa <i>et al</i> <sup>[3]</sup>	<i>Hepatogastroenterol</i>	November-December 2002	Japan	139	HCC	The mortality within 30 postoperative days was 2.2%, and complication morbidity was 40.2%
Descottes <i>et al</i> <sup>[4]</sup>	<i>Surg Endosc</i>	January 2003	France	87	Benign liver tumors	There was no postoperative mortality, and the postoperative complication rate was 5% (laparoscopic liver resection)
Dimick <i>et al</i> <sup>[5]</sup>	<i>Arch Surg</i>	January 2003	United States	569	Malignant or benign liver disease	The overall in-hospital mortality rate was 4.8%
Benzoni <i>et al</i> <sup>[6]</sup>	<i>Hepatobiliary Pancreat Dis Int</i>	November 2006	Italy	287	HCC or liver metastasis	In-hospital mortality rate was 4.5%, and the morbidity rate was 47.7%
Benzoni <i>et al</i> <sup>[7]</sup>	<i>Hepatogastroenterol</i>	January-February 2007	Italy	134	HCC	In-hospital mortality rate was 7.4%, and the morbidity rate was 47.7%
Mullen <i>et al</i> <sup>[8]</sup>	<i>J Am Coll Surg</i>	May 2007	United States	1059	Noncirrhotic patients	The complication rate was 43%, and the 90-d all-cause mortality rate was 4.7% (1.9% patients died of causes unrelated to the liver)
McKay <i>et al</i> <sup>[9]</sup>	<i>Ann Surg Oncol</i>	May 2008	Canada	1107	Liver tumor	In-hospital mortality rate was 6.0%, and an overall complication rate was 46%
Feng <i>et al</i> <sup>[10]</sup>	<i>World J Gastroenterol</i>	December 2008	China	827	Benign hepatic lesion	In-hospital mortality rate was 0.24%, and the postoperative complication rate was 13.54%
Tomuş <i>et al</i> <sup>[11]</sup>	<i>Chirurgia (Bucur)</i>	May-June 2009	Romania	50	Benign hepatic lesion	There was no mortality, and the morbidity rate was 18%
Cescon <i>et al</i> <sup>[12]</sup>	<i>Ann Surg</i>	June 2009	Italy	1500	Malignant or benign disease	Overall mortality was 3%, and the morbidity was 22.5%
Huang <i>et al</i> <sup>[13]</sup>	<i>Chin Med J (Engl)</i>	October 2009	China	2008	Malignant or benign liver disease	The overall hospital mortality was 0.55%, and the overall postoperative complication rate was 14.44%
Mathur <i>et al</i> <sup>[14]</sup>	<i>J Gastrointest Surg</i>	August 2010	United States	3960	Liver tumor	The overall mortality rate was 2.5%, and the overall complication rate was 23.3%
Sato <i>et al</i> <sup>[15]</sup>	<i>J Gastroenterol</i>	October 2012	Japan	5270	HCC	In-hospital mortality was 2.6%, and the postoperative complication rate was 14.5%
Dan <i>et al</i> <sup>[16]</sup>	<i>Chirurgia (Bucur)</i>	November-December 2012	Romania	133	Benign or malignant tumors	The overall mortality rate was 2.25%

HCC: Hepatocellular carcinoma.

manipulation of the hepatic vein root or trauma to the diaphragm, and increased intrathoracic pressure and vena cava pressure which may lead to bleeding; and (3) vascular sutures loosened or fallen off, an event which usually is ascribed to elevated pressure in the vena cava from patients' body movement, such as turning over or coughing severely. Detachment of the ligature on the short hepatic veins may cause a gap in the vena cava wall. Postoperative intraperitoneal hemorrhage usually occurs within 48 h, and from the residual liver's surface or the diaphragm. Thorough intraoperative hemostasis is critical and must be ascertained before the operation is concluded. When the root of the hepatic vein is manipulated during the operation, hemorrhage from the vein or anterior to the inferior vena cava should be carefully sought by increasing the intrathoracic pressure artificially. Mattress sutures with hepatic needles should be used for the hemostasis, and the traumatized surface can be covered with hemostasis film, gelatin sponge, biological glue, or omentum as means of achieving additional hemostasis<sup>[42]</sup>. The pres-

ence of persistent bloody drainage might indicate that intraperitoneal clots have formed, which may occlude abdominal drains, leading to abdominal distention. Close monitoring of vital signs and transfusion of whole blood, platelets, and plasma are usually recommended as long as the patient's blood pressure and pulse remain stable. Otherwise, secondary open surgery should be considered<sup>[43]</sup>. We recommend that open surgery to attain hemostasis be performed if blood loss exceeds 1000 mL/h for more than eight hours. In summary, correct timing for operations on infected liver sections, careful manipulation during operation, and thorough hemostasis and drainage are critical for success in attaining hemostasis.

### Coagulation disorders

Five common causes of coagulation disorders associated with hepatectomy are: (1) functional failure of the residual liver due to prolonged ischemia, especially in the presence of cirrhosis<sup>[44]</sup>; (2) massive intraoperative bleeding, or blood transfusion of more than 4000 mL;

(3) consumption of coagulation factors and platelets due to severe infection; (4) overdose of heparin after hepatic artery or portal vein catheterization; and (5) cardiopulmonary bypass or extracorporeal circulation<sup>[45,46]</sup>. Coagulation time, prothrombin time, platelet count, and fibrinogen level should be tested to aid in making the diagnosis of a postoperative coagulation disorder, and the 3P test may be performed if necessary<sup>[47]</sup>. Expansion of the circulating blood volume and transfusion of fresh blood should be carried routinely once a coagulation disorder is confirmed, and prompt administration of fibrinogen, prothrombin complex, fresh platelets, and plasma cold precipitates also is important<sup>[48-50]</sup>. Protamine can be administered to neutralize the heparin if it has been overdosed.

### Gastrointestinal tract bleeding

The common causes of gastrointestinal tract bleeding after hepatectomy are: (1) stress ulcer, the most common; (2) portal hypertension due to liver cirrhosis; and (3) congestion of gastrointestinal organs because of secondary portal hypertension due to the limited volume of the residual liver. Gastrointestinal tract bleeding usually occurs within two weeks after operation. It may be manifested by the passage of brown or bloody drainage, hematemesis, melena, deterioration of vital signs, and abdominal pain. If the bleeding is mild, nasogastric suctioning and administration of proton pump inhibitors and hemostatic drugs may be adequate treatment. Somatostatin and ulinastatin may be given if the bleeding is massive. Operation should be considered if the blood pressure and pulse are unstable, or if hemorrhage persists after 48 h of aggressive treatment<sup>[51-54]</sup>.

### Biliary tract hemorrhage

Iatrogenic bile duct injury is the most common cause of biliary tract hemorrhage after hepatectomy. Surgical maneuvers, including operating in the hepatic portal region, bile duct exploratory surgery, and placement of T-tubes in the biliary ducts could result in biliary tract hemorrhage. Other common causes are mucosal erosion ulcer and coagulation disorders due to biliary tract infection and inflammation. The major manifestations of biliary tract hemorrhage are right upper quadrant gripping pain, upper gastrointestinal bleeding, and obstructive jaundice. Usually, bleeding of this kind can be treated effectively with appropriate hemostasis, antibiotics, and supportive measures. For patients who have massive biliary tract bleeding or whose bleeding site is unclear (after hepatic artery angiography), explorative operation should be carried out<sup>[55-59]</sup>.

## BILE LEAKAGE

The incidence of bile leakage ranges from 4.0% to 17%<sup>[60-63]</sup>. Common causes of postoperative bile leakage are: (1) truncation of the distal bile duct in the residual liver, the most common cause; (2) leakage at the bile duct-intestinal anastomosis, or incomplete suture around the

T-tube; and (3) injury of the bile duct from inappropriate surgical technique. A retrospective analysis by Yoshioka *et al.*<sup>[64]</sup> of 505 hepatectomy cases found that the incidence of bile leakage was 6.7%, with three independent risk factors: (1) multiple hepatectomy ( $P = 0.002$ , OR = 3.439; 95%CI: 1.552-7.618); (2) traumatized liver surface  $\geq 57.5 \text{ cm}^2$  ( $P = 0.004$ , OR = 5.296; 95%CI: 1.721-16.302); and (3) intraoperative bleeding  $\geq 775 \text{ mL}$  ( $P = 0.01$ , OR = 2.808; 95%CI: 1.280-6.160)<sup>[64]</sup>. Another analysis by Sadamori *et al.*<sup>[65]</sup> of 359 hepatectomy cases found that operative time  $\geq 300 \text{ min}$  was an independent risk factor for bile leakage after hepatectomy. To help predict if postoperative bile leakage will occur, the residual liver can be covered with wet gauze, which may show the presence of minimal bile seepage. To help avoid postoperative bile leakage, biological glue can be applied to the surface of the residual liver, and a C tube can be placed in the cystic duct for decompression<sup>[66,67]</sup>. Intraoperatively, bile leakage might be revealed with the use of indocyanine green fluorescein<sup>[68-70]</sup>. Close postoperative monitoring is mandatory and should include observing for abdominal pain, rebound tenderness, muscle tension, and bile leakage from the drainage tube. Bile leakage also may be evident by the presence of bile in the peritoneal drainage (the concentration of bilirubin in the bile will be higher than in serum). In addition, computed tomography (CT) visualization can be used to determine if the bile duct is occluded and, if so, where the occlusion is located. A drainage tube can remain in the bile duct if there is no sign of peritonitis; the bile leakage may resolve spontaneously within two months. However, if peritonitis develops, open surgery should be performed as soon as possible for thorough cleaning of the abdominal cavity and repair of the damaged common bile duct. Antibiotics may be administered for control of infection, and supportive treatment should be given as usual after a major operation<sup>[71,72]</sup>. It has been reported that bile leakage occurred in 14 of 96 patients who underwent hepatectomy; nine were treated successfully without operation, but five required a second operation. In general, non-operative treatment was sufficient if the results of ERCP and CT were negative for bile leakage, but operative intervention was needed if conservative therapy failed<sup>[73]</sup>.

## LIVER FAILURE

Liver failure is a severe postoperative complication of hepatectomy. It is closely associated with active hepatitis, cirrhosis, limited residual liver tissue, massive intraoperative hemorrhage, the mode and duration of hepatic portal vein occlusion, the kind of anesthesia used, and perioperative medication used. An incidence of liver failure after hepatectomy of about 0.70%-33.83% has been reported<sup>[74-77]</sup>, and the failure was related to inadequate residual liver tissue and functional capacity<sup>[78,79]</sup>. Comprehensive therapy for liver failure includes postoperative supplementation with albumin, fibrinogen or prothrombin complex; intravenous nutrition; and transfusion of

fresh blood. Prognosis is poor if coagulation disorders develop. Presently, the most effective therapy for liver failure is liver transplantation, but it is associated with a high mortality rate in patients with liver cirrhosis and, therefore, it remains a controversial treatment choice in this circumstance<sup>[80-86]</sup>. Generally, prevention of liver failure is felt to be more important than treatment of it. Some common preventive measures are: careful preoperative assessment of the liver's functional reserve and institution of measures to improve the liver function. Prevention of intraoperative bleeding and the need for blood transfusion also are important in preventing liver failure. In one report, the incidence of postoperative complications increased significantly when the intraoperative blood loss exceeded 1200 mL<sup>[11]</sup>. Several methods can be used to reduce the chance of intraoperative bleeding: CUSA<sup>[87-89]</sup>, heat solidification technology<sup>[90-93]</sup>, reduction of central venous pressure<sup>[94-96]</sup>, and blocking of hepatic portal blood inflow (with or without control of hepatic blood outflow)<sup>[97-101]</sup>. For patients with liver cirrhosis, the volume of residual liver and the time of portal occlusion must be strictly assessed. Also, the method used for occluding blood flow to the liver must be appropriately selected. It has been recommended that half hepatic blood flow occlusion should be used for patients with cirrhosis, and hepatic blood inflow occlusion without hemihepatic artery control (hemi-hepatic artery-preserved portal occlusion) used if half occlusion is difficult or inadequate. Hepatic blood inflow occlusion without hemihepatic artery control is simple for operation, with less damage to the liver function; more importantly, the effect of the blood flow blocking is equivalent to the half-hepatic blood flow blocking<sup>[102,103]</sup>. The procedure for inflow occlusion is the following: the hepatic artery is exposed first, to separate the right and left hepatic arteries from the root of the artery. To restrict blood flow to the right half of the liver, the hepatic portal vein, bile duct, and the right hepatic artery should be tightened together with a catheter; the opposite arrangement is used for restricting blood flow to the left half of the liver, except that the left hepatic artery, instead of the right hepatic artery, is occluded<sup>[98]</sup>. It is important that the patient receive sufficient oxygen throughout the perioperative period, and that hepatotoxic drugs are avoided.

After hepatectomy, the patient should be closely monitored, with particular attention to abnormalities in levels of consciousness, liver function, the volume and character of drainage fluid, acid-base balance, and serum lactic acid levels. In general, during the first postoperative day, the ideal levels of serum hepatic transaminases, total bilirubin, and prothrombin activity can be expected to remain below 1000 IU/mL, about 2 mg/dL, and about 50%, respectively. Acidosis is very common in liver failure, so the level of serum lactic acid should be carefully monitored. Serum bilirubin level should rapidly decrease; if the level increases abruptly after the second postoperative day the risk of hepatic failure increases. Currently, there is not a unified definition of liver failure after hepa-

tectomy. The international hepatic surgery research team has proposed a definition based on the normal postoperative course of serum bilirubin concentration and international normalized ratio (INR), reflecting the ability of the liver to maintain its synthetic, excretory, and detoxifying functions. Postoperative liver failure is defined as an increased INR and hyperbilirubinemia (according to the normal limits of the local laboratory) on or after postoperative day 5<sup>[104]</sup>. The severity of post-hepatectomy liver failure is graded based on its effect on clinical management; grade A failure requires no change in the patient's clinical management; grade B failure requires deviation from the usual management but does not require invasive therapy; grade C requires invasive treatment<sup>[104]</sup>.

## CONCLUSION

In conclusion, hepatectomy still has significant associated complications and mortality. These problems are closely related to surgical manipulations, anesthesia, preoperative evaluation and preparation, and postoperative observation and management. The safety profile of hepatectomy probably can be improved if the surgeons and medical staff involved have comprehensive knowledge of the expected complications and expertise in their management.

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## Splanchnic-aortic inflammatory axis in experimental portal hypertension

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steatosis and changes in lipid and carbohydrate metabolism similar to those produced in chronic inflammatory conditions described in metabolic syndrome in humans. Dysbiosis and bacterial translocation in this experimental model suggest the existence of a portal hypertensive intestinal microbiome implicated in both the splanchnic and systemic alterations related to prehepatic portal hypertension. Among the systemic impairments, aortopathy characterized by oxidative stress, increased levels of proinflammatory cytokines and profibrogenic mediators stand out. In this experimental model of long-term triple portal vein ligated-rats, the abdominal aortic proinflammatory response could be attributed to oxidative stress. Thus, the increased aortic reduced-nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase activity could be associated with reactive oxygen species production and promote aortic inflammation. Also, oxidative stress mediated by NAD(P)H oxidase has been associated with risk factors for inflammation and atherosclerosis. The splanchnic and systemic pathology that is produced in the long term after triple partial portal vein ligation in the rat reinforces the validity of this experimental model to study the chronic low-grade inflammatory response induced by prehepatic portal hypertension.

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### Abstract

Splanchnic and systemic low-grade inflammation has been proposed to be a consequence of long-term prehepatic portal hypertension. This experimental model causes minimal alternations in the liver, thus making a more selective study possible for the pathological changes characteristic of prehepatic portal hypertension. Low-grade splanchnic inflammation after long-term triple partial portal vein ligation could be associated with liver steatosis and portal hypertensive intestinal vasculopathy. In fact, we have previously shown that prehepatic portal hypertension in the rat induces liver

**Key words:** Portal hypertension; Inflammation; Aortopathy; Hepatic steatosis

**Core tip:** Triple partial portal vein ligation in the rat induces in the long term (22 mo) both splanchnic alterations, *i.e.*, liver steatosis and portal hypertensive intestinal vasculopathy associated with a portal hypertensive microbiome, and systemic alterations, *i.e.*, a wound-like inflammatory aortic response. These alterations support this experimental model of prehepatic portal hypertension for studying the pathophysiological mechanisms involved in the low-grade inflammatory response produced.



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## INTRODUCTION

Portal hypertension is the most severe complication that develops in cirrhotic patients and is a leading cause of mortality worldwide<sup>[1]</sup>. Ascites, hepatorenal syndrome, life-threatening gastroesophageal bleeding, portosystemic encephalopathy and sepsis, derived from shunting of portal blood into the systemic circulation through neoformed collateral vessels, are the most serious and frequent clinical complications<sup>[1]</sup>.

Portal hypertension is defined as an increase in portal blood pressure and is determined from the hepatic venous pressure gradient or pressure difference between the portal vein and the inferior vena cava<sup>[2]</sup>. The impairments arising from this pathological increase in portal pressure constitute the portal hypertensive syndrome<sup>[3]</sup>.

## EXPERIMENTAL PORTAL HYPERTENSIVE MODEL

The partial portal vein ligation experimental model in the rat is generally used to study portal hypertension since it has the lowest degree of hepatic impairment because portal hypertension is extrahepatic<sup>[4]</sup>. The surgical technique is simple and is based on making a calibrated stenosis of the portal vein<sup>[5]</sup>. If it is assumed that the intensity of the portal hypertension is determined by the resistance to the inflow produced by the constriction of the portal vein, this model of prehepatic portal hypertension could be improved by increasing the initial resistance to blood flow. With this objective in mind, we have modified this surgical technique by increasing the length of the stenosed portal tract with three equidistant calibrated stenosis<sup>[6]</sup>.

This experimental model causes minimal alternations in the liver, thus making a more selective study possible for the pathological changes characteristic of prehepatic portal hypertension<sup>[4,6]</sup>. The experimental model of partial portal vein ligation is generally studied in the short-term, *i.e.*, 2-4 wk<sup>[4]</sup>. However, studying the late phases could be of great interest since the mechanisms involved and the related complications could be more similar to those found in chronic liver diseases in humans<sup>[1,2]</sup> which are related to the chronicity of portal hypertension, among other factors.

## INFLAMMATORY RESPONSE RELATED TO PORTAL HYPERTENSION

Much evidence shows how inflammation contributes to

the initiation and maintenance of portal hypertension<sup>[7,8]</sup>. Furthermore, early and chronic partial portal vein ligated-rats show hemodynamic and metabolic impairments, where the etiopathogeny is of an inflammatory nature<sup>[7,8]</sup>. Consequently, it could be hypothesized that chronic hemodynamic, vascular and metabolic changes in rats with prehepatic portal hypertension could have an inflammatory origin, most probably subsequent to splanchnic inflammation. In this way, the endothelial inflammatory mechanotransduction induced by portal hypertension could be the first step in the production of an inflammatory response in the intestinal wall. Thus, the early splanchnic endothelial disorder could induce, in turn, an inflammatory intestinal phenotype that would be linked to both phenomena: the gut microbiota alteration as well as the vasomotor impairments that are responsible for the splanchnic hyperdynamic circulation (Figure 1).

Furthermore, prehepatic portal hypertension induces the development of portal hypertensive enteropathy with inflammatory cell infiltration, particularly mast cells<sup>[9,10]</sup>, which is reduced by the prophylactic administration of anti-inflammatory drugs, like budesonide and ketotifen<sup>[8,11]</sup>. Since the basic structural alteration found in portal hypertensive enteropathy is angiogenesis, the very appropriate name of "hypertensive portal intestinal vasculopathy" has been proposed<sup>[12]</sup>.

The formation of new blood vessels could be a key mechanism in the pathogenesis of prehepatic portal hypertension<sup>[8]</sup>. Mast cells are involved in the regulation of physiological and pathological vasculogenesis by producing mediators, such as heparin, histamine, tryptase, transforming growth factor- $\beta$ 1, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins and cytokines, such as vascular endothelial growth factor<sup>[13]</sup>. The ability of mast cells to promote the synthesis and selective release of different angiogenic mediator molecules<sup>[14]</sup> would explain their participation in the splanchnic remodeling related to experimental prehepatic portal hypertension<sup>[8-10]</sup>. Lastly, intestinal mast cells are also a potent source of multiple chemokines and play an important role in immune regulation<sup>[15,16]</sup>.

We have previously shown that prehepatic portal hypertension in the rat induces liver steatosis and causes changes in lipid and carbohydrate metabolism similar to those produced in chronic inflammatory conditions described in metabolic syndrome in humans<sup>[17-19]</sup>. Long-term portal hypertensive rats show a decrease in plasma adrenocorticotrophic hormone and corticosterone<sup>[20]</sup>. Glucocorticoids, such as cortisol and corticosterone, are pluripotent hormones that are vital in the host adaptation to stress. They are also essential for maintaining normal vascular tone, endothelial integrity and vascular permeability. Thus, the decrease in corticosterone in portal hypertensive rats could have deleterious effects on vascular systems. In addition, cortisol clearance is increased in individuals with fatty liver, and cortisol clearance is in turn inversely correlated with insulin sensitivity<sup>[21]</sup>. Therefore, in rats with portal hypertension, in which liver steatosis is present, a decreased stress responsiveness could be re-

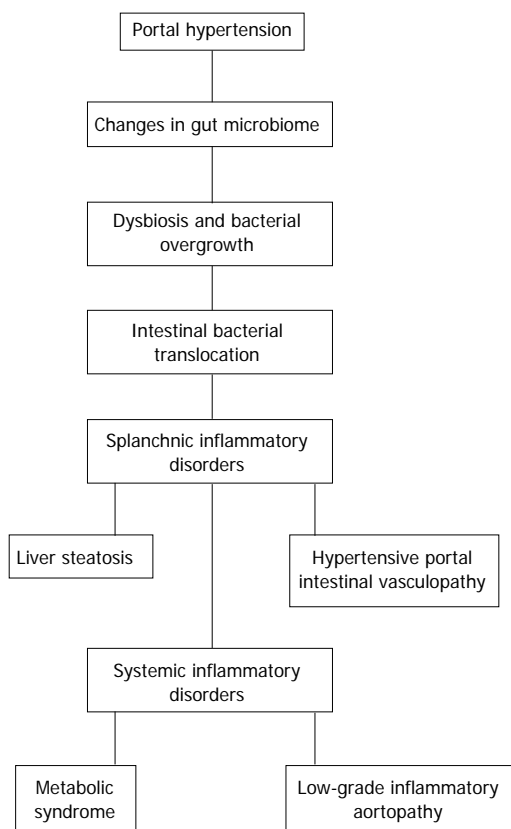


Figure 1 Splanchnic and systemic alterations in rats with prehepatic portal hypertension produced by triple partial portal vein stenosis.

lated to an impaired metabolic feedback system. The decreased neuroendocrine response to stress and systemic chronic inflammation would be another link between disordered lipid metabolism and inflammation in the evolution of this experimental model<sup>[22]</sup>. So, rats with long-term portal hypertension presenting systemic low-grade inflammation and decreased responsiveness to stress could inappropriately switch carbohydrate metabolism to predominant lipid metabolism, thus inducing body energy imbalance and ultimately, hepatic steatosis and metabolic syndrome<sup>[18,19]</sup>. Consequently, data from animal models proved that mast cells are directly involved in diet-induced obesity, diabetes and metabolic syndrome<sup>[23]</sup>.

The association of liver steatosis and metabolic syndrome with inflammation is well documented<sup>[24]</sup>. Hence, we could hypothesize that inflammation, probably of splanchnic origin, could be the pathophysiologic link between the metabolic syndrome with liver steatosis and aortic vascular disease in portal hypertensive rats. The association between metabolic and atherogenic alterations with a proinflammatory aortic phenotype could suggest the possibility that portal hypertension might constitute a novel risk factor for cardiovascular disease<sup>[20,25]</sup> (Figure 1).

## PORTAL HYPERTENSIVE INTESTINAL MICROBIOME

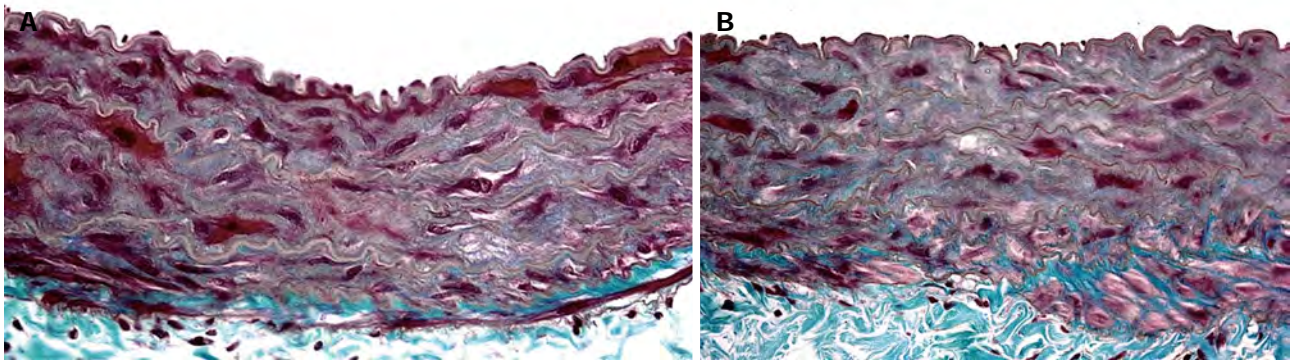
Bacterial intestinal translocation occurs in acute portal

hypertension<sup>[26]</sup> and in chronic portal hypertension in the adult partial portal vein ligated rat<sup>[27]</sup> when there are associated precipitating factors, such as hemorrhagic shock<sup>[28]</sup>. However, in chronic (1 mo) prehepatic portal hypertension by triple partial portal vein ligation, there are gut microflora alterations with less positive cultures of *Enterococci* and lactic acid bacteria, associated with bacterial translocation to the mesenteric lymph nodes<sup>[29]</sup>. Bacterial overgrowth in the intestinal tract may be the most important factor in bacterial translocation, in particular when associated with splanchnic inflammation and increased intestinal permeability, secondary to portal hypertension<sup>[29]</sup>. It has also been proposed that bacterial translocation may render the gut a “cytokine-releasing” organ that, at the same time, would induce nitric oxide overproduction and the development of the hyperdynamic circulatory state. At the same time, this is one of the progressive characteristics of portal hypertension<sup>[30]</sup>. Bacterial overgrowth could also be caused by delayed transit, mucosal hypoperfusion and oxidative damage, which increases intestinal permeability and induces the transmural passage of bacteria in portal hypertensive rats<sup>[31,32]</sup>.

Although microbial communities reside on all mammal body surfaces, including the skin and respiratory, gastrointestinal and genitourinary tracts, the largest collection of microbes reside in the gut<sup>[33]</sup>. The intestinal microbiome is considered more than just a simple organ of the mammal body. Cooperative interactions between intestinal microbes and their hosts typically involve microbial participation in host functions such as defense, metabolism, and reproduction<sup>[34]</sup>. However, communications between the host and its gut microbiota are altered in pathophysiological processes, especially if associated with inflammation, including portal hypertension<sup>[35]</sup>. Diseases mediated by the inflammatory response could induce a change in the relationship of the rat body with gut microbiota, the significance of which is unknown<sup>[35]</sup>.

Inflammatory conditions, such as splanchnic inflammation related to portal hypertension, could induce a change in the mammalian organism and gut microbiota relationship. In particular, splanchnic inflammation not only could alter gut microbiota composition, but also cause epithelial and endothelial permeability of the intestinal bacteria to increase bacterial products, such as toxins<sup>[34]</sup>. Bacterial translocation secondary to portal hypertension could lead to bacterial overgrowth and disruption of gut homeostasis<sup>[29]</sup>. This results in a “leaky gut” syndrome, with translocation of gut bacteria and bacterial products, also called pathogen-associated molecular patterns or PAMPs, to systemic sites, that finally results in systemic complications<sup>[34-37]</sup>.

Gut microbiota in rats with portal hypertension could contribute to the development of liver steatosis and metabolic syndrome. The gut microbiota may be involved in hepatologic conditions including non-alcoholic fatty liver disease<sup>[33]</sup>. Thus, bacterial products, including endotoxins, can affect Kupffer cells, hepatocytes and hepatic stellate cells, which participate in the initiation and progression of non-alcoholic fatty liver disease<sup>[38]</sup>. Gut microbiota



**Figure 2** Aortic abdominal wall microscopic images in long-term (22 mo) sham-operated rat (A) and in a triple partial portal vein ligated-rat (B). The aortic wall in the rat with portal hypertension is enlarged, has more fibrosis, with collagen deposition in the middle layer, a greater loss of the smooth muscular cell nucleus and much thinner elastic fibers than in the aortic wall of the sham-operated rat. They are also distributed in an irregular manner (Masson,  $\times 40$ ).

could also contribute to the development of hypertensive portal intestinal vasculopathy. Recent results suggest that the increased intestinal vascularization is mediated through microbiota-induced angiopoietin-1 expression in the intestinal epithelium<sup>[39]</sup>.

Gut microbiome responses to portal hypertension could be a central event in the pathogenesis of splanchnic, *i.e.*, liver steatosis and enteropathy, and systemic inflammatory conditions. However, it has been also suggested that microbiome changes could alter host-microbiome interactions to mitigate disease<sup>[33]</sup> (Figure 1).

## WOUND-LIKE INFLAMMATORY AORTIC RESPONSE

Long-term experimental prehepatic portal hypertension represents a risk factor of an inflammatory nature for aortic disease development<sup>[20,25]</sup>. Triple partial portal vein ligation in the rat induces an abdominal aortic inflammatory response 22 mo after the operation. These portal hypertensive rats show significant histological changes, in particular in the middle layer of the aortic wall. The elastic fibers lose their orderly circumferential arrangement. The interstitial connective tissue is enlarged with fibrosis and associated with a dramatic decrease in the number of smooth muscle cells. Finally, immature collagen also increases with the degeneration of connective tissue<sup>[20]</sup> (Figure 2).

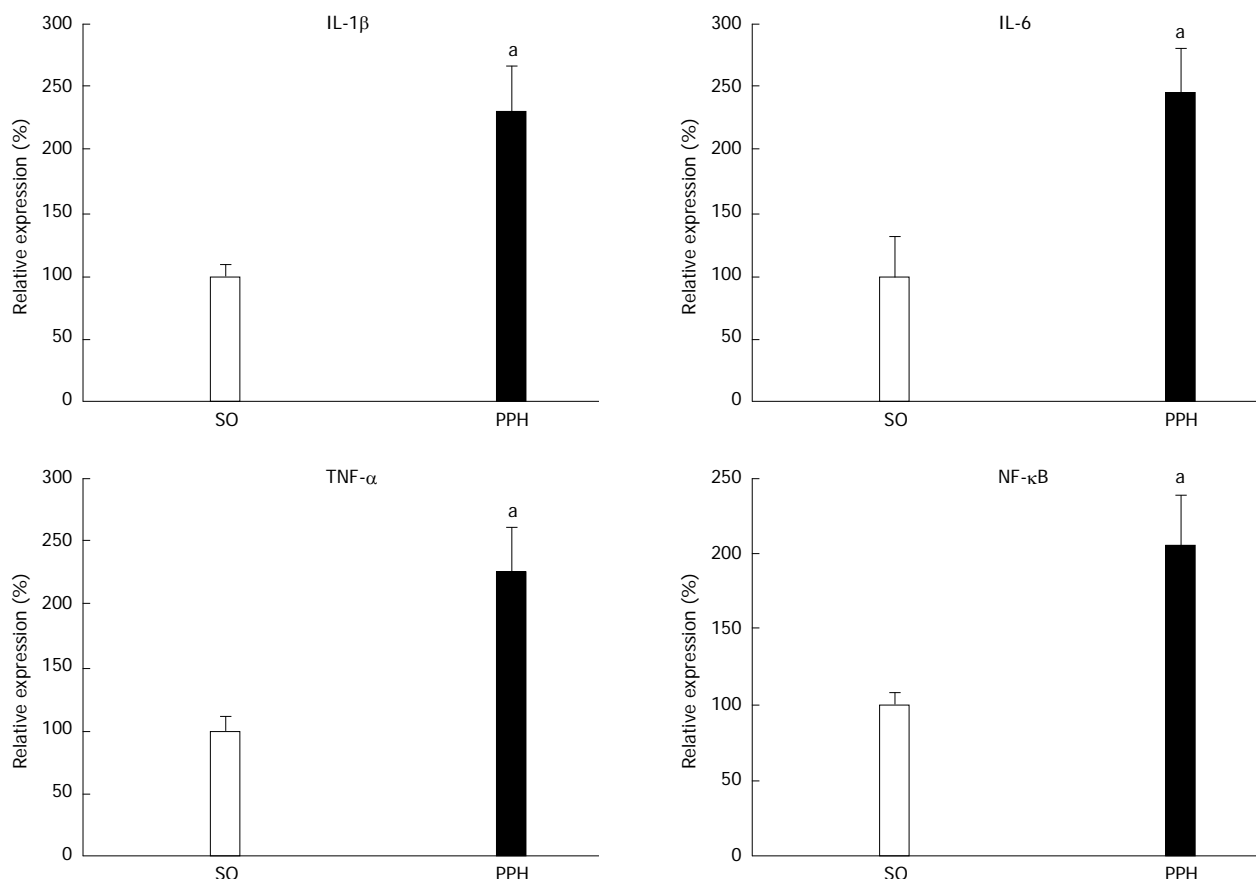
In this experimental model of long-term triple portal vein ligated rats, the abdominal aortic proinflammatory response could be attributed to oxidative stress. In this way, the increased aortic reduced-nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase activity<sup>[20]</sup> could be associated with reactive oxygen species production and promote aortic inflammation<sup>[40]</sup>. Also oxidative stress mediated by NAD(P)H oxidase has been associated with risks factors for inflammation and atherosclerosis<sup>[41]</sup>.

In chronic portal hypertensive rats, over-activation of endothelial nitric oxide synthase (eNOS) might cause aortic nitric oxide overproduction<sup>[20,25]</sup>. Upregulation of eNOS has also been seen in the aorta of short-term por-

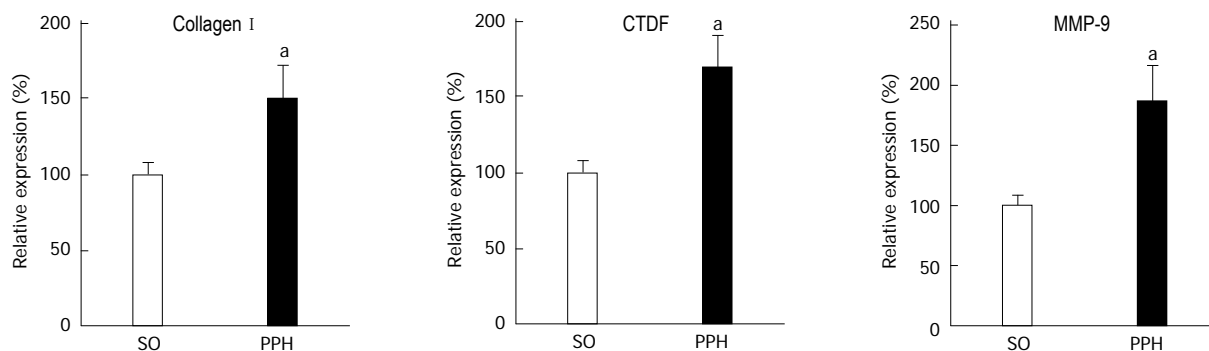
tal vein stenosed and biliary cirrhotic rats, respectively<sup>[42]</sup>. It has been suggested that an increased basal release of nitric oxide has a major role in the pathogenesis of vasodilation and vascular hypocontractility associated with portal hypertension<sup>[7]</sup>. Lipopolysaccharide (LPS) administration to cirrhotic rats increases aortic eNOS activation but, on the contrary, decreases eNOS protein expression and activity in superior mesenteric arteries. These results may explain the worsening of the hyperdynamic state in cirrhosis during septic shock by direct LPS-induced eNOS activation in large systemic vessels, and its inhibition in concomitant small splanchnic vasculature<sup>[43]</sup>.

The essential role of reactive oxygen species in the chronic inflammatory response has led to the view that reactive oxygen species promote inflammation<sup>[44]</sup>. Reactive oxygen species can increase the expression of inducible genes leading to the synthesis of cytokines, chemokines, chemokine receptors and adhesion molecules. These actions rely on transcription factors, such as nuclear factor  $\kappa$ B (NF- $\kappa$ B)<sup>[44]</sup>. Aortic overproduction of proinflammatory cytokines, including TNF- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), and IL-6 in chronic portal hypertensive rats, associated with an increased NF- $\kappa$ B/NF- $\kappa$ B inhibitor (I $\kappa$ B) ratio supports the existence of a proinflammatory abdominal aortic response. If so, reactive oxygen species or TNF- $\alpha$  could induce activation of the I $\kappa$ K (I $\kappa$ B kinase) complex resulting in phosphorylation of I $\kappa$ B, subsequent translocation of NF- $\kappa$ B to the nucleus and expression of NF- $\kappa$ B responsive genes (Figure 3).

Proinflammatory cytokines, cytokine-dependent pathways and immune cells have been implicated in the development of cardiovascular diseases, *i.e.*, atherosclerosis, coronary artery disease, chronic heart failure and hypertension<sup>[44]</sup>. Thus, a large body of evidence supports the involvement of common proinflammatory cytokines in the development and progression of a systemic low-grade inflammation affecting the cardiovascular system. In addition, these low-grade inflammatory cardiovascular diseases can be aggravated when a new inflammatory process, either of infectious origin or autoimmune nature, is added<sup>[45,46]</sup>. This is the reason why it could also be considered that prehepatic portal hypertension produces



**Figure 3 Aortic inflammatory mediators in triple partial vein ligated-rats at 22 mo after postoperative evolution.** Increased aortic mRNA expression of tumoral necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6, are associated with the increased expression of mRNA levels of the nuclear factor  $\kappa$ B (NF- $\kappa$ B)/NF- $\kappa$ B inhibitor (I $\kappa$ B) ratio. SO: Sham-operated rats; PPH: Prehepatic portal hypertensive rats. <sup>a</sup> $P < 0.05$ , statistically significant value in regards to SO-rats.



**Figure 4 Aortic profibrogenic mediators in triple partial vein ligated-rats at 22 mo of postoperative evolution.** Increased abdominal aortic expression of matrix metalloproteinase (MMP)-9, collagen I and connective tissue growth factor (CTGF). SO: Sham-operated rats. PPH: Prehepatic portal hypertensive-rats. <sup>a</sup> $P < 0.05$ , statistically significant value in regards to SO-rats.

a low degree inflammatory cardiovascular response, which could be aggravated when a new inflammatory process (acute-over-chronic) is added, particularly infections or hepatic insufficiency<sup>[8]</sup>.

Mast cells stand out among the potential mediators of the low-grade inflammatory response supposedly involved in metabolic and vascular diseases in the experimental model of prehepatic portal hypertension<sup>[3,9]</sup>. Nonetheless, these results obtained in the short-term evolution of portal hypertensive rats cannot be extrapolated to long-term portal hypertensive rats. Thus, a study of

the role of the splanchnic subpopulations of mast cells in chronic portal hypertensive rats would be interesting, given that the gut, particularly with impaired intestinal barrier function, plays an important pathophysiological role in chronic inflammation in cardiovascular diseases<sup>[47]</sup>. In particular, mast cells play a key role in experimental atherosclerosis and can modulate the inflammatory aortic response through numerous proinflammatory mediators, including TNF- $\alpha$ , IL-6 and metalloproteinases<sup>[48,49]</sup>. In addition, mast cell chymase also functions as an angiotensin-converting enzyme, particularly in rodents and there-



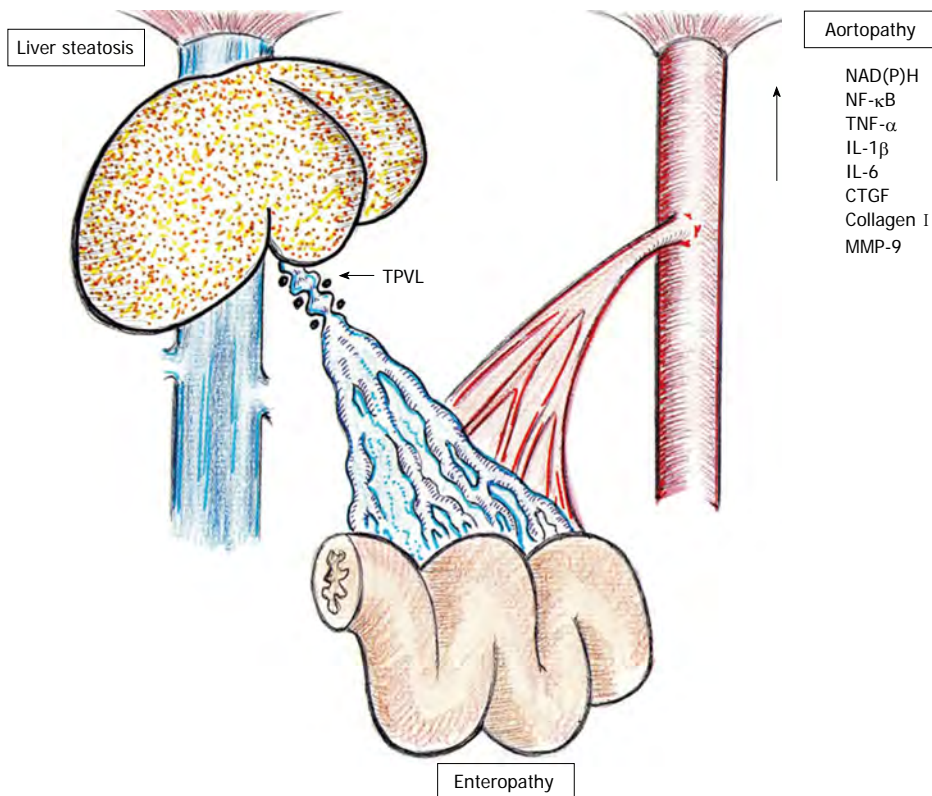


Figure 5 Chronic splanchnic alterations, liver steatosis and enteropathy, secondary to long-term triple partial portal vein stenosed rats are associated with oxidative stress, inflammatory cytokines and profibrogenic mediators in abdominal aorta. NAD(P)H: Reduced-nicotinamide-adenine dinucleotide phosphate; MMP: Matrix metalloproteinases; TPVL: Triple partial portal vein stenosed; NF-κB: Nuclear factor κB; TNF-α: Tumor necrosis factor-α; IL: Interleukin; CTGF: connective tissue growth factor; MMP-9: Matrix metalloproteinase 9.

fore contributes to aortic fibrosis<sup>[50]</sup> (Figure 4).

Lastly, enhanced aortic mRNA expression of oxidative and inflammatory mediators are associated with increased aortic expression of collagen I and connective tissue growth factor (CTGF) in long-term portal hypertensive rats<sup>[20,25]</sup>. Increased aortic CTGF expression could regulate aorta collagen remodeling, and therefore enhance the synthesis of extracellular matrix proteins, particularly type I collagen. In turn, CTGF could activate the NF-κB pathway and increase proinflammatory gene expression<sup>[51]</sup>. These results suggest the existence of a proinflammatory and a profibrotic aortic phenotype in long-term prehepatic portal hypertensive rats (Figures 4 and 5).

## CONCLUSION

In summary, rats with long-term portal hypertension with liver steatosis and hypertensive portal intestinal vasculopathy also suffer a low-grade abdominal aortic inflammation associated with fibrosis.

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## Identification and characterization of a novel bipartite nuclear localization signal in the hepatitis B virus polymerase

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### Abstract

**AIM:** To characterize the nuclear import of hepatitis B virus (HBV) polymerase (P) and its relevance for the viral life cycle.

**METHODS:** Sequence analysis was performed to predict functional motives within P. Phosphorylation of P was analyzed by in vitro phosphorylation. Phosphorylation site and nuclear localization signal (NLS) were destroyed by site directed mutagenesis. Functionality of the identified NLS was analyzed by confocal fluorescence microscopy and characterizing the karyopherin binding. Relevance of the structural motives for viral life cycle was studied by infection of primary *Tupaia* hepatocytes with HBV.

**RESULTS:** We identified by sequence alignment and functional experiments a conserved bipartite NLS con-

taining a casein kinase II (CK II) phosphorylation site located within the terminal protein domain (TP) of the HBV polymerase. Inhibition of CK II impairs the functionality of this NLS and thereby prevents the nuclear import of the polymerase. Binding of the import factor karyopherin- $\alpha$ 2 to the polymerase depends on its CK II-mediated phosphorylation of the bipartite NLS. In HBV-infected primary *Tupaia* hepatocytes CK II inhibition in the early phase (post entry phase) of the infection process prevents the establishment of the infection.

**CONCLUSION:** Based on these data it is suggested that during HBV infection the final import of the genome complex into the nucleus is mediated by a novel bipartite NLS localized in the TP domain of HBV polymerase.

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**Key words:** Hepatitis B virus; Nuclear localization signal; Casein kinase II; Trafficking; Replication

**Core tip:** The mechanism mediating import of the hepatitis B virus (HBV) genome into the nucleus is still not fully understood. We describe the identification and characterization of a bipartite nuclear localization signal (NLS) in the HBV polymerase that harbours a phosphorylation site for casein kinase II (CK II). Integrity of the phosphorylation site is crucial for the functionality of the NLS. Moreover, inhibition of CK II prevents karyopherin- $\alpha$ 2 from binding to the polymerase and thereby the import of the polymerase is impaired. Analysing the viral life cycle we observed that inhibition of CK II blocks the import of the genome into the nucleus resulting in impaired cccDNA formation and so the establishment of the viral infection is prevented.



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## INTRODUCTION

Infection with human hepatitis B virus (HBV) can cause acute or chronic inflammation of the liver. At present there are about 400 million chronically infected people worldwide. Moreover, persistently infected individuals have an increased risk of developing primary hepatocellular carcinoma (HCC)<sup>[1-6]</sup>. HBV is the prototype member of the hepadnaviridae family, which encompasses members isolated from woodchucks, ground squirrels and avian viruses isolated from *e.g.*, pekin duck, grey heron and stork.

The HBV polymerase (P) has four domains (Figure 1A). The terminal protein domain (TP) contains the tyrosine residue that primes DNA synthesis and covalently links P to the viral DNA<sup>[7-9]</sup>. The spacer domain has no known function other than to connect the terminal protein domain with the rest of P. The spacer domain, however, harbors at aa position 320 a thrombin cleavage site. This generates the possibility that not a full length protein, but a truncated polymerase is linked to the HBV genome due to intracellular proteolytic processing<sup>[10]</sup>. The reverse transcriptase domain and the RNase H domain contain the two enzymatic active sites catalyzing the reverse transcription of the RNA template to DNA and degradation of the RNA template.

Regarding the life cycle of HBV one open question concerns the fate of the viral nucleocapsid after the virus has entered the cell. There is evidence that the virus enters the cell by receptor mediated endocytosis and at the end of this process the nucleocapsid is released into the cytoplasm<sup>[11,12]</sup> and transported towards the nuclear pore complex<sup>[13,14]</sup>. Productive viral infection requires the transport of the HBV genome into the nucleus where the conversion into cccDNA occurs<sup>[15]</sup>.

The phase of nuclear entry is not fully understood. It is discussed that the intact viral capsid shuttles the genome-polymerase complex into the nuclear basket of the nuclear pore complex<sup>[16-18]</sup> where a partial disassembly of the DNA-loaded nucleocapsid leads to a release of the polymerase-linked genome<sup>[13,19-21]</sup>. The polymerase-genome complex is too big for free diffusion through the nuclear pore complex.

Due to the facts that although P is too big for free diffusion through the nuclear pore complex the polymerase-genome complex is imported into the nucleus and that a fraction of P protein is found within the nucleus<sup>[22-24]</sup> we examined the P protein for conserved motifs that could play a role for nuclear import and aimed

to characterize the nuclear import of HBV polymerase-genome complex.

## MATERIALS AND METHODS

### Cell lines and culture conditions

The human hepatoblastoma cell lines HuH-7<sup>[25]</sup> and HBV producing cell line HepG2.2.15<sup>[26]</sup> were cultured in D-MEM medium containing 10% (v/v) fetal calf serum (FCS), 500 U/L penicillin and 100 mg/L streptomycin (PAA, Pasching, Austria). Inducible HBV producing cell line HepAD38<sup>[27]</sup> were cultivated like HepG2.2.15 but with 400 mg/L G418, 50 µmol/L hydrocortisone and 2.5 mg/L insulin (Sigma-Aldrich, Sneeze, Germany), additionally.

### Subcellular fractionation

Subcellular fractionation was performed as described<sup>[28,29]</sup>.

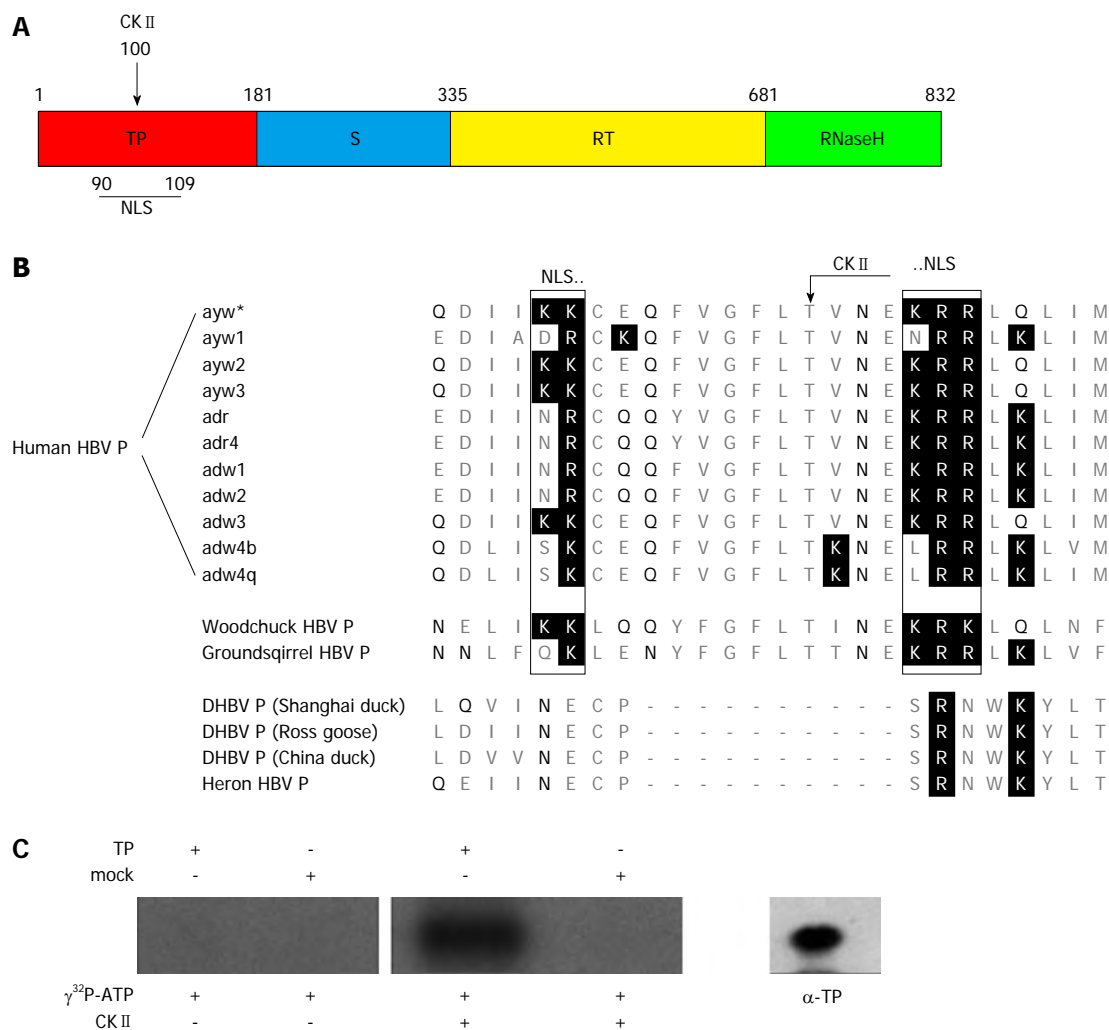
### Infection of primary hepatocytes

Primary *Tupaia belangeri* hepatocytes were isolated, cultivated and infected as described<sup>[30,31]</sup>. Trypsin treatment for removal of attached viral particles was performed as described<sup>[12,31-33]</sup>. HBeAg and HBsAg synthesis were analysed 120 h after infection.

### Generation of expression constructs

Plasmids were sub-cloned in *Escherichia coli* strain DH5α. The relevant mutations in the listed primer sequences are highlighted, restriction sites underlined and the corresponding backward primer sequences of mutation primers are reverse complementary to the forward primer if not cited otherwise.

The 1.2 fold HBV genome pJO19 (subtype ayw, genotype D) was derived from plasmid pSM2<sup>[26]</sup> by a stepwise truncation of the plasmid with *BseA* I and *Aat* II. Mutant versions of the wild type genome with alterations in the polymerase coding sequence were generated based on pJO19: The CK II recognition site deficient genome pJO19[T100I] was generated using primer ΔCK II\_fw (5'-CAG TTT gTA ggC CCA CTC ATA gTT AAT gAg AAA AgA AgA TTg CAA TTA ATT ATg CCT gCC), the pseudophosphorylated genome pJO19[T100D] was generated with primer \*CK II\_fw (5'-CAG TTT gTA ggC CCA CTCg ACg TTA ATg AgA AAA gAA gAT TgC AAT TAA TTA TgC CTg CC), the genome with an inactivated NLS pJO19[K105D,K106S] was generated with forward primer ΔNLS\_fw (5'-ggC CCA CTC ACA gTT AAT gAg CAg TCT AgA TTg CAA TTg ATT ATg CCT g). GFP wild type expression was obtained by pEGFP-N1 (BD, Heidelberg, Germany). The N-terminal fusions of NLS signals to GFP were generated from a modified version of pEGFP-N1 (pJO21). In pJO21 the first base of the transcriptional START of the GFP reading frame was deleted by site directed mutagenesis to prevent wild type GFP expression using primer GFP Δstart\_fw (5'-CCA CCg gTC gCC ACC Tgg TgA gCA



**Figure 1** Sequence alignment of the hepatitis B virus polymerase from various virus subtypes and species. **A:** Scheme of the hepatitis B virus (HBV) polymerase showing the different domains. The terminal protein domain (TP) is shown in red, the spacer domain (S) in blue, the reverse transcriptase domain (RT) in yellow and the RNase H domain in green. The numbers designate the amino acids referred to HBV genotype D. The positions of the casein kinase II (CK II) phosphorylation site and of the bipartite nuclear localization signal (NLS) identified in this study (see Figure 1B) are indicated; **B:** This figure shows amino acid alignment of Q86-M111 referred to the sequence of subtype ayw\*, which was used in this study. Basic amino acids are highlighted by a black background and polar ( $\delta^+$ ) amino acids are highlighted by black letters. A conserved protein kinase CK II recognition site (arrow) was identified in orthohepadnaviruses at Thr100 (protein kinase CK II: T/S-X-X-E/D; X = any amino acid). A bipartite nuclear localization signal was identified in *orthohepadnaviruses*, which is flanking the CK II recognition site by its two basic amino acid clusters (rectangles). All identified putative motifs were not found in the aligned P proteins of the compared *avihepadnaviruses*; **C:** Purified TP domain and mock purified proteins (empty vector products) were incubated with [ $\gamma$ - $^{32}$ P] ATP and recombinant protein kinase CK II. To control auto-phosphorylation TP domain was incubated with [ $\gamma$ - $^{32}$ P] ATP in absence of the kinase. The protein specificity was verified by Western blotting using a TP specific antibody ( $\alpha$ -TP) on a separate lane. All experiments were performed in triplicate. One representative is shown.

Agg gCg Agg). The plasmid pNLS<sub>NP</sub>-GFP was generated by amplifying human nucleoplasmin from a cDNA library. The polymerase chain reaction (PCR) product was flanked by terminal *Bgl*/II sites, which were generated by primer N-NLS-3fw (5'-TTT AgA TCT gTT CAg ggC CAg TgC) and primer N-NLS-3bw (5'-TTT AgA TCT TTT TAC TTT TTT CTg Tgg). The PCR product was ligated to the *Bam*HI cut pJO21. An engineered *Bgl*/II site followed by an optimized Kozak sequence<sup>[34]</sup> with transcriptional START was inserted immediately upstream of the NLS sequence by site directed mutagenesis using forward primer N-NLS-4fw (5'-ggA TgT gAA ACT CTT AAg TAg ATC TCg CCA CCA Tgg gAA AgC ggT CTg CCC CTg g). The dispensable upstream sequence was removed by a *Bgl*/II digest. Analogue to pNLS<sub>NP</sub>-GFP, the plasmid pNLS<sub>TP</sub>-GFP was generated by amplifying

the putative NLS of the TP domain from pJO19 using forward primer TP-NLS-3fw (5'-CCC ggA TCC ATg CCC CTA TCC TAT CAA CAC) and backward primer TP-NLS-3bw (5'-TTT AgA TCT TCT TTT CTC ATT AAC Tg). The upstream *Bgl*/II site, the Kozak signal and the transcriptional START was inserted using primer TP-NLS-4fw (5'-CCT AAT ATA CAT TTA CAC CAA gAC AgA TCT CgC CAC CAT ggT gAA AAA ATg TgA ACA gTT TgT Agg C).

The P-expression constructs were generated based on pJO19 or the corresponding mutants by PCR and subcloned in the pCDNA.3 eukaryotic expression vector.

#### Site-directed mutagenesis

was performed as described<sup>[35]</sup> by amplification of the whole plasmid using *Pfu* Turbo Hotstart DNA-Poly-

merase (Invitrogen, Karlsruhe, Germany). All synthetic oligonucleotides are purchased by Tib-Molbiol, Berlin, Germany.

### Purification of recombinant proteins

The coding sequence for the TP domain (amino acid 1-181) of HBV polymerase was amplified by PCR and inserted into the eubacterial expression vector pQE60 (Qiagen, Hilden, Germany), which encodes a C-terminal His-tag. Expression was performed at room temperature to reduce the formation of inclusion bodies. The soluble fraction of recombinant TP was purified by affinity chromatography on a Ni-NTA column under native conditions as described recently<sup>[36]</sup>. TP protein inclusion bodies were solved using 6 mol/L guanidine hydrochloride. Ni-NTA affinity purification under denaturing conditions was performed as described<sup>[37]</sup>. For further purification the TP containing fractions were pooled, dialyzed to buffer A<sub>MS</sub> (6 mol/L urea, 20 mmol/L sodium acetate, 2% (v/v) ethanol, pH 5.5) and polished by cationic exchange chromatography using a pre-packed Tricorn MonoS column (GE Healthcare, Freiburg, Germany). The elution was performed by a linear gradient over 20 column volumes (cv) between buffer A<sub>MS</sub> and A<sub>MS</sub> containing 1 mol/L sodium chloride.

### In vitro phosphorylation

experiments were performed using highly purified *E. coli* produced terminal protein domain dialyzed against kinase buffer (25 mmol/L Tris-HCl, 25 mmol/L beta-glycerophosphate, 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L DTT, pH 7.5). Phosphorylation was started by addition of 10 µCi [ $\gamma$ -<sup>32</sup>P] ATP and recombinant human CK II (Merck, Darmstadt, Germany). After 30 min incubation at 30 °C the reaction was stopped by addition of SDS sample buffer and heat treatment (5 min, 95 °C). Proteins were separated by 12% (v/v) SDS-PAGE and detected by autoradiography.

On column phosphorylation of was performed using polished, denatured TP from the cationic exchange chromatography. After addition of 20 mmol/L 2-mercaptoethanol and 100 mmol/L Tris, pH 8 the TP containing fraction was incubated for 1 h at room temperature with 2 cv Ni-NTA agarose, which was pre-washed with buffer A<sub>D</sub> (6 mol/L urea, 100 mmol/L Tris, pH 8.0). The coupling efficiency was 90%, which was determined by optical density at 280 nm. Equal amounts of TP-agarose were loaded on two empty chromatography columns. A controlled refolding of TP was initiated by a 30 cv linear gradient of buffer A<sub>D</sub> to buffer R (20 mmol/L Tris, 134 mmol/L sodium chloride, 10% (v/v) glycerol, 10% (v/v) sucrose, 20 mmol/L 2-mercaptoethanol, 0.1% (v/v) Tween-20, pH 7.5). The buffer was changed by a 10 cv linear gradient to buffer K (20 mmol/L Tris, 50 mmol/L potassium chloride, 10 mmol/L imidazole, 20 mmol/L 2-mercaptoethanol, 20 mmol/L beta-glycerol phosphate, 0.1 mmol/L sodium ortho-vanadate, 0.1% (v/v) Tween-20, pH 7.5). 2500 U recombinant protein kinase CK II (Merck, Darmstadt, Germany) was injected together with 200 µmol/L GTP in buffer K and the

column was incubated for 3 h at 28 °C. The reaction was stopped by washing the column with 5 cv buffer K.

### Binding partner fishing

Six confluent grown 175 cm<sup>2</sup> culture flasks of HuH-7 cells were lysed by sonification in TBS buffer including protease inhibitor cocktail (1 mmol/L PMSF, 5 mg/L aprotinin, 1 mg/L pepstatin, 4 mmol/L leupeptin, 1 mmol/L EDTA). The crude lysate was cleared by centrifugation at 20000 rpm in a TST41 rotor. The lipid content of the supernatant was reduced by precipitation of the proteins at 75% (w/v) ammonium sulfate. The protein pellet was resolved in TBS buffer and desalted by gel filtration using a HiTrap Desalting column (GE Healthcare, Freiburg, Germany). The desalted 75% (w/v) ammonium sulfate fraction of HuH-7 cell lysate was diluted in buffer B (20 mmol/L Tris, 25 mmol/L beta-glycerol phosphate, 1 mmol/L ortho-vanadate, 20 mmol/L 2-mercaptoethanol, 0.1% (v/v) Tween-20, pH 7.5). Equal amounts of this protein solution were injected to the two terminal protein bound columns and to a blank column only loaded with nickel-agarose. After washing the three columns for 5 cv with buffer B the binding partners were eluted by 1 mol/L sodium chloride and analyzed by western blotting using antibody karyopherin- $\alpha$ 2 (C-20) purchased from Santa Cruz, Heidelberg, Germany.

### Kinase inhibitor experiments

HepG2.2.15 cells were seeded in 6-well plates with an initial density of  $5 \times 10^5$  cells/well. Three days after cell seeding CK II inhibitor DMAT (Merck, Darmstadt, Germany) was added to the consumed cell culture medium for 2.5 h. After washing with phosphate buffered saline the cells were incubated for additional 18 h with consumed cell culture medium from the non-HBV producing cell line HuH-7, supplemented with the same concentration of DMAT as already pre-treated. The concentration of the solvent dimethyl sulfoxide (DMSO) was kept at 0.7% (v/v) in all investigated samples.

### HBV quantification

Virus genomes were extracted from cell culture supernatant using High Pure Viral Nucleic Acid Kit and determined by LightCycler PCR (Roche, Mannheim, Germany) using a HBx specific probe<sup>[38]</sup>. cccDNA was extracted from HBV infected *Tupaia* hepatocytes according standard protocols for genomic DNA extraction with phenol/chloroform<sup>[39]</sup>. Southern blotting of HBV DNA using a HBV specific <sup>32</sup>P labeled probe was performed as described<sup>[31]</sup>.

### Endogenous polymerase reaction

HuH-7 cells ( $3 \times 10^5$ ) were seeded in 6-well plates and transfected with 2 µg HBV DNA using Fugene 6 (Roche, Mannheim, Germany). The enveloped viral particles were precipitated 5 d after transfection by sheep anti-HBs polyclonal serum (kindly gift from Klaus-H. Heermann, University Goettingen, Dept. Virology, Germany) and swollen protein-A sepharose beads (Sigma-Aldrich,

Sleeze, Germany) from the cell culture supernatant. The endogenous polymerase reaction (EPR) reaction was performed as described<sup>[40]</sup> using 10  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] dCTP (GE Healthcare, Freiburg, Germany) for the labeling.

### Confocal laser scanning microscopy

HuH-7 cells ( $5 \times 10^4$ ) were grown on cover slides in 24-well plates and fixed with 4% formaldehyde/PBS for 30 min at 25 °C. For visualization of actin filaments, the cells were stained with FITC-labelled phalloidin (Sigma, Munich, Germany). Staining was performed as described<sup>[41,42]</sup>. Rabbit-derived polyclonal TP-specific or spacer domain-specific sera were used for detection of P. Confocal laser scanning microscopy (CLSM) immunofluorescence was performed using the Zeiss LSM 510 microscope (Zeiss, 20  $\times$  and 63  $\times$  objectives).

## RESULTS

### Identification of conserved motifs on HBV polymerase

Previous data of our lab based on cell permeable HBV capsids<sup>[13]</sup> and studies by M Kann's lab<sup>[21]</sup> argue against the concept that intact viral capsids<sup>[17]</sup> or HBcAg shuttles the genome-polymerase complex into the nucleus<sup>[16,20]</sup>. Based on the data from the TLM-nucleocapsid model system it can be assumed that a partial disassembly of the capsid occurs within the nuclear pore complex or in a perinuclear domain that leads to a release of the genome complex. This raises the question about the final import of the polymerase linked genome into the nucleus.

Sequence analysis of HBV polymerase subtype ayw predicted the existence of a bipartite nuclear localization signal within the terminal domain (TP) (amino acid K90-K91, K104-R106) (Figure 1A). Moreover, a phosphorylation site for CK II (T100) was found within the putative NLS (Figure 1B). The family hepadnaviridae encompasses two genera: orthohepadnavirus and avihepadnavirus. Further analysis revealed that the bipartite NLS and the enclosed CK II phosphorylation site are conserved within the *orthohepadna* viruses but not within the *avihepadna* viruses (Figure 1C). The existence of a functional NLS would enable the transfer of the genome-polymerase complex through the nuclear pore complex into the nucleus.

### TP domain is phosphorylated by CK II *in vitro*

To control experimentally whether the predicted phosphorylation site indeed can be phosphorylated by CK II *in vitro* phosphorylation was performed. Thereto, highly purified recombinant TP domain was incubated with [ $\gamma$ -<sup>32</sup>P] ATP in the presence of CK II. To exclude any phosphorylation by contaminating kinases the purified TP domain was incubated as described above, but CK II was omitted. As an additional control a mutated TP domain was used in which the predicted CK II phosphorylation site was destroyed by a T to A conversion at aa position 100. Figure 1B shows a significant specific phosphorylation of the TP domain only if CK II is present.

In case of the controls no significant phosphorylation was observed. To confirm the identity of the phosphorylated species with the TP domain Western blotting analysis was performed (Figure 1C, right panel). This indicates that the predicted kinase recognition site on the terminal protein is indeed accessible for phosphorylation.

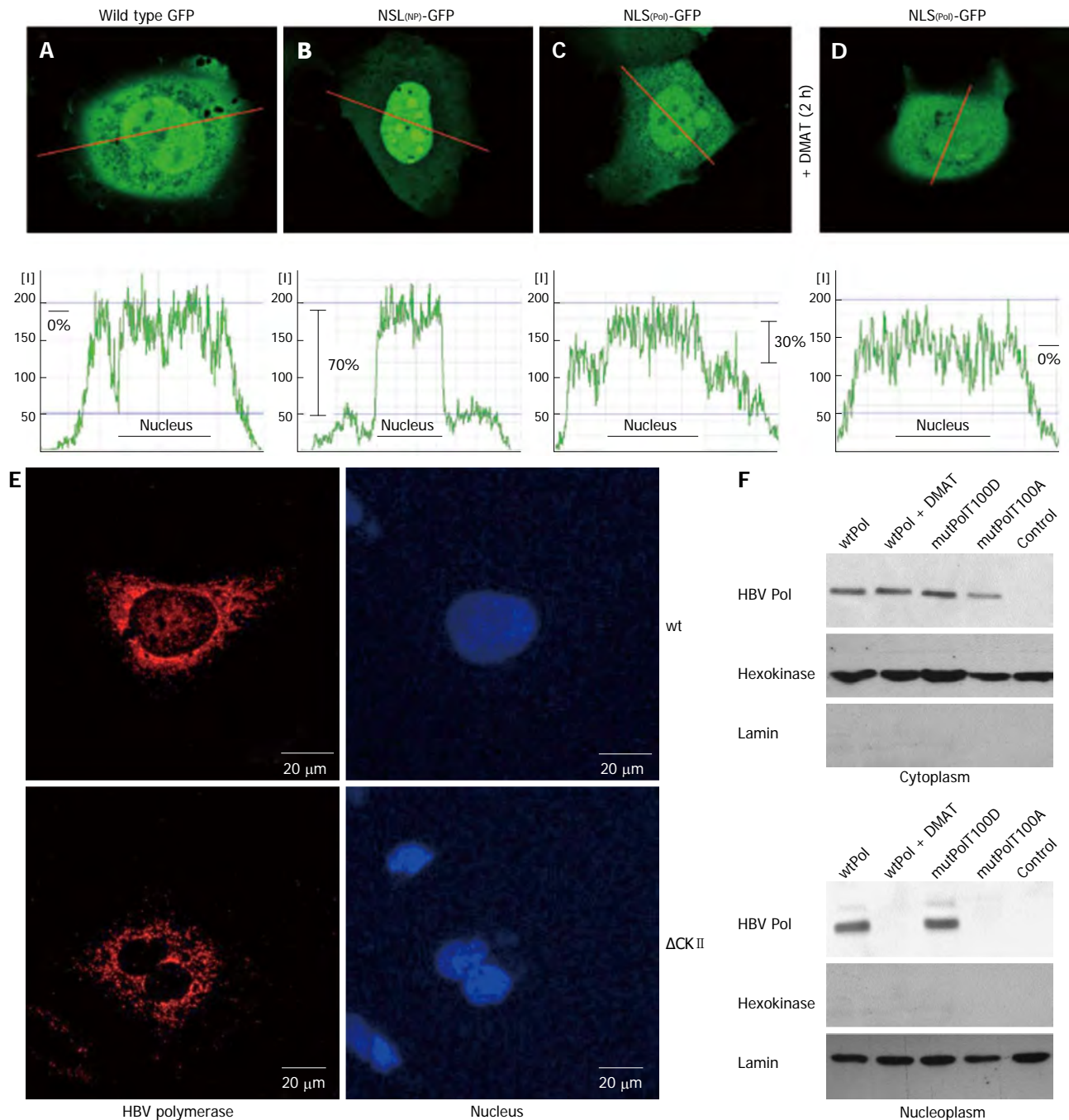
### P protein harbors a functional bipartite NLS, which depends on phosphorylation

To study the functionality of the TP-derived putative bipartite NLS HuH-7 cells were transfected with an expression plasmid encoding for a fusion protein of the putative NLS and GFP (NLS<sub>P</sub>-GFP). As a positive control served a 17 aa long prototype NLS (K142 to K158) derived from human nucleoplasmin (gi114762) fused to the amino terminus of GFP (NLS<sub>NP</sub>-GFP). The intracellular distribution of the GFP fluorescence was quantified by confocal laser scan microscopy in living cells. Compared to wild type GFP expression, which was found evenly distributed within the cell (Figure 2A), the level of NLS<sub>P</sub>-GFP was approximately 30% higher in the nucleus than in the cytosol (Figure 2B). In case of the positive control (NLS<sub>NP</sub>-GFP) an about 75% elevated level of GFP specific fluorescence in the nucleus was observed (Figure 2C). This confirms that the predicted sequence indeed acts as a functional NLS. To analyze a putative relevance of CK II-dependent phosphorylation for the functionality of the TP-derived NLS, NLS<sub>P</sub>-GFP producing cells were incubated for 2 h with CK II inhibitor DMAT prior analysis by confocal microscopy. The quantification of GFP fluorescence revealed that presence of the CK II inhibitor prevented the directed nuclear enrichment of the NLS<sub>P</sub>-GFP (Figure 2D). Comparable results were obtained for cells expression the NLS<sub>P</sub>-GFP(T100A) mutant. An equal distribution comparable to GFP was observed (data not shown).

To study the relevance of the identified bipartite NLS for the subcellular distribution of the HBV polymerase cells were transfected with an expression construct encoding for HBV polymerase and analyzed by confocal immunofluorescence microscopy or subjected to cell fractionation. The immunofluorescence microscopy shows that in HBV P overproducing cells a fraction was found within the nucleus. However, in cells overexpressing the T100A mutant that destroys the CK II phosphorylation site the nuclear-localized fraction disappeared and the P was exclusively found in the cytoplasm (Figure 2E). Western blotting analysis of the cytoplasmic and of the nuclear fraction confirmed that in addition to the cytosolic fraction a significant amount of P was detectable in the nucleus. However in cells treated with DMAT or overexpressing the T100A mutant of the putative CK II phosphorylation site no P-specific signal was detectable in the nuclear fraction (Figure 2F).

Taken together these results indicate that the HBV polymerase harbors a bipartite nuclear localization signal which functionality is dependent on CK II-mediated phosphorylation.



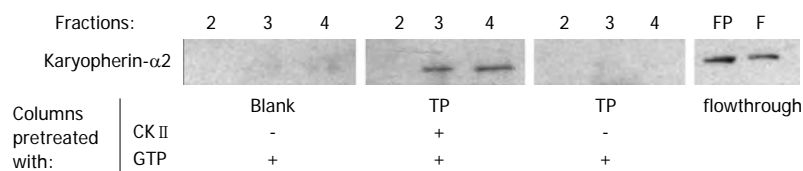


**Figure 2** Hepatitis B virus polymerase harbors a functional nuclear localization signal in the terminal protein domain. A-D: HuH-7 cells were transfected with (A) the negative control wild type green fluorescent protein (GFP) (pEGFP-N1), (B) a positive control: GFP fused to a prototype bipartite nuclear localization signal (NLS) of human nucleoplasmin, (C) GFP fused to the putative bipartite NLS of hepatitis B virus (HBV) P protein, (D) the same as (C) but cells were treated with  $10 \times \text{IC}_{50}$  of casein kinase II (CK II) inhibitor 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) 2 h prior analysis. The fluorescence was measured in living cells by confocal laser scan analysis. The central layer (out of 6) was quantitated along the red indicated line and displayed in the corresponding graph of the lower panel as relative fluorescence intensity [I]. Differences of mean fluorescence intensities in the cytoplasm and within the nucleus (indicated as black line in the graph) were calculated and are indicated in percent. One representative cell for each fusion protein is shown; E: Confocal immunofluorescence microscopy of HuH-7 cells transfected with an expression vector encoding wt P or the mutant  $\Delta\text{CK II}$  (= T100I) that is not phosphorylated by CK II. For detection of P (red) a rabbit-derived spacer domain-specific serum was used. Nuclei were stained with DAPI (blue); F: HuH-7 cells were transfected with the indicated expression vectors and left untreated or treated with  $10 \times \text{IC}_{50}$  of CK II inhibitor DMAT 5 h prior analysis. Transfection with pCDNA.3 served as control. Cells were lysed, the cytosolic and nuclear fraction were isolated by differential centrifugation and analyzed by western blotting. For detection of P a TP-domain specific serum was used. Detection of hexokinase and of lamin served as loading control and as control for the purity of the subcellular fractions. All experiments were performed in triplicate. One representative is shown.

### Binding of karyopherin- $\alpha 2$ to TP depends on CK II mediated phosphorylation

Karyopherin- $\alpha 2$  is an essential factor for NLS-mediated nuclear import. Therefore, it was investigated whether the

data described above are reflected by an increased binding of karyopherin- $\alpha 2$  to *in vitro* phosphorylated TP as compared to unphosphorylated TP. Equal amounts of terminal protein domain were immobilized on two columns.



**Figure 3 Binding of karyopherin-α2 to terminal protein depends on casein kinase II mediated phosphorylation.** The interaction of soluble protein fraction of HuH-7 cells to immobilized terminal protein (TP) domain was investigated by western blotting of the sodium chloride eluted fractions 2-4. Karyopherin-α2 bound only to the casein kinase II (CK II) pre-treated TP column (upper panel). FP: Flow through of the CK II treated TP column; F: Flow trough of the untreated TP column.

One column was *in vitro* phosphorylated by CK II and its substrate GTP the other and a blank control column were treated equally without the addition of the kinase. The pretreated columns were equilibrated and loaded with the desalted 75% (w/v) ammonium sulfate fraction of HuH-7 cell lysate. Finally, binding partners were eluted by sodium chloride and the eluted fractions were analyzed by Western blotting using a karyopherin-α2 specific antibody. Interestingly, karyopherin-α2 was only found in the eluate of the column with CK II pre-treated TP (Figure 3). This indicates clearly that *in vitro* binding of karyopherin-α2, the key enzyme for nuclear import, is dependent on CK II phosphorylation of the terminal protein.

#### **Inhibition of CK II impairs HBV replication in primary Tupaia hepatocytes**

To study the relevance of the NLS and of the CK II phosphorylation site for the HBV life cycle the HBV P protein was mutated based on a recombinant 1.2 fold HBV genome (subtype ayw) by site directed mutagenesis. The changes in the DNA sequence did not affect other reading frames or regulatory elements.

In the NLS-deficient mutant (ΔNLS) the NLS is inactivated by manipulating the basicity of the downstream cluster to K105D and K106S. The putative CK II recognition site on the P protein is destroyed by a T100I substitution (ΔCK II), whereas a pseudo-phosphorylated mutant was generated by a T100D conversion (CK II\*). However, the attempt to produce mutant virus by transfection of HepG2 or HuH-7 cells with the respective 1.2 fold genomes failed in case of ΔNLS and of the ΔCK II mutant (Figure 4A). In both cases significant less virus was produced (about hundredfold) as compared to the wt genome or the mutant encoding the CK II\* mutant. In a transfection experiment the nuclear import is not the limiting step. It can be concluded that mutations affecting the integrity of the NLS motive or of the CK II phosphorylation site cause secondary effects affecting the functionality of the polymerase. Therefore a direct analysis of the relevance of the NLS and of the CK II substrate domain based on mutated virus was not possible.

The *in vitro* data described above had shown that the functionality of the NLS identified in the TP-domain depends on the CK II-dependent phosphorylation (Figure 2). To study the relevance of CK II-dependent phosphorylation of the TP domain for HBV life cycle primary Tupaia hepatocytes were infected with wtHBV particles for 8 h. Adherent HBV particles were removed

by trypsin treatment as described above. After infection the cells were grown for 36 h in the presence of the cell permeable small molecular CK II inhibitor DMAT and the virus replication was analyzed by quantification of HBsAg and HBeAg secretion (Figure 4B). Moreover secreted viral particles were quantified by real time PCR. Both approaches revealed that inhibition of CK II by DMAT caused a strong and significant reduction of virus replication. This was further confirmed by analysis of cccDNA formation in infected PTHs. Inhibition of CK II by increasing concentrations of DMAT abolishes cccDNA formation (Figure 4B).

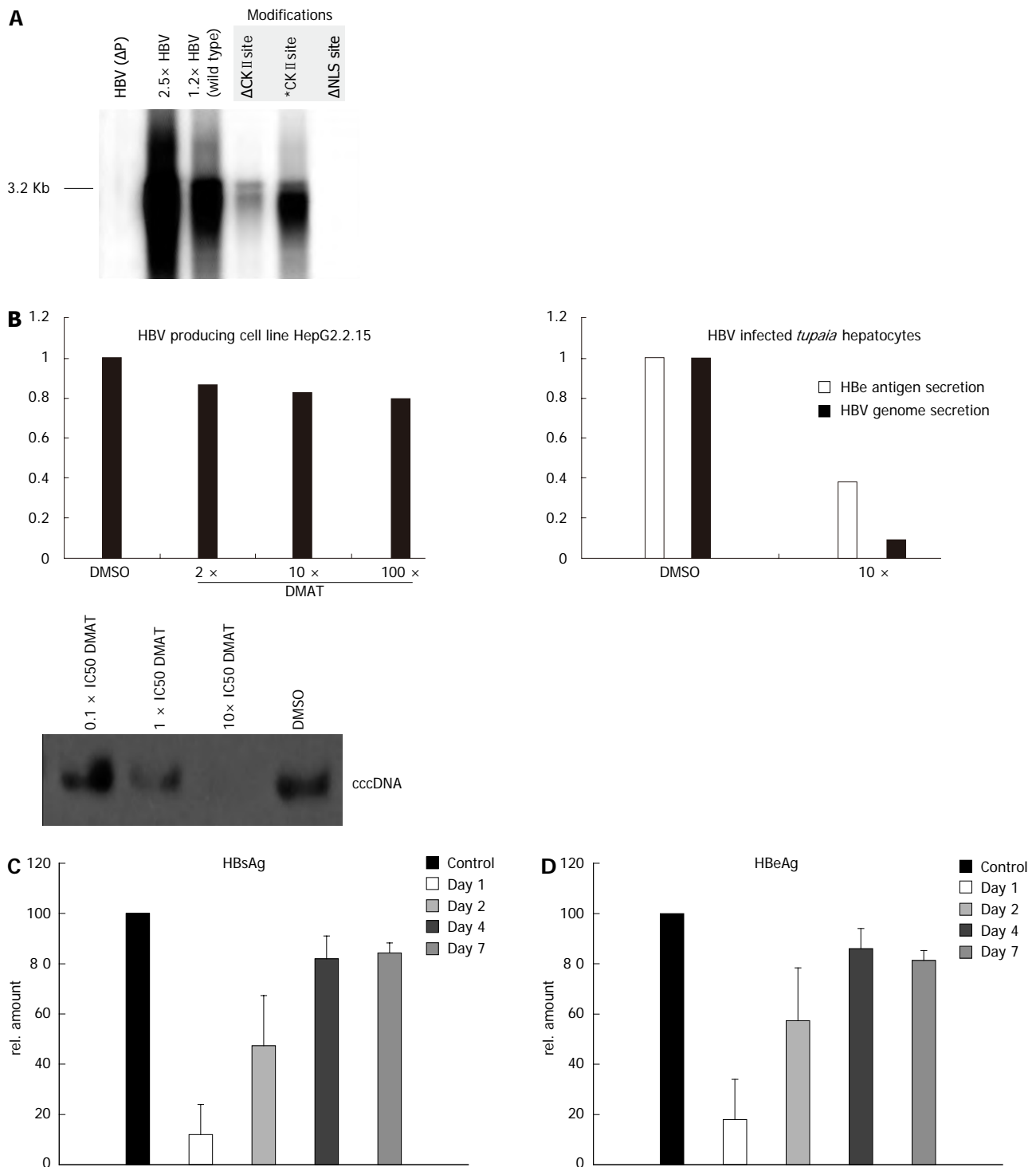
For a more detailed analysis primary Tupaia hepatocytes were infected as described above. One, two, four and seven days after infection the hepatocytes were grown for 36 h in the presence of the cell permeable small molecular CK II inhibitor DMAT and the virus replication was analyzed by quantification of HBsAg and HBeAg secretion (Figure 4C and D). Both approaches revealed that inhibition of CK II by DMAT at 1 and 2 d after infection caused a strong and significant reduction of virus replication, while inhibition after 4 and 7 d *pi* resulted only in a small reduction of virus replication.

To control the specificity of the observed effect the constitutively HBV expressing cell line HepG2.2.15 were instrumental. This cell line harbors a stably integrated HBV genome. Due to the stable integration of the genome the re-import of *de novo* synthesized genomes plays a minor role for maintaining the pool of transcriptional templates. Therefore, inhibition of polymerase import should exert a small effect. HepG2.2.15 cells were treated with DMAT, HBeAg and HBsAg secretion were analyzed by ELISA and virus secretion was quantified by LightCycler PCR (Figure 4B). Under these conditions inhibition of CK II with DMAT did slightly but not significantly reduce virus secretion as compared to the solvent control (Figure 4B). Comparable results were obtained for the cell line HepAD38 (data not shown).

Taken together these data provide indirect evidence that the functionality of the NLS in the TP domain of P is required for the import of the viral genome into the nucleus.

## **DISCUSSION**

It is well as established that HBV replicates its genome inside the nucleus (recent reviews<sup>[18,43,44]</sup>). This raises the question about the post entry transport of the genome complex to and about its final import into the nucleus.



**Figure 4** Casein kinase II inhibition impairs hepatitis B virus replication in infected primary *Tupaia* hepatocytes. **A:** HuH-7 cells were transfected with mutant versions of a 1.2 fold hepatitis B virus (HBV) genome. After 5 d the secreted viral particles were precipitated with a HBs specific antibody and the containing 3.2 kb HBV genomes were visualized by the radioactive tracer [ $\alpha$ - $^{32}$ P] dCTP incorporated using the endogenous polymerase activity. The unphosphorylated form of the casein kinase II (CK II) recognition site in the P protein was simulated by a T100I substitution ( $\Delta$ ) and the pseudo-phosphorylation was simulated by a T100D substitution (\*) in the 1.2 fold HBV wild type genome. The nuclear localization signal (NLS) was inactivated by mutating the downstream basic cluster (K105D and K106S) on the P protein. A 2.5  $\times$  HBV genome and a P deficient genome [HBV (P-)] served as controls; **B:** Primary *Tupaia* hepatocytes were infected with HepAD38 derived HBV and post infection treated for 36 h with solvent DMSO or 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) ( $10 \times \text{IC}_{50}$ ). Twelve days after infection the HBV genome secretion was measured by Lightcycler polymerase chain reaction. The cccDNA content of infected *Tupaia* hepatocytes that were incubated for 36 h with the indicated amounts of DMAT was visualized by Southern blot using a HBV specific probe. The specificity of DMAT incubation was analyzed by 2 h inhibitor pre-treatment of the stably HBV transfected cell line HepG2.2.15 followed by treatment of 36 h with  $10 \times \text{IC}_{50}$  CK II inhibitor DMAT ( $\text{IC}_{50}$  in rat liver = 150 nmol/L) and genome secretion was compared to the solvent control DMSO measured by Lightcycler PCR. All experiments were performed in triplicate. The figure shows one representative experiment; **C, D:** Primary *Tupaia* hepatocytes were infected with HepAD38-derived HBV and at day 1, day 2, day 4 and day 7 treated for 36 h DMAT ( $10 \times \text{IC}_{50}$ ). Twelve days after infection the HBV replication was measured by HBeAg or HBsAg-specific enzyme linked immunosorbent assay. The bars represent the standard deviation.

Previous reports discussed whether the final import of the assembled nucleocapsid in the nucleus harboring the genome complex can occur<sup>[17]</sup>. More recent reports provide evidence that the mature nucleocapsid disassembles in the nuclear pore complex and the final import of the genome complex could be mediated by an association with HBcAg oligomers that harbor in their C-terminal domain a NLS<sup>[20,21,44-47]</sup>. For DHBV it was described that completion of plus-strand DNA synthesis triggers genomic DNA deproteinization and conformational changes of the nucleocapsid. This could lead to the exposure of a NLS within the core and thereby could enable the import of the rcDNA<sup>[48]</sup>. In this context it is interesting to mention that the presence of the identified NLS is not conserved for the genus avihepadnavirus.

Recently we developed cell permeable HBV nucleocapsids as a vehicle for gene transfer (Brandenburg *et al.*<sup>[13]</sup>). Based on this system it was observed that neither HBcAg dimers nor nucleocapsids were visible in the nucleus of TLM-nucleocapsid treated cells although an efficient expression of the packaged, P-linked genome occurred suggesting that neither the nucleocapsid nor HBcAg dimers mediate the final import of the genome complex. Therefore the question arose about an alternative import mechanism: with about 90 kDa the covalent complex of HBV polymerase and genome clearly exceeds the size that freely can pass the nuclear pore complex.

In this context it is interesting that previous *in vitro* experiments have shown that the HBV genome complex can be efficiently imported into the nucleus. However if the complex is deproteinized, the naked genome fails to enter efficiently the nucleus<sup>[23]</sup>. These data suggest that the genome-linked polymerase could be relevant for the nuclear entry process.

The bipartite NLS identified in the TP domain of P could mediate the entry of the genome complex into the nucleus. The functional analysis of the genome complex revealed that the predicted NLS indeed has the potential to act as a nuclear localization signal. However, compared to other nuclear localization signals the TP-derived NLS is not a strong signal. This might reflect the different functions of P<sup>[18]</sup>. On the one hand P recognizes in the cytoplasm the *de novo*-synthesized 3.5 kb mRNA<sup>[43,49]</sup> and on the other hand the genome associated Pol is assumed to mediate by its NLS the entry of the genome complex into the nucleus. It is obvious that a too strong NLS signal might counteract the RNA-recognizing function in the cytoplasm. Yet, it was described that in duck HBV replicating cells in addition to the encapsidated polymerase non-encapsidated polymerase exists<sup>[24]</sup>. The major fraction of the non-encapsidated duck HBV polymerase is found in the cytoplasm a smaller fraction however can be detected within the nucleus<sup>[24]</sup>. Interestingly, in cells overexpressing HBV polymerase in the absence of other viral proteins a fraction of HBV polymerase is found within the nucleus, co-localized with the p11 protein of PML bodies<sup>[22]</sup>.

A further interesting feature is the CK II phosphorylation site localized in the center of the bipartite NLS.

The functionality of the HBV polymerase-derived NLS depends on the CK II-mediated phosphorylation. CK II is not a very tightly regulated kinase<sup>[50]</sup>. It can be assumed that CK II exerts a housekeeping phosphorylation function<sup>[51]</sup>. If subcellular localization and function of HBV polymerase is subjected to a tight control, it is not likely that CK II exerts this function. It is tempting to speculate that phosphatases could play an important role to regulate the subcellular localization and thereby function of HBV polymerase. CK II phosphorylation is reported to influence subcellular localization of various nuclear proteins<sup>[52]</sup>. For example CK II phosphorylation upstream of the NLS of simian virus 40 T-antigen enhances its nuclear import up to 40 fold<sup>[53]</sup>. But immediate phosphorylation one or two amino acid upstream of the crucial amino acid of classical monopartite NLS seems to have inhibitory effects on karyopherin binding due to a disturbance of the NLS basicity<sup>[54]</sup>. In case of bipartite nuclear localization signals this correlation is not evident. For example the spacer of the functional bipartite NLS of the *Agrobacterium tumefaciens* protein nopaline contains four negative charged aspartates, one even immediate located at the downstream basic cluster<sup>[55]</sup>. On the other hand an increase of the hydrophobicity of the 10-12 amino acid spacer seems to decrease its functionality<sup>[56]</sup>.

In transfection experiments it was found that destruction of the NLS or of the CK II-site almost completely abolishes HBV replication. Under these experimental conditions the import of the viral genome into the nucleus does not represent the limiting step. The plasmid DNA freely moves into the nucleus. However this observation suggests that perturbation of this domain has further effects on the polymerase function. Since the  $\Delta$ NLS and  $\Delta$ CK II-mutants were replication deficient it was not possible to study the replication of the respective mutant viruses. The *in vitro* data however have shown that the functionality of the TP-derived NLS requires the functionality of CK II. Based on this it could be shown that inhibition of CK II in the early phase of the infection abolished the establishment of HBV infection while inhibition of CK II in a later phase of the infection or in a stable system had no effect on HBV replication.

In conclusion we demonstrate the presence of a bipartite NLS within the TP domain of P and provide evidence for a novel model describing the import of the HBV genome into the nucleus.

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## COMMENTS

### Background

Human hepatitis B virus (HBV) enters the cell by receptor mediated endocytosis and at the end of this process the nucleocapsid that harbours the viral genome is released into the cytoplasm and transported towards the nuclear pore complex. Establishment of a productive viral infection requires the transport of the HBV genome that is covalently linked to the polymerase into the nucleus. In the



nucleus the partial- double stranded DNA genome is converted to covalently closed circular (ccc) double stranded DNA.

### Research frontiers

The phase of nuclear entry is not fully understood. It is discussed that the intact viral capsid shuttles the genome-polymerase complex into the nuclear basket of the nuclear pore complex. Here, after a partial disassembly of the nucleocapsid the polymerase-linked genome is released. The polymerase-genome complex however is too big to pass freely through the nuclear pore complex. This raises the question about the import mechanism.

### Innovations and breakthroughs

The identification and characterization of a bipartite NLS in the HBV polymerase that harbours a phosphorylation site for casein kinase II (CK II) is described in this manuscript. The integrity of the phosphorylation site is crucial for the functionality of the NLS. Moreover, inhibition of CK II prevents karyopherin  $\alpha 2$  from binding to the polymerase. Thereby the import of the polymerase is impaired resulting in inhibited cccDNA formation that prevents the establishment of the viral infection. The data identify novel structural and functional prerequisites for the establishment of HBV infection.

### Applications

The data describe a potential novel target for antiviral that could block the establishment of a HBV-infection.

### Peer review

In this work, they identified a novel NLS located in the terminal protein domain of HBV polymerase and defined a CK II phosphorylation site (threonine) which is adjacent to the NLS. This paper is well written.

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## Addicts with chronic hepatitis C: Difficult to reach, manage or treat?

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### Abstract

**AIM:** To assess the acceptance, safety and efficacy of care and treatment for chronic hepatitis C (CHC) in drug addicts.

**METHODS:** We designed a multidisciplinary, phase IV prospective cohort study. All illicit drug users (IDUs) visited a Territorial Addiction Service (SerT) in the District of Brescia, and hepatitis C antibody (HCVAb) testing positive were offered as part of a standardised hepatologic visit in our Gastroenterology Unit. Patients with confirmed CHC and without medical contraindications were administered peginterferon alfa-2b 1.5 µg/kg per week plus ribavirin (800-1400 mg/d) for 16-48 wk. All IDUs were unselected because of ongoing addiction and read and signed an informed consent form.

Virologic responses at weeks 4 and 12 of therapy, at the end of treatment and 24 wk after the end of treatment were the main measures of efficacy. Adherence was estimated according to the 80/80/80 criteria.

**RESULTS:** From November 2007 to December 2009, 162 HCVAb+ IDUs were identified. Sixty-seven patients (41% of the initial cohort) completed the diagnostic procedure, and CHC was diagnosed in 54 (33% of the total). Forty-nine patients were offered therapy, and 39 agreed (80% of acceptance rate). The prevalent HCV genotype was type 1, and the HCV RNA baseline level was over 5.6 log/mL in 61% of cases. Five patients dropped out, two because of severe adverse events (SAEs) and three without medical need. Twenty-three and 14 patients achieved end of treatment responses (ETRs; 59%) and sustained virologic responses (SVRs; 36%), respectively. Thirty-one patients were fully compliant with the study protocol (80% adherence). The prevalence of host and viral characteristics negatively affecting the treatment response was high: age over 40 years (54%), male gender (85%), overweight body type (36%), previous unsuccessful antiviral therapy (21%), HCV genotype and viral load (60% and 62%, respectively), earlier contact with HBV (40%) and steatosis and fibrosis (44% and 17%, respectively). In a univariate analysis, alcohol intake was associated with a non-response ( $P = 0.0018$ , 95%CI: 0.0058-0.4565).

**CONCLUSION:** Drug addicts with CHC can be successfully treated in a multidisciplinary setting using standard antiviral combination therapy, despite several "difficult to reach, manage and treat" characteristics.

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**Key words:** Chronic hepatitis C; Addiction; Antiviral therapy; Interferon; Multidisciplinary



**Core tip:** The paper reports results from a clinical trial on the management of chronic hepatitis C (CHC) in illicit drug users (IDUs). Two key elements characterise the trial: (1) the study was performed by a multidisciplinary team; and (2) the patients were unselected because of ongoing addiction. We assessed the acceptance of care and treatment for CHC among IDUs, who are classically considered to be a “difficult to reach and manage” group. For the IDUs accepting antiviral treatment, we analysed results on safety, efficacy and adherence and on the prevalence of negative prognostic factors affecting the virologic response to address whether IDUs are also “difficult to treat” patients.

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## INTRODUCTION

Hepatitis C virus (HCV) is estimated to chronically infect more than 180 million people worldwide, with approximately 4 million carriers in Europe alone<sup>[1]</sup>. The prognosis of chronic hepatitis C (CHC) is related to fibrosis progression, and the development of cirrhosis varies from 5%-25% over an average period of 30 years<sup>[2]</sup>. According to a recently validated mathematical model, morbidity and mortality from HCV are expected to rise in 2010 and to peak between 2030 and 2035<sup>[3]</sup>. The main reasons for this negative forecast are the low rates of screening for HCV and of treatment for CHC. The World Health Organization has defined chronic infection with HCV as a public health problem of primary importance, and during a consensus meeting in May 2010, all health authorities were invited to strive to prevent, identify and rapidly treat the infection<sup>[1]</sup>.

In developed countries, HCV is mainly transmitted by needles during drug injections, and illicit drug users (IDUs) are considered to be the largest group affected by HCV, accounting for 20%-50% of cases of chronic infection<sup>[4]</sup>. A recent paper estimating viral transmission showed, for the first time, that HCV “super-spreading” is led by IDUs. According to this estimation model, each infected IDU is likely to infect approximately 20 other people, half of whom will be infected within 2 years of the initial infection<sup>[5]</sup>.

International authorities on liver diseases (*i.e.*, the National Institutes of Health since 2002, the American Gastroenterological Association since 2006 and the American Association for the Study of the Liver since 2009) recommend the treatment of CHC in IDUs and encourage clinical studies in chronically infected IDUs “to evaluate the safest and most effective treatment, factors favouring compliance, risk of relapse, side-effect profile and the

impact of methadone maintenance treatment”<sup>[6-8]</sup>.

Despite international recommendations, several barriers to treating IDUs persist not only among physicians but also among IDUs<sup>[9,10]</sup>. Physicians’ concerns mainly include IDUs’ chaotic lifestyle; IDUs’ possibly poor adherence to treatment; difficulties in the management of the psychiatric side effects of treatment, which are believed to be more frequent among IDUs; and the risk of re-infection after HCV eradication<sup>[10]</sup>. The risk of relapse into addiction due to interferon (IFN)-driven mood changes and the use of needles in CHC therapy, is also described as a relative contraindication to IFN treatment in IDUs, although the data from prospective trials on this risk are scarce<sup>[11,12]</sup>. Concerns about antiviral therapy for CHC are also present among IDUs: their conception of illness and death is often different, for cultural reasons, from the beliefs of the general population, and information on the natural history and treatment challenges of HCV is inexact or incomplete<sup>[10]</sup>. Moreover, factors including precarious working conditions, a lack of fixed abode, undocumented migrant status and social isolation can affect access to care and treatment for liver diseases, and special efforts may be necessary to reach IDUs in their social environment<sup>[13]</sup>. In our recent review on the subject<sup>[14]</sup>, we report that due to the barriers to treatment, most published prospective trials on the treatment of CHC in IDUs involve limited numbers of patients, ranging from 11-71, have no standardised intervention protocol and are mainly restricted to abstinent patients.

As an overview, IDUs are perceived as patients who are difficult to reach for social reasons and difficult to manage because of lifestyle. Moreover, no previous study has assessed whether IDUs are also difficult to treat because of the presence of negative prognostic factors, either viral or host-related, affecting the rate of success of antiviral therapy. For these difficulties to be addressed, and to successfully be able to contact, manage and treat these patients, a multidisciplinary approach is mandatory. This approach should involve health professionals engaged in the management of addiction and dedicated hepatologists with a highly personalised approach to patient care.

We performed a prospective clinical study designed to maximise IDUs’ access to treatment for HCV infection by involving both the physicians directly engaged in the management of addiction and the specialised hepatologist in our unit and by avoiding “a priori” exclusion criteria for antiviral therapy of active IDUs. The main objectives of the study were to specifically evaluate the rate of access to clinical care; the acceptance, safety and efficacy of antiviral treatment for CHC; and the prognostic factors for responses to standard antiviral therapy in a large cohort of IDUs, who were unselected because of ongoing addiction.

## MATERIALS AND METHODS

### Study design

We designed a multidisciplinary, phase IV prospective



cohort study. The multidisciplinary approach was ensured by close collaboration between six Territorial Addiction Services (SerT) of the Local Health Authority of the District of Brescia (ASL) and our Gastroenterology Unit (GU, Spedali Civili and University of Brescia). The physicians of the SerT were responsible for the identification of patients with hepatitis C antibody (HCVAb) positivity among those individuals visiting the SerT clinic. Based on the protocol definition, patients with “ongoing addiction problems”, actively using illicit drugs and/or alcohol or in a supportive/substitution treatment program, were all considered to be subjects. The SerT physicians were also responsible for collecting all demographic, social, psychological and addiction data in a standardised case report form. The patients selected by the SerT physicians were instructed to call a dedicated telephone number to make an appointment for an initial standard hepatologic evaluation in the GU, including a medical visit, laboratory tests and ultrasound evaluation. The aims of the hepatologic evaluation were to confirm HCV-related chronic hepatitis, to assess the severity of liver disease and to evaluate eligibility for antiviral treatment. A liver biopsy was not routinely performed. The patients with confirmed CHC and meeting standard criteria for HCV therapy<sup>[8]</sup> were offered antiviral treatment with pegylated interferon and ribavirin, according to the study protocol for treatment, and were asked to sign an informed consent form. To improve adherence, a mobile telephone number was activated for all patients on antiviral treatment, with a physician on call every day from 8 a.m. to 1 p.m.

### Selection criteria

The inclusion criteria were as follows: over 18 years of age, HCV RNA detected with a sensitive polymerase chain reaction (PCR) (cut-off of determination 50 IU/mL; COBAS Amplicor HCV test, Roche Diagnostics, Branchburg, NJ, United States) and confirmed on at least two occasions over a period of 6 mo, compensated liver disease (Child-Pugh score  $\leq 5$ ), absence of major medical contraindications to antiviral therapy (including malignancies, severe cardiac illness and uncontrolled psychiatric condition), willingness to avoid pregnancy during the entire treatment period and during the 6 mo after the last ribavirin dose intake, ability to read and sign a written informed consent form and willingness to adhere to the study protocol for treatment. Patients with suspected or confirmed idiosyncratic reactions to interferon or ribavirin were excluded.

### Treatment protocol

The study protocol for treatment consisted of peginterferon alfa-2b (12 kDa) 1.5  $\mu\text{g/kg}$  per week plus ribavirin 800-1400 mg according to body weight (800 mg for  $< 65$  kg, 1000 mg for 65-80 kg, 1200 mg for 81-105 and 1400 mg for  $> 105$  kg) divided into two daily administrations.

The duration of the treatment was 24 wk for HCV genotypes 2/3 and 48 wk for HCV genotypes 1/4. The achievement of a rapid virologic response (RVR; HCV

RNA  $< 50$  IU/mL at week 4 of therapy) was regarded as an indication for short-term therapy in all naïve patients fulfilling the criteria of no dose reduction during the first 4 wk of therapy, low baseline viral load (HCV RNA  $< 600000$  IU/mL) and the absence of cirrhosis. The short-term scheme consisted of 16 wk for HCV genotypes 2/3 and 24 wk for HCV genotypes 1/4. Treatment was discontinued prematurely, according to international rules<sup>[8]</sup> (at week 12 if the HCV RNA level drops by  $< 2$  Log and at week 24 if the HCV RNA level is  $> 50$  IU/mL); in the case of virologic breakthrough; in the presence of severe adverse events (SAEs); or upon patients' request, with no need for explanation. In the case of a virologic breakthrough, HCV genotyping [line probe assay (LIPA), Bayer HealthCare, Tarrytown, NY, United States] was performed to exclude mixed/new HCV infections.

Adverse reactions to interferon and/or ribavirin were managed according to international guidelines<sup>[8]</sup>, and the use of erythropoietin and leucocyte growth factors was allowed, according to the current Italian Drug Agency (AIFA) recommendations.

The timetable of the study was as follows: a medical visit including a general physical examination, an assessment of body mass index, the administration of AUDIT-C for screening for at-risk alcohol-related behaviour and a Hamilton Test for scoring anxiety and depression. The measurement of the complete blood count and the alanine aminotransferase (ALT), aspartate aminotransferase (AST) and  $\gamma$ -glutamyl transferase (GGT) levels was requested every 4 wk during treatment and 24 wk after the end of therapy. Blood tests, including for thyroid function (FT4 and TSH), autoimmunity (ANA, AMA, LKM and ASMA) and liver function (albumin, PT and bilirubin), were mandatory at the beginning and every 12 wk of treatment. Quantitative and qualitative assays for HCV RNA were requested at week 0-4-12 and at week 4-12-24 of treatment, at the end of therapy and 24 wk after the end of therapy, respectively.

### Outcome measures

Access to liver care was calculated as the proportion of IDUs attending the first hepatologic visit and completing the diagnostic procedure among all HCVAb+ IDUs screened by the SerT and showing interest in this opportunity. Access to therapy was evaluated as the proportion of patients starting antiviral therapy among the eligible patients. The main measure of safety was the rate of withdrawal for SAEs, according to the Common Terminology Criteria for Adverse Events v3.0<sup>[15]</sup>. The main outcome measure of efficacy was the sustained virologic response (SVR; HCV RNA persistently  $< 50$  IU/mL 24 wk after treatment discontinuation) among the treated patients. Secondary efficacy measures were the achievement of an RVR, an early virologic response (EVR; HCV RNA  $< 50$  IU/mL at week 12 of therapy) and an end of treatment response (ETR; HCV RNA  $< 50$  IU/mL at the end of therapy). Adherence was estimated according to the 80/80/80 criteria (80% of pegylated interferon,

**Table 1** Main baseline characteristics of 162 hepatitis C antibody+ illicit drug users selected by physicians operating in six Territorial Addiction Service in the District of Brescia and comparison with 39 hepatitis C antibody+ illicit drug users accepting antiviral therapy *n* (%)

Patient characteristics	Selected by SerT ( <i>n</i> = 162)	Accepting therapy ( <i>n</i> = 39)	<i>P</i> value
Male gender	135 (83)	27 (69)	0.8152
Age, yr, mean $\pm$ SD	38 $\pm$ 7	39 $\pm$ 6	0.8888
Spoken language:	152 (94)	33 (85)	0.0912
Italian			
Place of birth			0.1502
Italy	147 (91)	32 (82)	
EU	5 (3)	2 (5)	
Non-EU	10 (6)	5 (13)	
Level of education	( <i>n</i> = 151)		0.8320
$\leq$ 8 yr of school	118 (79)	30 (77)	
High school diploma	32 (21)	8 (21)	
University degree	1 (0)	1 (2)	
	( <i>n</i> = 149)		1.0000
Unemployed	49 (33)	13 (33)	
Chronic associated conditions	41 (25)	11 (28)	0.6888
Cardiovascular	5 (3)	2 (5)	
Respiratory	4 (2)	1 (3)	
Allergic	2 (1)	1 (3)	
Psychiatric	19 (12)	3 (8)	

SerT: Territorial Addiction Service.

80% ribavirin cumulative dosage and 80% of the duration of therapy<sup>[16]</sup>).

### Ethics

The entire study design was evaluated by the Ethics Committee of Spedali Civili of Brescia and fully approved on July 31<sup>st</sup>, 2007. The study was registered with EudraCT, number 2008-001283-37.

### Statistical analysis

Treatment efficacy was measured according to the intention to treat (ITT) criteria, including all patients who had received at least one dose of interferon and one dose of ribavirin after signing the informed consent form. For statistical analyses, an unpaired t-test and Fisher's exact test were used when appropriate using GraphPad Prism, version 5.0 (Graph Pad Software, Inc., San Diego, CA, United States). Logistic regression analysis was performed to assess the effect of baseline features on efficacy and was completed using Stata software (version 7, StataCorp LP, College Station, TX, United States). A *P* value < 0.05 was accepted to reject the null hypothesis.

## RESULTS

### Identification of the cohort

From November 2007 to December 2009, a cohort of 162 HCVAb+ IDUs was identified by six SerT in the District of Brescia. Most patients were Italian males with a low level of education. At the time of recruitment, one third of the patients were unemployed, and one quarter

**Table 2** Type of addiction and opiate substitution treatment among the hepatitis C antibody+ illicit drug users selected by the Territorial Addiction Service and comparison with hepatitis C antibody+ illicit drug users accepting antiviral therapy *n* (%)

	Selected by SerT ( <i>n</i> = 162)	Accepting therapy ( <i>n</i> = 39)	<i>P</i> value
Alcohol			
Active	11 (7)	4 (10)	0.4972
Partial remission	5 (3)	0 (0)	0.5867
Total remission	23 (14)	10 (26)	0.0944
Cannabis			
Active	7 (11)	2 (5)	0.0590
Partial remission	3 (2)	1 (3)	0.5811
Total remission	4 (2)	3 (8)	0.1344
Cocaine			
Active	33 (20)	3 (8)	0.0038
Partial remission	6 (4)	1 (3)	1.0000
Total remission	39 (24)	12 (31)	0.4150
Heroin			
Active	48 (30)	6 (15)	0.1059
Partial remission	19 (12)	5 (13)	0.7885
Total remission	74 (46)	23 (59)	0.0647
Duration of intravenous drug use, yr, mean $\pm$ SD (range)	( <i>n</i> = 98) 13 $\pm$ 8 (7-34)	( <i>n</i> = 33) 13 $\pm$ 9 (6-32)	0.8588
Opiate substitution treatment	126 (78)	28 (72)	0.4089
Methadone, mg, mean $\pm$ SD	107 (66), 41 $\pm$ 22	19 (60), 46 $\pm$ 26	0.0642
Buprenorphine, mg, mean $\pm$ SD	19 (12), 5 $\pm$ 3	9 (23), 6 $\pm$ 4	0.0751

A smoking habit was concomitant in 100% of patients: < 5 cigarettes/d in 4%, 5-10 cigarettes/d in 12%, 11-20 cigarettes/d in 54% and > 20 cigarettes/d in 30%. SerT: Territorial Addiction Service.

had comorbidities (Table 1). The prevalent type of addiction was intravenous injection of heroin, with a mean duration of 13 years. Most patients were on opiate substitution treatment, with 66% on methadone and 12% on buprenorphine. All patients in methadone maintenance therapy received a dose lower than 100 mg/d (Table 2). Most IDUs who received information about HCV infection from health operators, the press or television were not confident about their knowledge and had moderate worries about the side effects of HCV therapy (Table 3).

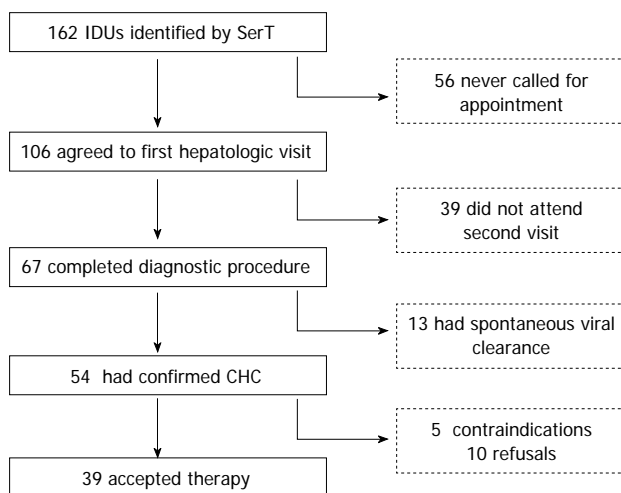
### Access to care and treatment

Patient disposition, according to the study protocol, is reported in Figure 1. Access to the first hepatologic work-up was observed in 106 patients, which was 65% of the initial cohort. Although 56 IDUs expressed interest in the opportunity for a dedicated medical examination to a SerT doctor, these individuals never called our clinic for an appointment. Sixty-seven patients completed the diagnostic procedure, or 41% of the initial cohort, corresponding to 63% of patients visiting our clinic for an initial evaluation. Patients who did not adhere to the diagnostic protocol (39 IDUs) were all contacted by telephone by a physician (BZ), and these patients all preferred to postpone the medical procedures because of

**Table 3** Attitudes toward/knowledge about hepatitis C virus infection among the hepatitis C antibody+ illicit drug users selected by the Territorial Addiction Service in comparison with hepatitis C antibody+ illicit drug users accepting antiviral therapy *n* (%)

Patient attitudes/knowledge	Selected by SerT ( <i>n</i> = 162)	Accepting therapy ( <i>n</i> = 39)	<i>P</i> value
Source of HCV information	( <i>n</i> = 150)	( <i>n</i> = 33)	NS
Other HCV patients	44 (29)	11 (33)	
Health operators	72 (48)	18 (55)	
Press	54 (36)	14 (42)	
Web	15 (10)	6 (18)	
Television	62 (41)	16 (48)	
None	25 (17)	6 (18)	
Feelings toward information			
Complete	( <i>n</i> = 139) 72 (52)	( <i>n</i> = 32) 15 (47)	0.6964
Confident	( <i>n</i> = 131) 30 (23)	( <i>n</i> = 31) 16 (52)	0.0033
Reassuring	( <i>n</i> = 130) 68 (52)	( <i>n</i> = 28) 14 (50)	0.8381
Attitudes toward HCV therapy			
Total fright	( <i>n</i> = 129) 3 (2)	( <i>n</i> = 29) 0 (0)	1.0000
Moderate worries	( <i>n</i> = 141) 102 (78)	( <i>n</i> = 32) 25 (78)	0.5271
Positive expectations	( <i>n</i> = 125) 70 (56)	( <i>n</i> = 28) 18 (64)	0.5271

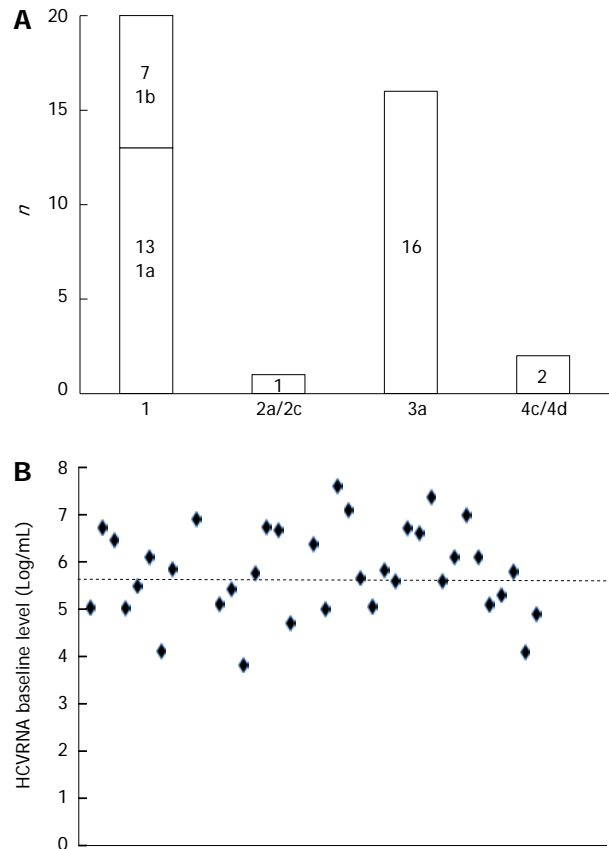
HCV: Hepatitis C virus; SerT: Territorial Addiction Service.



**Figure 1** Patient disposition according to the study protocol. CHC: Chronic hepatitis C; IDU: Illicit drug user; SerT: Territorial Addiction Service.

other priorities.

CHC was confirmed in 54 IDUs (33% of the total), of which 13 patients had a confirmed HCV RNA-negative test, with an estimated rate of spontaneous clearance of 19%. Five patients had medical contraindications to specific antiviral therapy (two cases of decompensated cirrhosis, one case of hepatocellular carcinoma, one case of pregnancy and one case of uncontrolled severe psychiatric illness). The remaining 49 patients were offered specific treatment for CHC, and 39 accepted and signed the informed consent form (80% acceptance rate). Two patients never started treatment after signing the informed consent form and were thus excluded from the ITT analysis, and two patients, one with HCV genotype 1a and the other with HCV genotype 3a, relapsed after the end of therapy and asked for a second cycle of anti-



**Figure 2** Hepatitis C virus genotypes (A) and baseline hepatitis C virus RNA levels (B) in 36 patients (the dotted line indicates the 5.6 Log/mL cut-off value for high viral load).

ral therapy (after a 6-mo wash-out period). We therefore report results for 39 treatments in 37 patients.

### Baseline characteristics of treated patients

The virologic features of the treated patients are reported in Figure 2. The most represented HCV genotype was type 1 (13 patients with 1a and seven with 1b). The HCV RNA baseline level, available in 36 of 39 patients, was over 5.6 Log/mL in 22 cases (61%). In total, 36% of our patients were active illicit drug users, mainly using heroin; approximately one third had a history of depression; one quarter had a pathologic Hamilton score for anxiety or depression; four patients were addicted to alcohol; and seven patients had an AUDIT-C at-risk score (Table 4). As reported in Table 5, several prognostic factors negatively affecting the outcome of antiviral therapy for CHC were well represented among our treated IDUs. These factors included age over 40 years, male gender, overweight body type, previous unsuccessful HCV antiviral treatment, unfavourable HCV virologic genotype or viral load, earlier contact with HBV, steatosis and progression to cirrhosis.

### Safety

Two SAEs occurred during the study period, leading to therapy discontinuation: a case of psychosis and a case of pneumonia with suspected tuberculosis at weeks 4 and 10, respectively. Three patients dropped out without

**Table 4** Main baseline clinical and laboratory characteristics of treated illicit drug users

Characteristics	n = 39
BMI (kg/m <sup>2</sup> ), M (range)	24.3 (17.6-34.6)
Duration of HCV infection (yr), M (range)	5 (1-21)
Duration under 1 yr	14 (36)
Duration of IDU status (yr), M (range)	12 (1-32)
Active IDU	14 (36)
History of depression	11 (28)
Pathologic Hamilton score	
Anxiety	10 (26)
Depression	8 (21)
AUDIT-C at-risk score	7 (18)
Leucocytes (n/mm <sup>3</sup> ), M (range)	6960 (3960-11960)
Haemoglobin (g/dL), M (range)	15.5 (11.8-17.7)
Platelets	224 (106-421)
ALT index (value/u.l.n.), M (range)	2.5 (0.5-16.4)
AST index (value/u.l.n.), M (range)	2.0 (0.6-6.6)
GGT index (value/u.l.n.), M (range)	1.2 (0.3-13.9)

HCV: Hepatitis C virus; IDU: Illicit drug user; M: Male; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyltransferase.

**Table 5** Main baseline features potentially affecting the response to antiviral therapy in treated illicit drug users

Features	Prevalence
Age over 40 yr	54%
Males	85%
BMI over 25 kg/m <sup>2</sup>	36%
Previous unsuccessful interferon treatment	21%
Unfavourable HCV genotype (1 or 4)	60%
HCV viral load > 5.6 Log (IU/mL)	62%
HBcAb positivity	40%
Ultrasonography suggestive of steatosis	44%
Ultrasonography suggestive of cirrhosis	17%

HBcAb: Hepatitis B core antibody; HCV: Hepatitis C virus; BMI: Body mass index.

medical need and were lost to follow-up; all dropouts occurred within the first 8 wk of antiviral treatment. Sixteen (41%) and 17 (44%) patients needed a dose adjustment of pegylated interferon and ribavirin, respectively. In six patients (15%), the use of erythropoietin was offered. The use of leucocyte grown factors was not necessary for any patient. One patient became pregnant during the 6 mo after the end of therapy and decided on an abortion for personal reasons.

### Efficacy

In the ITT analysis, 23 patients achieved an ETR (59%), and nine (23%) relapsed during the 6 mo after the end of therapy. Fourteen patients achieved an SVR (36%), seven of whom were infected with an unfavourable HCV genotype (Figure 3). The HCV RNA serologic clearance rates at weeks 4 and 12 are reported in Figure 4. Short-term therapy was offered to nine patients, according to the study protocol, and did not negatively affect the SVR rate based on univariate analysis.

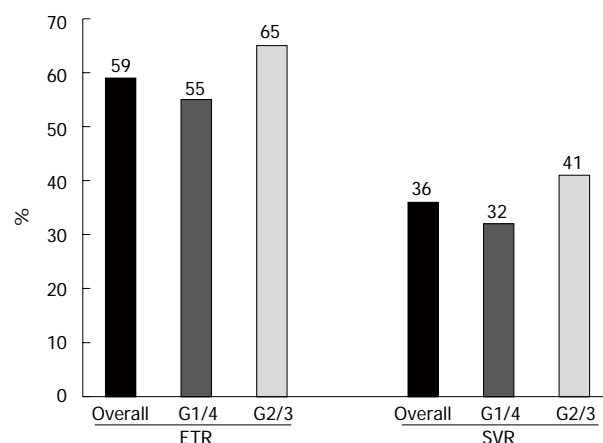


Figure 3 Percentage of end of treatment responses and sustained virologic responses in the entire cohort and according to Hepatitis C virus genotype (G). ETR: End of treatment response; SVR: Sustained virologic response.

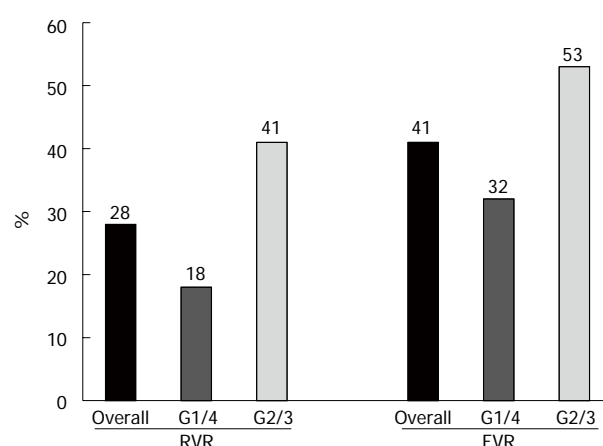


Figure 4 Percentage of rapid virologic responses and early virologic responses in the entire cohort and according to Hepatitis C virus genotype (G). RVR: Rapid virologic response; EVR: Early virologic response.

### Adherence and prognostic factors for response

According to our adherence definition, 31 patients (80%) were compliant with the study protocol.

In the univariate logistic regression analysis, the active use of cocaine and/or heroin, ongoing substitution treatment, the type of substitution treatment, the presence of comorbidity, spoken language and male gender did not affect the rate of the SVR, whereas alcohol intake was associated with a non-response ( $P = 0.0018$ , 95%CI: 0.0058-0.4565), independent of the dose and type of alcoholic beverage.

## DISCUSSION

Our study indicates that antiviral treatment for CHC in IDUs is safe and effective and that a multidisciplinary approach is a key element of the care of such patients. We have considered three main aspects of this issue to understand whether these patients are, as generally perceived, difficult to reach, manage or treat.



### Are IDUs difficult to reach?

IDUs, together with migrants and prison inmates, are regarded as special population groups. As recommended by an official position paper on behalf of several Italian scientific societies<sup>[13]</sup>, in such vulnerable people, specific intervention is mandatory to identify, prevent and treat chronic viral infections of the liver. In our approach, collaboration with the territorial services involved in the care of addicts was the key means of reaching IDUs (of whom 6%-15% were migrants) who were at risk of exclusion from medical care for social reasons. In total, 65% of IDUs with HCVAb positivity and identified by physicians of the SerT agreed to and received a dedicated medical visit that, even in the case of patients not receiving treatment, provided an instructive opportunity for counselling on HCV transmission, the prevention of liver complications, healthy lifestyle and available therapeutic protocols. In contrast, 56 IDUs (35% of the total cohort identified by the SerT) never called for an appointment, despite an initial statement of interest in the project. Moreover, after the first medical evaluation, 39 patients (37% of the patients who agreed to the first visit) never completed the diagnostic procedure, even after encouragement by direct telephone contact with a physician. Because of incomplete procedures, clinical and laboratory data were not sufficient to confirm an active HCV infection and to stage liver disease in 95 patients (59% of the initial cohort). Such a finding indicates that difficulties in reaching and motivating this population of patients persist even in the context of a well-organised multidisciplinary approach.

### Are IDUs difficult to manage?

Concerns about treating IDUs are mainly due to suspicion of low adherence, the risk of SAEs (typically psychiatric) and the inability to follow therapeutic prescriptions<sup>[10]</sup>. In our study, adherence was high and comparable to the adherence reported for clinical trials in the general population<sup>[17,18]</sup>. The use of a psychiatric questionnaire to monitor depression and anxiety was well accepted; one patient received antidepressant therapy before starting antiviral treatment, and paroxetine was offered to another patient after 12 wk of antiviral treatment. The patient with a psychotic reaction completely recovered after the withdrawal of antiviral treatment without consequences or the need for psychiatric drugs. These data on the psychiatric safety of CHC treatment in IDUs, as previously suggested by other studies<sup>[19-22]</sup>, are encouraging.

An important feature of our study was the inclusion of people who were actively addicted, with no period of mandatory abstinence; 36% of our enrolled patients continued to use illicit drugs (mainly heroin and cocaine) during the study protocol. Despite this “difficult to manage” characteristic, the data on safety, efficacy and adherence are encouraging. Moreover, logistic regression failed to demonstrate a negative correlation with the viral response to therapy in patients who were actively addicted during antiviral treatment. Only alcohol consumption was relat-

ed to a lower SVR rate, and this finding confirms the role of alcohol consumption in the impairment of antiviral treatment efficacy, which has already been demonstrated in the general population<sup>[23]</sup>.

Although IDUs are considered to be poorly motivated to undergo medical care, the multidisciplinary setting and strict collaboration among the different physicians involved in the care of the IDUs led to a high rate of access to therapy; 76% of patients with confirmed CHC started treatment. Such a rate was markedly higher than the rate previously reported in studies in the general population<sup>[10]</sup>.

### Are IDUs difficult to treat?

Adherence to treatment was high (80%), and despite few withdrawals for safety reasons, the overall SVR of 36% was lower than expected for the general population. This “efficacy” goal must be observed in light of several “difficult to treat” characteristics in our study population<sup>[24]</sup>. Among our IDUs, viral features such as HCV genotype 1 and a high baseline level of viremia were prevalent. Moreover, 40% of our patients tested positive for HBcAb<sup>[25]</sup>. Male gender, an overweight body type and an age over 40 were frequent. Other unfavourable factors affecting the virologic response were a relatively high prevalence of steatosis and cirrhosis in 44% and 17%, respectively, of patients. Most patients had been addicted for over 10 years, and no patient was identified and treated during the acute phase of the infection. In total, 21% of patients experienced the failure of at least one antiviral treatment for CHC. A few of these features are not modifiable (HCV genotype, viral load and gender), whereas other features could be modified by a more prompt strategy of intervention (younger age, shorter duration of infection and lower score of fibrosis).

In conclusion, IDUs with HCV-related CHC, actively using illicit drugs and/or opioid substitution treatment, can be successfully treated in a multidisciplinary setting with a standard antiviral combination of ribavirin and pegylated interferon, with good adherence and a good safety profile. IDUs’ “difficult to reach, manage and treat” characteristics should not be used to contraindicate antiviral therapy. An appropriate multidisciplinary setting is a key factor in overcoming the “difficult” characteristics of these patients, with a strategic aim of reducing HCV circulation in the largest reservoir of this viral infection. Whether treatment will benefit from upcoming new antiviral agents is currently under study in our unit.

## ACKNOWLEDGMENTS

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## COMMENTS

## Background

Hepatitis C virus (HCV) infection is a common condition worldwide with prevalence of 3%. Illicit drug users (IDUs) are regarded as an important reservoir of this infection and as “super-spreaders”. HCV infection is a progression disease possibly leading to chronic liver disease and ultimately to end stage liver disease. Is therefore important to identify strategy to eradicate infection particularly in the reservoir-population? Concerns about therapy of HCV infection in these populations are present in both physicians and IDUs.

## Innovations and breakthroughs

The investigators report that within a multidisciplinary setting involving both liver and addiction specialists nearly half of identified HCV+ IDUs accept hepatologic counseling and nearly a quarter accept treatment. Eighty percent of treated patients are adherent to treatment according to 80/80/80 rule. Sustained virological response is achieved in a proportion similar of that reported in registration trials, is not influenced by ongoing addiction, but is negatively affected by alcohol consumption. Incidence of psychiatric and organic side effects is not different from that reported in the general population.

## Applications

This article supports the concept that barriers to HCV therapy of IDUs can be overcome in the context of a multidisciplinary team, and that in this clinical context adherence and efficacy of therapy is similar as in the general population. The study highlights the point that the risk of HCV spreading by the super-spreaders IDUs can be reduced and that their habits can not be used as an argument to withhold antiviral therapy.

## Peer review

The study includes challenging for ethical difficulty of HCV treatment. It's very interesting and authors may applaudable effort on this study. Of course, opposite opinions for HCV treatment on addicts may exist, nonetheless this study indicates possibility of HCV treatment for some addicts if patients can receive enough support from medical profession. Although this study may raise an ethical issue, it will give a strong impact to readers and make fascinating reading.

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## Expression of hepatitis B virus 1.3-fold genome plasmid in an SV40 T-antigen-immortalized mouse hepatic cell line

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### Abstract

**AIM:** To investigate the expression of the hepatitis B virus (HBV) 1.3-fold genome plasmid (pHBV1.3) in an immortalized mouse hepatic cell line induced by SV40 T-antigen (SV40T) expression.

**METHODS:** Mouse hepatic cells were isolated from mouse liver tissue fragments from 3-5 d old Kunming mice by the direct collagenase digestion method and cultured *in vitro*. The pRSV-T plasmid was transfected into mouse hepatic cells to establish an SV40LT-immortalized mouse hepatic cell line. The SV40LT-immortalized mouse hepatic cells were identified and transfected with the pHBV1.3 plasmid. The levels of hepatitis B sur-

face antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the supernatant were determined by an electrochemiluminescence immunoassay at 24, 48, 72 and 96 h after transfection. The expressions of HBsAg and hepatitis B c antigen (HBcAg) in the cells were investigated by indirect immunofluorescence analysis. The presence of HBV DNA replication intermediates in the transfected cells and viral particles in the supernatant of the transfected cell cultures was monitored using the Southern hybridization assay and transmission electronic microscopy, respectively.

**RESULTS:** The pRSV-T plasmid was used to immortalize mouse hepatocytes and an SV40LT-immortalized mouse hepatic cell line was successfully established. SV40LT-immortalized mouse hepatic cells have the same morphology and growth characteristics as primary mouse hepatic cells can be subcultured and produce albumin and cytokeratin-18 *in vitro*. Immortalized mouse hepatic cells did not show the characteristics of tumor cells, as alpha-fetoprotein levels were comparable ( $0.58 \pm 0.37$  vs  $0.61 \pm 0.31$ ,  $P = 0.37$ ). SV40LT-immortalized mouse hepatic cells were then transfected with the pHBV1.3 plasmid, and it was found that the HBV genome replicated in SV40LT-immortalized mouse hepatic cells. The levels of HBsAg and HBeAg continuously increased in the supernatant after the transfection of pHBV1.3, and began to decrease 72 h after transfection. The expressions of HBsAg and HBcAg were observed in the pHBV1.3-transfected cells. HBV DNA replication intermediates were also observed at 72 h after transfection, including relaxed circular DNA, double-stranded DNA and single-stranded DNA. Furthermore, a few 42 nm Dane particles, as well as many 22 nm subviral particles with a spherical or filamentous shape, were detected in the supernatant.

**CONCLUSION:** SV40T expression can immortalize mouse hepatic cells, and the pHBV1.3-transfected SV40T-immortalized mouse hepatic cell line can be a new *in vitro* cell model.



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**Key words:** SV40 T-antigen; Mouse hepatic cell; Hepatitis B virus 1.3-fold genome plasmids; Immortalized; Liposomes; Transfection

**Core tip:** This study established a new immortalized mouse hepatic cell line through the transfection of the pRSV-T plasmid. SV40 T-antigen (SV40LT)-immortalized mouse hepatic cells had the same morphology and biological characteristics as primary mouse hepatic cells. SV40LT-immortalized mouse hepatic cells could be transfected with the pHBV1.3 plasmid, which caused the hepatitis B virus (HBV) genes to replicate in SV40LT-immortalized mouse hepatic cells. The expressions of hepatitis B surface antigen and hepatitis B c antigen, as well as the presence of HBV DNA replication intermediates, were observed in the pHBV1.3-transfected cells. This cell model will contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

Song XG, Bian PF, Yu SL, Zhao XH, Xu W, Bu XH, Li X, Ma LX. Expression of hepatitis B virus 1.3-fold genome plasmid in an SV40 T-antigen-immortalized mouse hepatic cell line. *World J Gastroenterol* 2013; 19(44): 8020-8027 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8020.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8020>

## INTRODUCTION

Chronic hepatitis B (CHB) is a severe public health problem that affects more than 400 million people worldwide and causes more than one million deaths annually<sup>[1]</sup>. Recent studies have shown that the correlation between serum hepatitis B virus (HBV) DNA levels and the risk of developing cirrhosis and hepatocellular carcinoma (HCC) is stronger than other baseline or virologic parameters<sup>[2]</sup>. The ultimate long-term goal of therapy is to achieve a “durable response” to prevent hepatic decompensation, reduce or prevent progression to cirrhosis and/or HCC, and prolong survival<sup>[3]</sup>. Moreover, it has now become clear that continuous suppression of HBV replication can revert liver fibrosis or even cirrhosis in most patients<sup>[4]</sup>.

Treatment of hepatitis B depends on several factors, such as the stage of disease, the presence or absence of the “e” antigen, and the potential for drug resistance and subsequent inability to use a medicine, particularly in the advanced stages of chronic disease of the liver. Therefore, it is very important to evaluate these factors at the time the decision is made regarding the type and duration of treatment<sup>[5]</sup>. Interferon alpha-2a and interferon alpha-2b (IFN 2a and 2b)-based therapies have been used for many years as the preferential treatment approaches for cases with low levels of HBV DNA and high levels of alanine aminotransferase (ALT)<sup>[6-8]</sup>. The goal of treatment

is to activate an immune response leading to hepatitis B e antigen (HBeAg) seroconversion<sup>[9]</sup>. This type of treatment, as the first option to modulate the immune system, aims to achieve elimination or remission.

However, this course of treatment results in a high cost, and also produces many adverse side effects, such as anaemia, a significant decrease in hemoglobin, vomiting, cold sweats and nausea<sup>[10]</sup>. Previous studies have reported that only about one out of three patients receives IFN therapy<sup>[11-13]</sup>. Furthermore, nucleoside analogues cannot completely eliminate the virus, and may lead to the mutation of the virus<sup>[14]</sup>. Thus, the development of new antiviral treatments remains a major research task. Additionally, new suitable HBV-infected animal cell models are urgently required to evaluate new treatment strategies. Therefore, this study aimed to establish a new immortalized mouse hepatic cell line induced by SV40 T-antigen (SV40T) expression, and to investigate the expression of the HBV 1.3-fold genome plasmid (pHBV1.3) in the established SV40T-immortalized mouse hepatic cell line.

## MATERIALS AND METHODS

### *Establishment of SV40T-immortalized mouse hepatic cell line*

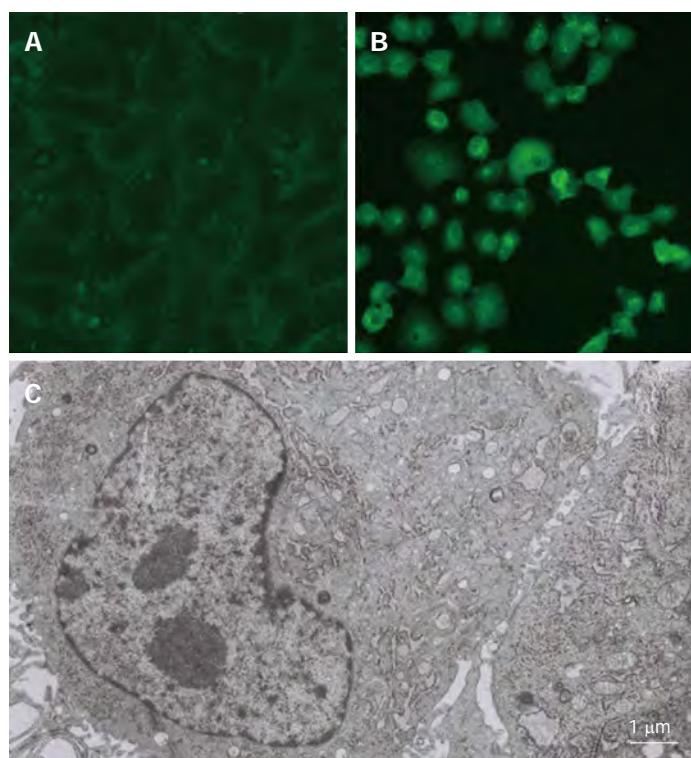
Kunming mice (3-5 d old) were provided by the clinical drug trial-based Animal Laboratory of Shandong University. Livers were collected from these mice, and mouse hepatic cells were isolated from the liver tissue fragments by the direct collagenase digestion method<sup>[15]</sup>. The isolated cells were cultured in the 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin and 100 mg/mL streptomycin, and placed in the incubator with 5% CO<sub>2</sub> and 37 °C. All animals received humane care in compliance with the Principles of Laboratory Animal Care. The protocol was approved by the Animal Care and Use Committee.

The pRSV-T plasmid<sup>[16]</sup> was provided by Professor Reddel RR of the Australia Children's Medical Research Institute. The pRSV-T plasmid was transfected into primary mouse hepatic cells according to the instructions of the liposome transfection kit (Invitrogen, Grand Island, United States). Twenty-four hours later, the 1640 culture medium with 10% FBS was added. Forty-eight hours later, it was replaced by the 1640 culture medium supplemented with 10% FBS and 500 µg/mL G418. Cells were passaged every 5 d at a ratio of 1:2.

An SV40 monoclonal antibody (Thermo, Waltham, United States) was used to detect the SV40T antigen and its distribution in SV40T-transfected cells by the indirect immunofluorescence assay. Primary mouse hepatic cells were employed as the negative control.

An inverted phase contrast microscope and an electron microscope were used to observe the morphology and ultrastructure of the SV40T-transfected mouse hepatic cells.

The supernatants of primary and immortalized mouse hepatic cell cultures were collected. The levels of ALT,



**Figure 1** SV40 T-antigen-immortalized mouse hepatic cells ( $\times 200$ ). A: SV40 T-antigen (SV40T)-immortalized mouse hepatic cells visualized by an inverted phase contrast microscope; B: SV40T antigen immunofluorescence in mouse hepatic cells; C: SV40T-immortalized mouse hepatic cells visualized by an electron microscope.

aspartate aminotransferase (AST) and alpha-fetoprotein (AFP) were determined by an automatic biochemical analyzer (Beckman, Boulevard Brea, United States). Primary cultured mouse hepatic cells were employed as the control.

After total RNA extraction of primary and SV40T-transfected hepatic cells with an RNA extraction kit (Invitrogen), reverse transcription polymerase chain reaction (RT-PCR) was used to determine albumin (ALB) mRNA levels as previously described<sup>[17]</sup>.

Western blotting was used to detect the presence of cytokeratin-18 (CK-18) in primary and SV40T-transfected mouse hepatic cells (22<sup>nd</sup> generation). Rabbit anti-mouse cell CK-18 was the primary antibody employed and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (IgG) was used as the secondary antibody. These two antibodies were purchased from Boster Biological Technology, Ltd (Wuhan, China).

### Transfection

pHBV1.3<sup>[18]</sup> was provided by Professor Yin-Ping Lu of Huazhong University of Science and Technology. pHBV1.3 contains a 1.3-fold HBV genome (ayw subtype). Following the instructions of the Lipofectamine 2000 transfection kit (Invitrogen), the pHBV1.3 plasmid was transfected into SV40T-immortalized cells (22<sup>nd</sup> generation).

### Electrochemiluminescence

The supernatants of the pHBV1.3-transfected cell cultures were collected at different times. An AXSYM automatic electrochemiluminescence immunoassay analyzer (Abbott) was used to quantify the levels of HBsAg and

HBcAg.

### Indirect immunofluorescence

pHBV1.3-transfected cells were seeded in 24-well plates. The cells were washed with PBS three times and fixed with 4% paraformaldehyde at 4 °C for 20 min. The cells were then washed with PBS three times again and incubated with PBS-diluted 10% goat serum for 30 min. Then the indirect immunofluorescence assay was performed using a fluorescence kit. Mouse anti-HBcAg and mouse anti-HBsAg were purchased from Millipore Corporation, while fluorescein Isothiocyanate-conjugated goat anti-mouse IgG was purchased from Southern Biotech (Birmingham, United States).

### Southern hybridization and transmission electronic microscopy

At 72 h post-transfection, a DNA extraction of pHBV1.3-transfected cells was performed for Southern hybridization analysis. The probe was the digoxigenin-labeled 3.2 kb HBV DNA<sup>[19]</sup>. The Southern kit was purchased from Roche (Indianapolis, United States). Southern hybridization was carried out according to the manufacturer's instructions.

At 72 h after transfection, the supernatants of pHBV1.3-transfected cell cultures were collected. JEOL transmission electronic microscopy (JEM-1200EX Electron Microscope) was used to visualize the cells and photographs were taken.

### Ethics statement

Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, Shandong University, and with the

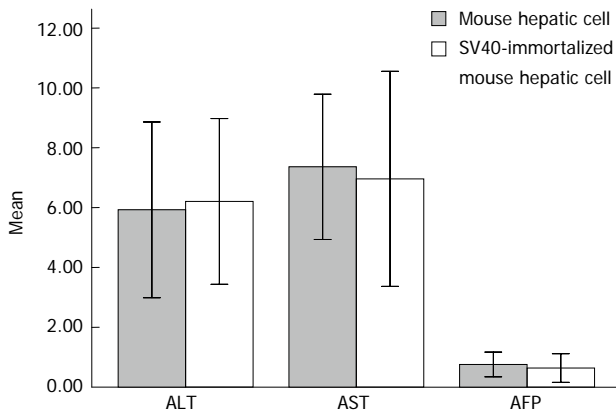


Figure 2 Levels of alanine aminotransferase, aspartate aminotransferase and alpha-fetoprotein in the cell culture supernatant. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP:  $\alpha$ -fetoprotein.

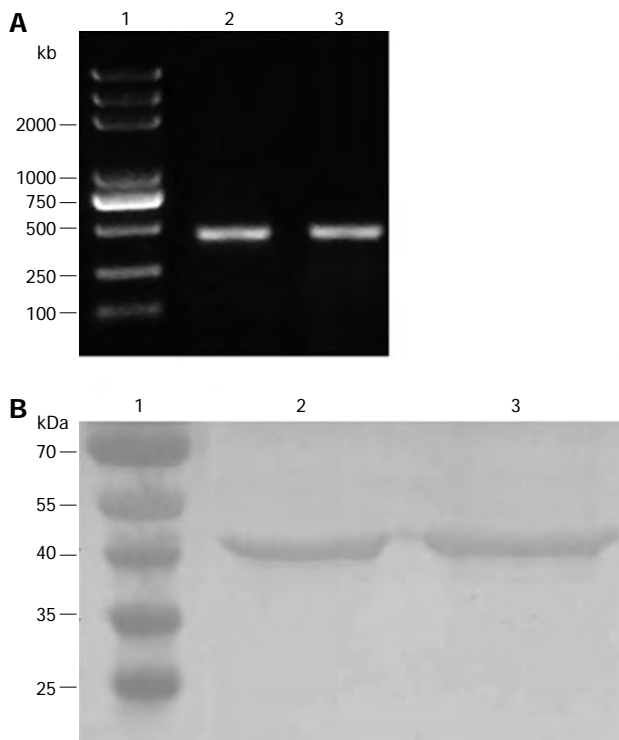


Figure 3 Electrophoresis and Western blotting. A: Electrophoresis of albumin (ALB) reverse transcription polymerase chain reaction products (1: markers; 2: primary mouse hepatic cells; 3: immortalized mouse hepatic cells at 22<sup>nd</sup> generation); B: ALB by Western blotting (1: Markers; 2: Primary mouse hepatic cells; 3: Transfected mouse hepatic cells at 22<sup>nd</sup> generation).

1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington DC, United States). The study was approved by the Institutional Animal Care and Use Committee at Shandong University (approval no. SDU003341201).

### Statistical analysis

The data are presented as means  $\pm$  SD. Comparisons between groups of data were performed using Student's *t*

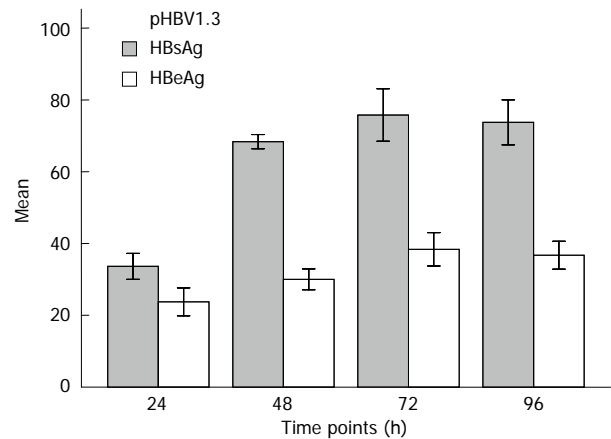


Figure 4 Levels of hepatitis B surface antigen and hepatitis B e antigen in the cell culture supernatant. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; pHBV1.3: Hepatitis B virus 1.3-fold genome plasmids.

test. A difference with *P* value  $< 0.05$  was considered to be statistically significant. Data were analyzed with the SPSS 11.0 statistical software package (SPSS Inc.; Chicago, IL, United States).

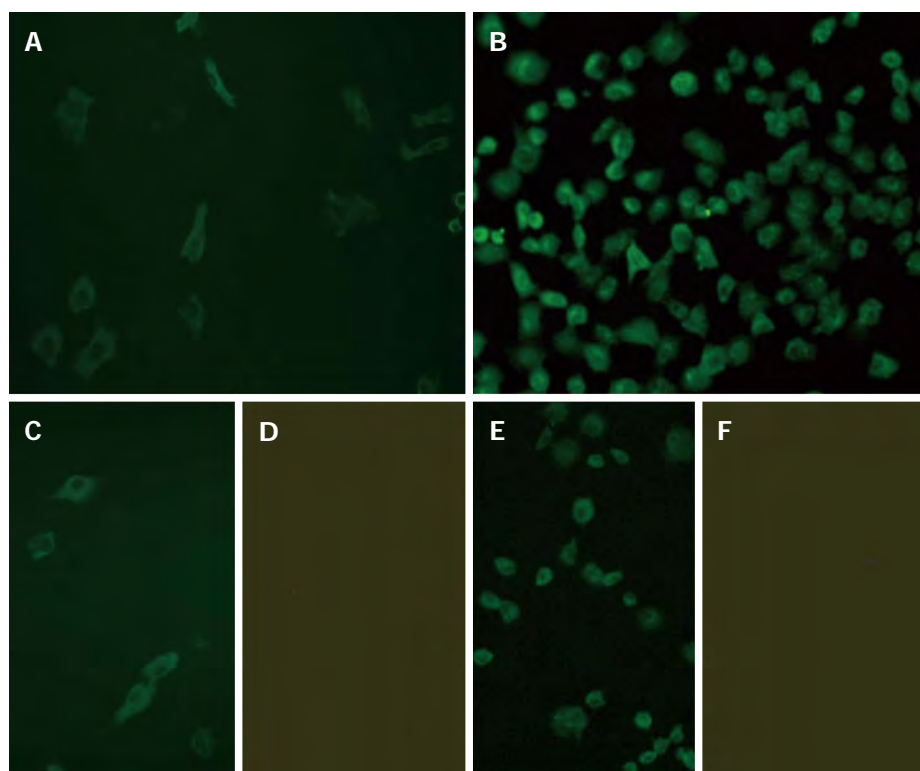
## RESULTS

### Evaluation of SV40T-immortalized mouse hepatic cell line

The epithelial cell-like positive clones were found 30 d after the mouse hepatic cells were transfected with a SV40T-expressing plasmid (pRSV-T) by lipofection; these cells were an adherent monolayer and flat-shaped and presented in a polygonal, cluster-like multi-cell arrangement (Figure 1A). The SV40T mouse hepatic cells displayed the typical morphology and structure of hepatic cells, and many glycogen granules, mitochondria and endoplasmic reticulum structures were clearly visible under the electron microscope (Figure 1C). Furthermore, the splitting dual-core cells reflected the *in vitro* proliferation and differentiation processes of the transfected hepatic cells (Figure 1C). Cells were passaged every five d at a ratio of 1:2 for 38 generations, and no change in cell morphology was observed.

After SV40T transfection, the SV40 T-antigen immunofluorescence of the mouse hepatic cells gradually increased, and was visible 30 d after transfection. Matte-like fluorescence could be clearly detected in the cytoplasm, along with granular-like fluorescence in the nucleus (Figure 1B).

The quantified levels of ALT, AST and AFP in the supernatant of the cultures are shown in Figure 2. The levels of ALT, AST and AFP in the supernatant of mouse hepatic cell and SV40T-transfected hepatic cell cultures were  $5.93 \pm 1.47$  *vs*  $6.21 \pm 1.38$  ( $t = 0.481$ ,  $P = 0.636$ ),  $7.36 \pm 1.21$  *vs*  $6.96 \pm 1.79$  ( $t = 0.643$ ,  $P = 0.527$ ) and  $0.76 \pm 0.21$  *vs*  $0.65 \pm 0.24$  ( $t = 1.318$ ,  $P = 0.201$ ), respectively ( $n = 12$ ). No significant difference in the levels of ALT, AST and AFP was observed between the mouse hepatic cell and SV40T-transfected hepatic cell cultures (*P*



**Figure 5** Analysis of hepatitis B surface antigen and hepatitis B c antigen expression in hepatitis B virus 1.3-fold genome plasmids-transfected cells by immunofluorescence microscopy ( $\times 200$ ). A: Hepatitis B surface antigen (HBsAg) observed in the hepatitis B virus 1.3-fold genome plasmids (pHBV1.3)-transfected cells at 24 h post-transfection; B: Hepatitis B c antigen (HBeAg) observed in the pHBV1.3-transfected cells at 24 h post-transfection; C: HBsAg observed in HepG2.215 cells (positive control); D: HBsAg observed in untransfected SV40 T-antigen (SV40T)-immortalized cells (negative control); E: HBeAg observed in HepG2.215 cells (positive control); F: HBeAg observed in untransfected SV40T-immortalized cells (negative control).

> 0.05).

Following the total RNA extraction of SV40T-transfected hepatic cells (22<sup>nd</sup> generation) and RT-PCR, the ALB mRNA was apparent as a bright band at 475 bp (Figure 3A), indicating that SV40T-immortalized mouse hepatic cells had the ability to express ALB mRNA. Mouse hepatic cells were employed as the positive control.

Following the protein extraction of SV40T-transfected hepatic cells (22 generation), SDS-PAGE and Western blotting were carried out. Immunoblotting of SV40T-transfected hepatic cells demonstrated their expression of CK-18, and mouse hepatic cells, employed as the positive control, also displayed immunoreactivity for CK-18, as expected (Figure 3B).

#### **Expression of pHBV1.3 in SV40T-immortalized mouse hepatic cells**

The levels of HBsAg and HBeAg in the supernatant were monitored 24, 48, 72 and 96 h after pHBV1.3 transfection. The results of this analysis are shown in Figure 4. The levels of HBsAg and HBeAg in the supernatant continuously increased after transfection of pHBV1.3, though they both began to gradually decrease after 72 h.

The expression of HBsAg and HBeAg were observed in the pHBV1.3-transfected cells at 24 h after transfection by immunofluorescence microscopy, with expression reaching a peak at 72 h. HBsAg was mainly observed in

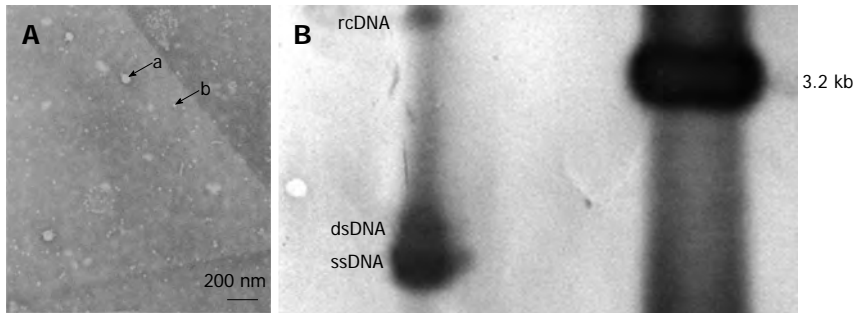
the cytoplasm, while HBeAg was detected in both the cytoplasm and nucleus, especially in the former (Figure 5). HBV DNA replication intermediates, including rcDNA, dsDNA and ssDNA, were also observed 72 h after transfection (Figure 6). Furthermore, a few 42 nm Dane particles, as well as many 22 nm subviral particles with a spherical or filamentous shape, were observed in the supernatant (Figure 6).

## **DISCUSSION**

The development of another HBV *in vitro* cell model would provide a very important tool to study the biological characteristics of HBV, the pathogenesis of hepatitis B, the mechanism of carcinogenesis by HBV infection, and carry out *in vitro* anti-HBV drug screening. 2.2.15 cells, a stable cell line which harbors the HBV genome, and can support HBV replication and the secretion of infectious virus particles<sup>[20-23]</sup>. However, the low expression of HBV was due to the low viral genome copy number integrated in the host cell chromosome. This drawback limits the application of this cell line as an *in vitro* cell model of HBV infection, especially as an infection model for antiviral drug-resistant mutant screening and investigation of biological characteristics<sup>[24]</sup>.

Simian vacuolating virus 40 (SV40), which was first obtained from cultured rhesus monkey kidney cells<sup>[25]</sup>, belongs to the *Papovaviridae* family<sup>[26]</sup>. The transfection of





**Figure 6** Expression of hepatitis B virus 1.3-fold genome plasmids in SV40 T-antigen-immortalized mouse hepatic cells. A: 42 nm Dane particles (a) and 22 nm subviral particles (b) in the supernatant; B: Hepatitis B virus (HBV) DNA replication intermediates in HBV 1.3-fold genome plasmids (pHBV1.3)-transfected cells 24 h after transfection. rc DNA: Relaxed circular DNA; dsDNA: Double-stranded DNA; ssDNA: Single-stranded DNA.

the SV40 early gene into cells is the most common method for cell immortalization. There are different opinions regarding the mechanism by which SV40LT causes cell immortalization<sup>[27,28]</sup>. This method has been found to be useful for a variety of human cell types<sup>[29]</sup>. The use of SV40 for cell immortalization has a long history. It has been used to immortalize epithelial cells of the bronchial intrahepatic bile duct, cervical cells and other cells<sup>[30-32]</sup>. Many studies of SV40T-immortalized cell lines have shown that the transduction of the *SV40T* gene can increase the growth rate of cells, while retaining the differentiation phenotype of many original cells and keeping the biological characteristics of the original cells, with the rare expression of a non-original gene<sup>[33,34]</sup>. These characteristics make the SV40T-immortalized cells suitable for use as an *in vitro* model. In this study, the pRSV-T plasmid was used to transfect the mouse hepatic cells to establish an SV40T-immortalized mouse hepatic cell line, which was selected through 500 µg/mL G418 screening.

Our experimental results showed that there were no significant differences in morphology between primary and SV40T-immortalized mouse hepatic cells. Furthermore, the same level of expression and secretion of ALB, as well as the CK-18 activity, was observed between the primary and SV40T-immortalized mouse hepatic cells (without any tumorigenic potential). At present, the SV40T-immortalized mouse hepatic cells have been passaged to the 38<sup>th</sup> generation.

The genome of the HBV 1.3-fold genome plasmid is smaller than that of the HBV 2.0-fold plasmid, and its efficiency of replication and expression is higher than that of the 1.2- and 1.1-fold plasmids<sup>[35]</sup>. The HBV 1.3-fold genome plasmid contains HBV 5'-end Enh I, Enh II, replication-origin (DR1, DR2), the former genome transcription start site, x and pre-C promoter, and x open reading frame. Hence, the HBV 1.3-fold genome plasmid is used most frequently. After Huh7 and HepG2 cells were transfected with the recombinant plasmid pHBV1.3, the *in vitro* replication and expression of the *HBV* gene could be detected, and high levels of HBsAg/HBeAg intermediates and transcripts involved in HBV DNA replication were also found<sup>[18,36]</sup>. In this study, the pHBV1.3 plasmid was transfected into SV40T-immortalized mouse hepatic cells. After transfection, the expression of HBsAg

and HBcAg was observed in the pHBV1.3-transfected cells. Additionally, HBV DNA replication intermediates, including relaxed circular DNA, double-stranded DNA and single-stranded DNA, were also observed 72 h after transfection. Furthermore, a few 42 nm Dane particles, as well as many 22 nm subviral particles with a spherical or filamentous shape, were observed in the supernatant.

In summary, our findings suggest that the expression of the *SV40T* gene immortalized a mouse hepatic cell line, and subsequent transfection of pHBV1.3 established a new *in vitro* cell model for anti-HBV drug research.

## COMMENTS

### Background

Chronic hepatitis B (CHB) is a severe public health problem, and treatment of hepatitis B poses many challenges, such as treatment for an advanced stage of disease and the potential for drug resistance. Thus, the development of new antiviral treatments remains a major research task, and new suitable hepatitis B virus (HBV)-infected *in vitro* cell models are urgently required to evaluate new therapeutic strategies. With this in mind, this study aimed to establish a new immortalized mouse hepatic cell line induced by SV40 T-antigen (SV40T) expression, and to investigate the consequences of HBV 1.3-fold genome plasmid (pHBV1.3) expression in this SV40T-immortalized mouse hepatic cell line.

### Research frontiers

SV40LT antigen has the ability to immortalize some animal cells. The genome of the HBV 1.3-fold genome plasmid is smaller than that of the HBV 2.0-fold plasmid, and its efficiency of replication and expression is higher than that of the 1.2- and 1.1-fold plasmids. The HBV 1.3-fold genome plasmid contains HBV 5'-end Enh I, Enh II, replication-origin (DR1, DR2), the former genome transcription start site, x and pre-C promoter, and x open reading frame. Hence, the HBV 1.3-fold genome plasmid is used most frequently. The genome of the HBV 1.3-fold genome plasmid has been shown to replicate in HepG2 and Hu7 cell lines. According to the authors, there has been no report concerning the expression of the HBV 1.3-fold genome plasmid in an immortalized mouse hepatic cell line.

### Innovations and breakthroughs

The SV40LT antigen immortalized mouse hepatic cells, which was not found in previous reports. In this study, a new immortalized mouse hepatic cell line was established through the transfection of the pRSV-T plasmid into primary mouse hepatic cells. The genome of the HBV 1.3-fold genome plasmid can replicate in human hepatoma cell lines (HepG2 or HUH7 cells). The authors successfully transfected the pHBV1.3 plasmid into the immortalized mouse hepatic cell line and observed the expression of HBV. The new cell model established in this study will contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

### Applications

This cell model will contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

## Peer review

This is a good basic study in which authors transfected the pRSV-T plasmid into primary mouse hepatic cells and established a new immortalized mouse hepatic cell line. The authors transfected the pHBV1.3 plasmid into the immortalized mouse hepatic cell line and observed the expression of HBV. This cell model would contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

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**S- Editor:** Gou SX **L- Editor:** Ma JY **E- Editor:** Zhang DN



## Evaluation of 4 three-dimensional representation algorithms in capsule endoscopy images

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### Abstract

**AIM:** To evaluate the three-dimensional (3-D) representation performance of 4 publicly available Shape-from-Shading (SfS) algorithms in small-bowel capsule endoscopy (SBCE).

**METHODS:** SfS techniques recover the shape of objects using the gradual variation of shading. There are 4 publicly available SfS algorithms. To the best of our knowledge, no comparative study with images obtained during clinical SBCE has been performed to date. Three experienced reviewers were asked to evaluate 54 two-dimensional (2-D) images (categories: protrusion/inflammation/vascular) transformed to 3-D by the aforementioned SfS 3-D algorithms. The best algorithm was selected and inter-rater agreement was calculated.

**RESULTS:** Four publicly available SfS algorithms were compared. Tsai's SfS algorithm outperformed the rest (selected as best performing in 45/54 SBCE images), followed by Ciuti's algorithm (best performing in 7/54 images) and Torreão's (in 1/54 images). In 26/54 images; Tsai's algorithm was unanimously selected as the best performing 3-D representation SfS software. Tsai's 3-D algorithm superiority was independent of lesion category (protrusion/inflammatory/vascular;  $P = 0.678$ ) and/or CE system used to obtain the 2-D images (MiroCam®/PillCam®;  $P = 0.558$ ). Lastly, the inter-observer agreement was good ( $\kappa = 0.55$ ).

**CONCLUSION:** 3-D representation software offers a plausible alternative for 3-D representation of conventional capsule endoscopy images (until optics technology matures enough to allow hardware enabled-"real" 3-D reconstruction of the gastrointestinal tract).

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**Key words:** Capsule endoscopy; Small-bowel; Three-dimensional; Software; Algorithm; Reconstruction; Technology; Advance

**Core tip:** Accurate three-dimensional (3-D) reconstruction of the gastrointestinal tract requires the use of stereo-cameras that can simulate human binocular vision. In the absence of such technology in capsule endoscopy, we rely on software approaches [such as the Shape-from-Shading (SfS) algorithms] to obtain 3-D representation of digestive tract structures. In the present study, we evaluated the use of 4 publicly available SfS in capsule endoscopy. 3 experienced/experts reviewers concluded that Tsai's approach is the best of the four available algorithms.

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Evaluation of 4 three-dimensional representation algorithms in capsule endoscopy images. *World J Gastroenterol* 2013; 19(44): 8028-8033 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8028.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8028>

## INTRODUCTION

Capsule endoscopy (CE) has changed our diagnostic approach for small-bowel diseases<sup>[1,2]</sup>. Although more accurate and of higher diagnostic yield than other modalities<sup>[3,4]</sup>, there are still occasions where pathology is either missed or misinterpreted<sup>[5-7]</sup>. Furthermore, reports have shown that three-dimensional (3-D) reconstruction can facilitate diagnosis by enhancing textural features of mucosal structures or intestinal abnormalities<sup>[8,9]</sup>. However, accurate 3-D reconstruction of the gastrointestinal (GI) tract requires the use of stereoscopic cameras that can simulate human binocular vision<sup>[10,11]</sup>. With the current level of technological investment in CE though *i.e.*, camera size, packaging constraints and power consumption, accurate 3-D imaging of the intestinal lumen in small-bowel capsule endoscopy (SBCE) is still unfeasible<sup>[9,12]</sup>.

Therefore, software approaches that offer 3-D representation of conventional monocular two-dimensional (2-D) CE frames have been developed<sup>[13]</sup> and proposed for use in CE<sup>[14]</sup>. Such approaches *e.g.*, Shape-from-Shading (SfS) algorithms, are members of a family of shape recovery algorithms called shape-from-X techniques (Figure 1)<sup>[13]</sup>. Given a single 2-D image, these algorithms recover the shape of objects using the gradual variation of shading<sup>[13]</sup>. Essentially, surface “reconstruction” with SfS is achieved through a mathematical representation that is inverted in order to recover dense surface distance and normal information by the gradual variation of shading<sup>[13]</sup>. We were able to retrieve 4 publicly available SfS algorithms<sup>[15-18]</sup>. To the best of our knowledge, no comparative study with images obtained during clinical SBCE has been performed to date<sup>[19]</sup>. We aimed to evaluate the 3-D representation performance of 4 publicly available SfS algorithms by comparing them with their equivalent 2-D images of small-bowel structures/lesions obtained during SBCE, in order to identify the algorithm more helpful in facilitating identification and distinction between lesion and surrounding mucosa.

## MATERIALS AND METHODS

Between January 2011 and January 2012, 262 SBCE procedures were performed at the Royal Infirmary of Edinburgh (tertiary referral centre for CE for the southeast of Scotland, United Kingdom) in 249 patients (mean age:  $52.6 \pm 12.1$  years), as already described elsewhere<sup>[9]</sup>. Out of them, 140 were performed with PillCam<sup>®</sup>SB2 (Given<sup>®</sup> Imaging Ltd., Yokneam, Israel) and 122 with MiroCam<sup>®</sup> (IntroMedic<sup>®</sup>Co, Seoul, South Korea). A total of 54 were selected images (27 obtained with MiroCam<sup>®</sup> and 27

with PillCam<sup>®</sup>SB) on the basis of the overall quality *i.e.*, brightness, absence of air bubbles, debris, or opaque luminal fluid and clarity of findings (lesions or structures). Thereafter, images were classified in the following image groups: (1) vascular lesions *i.e.*, angioectasias ( $n = 16$ ); (2) inflammatory lesions *i.e.*, ulcers, erosions, aphthae, cobblestone, fold and/or villous oedema ( $n = 18$ ); and (3) protruding lesions/structures *i.e.*, polyp/mass, nodular lymphoid hyperplasia, cluster of focal lymphangiectasia, chylous cysts, and ampulla of Vater, ( $n = 20$ ).

### 3-D image representation software

All selected images were reconstructed in 3-D by means of all 4 SfS algorithms. Three reviewers (Rondonotti E, Mandelli G, Koulaouzidis A) with extensive CE experience and blinded to each other participated in this study. In order to facilitate the evaluation process, a Mathworks<sup>®</sup> Matlab program with a graphic user interface (GUI) was developed (Figure 2; a video presenting the evaluation process is provided as supplementary material *via* this link: <https://dl.dropboxusercontent.com/u/7591304/EvaluationVideo.mov>). The program consisted of two windows in which the conventional 2-D SBCE image (Figure 2, single frame at the right side/window of the GUI screen) and its corresponding 3-D represented images (four, one for each of the 4 SfS under evaluation) are presented to the reviewer (Figure 2, left side/window of the GUI screen).

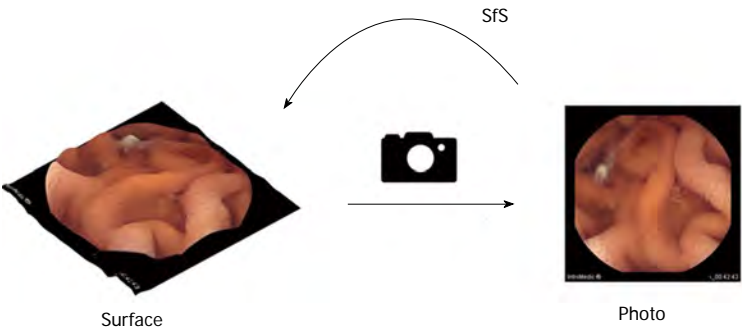
The 3-D SfS representations appeared in random order. The reviewers had the ability and freedom to rotate and zoom in each of the 3-D represented images. At the bottom of the GUI screen, a single “task request”: “Choose the 3-D representation you consider most helpful in distinguishing the finding (seen in 2-D) from the surrounding mucosa” appeared. This prompted reviewers to choose one among the four 3-D ‘reconstructed’ images, each generated by a different 3-D algorithm. After selecting the best SfS representation, the reviewer had to click “next” to proceed to the next case. This process was repeated until the program reached the last case after which each separate evaluation was concluded.

### Outcome measures

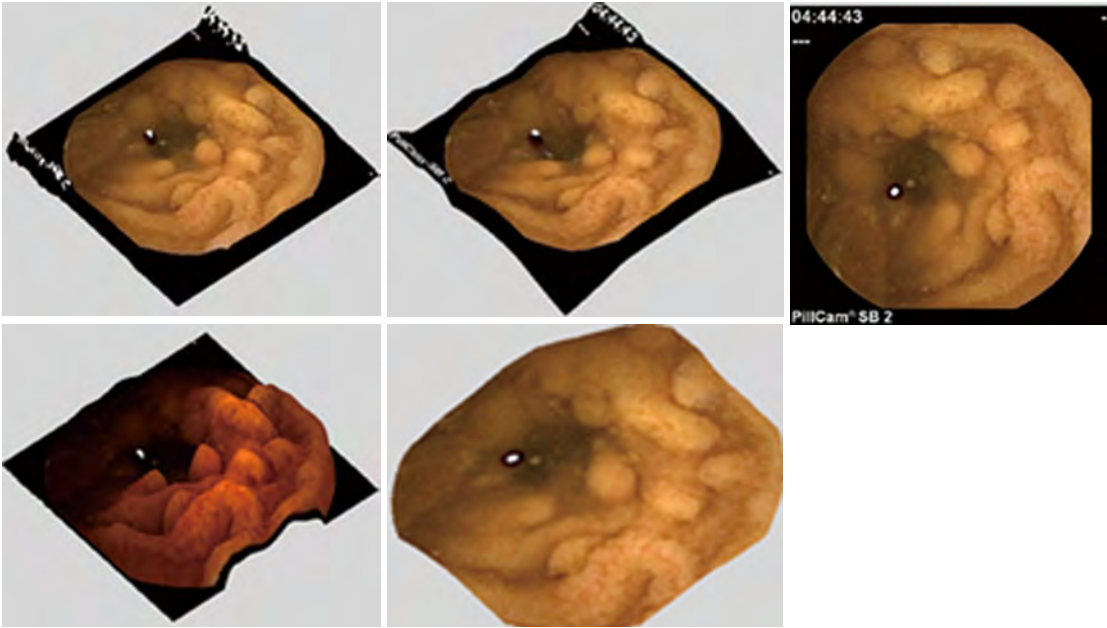
Reviewers were asked to evaluate 54 images. The following subgroup analyses were performed: (1) evaluation of 3-D representation according to the type of finding (vascular *vs* inflammatory *vs* protruding); and (2) evaluation according to the system generating the 2-D image (PillCam<sup>®</sup> *vs* Mirocam<sup>®</sup>). Furthermore, inter-observer agreement was calculated.

### Ethics consideration

This study was conducted in accordance with United Kingdom research ethics guidelines. After review by the local ethics committee further specific ethical review and approval were not required, as the study was considered an evaluation of previously collected endoscopy images, using data already obtained as part of regular clinical care<sup>[20]</sup>.



**Figure 1 Shape-from-Shading function.** Capturing a surface using a camera removes depth information. Shape-from-Shading (SfS) techniques try to reproduce the missing depth information from a given two-dimensional (2-D) image.



**Figure 2** For the evaluation phase, a Mathworks® Matlab program with a graphic user interface was developed. The program consists of two windows in which the conventional two-dimensional capsule endoscopy image (single frame at the right side/window of the graphic user interface screen) and its corresponding three-dimensional represented images (four, one for each of the 4 shape-from-shading under evaluation) were presented to the reviewer.

Table 1 Results of the Shape-from-Shading method per lesion category						
SfS method	Vascular		Inflammatory		Protrusion	
	PillCam®	MiroCam®	PillCam®	MiroCam®	PillCam®	MiroCam®
Tsai	7	7	7	6	8	10
Ciuti	1	0	1	0	1	4
Torreão	0	0	1	0	0	0
Barron	0	0	0	0	0	0
None selected	0	1	0	0	0	0

SfS: Shape-from-Shading.

Statistical analysis

For numerical variables, values are presented as mean ± SD. Where necessary, the Fisher exact test was calculated. A two-tailed *P* value < 0.05 was considered statistically significant. Inter-observer agreement was calculated using an online *kappa* calculator (available from <http://justus-randolph.net/kappa/>) which provides the calculation of Randolph's free-marginal multirater *kappa*<sup>[21]</sup>, applicable

when raters are not forced to assign a certain number of cases to each category. Values of *kappa* can range from -1.0 to 1.0, with -1.0 indicating perfect disagreement below chance, 0.0 indicating agreement equal to chance, and 1.0 indicating perfect agreement above chance. More specifically, the inte is classified per *kappa* as poor < 0.20, fair 0.2-0.40, good 0.41-0.60, very good 0.61-0.80 and, excellent 0.81-1.00<sup>[22]</sup>. All other statistical analyses were performed using a statistical package, StatsDirect, Stats-Direct Ltd, Altrincham, Cheshire, United Kingdom.

RESULTS

Of the 4 SfS algorithms, Tsai's 3-D algorithm outperformed the rest (selected as best in 45/54 images), followed by Ciuti's (best performing SfS in 7/54 images) and Torreão's (in 1/54 images); there was a single image for which each reviewer selected (as best performing) a different 3-D representation algorithm. Of note, not once was Barron's 3-D algorithm selected as best performing (Table 1, Figure 3).

In 26/54 images, Tsai's algorithm was unanimously

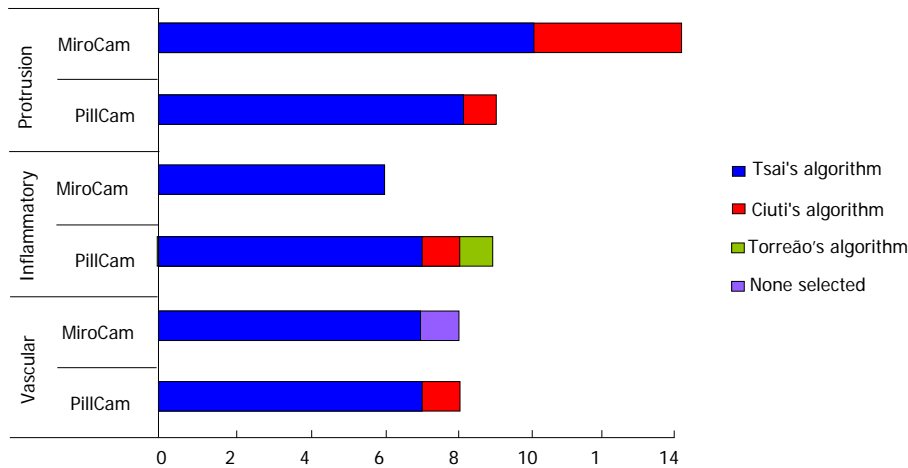


Figure 3 Assessment results for the 4 Shape-from-Shading algorithms per lesion category.

selected as the best performing 3-D representation SfS software. Tsai's 3-D algorithm superiority was independent of lesion category (protrusion/inflammatory/vascular;  $P = 0.678$ ) and/or CE system used to obtain the 2-D images (MiroCam®/PillCam®,  $P = 0.558$ ). Lastly, the inter-observer agreement was good ( $\kappa = 0.55$ ).

## DISCUSSION

In the present study, we compared the performance of 4 publicly available 3-D “reconstruction” algorithms<sup>[15-18]</sup> (SfS software) using 54 conventional 2-D CE images. The evaluation criterion was subjective *i.e.*, perceived visualisation improvement (3-D representations offered over the corresponding conventional 2-D images) by 3 experienced CE reviewers. Based on this evaluation, Tsai's algorithm is the 3-D representation model recommended for use in CE. This outcome directly supports Tsai's SfS model theoretical advantages: (1) able to produce good results for round surfaces, which are the case for most digestive tract shapes; and (2) it behaves quite well with bright surfaces<sup>[13]</sup>.

Depth information is an important aspect of human vision; it helps human brain to analyse and comprehend the surrounding environment. Images captured with conventional (non-stereoscopic) cameras “discard” the 3<sup>rd</sup> dimension (depth) as conventional cameras can only save 2 dimensions (height and width). Therefore depth information is lost; and moreover, most imaging algorithms perform less efficiently.

To date, engineers have not been able to equip capsule endoscopes with stereoscopic cameras for the following reasons: (1) packaging/space limitations; (2) low depth resolution of stereoscopic or time-of-flight cameras<sup>[22-24]</sup>; and (3) power consumption issues. However, it is almost certain that in the foreseeable future these hardware-related limitations will be overcome<sup>[11]</sup> and eventually 3-D CE will be a commodity. Nevertheless, until hardware changes are widely implemented, several efforts have been made to convert 2-D images into 3-D images (3-D representation or “reconstruction”) through software and dedicated algorithms. There are software algorithms that

offer a fair trade-off between 2-D images and hardware-enabled 3-D images. These algorithms are part of a family of shape recovery algorithms called Shape-from-X techniques<sup>[13]</sup>. Basically a SfS algorithm recovers the shape of objects, given a single monocular image, using the gradual variation of shading<sup>[8,13]</sup>.

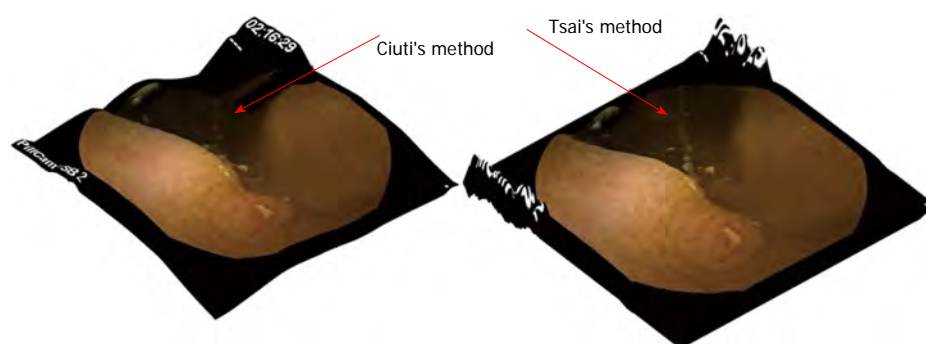
SfS algorithms can be divided into four groups: (1) minimization approaches<sup>[16-18]</sup>; (2) propagation approaches; (3) local approaches; and (4) linear approaches<sup>[15]</sup>. It is important to remember that each of the 4 SfS algorithms evaluated herein utilizes a different approach to recover the shape from a conventional 2-D image.

More specifically, Tsai *et al.*<sup>[15]</sup> described an repetitive update of the depth using a linear approximation of the reflectance function. Ciuti *et al.*<sup>[16]</sup> used a camera model with perspective projection and a light source close to the surface and away from the optical centre to measure depth. Torreão *et al.*<sup>[17]</sup> applied a linear-nonlinear biological model that mimics neuronal responses to estimate shape. Finally, Barron *et al.*<sup>[18]</sup> proposed a unified model for recovering shape, reflectance and optional illumination while using local smoothness, global scarcity or entropy, and the absolute colour of each pixel. Although Tsai's<sup>[14,15]</sup> method is very straightforward and to an extent simplistic, it provides satisfying results. Ciuti's *et al.*<sup>[16]</sup> algorithm, on the other hand, uses a more advanced model (incorporating a camera model with perspective projection) that makes things in the background appear further back than in Tsai's model (Figure 4).

Since for a given 2-D image, light source and surface shape are not known, these algorithms try to model how the 2-D image was created from the 3-D environment to finally produce an approximation this 3-D depth. The above modelling has a significant impact on the resulting 3-D representation. During SfS process additional constraints need to be applied on the surface shape parameters or the light conditions to find the surface characteristics.

In conclusion, we showed previously that 3-D representation software offers a plausible alternative for 3-D representation of conventional CE images (until optics technology matures enough to allow a hardware enabled-“real” 3-D reconstruction of the GI tract)<sup>[9]</sup>. In the pres-





**Figure 4** Ciuti's algorithm (left) and Tsai's method (right). Although Tsai's method is very straightforward and to an extent simplistic, it provides satisfying results. Ciuti's *et al*<sup>[18]</sup> algorithm, on the other hand, uses a more advanced model that makes things in the background appear darker than in Tsai's model.

ent study we compared 4 publicly available SfS methods. 3-D reconstruction is attracting interest in capsule endoscopy<sup>[8,9,14,25-28]</sup>, especially as newly developed and/or under development CE become available, with greater potential (due to imager and optics) for 3-D software<sup>[20]</sup>.

## COMMENTS

### Background

Over the past decade, conventional endoscope technology has advanced with the use of three-dimensional (3-D) cameras offering increased diagnostic and interventional capabilities. Unfortunately, due to hardware limitations, 3-D small-bowel capsule endoscopy (SBCE) is still an open technological challenge. It is aspired that 3-D SBCE will be able to offer similar benefits to conventional 3-D endoscopy. Therefore, information technology engineers suggested the use of software techniques (Shape-from-Shading, SfS) methods that simulate 3-D reconstruction *i.e.*, 3-D representation in SBCE images. To date, various SfS approaches have been proposed; each aims to retrieve depth information from 2-D images (shape recovery) through different mathematical transformations, hence offering different shape approximations.

### Research frontiers

The authors aimed to evaluate the 3-D representation performance of 4 publicly available SfS algorithms by comparing them with their equivalent 2-D images of small-bowel structures/lesions obtained during SBCE, in order to identify the algorithm more helpful in facilitating identification and distinction between the lesion and the surrounding mucosa.

### Innovations and breakthroughs

This study, in conjunction with further similar work in the field, is useful in the assessing the potential validity of integrating 3-D representation in capsule endoscopy reviewing software.

### Applications

Software-enabled 3-D representation is a promising approach that enables 3-D imaging at no additional cost. The authors have shown that SfS application leads to improved visualisation in SBCE and is likely to be of use in certain clinical scenarios, like the 'mass or bulge' question.

### Peer review

An interesting paper dealing with software and capsule endoscopy.

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## Predictors of *Clostridium difficile* infection severity in patients hospitalised in medical intensive care

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### Abstract

**AIM:** To describe and analyse factors associated with *Clostridium difficile* infection (CDI) severity in hospitalised medical intensive care unit patients.

**METHODS:** We performed a retrospective cohort study of 40 patients with CDI in a medical intensive care unit (MICU) at a French university hospital. We include patients hospitalised between January 1, 2007 and December 31, 2011. Data on demographics characteristics, past medical history, CDI description was collected. Exposure to risk factors associated with CDI within 8 wk before CDI was recorded, including previous hospitalisation, nursing home residency, antibiotics, antiseptics, and surgical procedures.

**RESULTS:** All included cases had their first episode of CDI. The mean incidence rate was 12.94 cases/1000 admitted patients, and 14.93, 8.52, 13.24, 19.70, and 8.31 respectively per 1000 admitted patients annually from 2007 to 2011. Median age was 62.9 [interquartile range (IQR) 55.4-72.40] years, and 13 (32.5%) were women. Median length of MICU stay was 14.0 d (IQR 5.0-22.8). In addition to diarrhoea, the clinical symptoms of CDI were fever ( $> 38^{\circ}\text{C}$ ) in 23 patients, abdominal pain in 15 patients, and ileus in 1 patient. The duration of diarrhoea was 13.0 (8.0-19.5) d. In addition to diarrhoea, the clinical symptoms of CDI were fever ( $> 38^{\circ}\text{C}$ ) in 23 patients, abdominal pain in 15 patients, and ileus in 1 patient. Prior to CDI, 38 patients (95.0%) were exposed to antibiotics, and 12 (30%) received at least 4 antibiotics. Fluoroquinolones, 3<sup>rd</sup> generation cephalosporins, coamoxiclav and tazocillin were prescribed most frequently (65%, 55%, 40% and 37.5%, respectively). The majority of cases were hospital-acquired ( $n = 36$ , 90%), with 5 cases (13.9%) being MICU-acquired. Fifteen patients had severe CDI. The crude mortality rate within 30 d after diagnosis was 40% ( $n = 16$ ), with 9 deaths (9 over 16; 56.3%) related to CDI. Of our 40 patients, 15 (37.5%) had severe CDI. Multivariate logistic regression showed that male gender [odds ratio (OR): 8.45; 95%CI: 1.06-67.16,  $P = 0.044$ ], rising serum C-reactive protein levels (OR = 1.11; 95%CI: 1.02-1.21,  $P = 0.021$ ), and previous exposure to fluoroquinolones (OR = 9.29; 95%CI:

1.16-74.284,  $P = 0.036$ ) were independently associated with severe CDI.

**CONCLUSION:** We report predictors of severe CDI not dependent on time of assessment. Such factors could help in the development of a quantitative score in ICU's patients.

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**Key words:** *Clostridium difficile*; Health-care associated infection; Hospital-acquired infection; Intensive care unit; Nosocomial infection; Severe *Clostridium difficile* infection

**Core tip:** We reported that male gender, rising serum C-reactive protein level, and previous exposure to fluoroquinolones were independently associated with severe *Clostridium difficile* infection (CDI) in medical intensive care unit. This could help in the development of a quantitative severity score that could fuel comparative effectiveness studies and prospective trials of CDI therapy in critically-ill patients.

Khanafer N, Touré A, Chambrier C, Cour M, Reverdy ME, Argaud L, Vanhems P. Predictors of *Clostridium difficile* infection severity in patients hospitalised in medical intensive care. *World J Gastroenterol* 2013; 19(44): 8034-8041 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8034.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8034>

## INTRODUCTION

*Clostridium difficile* (*C. difficile*) infection (CDI) has become a growing cause of nosocomial morbidity, high hospital costs and mortality in North America as well as in other areas of the world<sup>[1-6]</sup>. Hospital-acquired CDI has surpassed methicillin-resistant *Staphylococcus aureus* (*S. aureus*) in some hospitals as the leading source of healthcare-associated infections<sup>[7]</sup> and was ranked in the five most important scientific issues facing healthcare epidemiology<sup>[8]</sup>. Several mechanisms have been postulated to increase disease severity, including the emergence of specific strains with genetic polymorphisms that encode higher levels of bacterial toxins A and B as well as the production of a binary toxin<sup>[9-11]</sup>. Advanced age, severe co-morbidity, hospitalisation, antibiotic exposure, immunosuppressants and treatment with motility-influencing or acid-suppressive drugs have all been implicated as risk factors for CDI<sup>[12-17]</sup>.

The cumulative mortality attributable to CDI for all patients typically ranges from 5.5% to 6.9% but can reach 16.7% during severe outbreaks<sup>[18-24]</sup>. In the United States, *C. difficile* is now the 9<sup>th</sup> leading gastrointestinal cause of death<sup>[25]</sup>. CDI is more common in the intensive care unit (ICU) setting, with an overall incidence of roughly 4%<sup>[26]</sup>. Up to 20% of ICU patients who develop symptomatic

disease will progress to fulminant colitis with a mortality rate of nearly 60%<sup>[26]</sup>. In the United States, attributable costs range from \$2871 to \$4846 per case of primary CDI and from \$13655 to \$18067 for infection recurrence or relapse<sup>[18]</sup>, with annual expenditures in excess of \$3 billion<sup>[27]</sup>. A study of ICU patients disclosed gross costs of \$11353 for CDI compared to \$6028 without CDI<sup>[26]</sup>.

CDI among critically-ill patients usually presents as diarrhoea, abdominal pain, hypotension, electrolyte perturbations, and fever<sup>[2,3,10,21,28,29]</sup>. Several studies have examined factors related to CDI acquisition and mortality in different medical units<sup>[30,31]</sup>. To the best of our knowledge, factors associated with CDI severity in medical ICUs (MICU) are poorly documented. We undertook a one-center cohort investigation to analyse factors linked with CDI severity and to report the prognosis of CDI in hospitalised MICU patients.

## MATERIALS AND METHODS

### Study population

This retrospective cohort study was performed at an 860-bed university-affiliated public hospital in Lyon, France. All adult patients with CDI diagnosed in a MICU (15 beds) between January 1, 2007 and December 31, 2011 were included. Patients were followed up until the last point of hospital contact. Thirty-day in-patient mortality from any cause was chosen as the primary endpoint. According to French law, a study like this one does not require ethics committee approval because it is observational and derives from a surveillance database approved under national regulations (*Comité National Informatique et Liberté*)<sup>[32]</sup>. Protocol design was approved by the hospital's institutional review board.

### Data collection

After case identification, full medical files were reviewed and data collected through our institution's electronic medical database. The following data were analysed: age, sex, body weight, diagnosis on admission, co-morbidities, Glasgow coma score available on the day of ICU admission, nutritional status, parenteral nutrition administration, CDI symptoms and their duration, prior CDI history, results of microbiological tests, specific antibiotic therapy for CDI, and evolution of infection. Exposure to risk factors associated with CDI within 8 wk before CDI was recorded, including previous hospitalisation, nursing home residency, antibiotics, antisecretory drugs (proton pump inhibitors, PPI), and surgical procedures (endoscopy, percutaneous gastrostomy, nasogastric feeding, gastrointestinal surgery). Leukocyte count, C-reactive protein, and serum albumin values were collected on days -2 to +2 relative to day 0 (the day the diarrhoeal sample was tested for CDI). Patient outcomes were analysed until in-patient death or last point of hospital contact.

### Microbiological data

*C. difficile* testing was performed only on unformed stool samples from patients clinically suspected to have

CDI. Laboratory diagnosis of CDI was based on stool enzyme-linked immunosorbent assay (ELISA, Immuno-Card Toxins A and B, Meridian Biosciences, Cincinnati, OH, United States, Ref. 716060) coupled with toxigenic culture.

### Definitions

All definitions were selected as part of routine CDI surveillance. Bacteriological cases of CDI were defined as positive enzyme linked immunosorbent assay (ELISA) results and/or positive toxigenic culture. Clinical CDI severity was considered when patients met at least 1 of the following criteria: endoscopically- or histologically-proven colitis or CDI-related complications, such as toxic megacolon, intestinal perforation, colectomy, septic shock, CDI requiring admission to ICU or related death in 30 d. It should be noted, however, that there are currently no prospectively-validated severity scores for CDI. Recurrence was defined as a new episode of diarrhoea and positive toxin assay within 8 wk after a first correctly-treated episode.

According to French guidelines, a nosocomial CDI was assumed if diarrhoea onset took place more than 2 d after admission to hospital or if hospital admission occurred within 4 wk of discharge and indeterminate or unknown if the patient had been discharged from a healthcare facility within the previous 4-12 wk. Cases were defined as community-acquired if CDI signs presented in the absence of previous hospitalisation within the last 12 wk in out- or in-patients within the first 48 h of admission<sup>[33]</sup>. French health authorities currently adopt a cut-off period of 48 h post-admission to define hospital-acquired infections. We considered fever as core temperature  $> 38^{\circ}\text{C}$ , and leukocytosis as leukocyte count  $> 15 \times 10^9/\text{L}$ . Malnutrition was defined according to the national recommendations<sup>[34]</sup>. The incidence rate was calculated as the number of CDI in MICU per 1000 admitted patients.

### Statistical analysis

The data were analysed in 2 stages. First, univariate analysis identified significant differences between severe and non-severe CDI cases. Continuous variables were compared by the Mann-Whitney *U* test. The  $\chi^2$  or Fisher's exact test compared categorical variables. Second, a multivariate logistic regression model identified factors associated with CDI severity. The distribution of continuous variables was checked. All potential risk factors significant at the 0.2 level in univariate analysis were entered into the model. Multivariate analysis was performed with models that were judged a priori to be clinically sound. This was prospectively determined to be necessary to avoid producing spuriously significant results with multiple comparisons. The goodness-of-fit was assessed by the Hosmer-Lemeshow test. For all tests performed, 2-tailed *P* values  $< 0.05$  were regarded as denoting statistical significance. Statistical data were analysed with statistical package for the social sciences (version 17.0 for Windows, SPSS, Inc.,

Chicago, IL).

## RESULTS

A total of 40 adult patients suffering from CDI-related diarrhoea diagnosed in MICU from January 2007 and December 2011 were included. The mean incidence rate was 12.94 cases/1000 admitted patients, and 14.93, 8.52, 13.24, 19.70, and 8.31 respectively per 1000 admitted patients annually from 2007 to 2011 ( $P = 0.99$ ). The demographics and outcomes of these patients are summarised in Table 1. Median age was 62.9 [interquartile range (IQR) 55.4-72.40] years; 13 (32.5%) were women, and 24 (60%) were admitted directly to MICU. Median length of MICU stay was 14.0 d (IQR 5.0-22.8). Twenty-nine patients (72.5%) presented symptoms after MICU admission, and 15 patients (37.5%) developed CDI 10 d or less after MICU admission. Based on the inclusion criteria, ELISA was positive in 35 patients (87.5%), with the remaining 5 patients (12.5%) being diagnosed by toxigenic culture. Median time between onset of symptoms and microbiological diagnosis was 2 d for ELISA and 10 d for toxigenic culture. The mean interval between onset of symptoms and *C. difficile* laboratory test results was  $7.2 \pm 16.5$  d. The duration of diarrhoea was 13.0 (8.0-19.5) d. In addition to diarrhoea, the clinical symptoms of CDI were fever ( $> 38^{\circ}\text{C}$ ) in 23 patients, abdominal pain in 15 patients, and ileus in 1 patient. At the time of diagnosis, median leukocyte count was 14.4 (9.45-21.73), with leukocytosis ( $> 20 \times 10^9/\text{L}$ ) in 12 patients (30%). C-reactive protein was 117 mg/L (60-193), and albumin was 26.0 g/L (20.0-28.0). Prior to CDI, 38 patients (95.0%) were exposed to antibiotics, and 12 (30%) received at least 4 antibiotics. Fluoroquinolones, 3<sup>rd</sup> generation cephalosporins, coamoxiclav and tazocillin were prescribed most frequently (65%, 55%, 40% and 37.5%, respectively). During MICU stay, 12 patients received parenteral nutrition due to malnutrition and impossible intake. The majority of patients had hospital-acquired CDI (90%), with 5 cases (13.9%) being MICU-acquired. Metronidazole was administered as a single agent to 25 patients and vancomycin to 2 (5%). Eight patients (20%) received a combination of 2 CDI medications during the course of treatment. Five patients were given no antimicrobials against CDI.

Of our 40 patients, 15 (37.5%) had severe CDI. Table 1 shows characteristics of severe and non-severe patients. Univariate analysis showed that Glasgow coma score, gender, diabetes mellitus, previous exposure to fluoroquinolones, PPI or coamoxiclav, and C-reactive protein were statistically different between severe and non-severe patients. Multivariate analysis indicated that male gender, C-reactive protein levels, and fluoroquinolones were independently associated with severe CDI (Table 2).

The prognosis of CDI was good in 18 patients. A total of 12 patients (30%) experienced complications due to their infection with 2 cases (16.7%) of pseudomembranous colitis (PMC) and 4 cases (33.3%) of colitis. In one patient, CDI was marked by hyper-leukocytosis (53



**Table 1** Comparison of the characteristics of severe and non-severe *Clostridium difficile* infection patients hospitalised in medical intensive care unit between January 2007 and December 2011

	Total <i>n</i> = 40	Severe CDI <i>n</i> = 15	Non-severe CDI <i>n</i> = 25	<i>P</i> value
Age (yr)	62.9 (55.3-72.4)	59.52 (54.8-77.3)	64.27 (56.1-72.2)	0.99
Male gender	27 (67.5)	13 (86.7)	14 (56.0)	0.045
Origin of patient				0.61
Home	14 (35.0)	6 (40)	8 (32)	
Other ward and/or other hospital	26 (65.0)	9 (60)	17 (68)	
Diagnosis at MICU admission				0.39
Respiratory disease	15 (37.5)	3 (20)	12 (48)	
Septic shock	12 (30.0)	6 (40)	6 (24)	
Renal disease	3 (7.5)	1 (6.7)	2 (8)	
Gastrointestinal disease	3 (7.5)	1 (6.7)	2 (8)	
Neurological disease	3 (7.5)	1 (6.7)	2 (8)	
Other	4 (10.0)	3 (20)	1 (4)	
Clinical symptoms and biological features at diagnosis				
Fever	23 (57.5)	8 (53.3)	15 (60.0)	0.75
Abdominal pain	15 (37.5)	7 (46.7)	8 (32.0)	0.35
Duration of diarrhoea (d)	13.0 (8.0-19.5)	18 (5-29)	13 (8-17)	0.38
C-reactive protein (mg/L)	117 (60-193)	185 (73-339)	105 (39-127)	0.01
Albumin count (g/L)	26.0 (20.0-28.0)	23 (17-27)	26 (21-28)	0.30
Leukocyte count ( $\times 10^9/L$ )	14.4 (9.5-21.7)	17.9 (10.6-33.4)	12.4 (9.0-21.1)	0.17
Previous exposure to CDI risk factors within 8 wk before onset of symptoms				
Hospitalisation	28 (70.0)	10 (66.7)	18 (72.0)	0.72
Exposure to PPI	21 (52.5)	10 (66.7)	11 (44.0)	0.17
Chemotherapy	12 (30)	5 (33.3)	7 (28.0)	0.72
Gastrointestinal procedures	23 (57.5)	9 (60.0)	14 (56.0)	0.80
Antibiotic treatment	38 (95.0)	15 (100)	23 (92)	0.26
Cephalosporins 3 <sup>rd</sup> generation	22 (55)	8 (53.3)	14 (56)	0.87
Clindamycin	2 (5)	1 (6.7)	1 (4)	0.71
Coamoxiclav	16 (40)	8 (53.3)	8 (32)	0.18
Fluoroquinolones	26 (65)	13 (86.7)	13 (52)	0.026
Treatment				0.06
No treatment	5 (12.5)	2 (13.3)	3 (12.0)	
Only metronidazole	25 (62.5)	6 (40)	19 (76)	
Only vancomycin	2 (5)	2 (13.3)	0 (0)	
Metronidazole+vancomycin	8 (20)	5 (33.3)	3 (12.0)	
Duration of hospital stay (d) and outcomes				
LOS in hospital	27.0 (13.5-50.8)	16 (5-48)	28.0 (16.0-55.5)	0.26
LOS in MICU	14.0 (5.0-22.8)	8 (2-21)	16.0 (6.0-25.5)	0.27
Death in 30 d	16 (40)	9 (60)	7 (28)	0.046

Data represent *n* (%) of patients for categorical variables and median (interquartile range) for continuous variables. CDI: *Clostridium difficile* infection; LOS: Length of stay; MICU: Medical intensive care unit; PPI: Proton pump inhibitor; WBC: White blood cells.

g/L), PMC, renal failure and intestinal perforation. The patient died 56 d after CDI diagnosis.

Overall mortality was 52.5%; 12 patients expired in MICU and 9 in-hospital after MICU discharge. The mortality rate within 30 d after diagnosis was 40%; 9 deaths (56.3%) were CDI-related according to the physician in charge of the patient.

## DISCUSSION

*C. difficile* acquisition and severe CDI development are primarily associated with healthcare, although severe, community-acquired infections among persons previously thought to be at low risk have been reported<sup>[35,36]</sup>. CDI management has become more daunting over the past decade because of alarming increments in CDI incidence and severity. These increases have caused significant, concomitant escalation of the healthcare economic

burden from CDI and will likely translate into excessive ICU admissions and attributable mortality. Up to 20% of critically-ill patients may suffer from ileus without the diarrhoea typically associated with CDI<sup>[37]</sup>. The absence of diarrhoea coupled with the inability of critically-ill patients to communicate with care providers make the diagnosis of CDI extremely difficult<sup>[38]</sup>. The objectives of this study were to analyse factors associated with CDI severity and to describe the prognosis of CDI in hospitalised MICU patients.

Our investigation comprised 40 CDI patients diagnosed at a MICU between 2007 and 2011, with a mean incidence rate of 12.94 cases/1000 admitted patients. All included cases had their first episode of CDI. The majority were hospital-acquired (90%), with 5 cases (13.9%) being MICU-acquired. In this work, we compared the characteristics of a group of 15 cases of severe CDI with a group of 25 patients without severe CDI in our MICU.

**Table 2** Factors independently associated with severe *Clostridium difficile* infection among patients in medical intensive care unit

Variables	Unadjusted OR (95%CI)	P value	Adjusted OR (95%CI)	P value
Glasgow coma score	1.16 (0.99-1.36)	0.15	-	
Diabetes mellitus	4.89 (1.00-23.93)	0.04	-	
Previous PPI exposure	2.55 (0.67-9.66)	0.17	-	
Coamoxiclav (in the previous 8 wk)	2.43 (0.65-9.07)	0.18	-	
Fluoroquinolones (in the previous 8 wk)	6.0 (1.12-32.28)	0.026	9.29 (1.16-74.28)	0.036
C-reactive protein (mg/L; 10 mg/L increments)	1.10 (1.02-1.18)	0.014	1.11 (1.02-1.21)	0.021
Male gender	5.11 (0.95-27.55)	0.045	8.45 (1.06-67.16)	0.044

Exposure to fluoroquinolones, C-reactive protein level and gender were included in the multivariate model [The value of the likelihood was 34.56 with 3 df, and  $\chi^2$  test: 18.37 ( $P < 0.0001$ )]. OR: Odds ratios; PPI: Proton pump inhibitor.

In univariate analysis, gender, BMI, diabetes mellitus, fluoroquinolone use and C-reactive protein were associated with CDI severity. Multivariate logistic regression modelling showed that male gender, C-reactive protein, and previous exposure to fluoroquinolones were independently linked with severe CDI. Exposure to specific antimicrobial drugs, notably fluoroquinolones, clindamycin, and cephalosporins, has been linked to severe CDI in some studies<sup>[3,21]</sup> but not in others<sup>[14]</sup>.

Malnutrition, reported to be as high as 40%, is prevalent in ICU patients and is associated with increased morbidity and mortality<sup>[39]</sup>, but to the best of our knowledge, this observation has not been made in CDI patients. The majority of patients were not referred to a dietitian. Among patients consulting a dietitian, 87.5% required parenteral nutrition, which was not associated with 1-month survival in our study. This is consistent with the findings of a previous meta-analysis of 26 randomised trials<sup>[40]</sup>. The investigators showed that, in critically-ill patients, parenteral nutrition did not influence overall mortality.

Underlying illness is moderately associated with severe CDI<sup>[14]</sup>, an effect not observed in our study and could be related to the homogeneity of our study population. Recent investigations have disclosed a potential role of acid suppression in CDI acquisition and relapse<sup>[41,42]</sup>. Hardt *et al.*<sup>[43]</sup> noted an association between these agents and severe CDI, although their definition of severe CDI was different. Also, significant linkage has been reported in a recently-published paper<sup>[44]</sup>. This effect was not seen in our study, but may be related to our study population, and PPIs did not play a role in CDI severity in MICU. Previous works have identified few clinical characteristics that consistently predict severe CDI. Different findings, such as fever, abdominal pain, decreased albumin, and significant leukocytosis (often  $> 20$  g/L), are likely in severe colitis<sup>[45,46]</sup>. Such outcomes often precede multi-organ dysfunction and should prompt urgent consideration of CDI as a possible cause<sup>[47,48]</sup>. In our study, these variables were not different between severe and non-severe cases. Ananthakrishnan *et al.*<sup>[49]</sup> demonstrated that serum albumin  $< 3$  g/dL, haemoglobin  $< 9$  g/dL and creatinine  $> 1.5$  g/dL were independent predictors of severe CDI and may have prognostic significance in patients with inflammatory bowel disease. We also identified rising serum

C-reactive protein levels as being independently associated with severe CDI. As the distribution of C-reactive protein was normal, our multivariate result suggested that an increase by 10 mg/L lead to an increase of the risk of severe CDI by 10%. In fact, serum C-reactive protein was a far better predictor of severe CDI than white blood cell count, which has been implicated by others<sup>[43,50-52]</sup>. Perhaps more sensitive markers of inflammation, such as procalcitonin, might be especially useful in the evaluation of disease severity. Male gender was associated with severe CDI; to the best of our knowledge, this has not been found in other series. However, a similar effect was reported in a Canadian study, where women were less likely to develop severe CDI, but it was indicated by univariate analysis and was not significant<sup>[53]</sup>. Our study provides data on the initial treatment courses chosen by care providers. The majority of patients were treated with metronidazole. Only 7 (46.7%) with severe disease received vancomycin. However, information regarding antibiotherapy (duration and dosage) of CDI was not fully captured; thus, the treatment response could not be analysed in our study. Although current guidelines from the Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America urge vancomycin as first-line therapy in severe disease among adult patients<sup>[54]</sup>, a major portion of our study period predated the publication of these recommendations. In contrast, severe CDI was not associated with nursing home residency, the presence of hospital-acquired CDI or increasing age.

Our study has some limitations which need to be considered when interpreting the data. Our sample size was limited and the study was conducted in one single hospital. Therefore we would not be able to extrapolate our results to other groups. Other potential predictors of severe CDI were unable to provide complete risk scores. There was no validated definition of severe CDI; thus, we applied criteria of severe CDI without a scoring system. A larger, multi-center study would be required to validate any definition of severe CDI. Our patients were assembled from a MICU in a tertiary hospital and may not be generalisable to patients in community hospitals or outpatient settings. Our study population consisted of a significant proportion of patients with multiple co-morbidities, which may reflect tertiary care settings. However, these

centers may be ideal to investigate severe CDI, as patients at risk of severe disease are usually found in tertiary care facilities. The number of antibiotic days should be considered as a potential risk factor for severity, which was not available in our data. Nevertheless, we could not detail the antibiotic consumption. Instead we simply noted if antibiotics were used in the last 2 mo preceding CDI.

We performed this study with the aim of identifying factors that predict severe outcomes associated with CDI in MICU patients. Our results indicate that low C-reactive protein, male gender and previous use of fluoroquinolones are independent predictors of severe CDI in hospitalised MICU patients. In the majority of published studies, factors for a severity score index of CDI were assessed within 48 h after laboratory reporting of test results positive for *C. difficile*. This is problematic in terms of reproducibility in deciding the severity score index of CDI, because the time window from CDI diagnosis to the evaluation of severe CDI is variable. We reported predictors of severe CDI not dependent on the timing of their assessment except for C-reactive protein; in our study, however, values were obtained from the day of CDI diagnosis which made these results valid in clinical practice.

Identification of such factors would foster the development of a quantitative severity score that could drive comparative effectiveness investigations and prospective trials of CDI therapy in these patients. Clinicians need to maintain a high index of suspicion and must often rely on physical examinations and laboratory findings to make the diagnosis. Vancomycin is recognized as the first-line treatment of severe CDI and should be preferred in the ICU setting. Rigorous attention to infection control measures and vigorous antimicrobial stewardship are essential to prevent *C. difficile* transmission. Improved diagnostic methods and new therapeutic tools are required to help clinicians to manage severe CDI cases.

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## COMMENTS

### Background

*Clostridium difficile* infection (CDI) has become a growing cause of nosocomial morbidity, high hospital costs and mortality over the world. Several mechanisms have been postulated to increase disease severity, including the emergence of hypervirulent strains. Critically-ill patients are at particularly high risk of CDI due to the prevalence of multiple risk factors in the patient population. However, factors associated with CDI severity in medical intensive care unit (MICU) are poorly documented.

### Research frontiers

Current data are dealing with many aspects related to CDI. The list of hotspots, not exhaustive in any standards, would include measures of prevention, modalities of diagnosis and treatment, and standardization of basic definitions including severity and evaluation scales. Defining a set of approved prognostic factors would help us dealing with aforementioned topics.

### Innovations and breakthroughs

The authors reported predictors of severe CDI no matter the timing of assess-

ment except for C-reactive protein. Nevertheless, values were obtained from the day of CDI diagnosis which made these results valid in clinical practice.

### Applications

Identification of some factors would foster the development of a quantitative severity score that could drive comparative effectiveness investigations and prospective trials of CDI therapy in patients hospitalised in MICU. Intensivists need to maintain a high index of suspicion and must often rely on physical examinations and laboratory findings to make the diagnosis.

### Peer review

Risk factor assessment limited due to small sample size, but a tremendous time investment into the statistical analysis of this small sample makes the manuscript interesting. The study design is simple and reasonable and statistics are excellent.

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## Prognosis and follow-up of 135 patients with ischemic colitis over a five-year period

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**study** We analyzed prospectively 135 consecutive patients who met criteria for definitive or probable IC according to Brandt criteria, and follow up these patients during the next five years, retrospectively. Long-term results (recurrence and mortality) were evaluated retrospectively after a median interval of 62 mo (range 54-75 mo).

**RESULTS:** Estimated IC recurrence rates were 2.9%, 5.1%, 8.1% and 9.7% at years 1, 2, 3 and 5 years, respectively. Five-year survival was 69% (93 of 135) and 24% (10 of 42 patients) died for causes related to the IC. Among these 10 patients, 8 died in their first episode at hospital (4 had gangrenous colitis and 4 fulminant colitis) and 2 due to recurrence.

**CONCLUSION:** The five-year recurrence rate of IC was low. On the other hand, mortality during follow-up was high and was not associated with ischemic colitis.

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**Key words:** Colonic; Ischemic; Recurrence; Follow-up; Mortality

**Core tip:** The prognosis of patients with ischemic colitis is unknown. In this study we observed that recurrence rate of ischemic colitis was low (9.7% at 5 years). However, the mortality was high (31% at 5 years) and the only factor associated with mortality was age.

### Abstract

**AIM:** To study the prognosis (recurrence and mortality) of patients with ischemic colitis (IC).

**METHODS:** This study was conducted in four Spanish hospitals, participants in the Ischemic Colitis in Spain

Cosme A, Montoro M, Santolaria S, Sanchez-Puertolas AB, Ponce M, Durán M, Cabriada JL, Borda N, Sarasqueta C, Bujanda L. Prognosis and follow-up of 135 patients with ischemic colitis over a five-year period. *World J Gastroenterol* 2013; 19(44): 8042-8046 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8042.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8042>

## INTRODUCTION

Ischemic colitis (IC) has been estimated to account for approximately 3 in 1000 of all admissions to tertiary hospitals<sup>[1]</sup>. In a recent prospective study conducted in 24 Spanish hospitals, IC was the reason for 1.28 per 1000 hospital admissions<sup>[2]</sup>. According to the literature, the incidence in the general population is of 4.5-9.9 cases per 10<sup>5</sup> people/year and of 44 per 10<sup>5</sup> people/year for those above 40 years of age<sup>[3]</sup>. Many cases of IC (reversible forms) are ignored and undiagnosed.

The number of comorbid disorders ( $\geq 5$ )<sup>[4]</sup>, location in the right colon<sup>[5]</sup>, and certain clinical onset (gangrenous colitis and/or fulminant pancolitis)<sup>[2]</sup> are known to be associated with poor prognosis of the disease. However, only a few number of studies, have analysed the long-term prognosis of these patients. The objective of this study was to assess the prognosis (recurrence and mortality) of our patients with IC after first hospital admission.

## MATERIALS AND METHODS

We assessed the long-term recurrence and mortality of patients in four hospitals participating in the Ischemic Colitis in Spain (CIE) study (San Jorge Hospital in Huesca, Donostia Hospital in San Sebastian, La Fe Hospital in Valencia and Galdakao Hospital in Bizkaia). The CIE study is a prospective multicentre study which consecutively included all patients with diagnosis of IC between March 2005 and December 2006<sup>[2]</sup>.

For our study, patients follow-up was continued until June 2011. Data were obtained retrospectively from the outpatient clinic (gastroenterology and/or surgery) or by telephone using a questionnaire given to the patient and/or their families. The mean follow-up period was 62 mo (54-75 mo).

Patients were categorized as having definitive, probable, or possible IC according to the Brandt criteria<sup>[6-8]</sup>. The clinical pattern and outcome for each patient was categorised according to Brandt and Boley classification<sup>[6]</sup>: (1) reversible colopathy; (2) transient colitis; (3) chronic segmental IC; (4) gangrenous colitis; or (5) fulminant universal colitis.

The Ethics Committee of the Clinic Hospital of Barcelona approved the study protocol on 9 June 2005. All patients gave their written consent. The initial study protocol included, in all cases, a colonoscopy before the patient was released from the hospital. A second colonoscopy was not performed in asymptomatic patients and those with reversible colopathy or transient colitis, as these subclasses of disease heal spontaneously.

### Statistical analysis

To compare differences between the groups, the Fisher test was used for qualitative variables and the Student's *t*-test for quantitative variables. Differences were consid-

ered to be significant when *P* values were below 0.05.

## RESULTS

A total of 135 patients with who met IC criteria were included in the study. The diagnosis of IC was definitive in 74% and probable in 26% of these cases. The average age of our cohort was  $73 \pm 10$  years with a range of 17-90 years, and 50% of them were women. Of the 135 patients included in the study, 51 (38%) were classified with reversible colopathy; 29 (21%) transient colitis; 42 (31%) chronic segmental IC; 9 (7%) gangrenous colitis, and 4 (3%) fulminant universal colitis.

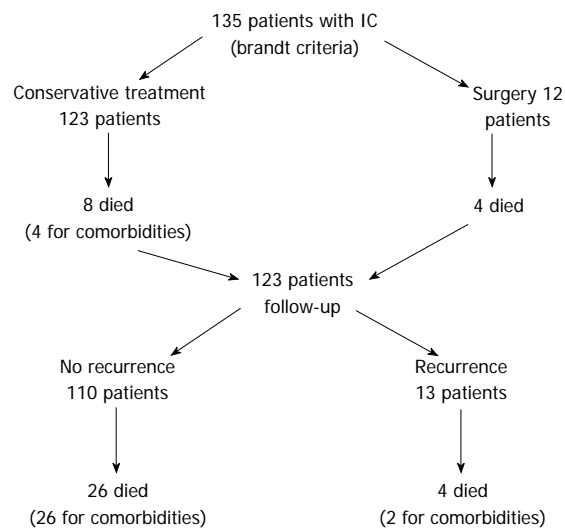
A total of 16 (12%) of the 135 patients had an unfavourable outcome namely death and/or the need for surgery. In the first episode at hospital, 12 patients (8.8%) died, 8 due to IC and 4 due to comorbidities after the acute episode had been solved (1-2 mo after) (Figure 1). Among the 8 who died from IC (3 fulminant universal colitis and 1 gangrenous colitis operated with a subtotal colectomy, and 1 fulminant universal colitis and 3 gangrenous colitis non-operated) the outcome reflected the severity of disease: gangrenous colitis (4/9, 44.4%) and fulminant pancolitis (4/4, 100%).

IC recurred during the follow-up period in 13 (9.7%) of the 123 patients (Tables 1 and 2). Patients with IC recurrence had a similar clinical onset at the first episode at hospital and at relapse. All these patients had colitis not gangrenous and had more frequently vomiting and abdominal pain in the first episode. These were non-gangrenous forms of colitis and located mainly in the left colon. Recurrence occurred in 4, 3, 4, 2 cases through the first, second, third and five year of follow-up, respectively.

Considering the course of the 123 patients, 30 (24.3%) of them died within 5 years, 28 due to comorbidities and only 2 due to recurrence and complications of the IC. The only factor that was associated increased mortality was age ( $72.5 \pm 11$  years *vs* 78.6 years;  $P < 0.001$ ). There were no differences by sex, nonsteroidal anti-inflammatory drugs (NSAIDs) intake or recurrence of colitis. Patients who died had more frequently hypertension 38.8% *vs* 22.4% ( $P = 0.06$ ). Among the 2 patients who died of recurrent IC, one, had reversible colitis, two months after stent insertion for chronic mesenteric ischemia, and the other had chronic segmental colitis, after 24 mo of mesenteric angina. The overall five-year survival was 69% (93 of 135 patients).

### Chronic segmental ischaemic colitis

The patients with chronic segmental IC were followed-up for six months with periodic colonoscopies and/or barium enemas. Of the 42 patients, 30 remained asymptomatic; 6 (14%) developed a stenosis; 3 (7%) had continuing or recurrent bloody diarrhoea and 1 (2%) suffered from persistent or chronic diarrhoea with protein-losing colopathy and serum albumin levels  $< 2.8$  g/L. After six



Study of 364 patients with ischemic colitis in Spain

Figure 1 Outcome of 135 patients with ischemic colitis after initial treatment. IC: Ischemic colitis.

Table 1 Clinical features and clinical pattern of the ischemic colitis *n* (%)

Symptoms	No recurrence <sup>1</sup> ( <i>n</i> = 110)	Recurrence <sup>2</sup> ( <i>n</i> = 13)	<i>P</i> value
Acute abdominal pain	76 (69)	12 (92)	< 0.05
Haematochezia	90 (82)	11 (85)	0.4
Diarrhoea	41 (37)	6 (46)	0.2
AUBD sequence	57 (52)	7 (54)	0.4
Vomiting	20 (18)	5 (38)	< 0.05

<sup>1</sup>Fulminant colitis 4, gangrenous colitis 8 and non-gangrenous colitis 98 (transient colitis 22, reversible colitis 42 and chronic segmental colitis 34);  
<sup>2</sup>Transient colitis 5, reversible colitis 4 and 4 chronic segmental colitis.  
AUBD: Sequence of abdominal pain, urgent desire to defecate, and bloody diarrhoea.

months none of these patients reported complications.

DISCUSSION

IC is the most common form of intestinal ischemia<sup>[9]</sup>. It is predominantly observed in elderly patients with varying comorbidities, though younger individuals may also be affected. The mean incidence of autopsy-verified fatal IC has been estimated to be 1.7/10<sup>5</sup> person years, rising to 23/10<sup>5</sup> person years in octogenarians<sup>[10]</sup>. In our study, the average age was 73 years, and clinical onset of IC was observed equally frequent in both women and men.

Clinical presentation in ischemic colitis varies, depending of the severity and extent of the disease. In general, the first symptoms of IC are haematochezia and acute abdominal pain<sup>[2,11-14]</sup>. Any part of the colon may be affected in IC although the left colon is the predominant location in approximately 75%-85% of patients<sup>[12-14]</sup>. Splenic flexure is involved in nearly one-

Table 2 Univariate analysis of variables in patients with ischemic colitis without recurrence versus patients with recurrence *n* (%)

	No recurrence ( <i>n</i> = 110)	Recurrence ( <i>n</i> = 13)	<i>P</i> value
Men/women	56/54	6/7	0.8
Age ≥ 65 yr	91 (83)	12 (92)	0.7
Age ≥ 80 yr	32 (29)	4 (31)	1
Hypertension	66 (60)	10 (77)	0.4
Patients under NSAID treatments	32 (29)	5 (38)	0.5
≥ 3 comorbidities diseases <sup>1</sup>	43 (39)	4 (31)	0.8
Location			
Pancolitis	4 (3)	0 (0)	1
≥ 2 locations	45 (41)	5 (38)	1
Caecum	6 (5)	1 (8)	0.5
Ascending colon	7 (6)	1 (8)	0.6
Hepatic flexure	8 (7)	1 (8)	1
Transverse colon	8 (7)	1 (8)	1
Splenic colon	23 (21)	3 (23)	1
Descending colon	45 (41)	2 (15)	0.08
Sigmoid colon	74 (67)	11 (85)	0.0001
Rectum	17 (15)	1 (8)	0.7
Clinical presentations			
Non-gangrenous	98 (89)	13 (100)	0.8
Gangrenous	8 (7)	0 (0)	0.6
Fulminant	4 (3)	0 (0)	1

<sup>1</sup>Comorbidities (diabetes, 44 patients; dyslipidaemia, 36; ischaemic heart disease, 34; cerebrovascular disease, 29; atrial fibrillation, 28; peripheral vascular disease, 24; congestive heart failure, 11; recent arterial hypertension, 11; malignancy, 3 and miscellaneous, 15). NSAID: Nonsteroidal anti-inflammatory drug.

quarter of patients<sup>[15-17]</sup>, and isolated right colon ischemia (IRCI) in about 10%-26% of cases<sup>[6,17]</sup>. Right-sided colonic ischemia tends to be more severe: about 60% of patients require surgery (four or five times more than with colitis in other areas). In our cohort, of the 10 patients with the right colon involved, 6 (5 with gangrenous colitis) required surgery.

It has been estimated that about 20% of patients with acute IC will require surgery with an associated mortality rate of up to 60%<sup>[18-21]</sup>. In our study, 12 (9%) of the 135 patients underwent surgery, 3 with universal fulminant colitis, 6 with gangrenous colitis, 2 with chronic segmental colitis and 1 with transient colitis. The gangrenous and fulminant universal colitis are associated with poorer prognosis than non-gangrenous forms of IC. Global mortality of IC is of 8%-10%. Gangrenous forms mortality usually reaches 30% and universal colitis is near 100%.

The rate of the recurrence of IC has been reported to be 10%-16% within five years<sup>[11,22]</sup>. To assess the recurrence, we evaluated the long-term outcomes in our patients (54-75 mo). Thirteen patients had recurrent symptoms. Two of them, one presented chronic segmental colitis and another with transient colitis did not have sigmoid colon involved. There were no statistically significant differences between the clinical presentation of IC and the involvement of different segments of the left colon. The estimated cumulative recurrence rates at years, 1, 2, 3 and 4/5 were 2.9%, 5.1%, 8.1% and 9.7%, respectively.



Some authors<sup>[23,24]</sup> have recommended that the following studies should be carried out on a prospective basis to assess potential etiologic factors that may increase the likelihood of recurrence: hypercoagulability workup, tests for connective tissue disorders, echocardiogram and holter, and magnetic resonance angiography. In this way, we may be able to detect structural heart diseases, fibromuscular dysplasia and other conditions that may predispose individuals for IC or to recurrence.

The question of whether patients should receive prophylactic treatment for recurrent IC after discharge from the hospital is important. Currently, our efforts should be addressed to control those factors that may contribute to develop IC such as intake of NSAID<sup>[25,26]</sup> and vasoactive drugs and arterial hypertension.

The limitations of this study are those of a retrospective analysis but prospective follow-up patients. The data collection was made by telephone or at the outpatient clinic and colonoscopy was undertaken only if symptoms were consistent with IC.

In summary, IC is associated with age and occurrence on the right-side markedly increases the risk of severe disease that requires surgery or leads to death. The mortality rate of IC is still high and the recurrence increases with time. In our sample, mortality due to IC at the first admission was 5.9% and 7.4% five years later. The overall rates of mortality, including comorbidities were 8.8% and 31.1% respectively.

## ACKNOWLEDGMENTS

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## COMMENTS

### Backgrounds

Ischemic colitis (IC) is the most common form of intestinal ischemia. IC is predominantly observed in elderly patients with varying comorbidities, although younger individuals may also be affected. IC was the reason for 1.28 per 1000 hospital admissions.

### Research frontiers

Few studies have assessed the long-term prognosis of these patients.

### Innovations and breakthroughs

IC is associated with age and occurrence on the right-side markedly increases the risk of severe disease that requires surgery or leads to death. The mortality rate of IC is still high and the recurrence increases with time. In our study, mortality due to IC at the first admission was 5.9% and 7.4% five years later. The overall rates of mortality, including comorbidities were 8.8% and 31.1% respectively.

### Applications

Patients with ischemic colitis should be monitored continuously to prevent decompensation during follow-up.

### Peer review

It is a clinical series of IC. It is well written.

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## Single balloon enteroscopy for endoscopic retrograde cholangiography in patients with Roux-en-Y hepaticojejunostomosis

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### Abstract

**AIM:** To evaluate single balloon enteroscopy in diagnostic and therapeutic endoscopic retrograde cholangiography (ERC) in patients with Roux-en-Y hepaticojejunostomosis (HJA).

**METHODS:** The study took place from January 2009 to December 2011 and we retrospectively assessed 15 patients with Roux-en-Y HJA who had signs of biliary obstruction. In total, 23 ERC procedures were performed in these patients and a single balloon videoen-

teroscope (Olympus SIF Q 180) was used in all of the cases. A transparent overtube was drawn over the videoenteroscope and it freely moved on the working part of the enteroscope. Its distal end was equipped with a silicone balloon that was inflated by air from an external pump at a pressure of  $\leq 5.4$  kPa. The technical limitations or rather the parameters of the single balloon enteroscope (working length - 200 cm, diameter of the working channel - 2.8 mm, absence of Albarran bridge) showed the need for special endoscopic instrumentation.

**RESULTS:** Cannulation success was reached in diagnostic ERC in 12 of 15 patients. ERC findings were normal in 1 of 12 patients. ERC in the remaining 11 patients showed some pathological changes. One of these (cystic bile duct dilation) was subsequently resolved surgically. Endoscopic treatment was initialized in the remaining 10 patients (5 with HJA stenosis, 2 with choledocholithiasis, and 3 with both). This treatment was successful in 9 of 10 patients. The endoscopic therapeutic procedures included: balloon dilatation of HJA stenosis - 11 times (7 patients); choledocholithiasis extraction - five times (5 patients); biliary plastic stent placement - six times (4 patients); and removal of biliary stents placed by us - six times (4 patients). The mean time of performing a single ERC was 72 min. The longest procedure took 110 min and the shortest took 34 min. This shows that it is necessary to allow for more time in individual procedures. Furthermore, these procedures require the presence of an anesthesiologist. We did not observe any complications in these 15 patients.

**CONCLUSION:** This method is more demanding than standard endoscopic retrograde cholangiopancreatography due to altered postsurgical anatomy. However, it is effective, safe, and widens the possibilities of resolving biliary pathology.

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**Key words:** Single balloon enteroscopy; Endoscopic retrograde cholangiography; Roux-Y hepaticojejunoanastomosis; Endoscopic diagnosis; Endoscopic treatment

**Core tip:** Endoscopic retrograde cholangiopancreatography (ERCP) represents a demanding method even in a normal anatomical situation. When a surgically altered gastrointestinal or pancreatobiliary anatomy is present, ERCP becomes even more demanding. Our retrospective study assessed diagnostic and therapeutic endoscopic retrograde cholangiography (ERC) using a single balloon enteroscope in 15 patients with Roux-en-Y hepaticojejunoanastomosis. A comparatively high success rate was achieved in both diagnostic (80%) and therapeutic (90%) ERC. This method is both time-consuming and technically demanding. However, it is an effective and safe method that widens the possibilities of resolving biliary pathology in these conditions.

Kianička B, Lata J, Novotný I, Dítě P, Vaníček J. Single balloon enteroscopy for endoscopic retrograde cholangiography in patients with Roux-en-Y hepaticojejunoanastomosis. *World J Gastroenterol* 2013; 19(44): 8047-8055 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8047.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8047>

## INTRODUCTION

Hepaticojejunoanastomosis (HJA) construction is a frequent method of surgical bypass for resolution of pathological conditions of the extrahepatic bile ducts, which enables bile drainage into the small intestine. These are the main groups of indications for HJA construction: (1) benign pathological processes in the papilla of Vater (VP) and terminal common bile duct, which cannot be resolved by endoscopy or surgical resection; (2) local inoperable pathological processes in the VP and distal part of the hepatocholedoch that cannot be resolved radically in patients who are able to undergo surgery; (3) stenoses of the distal part of the hepatocholedoch during expansion of the pancreas (both malignant and benign) stenosing the common bile duct; (4) iatrogenic or traumatic damage of the hepatocholedoch (*e.g.*, after laparoscopic cholecystectomy) leading to bile leak or stenosis that cannot be resolved by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC), leaving a proximal section of the hepatic duct long enough to enable anastomosis; (5) HJA stenosis; and (6) congenital bile duct anomaly with proximal section of the extrahepatic bile ducts in good condition.

Bile duct injuries during laparoscopic and open cholecystectomy belong to the most serious iatrogenic injuries, with high morbidity and mortality. The increasing

number of laparoscopic cholecystectomies has led to an increase in the number of bile duct injuries. Early perioperative detection of these injuries serves as the basis of successful reconstruction of the bile duct where HJA on Roux loop is the gold standard<sup>[1]</sup>. This procedure is nowadays a standard way of treating injuries of the bile duct and its consequences in the form of stenoses. HJA performed using Roux-en-Y loop (less frequently using omega loop) can be used universally. In general, using this method of bile duct reconstruction for any indication does not lead to mistakes. In contrast to simple end-to-end anastomosis, HJA can be used at any time, in the case of loss-making injury of the bile duct (*e.g.*, excision of its part) or in the case of thin and fine bile duct. HJA can be used in all recent injuries to the bile duct as well as in all adjustments of its stenoses (*i.e.*, in chronic conditions)<sup>[2]</sup>. It is controversial to perform reoperations for complications of laparoscopic procedures of the bile duct. For reoperations, it is possible to use the da Vinci robotic system, which offers a wide range of visualizing possibilities (fluoroscopy) and movability of instruments (endowrist)<sup>[3-5]</sup>. The main condition necessary for long-term successful diagnostic results and results of treatment of hepatobiliary diseases is the multidisciplinary approach of surgeons, endoscopists, and interventional radiologists.

One of the serious postoperative complications of HJA is stenosis, with the possible development of cholangitis. Surgery is frequently performed in the unfavorable conditions of inflammatory changes. It is therefore clear that the percentage of restenosis in the area of HJA is comparatively high and reaches about 7% even in the best institutions<sup>[6]</sup>.

The HJA construction (mainly on Roux-en-Y or less frequently on the omega intestinal loop) causes the bile duct orifice into the small intestine to become unreachable by ERCP performed in the standard way (*i.e.*, by lateroscope). That is why biliary drainage used to be provided using the transhepatic approach (*i.e.*, PTC) or surgically.

These patients with Roux-en-Y HJA and with signs of biliary obstruction have therefore in the past been a challenge for endoscopists due to the absence of endoscopic access to enterobiliary anastomosis<sup>[7,8]</sup>.

Single balloon enteroscopy has proved to be effective for deep intubation of the small intestine. The basic technique of performing single balloon enteroscopy has been described extensively in the literature<sup>[9,10]</sup>.

The use of a balloon enteroscope (initially a double balloon and recently also a single balloon) has resulted in achievement of enteroenteroanastomosis, and then bilioenteral anastomosis at the distal end of the afferent intestinal loop<sup>[11]</sup>. ERC was performed in our group of patients with Roux-en-Y HJA by single balloon enteroscopy. Using standard ERCP, in comparison with a lateroscope, one has to take account of certain technical limitations caused by the current balloon enteroscopes: (1) extreme working length of a single balloon enteroscope -



200 cm; (2) small diameter of the working channel of the single balloon enteroscope - 2.8 mm; and (3) absence of Albarran bridge in a single balloon enteroscope. These technical limitations or parameters show the necessity to use suitable endoscopic instrumentation.

ERCP is considered to be a technically demanding procedure of digestive endoscopy and the presence of surgically altered gastrointestinal or pancreatobiliary anatomy makes it even more difficult<sup>[12,13]</sup>.

The aim of this retrospective study was to analyze and evaluate our experience in using single balloon enteroscopy in diagnostic and therapeutic ERC in patients with Roux-en-Y HJA.

## MATERIALS AND METHODS

### Patients

The study took place from January 2009 to December 2011 and we retrospectively assessed 15 patients (7 men, average age: 55 years; 8 women, average age: 53 years) with Roux-en-Y HJA, who had signs of biliary obstruction.

### ERC procedure

Altogether 23 ERC procedures were performed in these 15 patients with Roux-en-Y HJA using a single balloon videenteroscope (Olympus SIF Q 180). Its working length was 200 cm, the outer diameter was 9.2 mm, and the diameter of the working channel was 2.8 mm. A transparent overtube was drawn over a single balloon enteroscope and it freely moved on the working part of the enteroscope. The overtube was 13.2 mm in diameter and 140 cm long. The distal end was equipped with a silicon balloon that was filled with air from an external pump up to a maximum pressure of 5.4 kPa. Inflation and deflation of this silicon balloon were performed by means of the external pump control.

The examination was performed after a 12-h fast. This endoscopic procedure was both time consuming and technically demanding and required the presence of an anesthesiologist. The mean time to perform the procedure was 72 min. The longest procedure took 110 min and the shortest took 34 min.

During the procedure, the patient lay on the left side and received intravenous sedation (mainly in various combinations) with: midazolam 1-5 mg, sufentanil 5-10 µg, and propofol 20-40 mg repeatedly, to a maximum dose of 200 mg. Buscopan was used after reaching the blind end of the afferent intestinal loop when looking for the HJA orifice.

As can be seen in the schematic image with Roux-en-Y HJA (Figure 1), if an endoscopist wishes to reach the target location, that is, the orifice of the HJA, he/she needs to cover a long distance using a single balloon enteroscope - namely the esophagus, stomach, duodenum, duodenojejunal flexure, proximal jejunum, enteroenteroanastomosis, and afferent intestinal loop, where, at its distal end, 5-6 cm before the blind end of the intestinal

loop, lies the orifice of the HJA. Non-ionic iodinated contrast medium (Omnipaque 300) was used for X-ray imaging of the biliary system.

As already mentioned above, the technical limitations or parameters of the single balloon enteroscope (working length - 200 cm, diameter of the working channel - 2.8 mm, absence of Albarran bridge) necessitate the use of special endoscopic instrumentation.

First, a cannula (width 6 Fr and length 330 cm, or width 7 Fr and length 312 cm; Cook Co., Bloomington, IN, United States) was used for cannulation of the orifice of the HJA and the adjoining bile ducts. Later, a triple-lumen extraction balloon was used more frequently for cannulation of the orifice of the HJA, which enabled simultaneous application of the contrast medium and insertion of the guidewire, which made cannulation significantly more effective. This triple-lumen extraction balloon is described below, and it is used, in addition to cannulation of the orifice of the HJA, for endoscopic extraction of choledocholithiasis.

An especially long guidewire of 600 cm (width 0.035 inches; Cook) was used specifically for this type of procedure using a single balloon enteroscope. HJA stenosis was endoscopically dilated by a bougie dilator (7 Fr) and by a balloon dilator (Cook Co., dilatation balloon type QBD, diameter 10 mm, balloon length 3 cm, designated for 2.8 mm diameter working channel, total length of instrument 320 cm). This balloon dilator was used for dilation under a pressure of 3 atm for 2 min.

Choledocholithiasis was endoscopically extracted using the above mentioned triple-lumen extraction balloon (Cook, TXR- HE, width 6.6 Fr, length 275 cm). Plastic biliary drains - width 7 Fr (and in 1 patient also 8.5 Fr), length 3-5 cm (Medinet or Cook or MSA) - were inserted endoscopically. Apart from others, Cook pusher, width 7 Fr, length 320 cm, was used. In cases of biliary obstruction, these inserted biliary drains were endoscopically removed using a polypectomy loop (Olympus).

### Ethics

The study was performed in accordance with the ethical criteria of the Declaration of Helsinki. The study was reviewed and approved by the Ethics Committee of St. Anne's University Hospital Brno. Written informed consent to perform diagnostic and therapeutic ERC using a single balloon enteroscope was obtained from all the patients.

## RESULTS

In the majority of patients (12/15), HJA construction was required for iatrogenic lesions of the common bile duct after laparoscopic cholecystectomy. In the remaining three patients HJA was required: (1) after resection of the head of the pancreas for chronic pancreatitis; (2) congenital malformation of the common bile duct; and (3) after orthotopic liver transplantation (OLT) for primary sclerosing cholangitis (PSC). Our patients are described

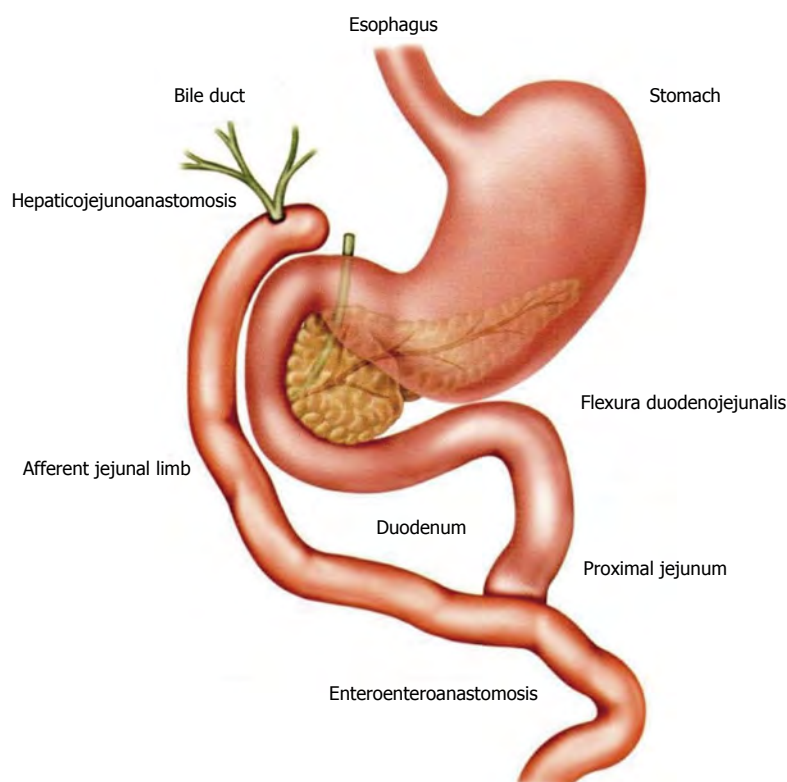


Figure 1 Schematic image of Roux-en-Y hepaticojejunostomosis<sup>[22]</sup>.

in Table 1 and a detailed description of each patient follows below.

#### Patient 1

ERC was performed three times in patient 1 (49-year-old woman). A narrow HJA stenosis was found during the first ERC procedure. Endoscopic balloon dilation of this HJA stenosis was subsequently performed, followed by endoscopic insertion of an 8.5 Fr plastic biliary drain into the bile duct, which completely bridged the HJA stenosis. Another ERC procedure was performed under the same conditions 1 mo later. No complications occurred during this month. Initially, an 8.5 Fr biliary drain was endoscopically extracted. After that, a control ERC was performed, showing that the original HJA stenosis was less prominent. Subsequently, repeat endoscopic balloon dilation of the HJA stenosis was performed. At the end of the second ERC session, an 8.5 Fr plastic biliary drain was repeatedly inserted into the bile duct. A third ERC was performed under the same conditions 1 mo later. An 8.5 Fr biliary drain was endoscopically extracted and a control ERC was performed, which showed a smaller HJA stenosis compared with the previous ERC. Another endoscopic balloon dilation of the small HJA stenosis was performed followed by control ERC. The results were satisfactory, showing almost no HJA stenosis. The procedure was then finished. Both the diagnostic and therapeutic ERC in this patient were therefore successful. The patient had no complications after the first ERC.

#### Patient 2

ERC was performed once in patient 2 (64-year-old woman) and there was only a slight manifestation of HJA stenosis. Endoscopic balloon dilation of this HJA stenosis was subsequently performed followed by control ERC, with satisfactory results and absence of HJA stenosis. Both the diagnostic and therapeutic ERC were therefore successful.

#### Patient 3

Successful diagnostic ERC was performed once in patient 3 (26-year-old woman) and showed cystic dilation of the bile duct. The condition was primarily resolved surgically, that is, without any attempt to perform endoscopic therapy.

#### Patient 4

The orifice of the HJA was found in patient 4 (60-year-old man). However, the attempt to probe the orifice was not successful, therefore, ERC was not performed. This was a case of cannulation failure, thus, the condition had to be resolved by PTC and percutaneous transhepatic drainage (PTD).

#### Patient 5

ERC was performed once in patient 5 (67-year-old man) and it showed slight choledocholithiasis. Endoscopic extraction of the choledocholithiasis using a balloon followed. Both the diagnostic and therapeutic ERC were

**Table 1 Detailed description of 15 patients with Roux-en-Y hepaticojejunostomosis**

<i>n</i>	Age/sex	Cause of HJA	Success of diagnostic ERC	Finding on diagnostic ERC	No. of ERC	Characteristics and No. of endoscopic therapeutic procedures
1	49/F	ILC after LCE	Yes	HJA stenosis	3	BD-3x, EBD-2x
2	64/F	Condition after RHP (CHP)	Yes	HJA stenosis	1	BD-1x
3	26/F	Congenital malformation choledochus (CBD)	Yes	Cystic dilatation choledochus (CBD)	1	Surgical solution
4	60/M	ILC after LCE	No	HJA found but not probed into	1 (SBE)	Solved by PTC and PTD
5	67/M	ILC after LCE	Yes	CDL	1	ECDL-1x
6	57/F	ILC after LCE	Yes	HJA stenosis + CDL	2	BD-1x, ECDL-1x
7	52/M	ILC after LCE	Yes	HJA stenosis + CDL	2	BD-1x, ECDL-1x
8	29/M	Condition after OLT (PSC)	Yes	HJA stenosis	3	BD-2x, EBD-2x
9	64/M	ILC after LCE	Yes	Normal ERC results	1	Without endoscopic treatment
10	48/F	ILC after LCE	Yes	HJA stenosis + CDL	2	BD-1x, ECDL-1x, EBD-1x
11	56/F	ILC after LCE	Yes	too narrow HJA stenosis	1	Therapeut. ERC impossible, condition solved by PTC and PTD
12	59/F	ILC after LCE	Yes	CDL	1	ECDL-1x
13	63/F	ILC after LCE	No	HJA not found at all	1 (SBE)	Solved by PTC and PTD
14	59/M	ILC after LCE	No	HJA found but not probed into	1 (SBE)	Solved by PTC and PTD
15	54/M	ILC after LCE	Yes	HJA stenosis	2	BD-2x, EBD-1x

BD: Balloon dilation; CDL: Choledocholithiasis; CHP: Chronic pancreatitis; EBD: Endoscopic insertion of plastic biliary drains; ECDL: Extraction of choledocholithiasis; ILC after LCE: Iatrogenic lesion of the common bile duct after laparoscopic cholecystectomy; RHP: Resection of the head of the pancreas; SBE: Single balloon enteroscopy; ERC: Endoscopic retrograde cholangiography; CBD: Common bile duct; M: Male; F: Female; PTC: Percutaneous transhepatic cholangiography; PTD: Percutaneous transhepatic drainage; HJA: Hepaticojejunostomosis; PSC: Primary sclerosing cholangitis.

therefore successful.

#### Patient 6

ERC was performed twice in patient 6 (57-year-old woman) and slight HJA stenosis and choledocholithiasis were found. Endoscopic balloon dilation of the HJA stenosis was performed during the first ERC, followed by endoscopic balloon extraction of the choledocholithiasis. Control ERC showed satisfactory results for the biliary tree. Both the diagnostic and therapeutic ERC were therefore successful.

#### Patient 7

ERC was performed twice in patient 7 (52-year-old man). The first ERC showed slight HJA stenosis and choledocholithiasis. Balloon dilation of the HJA stenosis was performed first, followed by endoscopic extraction of the choledocholithiasis using an extraction balloon. Control ERC showed satisfactory results in the biliary tree. Both the diagnostic and therapeutic ERC were therefore successful.

#### Patient 8

Patient 8 (29-year-old man) underwent HJA construction for a condition that developed after OLT for PSC. ERC was performed three times. The first ERC showed a narrow HJA stenosis. Endoscopic balloon dilation of the stenosis was subsequently performed, followed by endoscopic insertion of a 7 Fr plastic biliary drain into the bile duct, which completely bridged the HJA stenosis. The second ERC was performed under the same conditions 1 mo later. No complications occurred during that month. First, a 7 Fr biliary drain was endoscopically extracted. After that, control ERC was performed, showing that the original HJA stenosis was less prominent. Repeated

endoscopic balloon dilation of the HJA stenosis was performed. At the end of the second ERC session, a 7 Fr plastic biliary drain was inserted into the bile duct. Third, control ERC was performed under the same conditions 6 wk later. A 7 Fr biliary drain was endoscopically extracted and control ERC was performed, showing significant improvement of the HJA stenosis, which was in fact unnoticeable. Both the diagnostic and therapeutic ERC in this patient were therefore successful.

#### Patient 9

Successful diagnostic ERC was performed once in patient 9 (64-year-old man) and showed normal findings.

#### Patient 10

ERC was performed twice in patient 10 (48-year-old woman). The first ERC showed HJA stenosis and slight choledocholithiasis. Balloon dilation of the HJA stenosis was performed first, followed by endoscopic extraction of the choledocholithiasis using an extraction balloon. A 7 Fr plastic biliary drain was subsequently inserted into the bile duct, completely bridging the HJA stenosis. Control ERC in 4 wk (*i.e.*, during the second ERC session) showed satisfactory findings in the bile duct. Both the diagnostic and therapeutic ERC were therefore successful.

#### Patient 11

ERC was performed once in patient 11 (56-year-old woman) and prominent HJA stenosis was found. Therapeutic ERC, that is, insertion of a balloon or biliary drain into the HJA stenosis, was not possible because the stenosis was too narrow. This led to PTC and PTD. Successful diagnostic ERC was therefore performed once in this patient.

**Table 2** Diagnostic endoscopic retrograde cholangiography in patients with Roux-en-Y hepaticojejunostomosis

Results in ERC	No. of patients
Normal results	1
Cystic dilation of the bile duct	1
HJA stenosis	5
Choledocholithiasis	2
HJA stenosis + choledocholithiasis	3
Total	12

Cannulation success was reached in 12 of 15 patients [80% diagnostic endoscopic retrograde cholangiography (ERC) success rate]. HJA: Hepaticojejunostomosis.

### Patient 12

ERC was performed once in patient 12 (59-year-old woman) and slight choledocholithiasis was found. Endoscopic extraction of the choledocholithiasis using an extraction balloon was subsequently performed. Both the diagnostic and therapeutic ERC were therefore successful.

### Patient 13

The area of the distal end of the afferent intestinal loop was reached by single balloon enteroscopy in patient 13 (63-year-old woman), nevertheless the HJA was not found. ERC was therefore not performed. This was a case of cannulation failure, thus, the condition had to be resolved by PTC and PTCD.

### Patient 14

The orifice of the HJA was found in patient 14 (59-year-old woman), nevertheless, the attempt to probe the orifice was not successful. ERC was therefore not performed. This was a case of cannulation failure, thus, the condition had to be resolved by PTC and PTCD.

### Patient 15

ERC was performed once in patient 15 (54-year-old man). The first ERC showed HJA stenosis. Endoscopic balloon dilation of the stenosis was performed, followed by endoscopic insertion of a 7 Fr plastic biliary drain into the bile duct, which completely bridged the HJA stenosis. The second ERC was performed under the same conditions 1 mo later. No complications occurred during that month. First, a 7 Fr biliary drain was endoscopically extracted. After that, control ERC was performed, showing marked improvement, with the original HJA stenosis being almost unnoticeable. In spite of that, endoscopic balloon dilation of the area of the HJA stenosis was performed. At the end of the second ERC session, control ERC was performed, showing almost normal findings, which means that the original HJA stenosis was no longer noticeable. Both the diagnostic and therapeutic ERC in this patient were therefore successful.

### Summary of results

The results clearly show that cannulation was successful

in diagnostic ERC in 12 of 15 patients (80% diagnostic success rate). The diagnostic ERC findings in these patients are shown in Table 2. Normal ERC findings were present in one of the 12 patients. ERC in the remaining 11 patients showed some pathological findings. One of these cases (cystic dilation of the bile duct) was subsequently resolved by surgery.

Endoscopic treatment was started immediately after diagnostic ERC in the remaining 10 patients (5 with HJA stenosis, 2 with choledocholithiasis, and 3 with both conditions). This treatment was successful in nine patients (90% therapeutic success rate). Therapeutic ERC was not successful in the other patient due to an extremely narrow HJA stenosis. This condition was resolved by PTC and PTCD.

Endoscopic therapeutic procedures were performed as follows. Balloon dilatation of HJA stenosis was performed a total of 11 times in seven patients; choledocholithiasis extraction was performed a total of five times in five patients; plastic biliary stent insertion was performed a total of six times in four patients; and removal of a biliary stent inserted by our team was performed a total of six times in four patients.

Some of these endoscopic procedures are presented in Figures 2 and 3.

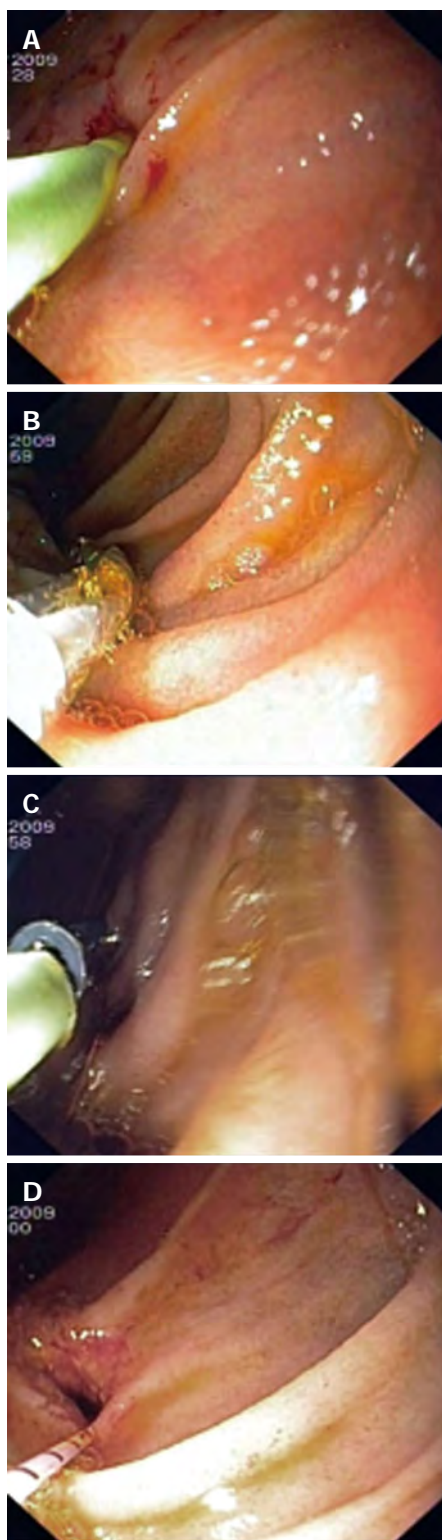
Cannulation failure was recorded in three of 15 patients. Causes of failure were: one patient in whom HJA was not found at all; and two cases in which HJA was found but not probed. All three cases were resolved using PTC and PTCD.

There were no complications in our group of 15 patients.

### Problems with ERC

It is also important to draw attention to some of the pitfalls that we encountered and resolved when performing ERC. (1) Fixing an overtube closely in front of the anastomosis in the area of an enteroenteroanastomosis is not recommended because the overtube, in case of the entrance to the afferent intestinal tube being at an acute angle, made insertion of the enteroscope harder; (2) The afferent intestinal loop could be identified by finding its blind end, and bilioenteral anastomosis can be found a few (5-6) centimeters before this blind end; (3) We suggest using endoscopic accessory of diameter no larger than 7 Fr in the working channel of the single balloon enteroscope (2.8 cm diameter). Based on our personal experience, the use of an 8.5 Fr dilator and biliary drain, or their insertion via the working channel, was difficult. A 7 Fr dilator was much easier to use for dilation and was followed by the use of a dilation balloon. Biliary drainage was, in indicated cases, easier to perform using two 7 Fr drains; and (4) It is advised to straighten the overtube during extraction of the enteroscope from the overtube by manipulation under skiascopic control but, at the same time, it is necessary to prevent the creation of curves that are small in diameter because they tend to break after the removal of the enteroscope, and make repeated insertion complicated.

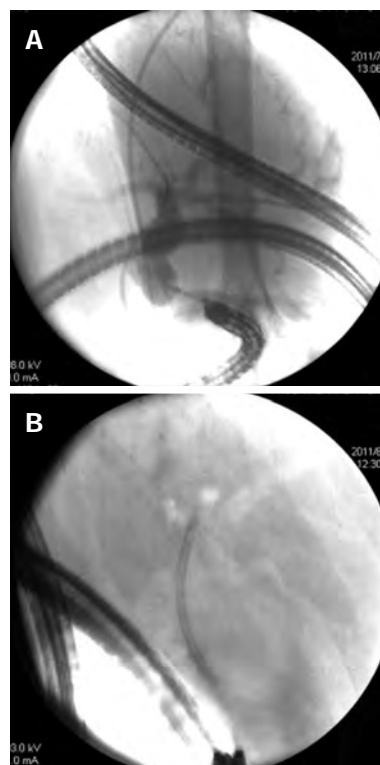




**Figure 2** Endoscopic image. A: Successful cannulation of a stenotic orifice of the hepaticojejunostomosis (HJA) (followed by successful cannulation of adjoining bile ducts); B: Dilation balloon in a deflated form successfully inserted down the guide wire into the area of the stenotic HJA; C: Last phase of a successful endoscopic insertion of a 7 Fr plastic biliary drain into the stenotic HJA and adjoining bile ducts; D: Highly satisfactory final effect of endoscopic treatment of the stenotic HJA (with inserted guide wire).

## DISCUSSION

Single or double balloon enteroscopy enables us to reach



**Figure 3** Endoscopic retrograde cholangiography performed by using single balloon enteroscopy. A: A patient with hepaticojejunostomosis (HJA) shows comparatively narrow HJA stenosis with suprastenotic dilation of bile ducts; B: The patient from Figure 3A in whom HJA stenosis was successfully bridged by endoscopic insertion of a 7 Fr plastic biliary stent.

even the more distant parts of the small intestine. It is therefore reasonable that this examination method began to be used also for reaching the orifice of the bile duct under conditions of altered anatomy after surgical procedures, when the bile ducts become unreachable by standard lateroscopy (*i.e.*, using conventional ERCP). Previously, pathological conditions (or biliary obstruction) had to be resolved surgically or using PTC<sup>[14]</sup>.

One of these most frequently resolved problems is bile duct pathology in patients with Roux-en-Y HJA (HJA stenosis, choledocholithiasis in the bile duct above the HJA, or both). If we know the type of surgical procedure, ERC using single balloon enteroscopy can be attempted in these cases<sup>[14]</sup>. Balloon enteroscopy can enable one to reach the afferent intestinal loop, identify the orifice of the HJA<sup>[15-17]</sup>, perform diagnostic ERC, and if indicated, also perform therapeutic ERC. Originally, the procedure was carried out with double balloon enteroscopy<sup>[7,18]</sup> and later also with single balloon enteroscopy<sup>[13,19-21]</sup>. There was a wide range of endoscopic therapeutic procedures performed. These procedures are also being performed in standard ERCP<sup>[11]</sup>. We used single balloon enteroscopy for diagnostic and therapeutic ERC in our group of 15 patients with Roux-en-Y HJA.

The results of diagnostic and therapeutic ERC using single balloon enteroscopy in patients with Roux-en-Y HJA in other endoscopic centers show that Dellon *et al*<sup>[20]</sup> were successful when performing ERC in three of four patients. Neumann *et al*<sup>[21]</sup> worked with 13 patients in

whom the diagnostic success rate was 62% and the therapeutic success rate 54%. The largest study to date was by Saleem *et al.*<sup>[12]</sup> who achieved a success rate of 78% (32 of 41 patients) in diagnostic ERC. Wang *et al.*<sup>[13]</sup> have recently described a group of 13 patients (16 procedures in total). Cannulation success rate was 81% (13 of 16 cases) for diagnostic ERC. Therapeutic ERC was necessary in 10 of these 13 patients and was successful in nine patients (90% therapeutic ERC success rate).

The diagnostic and therapeutic ERC success rates in our study were comparatively high and, at the same time, there were no procedure-related complications. Cannulation success was achieved in 12 of 15 patients (80% diagnostic ERC success rate). Endoscopic treatment was successful in nine of 10 patients (90% therapeutic ERC success rate). Our results in diagnostic and therapeutic ERC are comparable to those of other endoscopic centers dealing with this issue<sup>[12,13,20]</sup>.

As mentioned above, we encountered some pitfalls when performing ERC, which are not addressed in detail by other authors<sup>[7,12,19,20]</sup>. Nevertheless, they do consider the following: (1) avoidance of fixing an overtube closely in front of an enteroenteroanastomosis at the entrance of the enteroscope into the afferent loop; (2) identification of the afferent loop and the bilioenteral anastomosis; (3) using an endoscopic accessory of diameter no larger than 7 Fr in the working channel of the single balloon enteroscope (diameter 2.8 cm) - a suggestion based on our own experience; and (4) straightening the overtube during extraction of the enteroscope from the overtube, by manipulation under skiascopic control, and at the same time, preventing creation of curves that are small in diameter, because they tend to break after removal of the enteroscope and make repeated insertion complicated.

No complications, not even acute pancreatitis, appeared in our group of 15 patients. It might be that the altered anatomy of the gastrointestinal tract decreases the risk of complications associated with balloon enteroscopy<sup>[7]</sup>.

In conclusion, it can be stated that ERC using single balloon enteroscopy in patients with Roux-en-Y HJA is more difficult than standard ERCP, due to altered post-operative anatomy, and considerable endoscopic skill and experience is needed in order to perform ERC successfully. CRE requires a lot of time for individual procedures and the presence of an anesthesiologist is essential. The cannulation success rate reached in our group of patients was 80% (12 of 15 patients). Endoscopic treatment was successful in 90% (9 of 10 patients). Most ERC procedures in our group of patients were therapeutic (10 of 12 patients - *i.e.*, 83%). There were no complications in our patients. This method is highly demanding but, at the same time, effective and safe, significantly widening the possibilities of resolving biliary tract diseases.

## COMMENTS

### Background

Single balloon enteroscopy (SBE) was originally and still is used for endoscopic diagnosis and treatment of diseases of the small intestine. It was also intro-

duced in endoscopic retrograde cholangiography (ERC) in patients with Roux-en-Y hepaticojejunostomosis (HJA), especially in cases in which standard ERC (by lateroscopy) was unsuccessful, because it was impossible to reach the area of the HJA.

### Research frontiers

The need to continue developing and possibly improving equipment (both endoscopes and endoscopic accessories) is still present. They should be better adjusted to the needs of the ERC procedures in patients with Roux-en-Y HJA (enteroscopes with sideways or oblique optics with an elevator, shorter enteroscopes with wider channels specially designed for these procedures, and development of endoscopic accessories of greater length).

### Innovations and breakthroughs

When surgically altered gastrointestinal or pancreatobiliary anatomy is present, endoscopic retrograde cholangiopancreatography becomes even more demanding than in a normal anatomical situation. In spite of that, we managed to achieve a comparatively high success in diagnostic (80%) and therapeutic (90%) ERC using single balloon enteroscopy in our cohort of 15 patients with Roux-en-Y HJA, and there were no complications after ERC.

### Applications

ERC using single balloon enteroscopy in patients with Roux-en-Y HJA is time consuming and technically demanding. Nevertheless, it is also an effective and safe method that widens the possibilities of resolving biliary tract diseases. Previously, the only possibility of resolution was using percutaneous transhepatic cholangiography or a surgical approach.

### Peer review

The study reported high-quality results of diagnostic and therapeutic ERC using single balloon enteroscopy in patients with Roux-en-Y HJA. The study retrospectively evaluated 15 patients with Roux-en-Y HJA with signs of biliary obstruction. Altogether, 23 ERC procedures were performed without any complications.

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## Simultaneous follow-up of mouse colon lesions by colonoscopy and endoluminal ultrasound biomicroscopy

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### Abstract

**AIM:** To evaluate the potential use of colonoscopy and endoluminal ultrasonic biomicroscopy (eUBM) to track the progression of mouse colonic lesions.

**METHODS:** Ten mice were treated with a single azoxy-

methane intraperitoneal injection (week 1) followed by seven days of a dextran sulfate sodium treatment in their drinking water (week 2) to induce inflammation-associated colon tumors. eUBM was performed simultaneously with colonoscopy at weeks 13, 17-20 and 21. A 3.6-F diameter 40 MHz mini-probe catheter was used for eUBM imaging. The ultrasound mini-probe catheter was inserted into the accessory channel of a pediatric flexible bronchofiberscope, allowing simultaneous acquisition of colonoscopic and eUBM images. During image acquisition, the mice were anesthetized with isoflurane and kept in a supine position over a stainless steel heated surgical waterbed at 37 °C. Both eUBM and colonoscopic images were captured and stored when a lesion was detected by colonoscopy or when the eUBM image revealed a modified colon wall anatomy. During the procedure, the colon was irrigated with water that was injected through a flush port on the mini-probe catheter and that acted as the ultrasound coupling medium between the transducer and the colon wall. Once the acquisition of the last eUBM/colonoscopy section for each animal was completed, the colons were fixed, paraffin-embedded, and stained with hematoxylin and eosin. Colon images acquired at the first time-point for each mouse were compared with subsequent eUBM/colonoscopic images of the same sites obtained in the following acquisitions to evaluate lesion progression.

**RESULTS:** All 10 mice had eUBM and colonoscopic images acquired at week 13 (the first time-point). Two animals died immediately after the first imaging acquisition and, consequently, only 8 mice were subjected to the second eUBM/colonoscopy imaging acquisition (at the second time-point). Due to the advanced stage of colonic tumorigenesis, 5 animals died after the second time-point image acquisition, and thus, only three were subjected to the third eUBM/colonoscopy imaging acquisition (the third time-point). eUBM was able to detect the four layers in healthy segments of



colon: the mucosa (the first hyperechoic layer moving away from the mini-probe axis), followed by the muscularis mucosae (hypoechoic), the submucosa (the second hyperechoic layer) and the muscularis externa (the second hypoechoic layer). Hypoechoic regions between the mucosa and the muscularis externa layers represented lymphoid infiltrates, as confirmed by the corresponding histological images. Pedunculated tumors were represented by hyperechoic masses in the mucosa layer. Among the lesions that decreased in size between the first and third time-points, one of the lesions changed from a mucosal hyperplasia with ulceration at the top to a mucosal hyperplasia with lymphoid infiltrate and, finally, to small signs of mucosal hyperplasia and lymphoid infiltrate. In this case, while lesion regression and modification were observable in the eUBM images, colonoscopy was only able to detect the lesion at the first and second time-points, without the capacity to demonstrate the presence of lymphoid infiltrate. Regarding the lesions that increased in size, one of them started as a small elevation in the mucosa layer and progressed to a pedunculated tumor. In this case, while eUBM imaging revealed the lesion at the first time-point, colonoscopy was only able to detect it at the second time-point. All colonic lesions (tumors, lymphoid infiltrate and mucosal thickening) were identified by eUBM, while colonoscopy identified just 76% of them. Colonoscopy identified all of the colonic tumors but failed to diagnose lymphoid infiltrates and increased mucosal thickness and failed to differentiate lymphoid infiltrates from small adenomas. During the observation period, most of the lesions (approximately 67%) increased in size, approximately 14% remained unchanged, and 19% regressed.

**CONCLUSION:** Combining eUBM with colonoscopy improves the diagnosis and the follow-up of mouse colonic lesions, adding transmural assessment of the bowel wall.

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**Key words:** Ultrasound biomicroscopy; Animal model; Diagnostic imaging; Colonic neoplasm; Longitudinal study

**Core tip:** This paper employed imaging methods, endoluminal ultrasonic biomicroscopy (eUBM) associated to colonoscopy, in a longitudinal study to evaluate the progression of chemically-induced colonic lesions in mice, during a period of two months. The eUBM method complemented colonoscopy and enhanced the study, once the ultrasonic images allowed the detection of lesions underneath the epithelium. Potential future application of eUBM combined with colonoscopy could be in the monitoring of therapeutic efficacy of chemotherapeutic drugs *in vivo*.

M, Borges HL, Machado JC. Simultaneous follow-up of mouse colon lesions by colonoscopy and endoluminal ultrasound biomicroscopy. *World J Gastroenterol* 2013; 19(44): 8056-8064 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8056.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8056>

## INTRODUCTION

Colorectal cancer (CRC) has a high incidence in the world as it is the third most common cancer in men and women in developed countries<sup>[1]</sup>. It is estimated that more than 142000 people in the United States will be diagnosed with CRC in 2013<sup>[2]</sup>. In Europe, CRC is detected in approximately 413000 people each year, half of whom die during the course of the disease. Despite its high incidence and mortality rates, the majority of CRC-related deaths could be prevented through the implementation of powerful tools for CRC early detection and staging.

Currently, colonoscopy is the recommended screening method for CRC screening and follow-up, but it has some limitations. Studies have demonstrated that the detection of adenomas, serrated polyps and sessile serrated adenomas differs significantly among endoscopists<sup>[3]</sup>. Furthermore, colorectal neoplasms of a diminutive size (smaller than 10 mm) or nonpolypoid shape may be more easily overlooked during a routine colonoscopy<sup>[4-6]</sup>. The miss rate for CRC lesions may explain the high proportion (3.3%-12.4%) of proximal CRC that is diagnosed shortly after a clearing colonoscopy<sup>[7,8]</sup>. Therefore, the efforts of some research groups are focused on the development of other imaging methods that complement the results of a colonoscopy.

High frequency endoscopic ultrasonography (EUS) is a relatively new technique in which an ultrasonography probe is inserted into the accessory channel of a regular endoscope. EUS has the capacity to look deep below the lining of the colon and is a useful modality for transmural assessment of the bowel wall<sup>[9,10]</sup>. Usually, the ultrasound transducers used in EUS instrumentations operate at low frequencies (7.5-12 MHz), but higher ultrasound frequencies are also employed by using a mini-probe<sup>[11-13]</sup>. Higher ultrasound frequencies increase EUS resolution, allowing for the staging of colon tumors and the visualization of small colonic lesions. The use of high frequency mini-probe ultrasound for the diagnosis of mucosal and submucosal colorectal lesions and for the guidance of lesion resection has already been proposed as a safe and effective technique<sup>[13,14]</sup>. However, the role for high frequency mini-probe ultrasound in the routine diagnosis of colonic lesions has not been fully established<sup>[15-18]</sup>.

Animal models of diseases can be used to develop and evaluate new diagnostic tools before they are applied clinically. The development of non-invasive experimental imaging modalities allows for the study of the same ani-

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mal over time, enabling the investigation of disease development and therapeutic interventions. Mouse models of chemically induced CRC are highly reproducible, can be tested on animals with different genetic backgrounds and recapitulate human CRC. The use of an effective and valuable mouse model of chemically induced CRC can help investigators understand colonic tumorigenesis and to probe novel diagnostic platforms for use in clinical practice<sup>[19]</sup>.

Our group has previously used ultrasonic biomicroscopic (UBM) instrumentation, operating at 45 MHz, for *in vitro* imaging of chemically induced mouse CRC<sup>[20]</sup> to demonstrate that UBM is a feasible tool to identify the layers of mouse colon with adequate contrast between them and with sufficient resolution. Afterwards, endoluminal UBM (eUBM), operating at 40 MHz, was performed along with a colonoscopy, and simultaneous eUBM and colonoscopic images were generated *in vivo*<sup>[21]</sup>.

Recently, studies have verified the efficacy of UBM as a tool for longitudinal studies in mice: Harmon and co-workers<sup>[22]</sup> validated the use of 40 MHz extracorporeal UBM for carotid plaque development in mice; Tiwari *et al*<sup>[23]</sup> used a 40 MHz UBM for longitudinal monitoring of infliximab treatment efficacy in a mouse model of pancreatic cancer; Fernández-Domínguez *et al*<sup>[24]</sup> also used a 40 MHz UBM in a longitudinal study to evaluate the progression of fatty liver disease in mice; and Campos-Junior *et al*<sup>[25]</sup> analyzed the efficacy of UBM in the evaluation of induced ovarian follicular growth and ovulation in mice. Despite growing evidence confirming the efficacy of high frequency ultrasound in the monitoring of lesion progression, the ability of eUBM to diagnose colonic tumoral development in animal models has not yet been studied.

The present work comprises the use of eUBM instrumentation associated with colonoscopy in a longitudinal study to evaluate the progression of chemically induced colonic lesions in mice.

## MATERIALS AND METHODS

### Animals

Ten mice [*Mus musculus* (Linnaeus, 1758)] of both genders, with an average age of 7 wk, an average weight of 25 g, and  $p53^{+/+}$  and  $p53^{+/-}$  (heterozygous for tumor suppressor gene *Trp53*), were used. The mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, United States) and kept in the 129/SvJ background. We used the  $p53^{+/-}$  mice because *Trp53* mutations accelerate tumorigenesis in several tissues, including the colon<sup>[26]</sup>.

The animals were maintained at room temperature with the appropriate circadian cycle and diet. The Guide for Care and Use of Laboratory Animals (National Institutes of Health) was also considered.

Colon tumors were induced using a protocol (DAHE-ICB 042) approved by the Animal Care and Use Commit-

tee of the Biological Science Institute/Federal University of Rio de Janeiro. The studies involving colon imaging, such as eUBM combined with colonoscopy, were conducted under a protocol (71/08) approved by the Ethical Committee for Laboratory Animal Research/Federal University of Rio de Janeiro.

### Azoxymethane and dextran sulfate sodium carcinogenesis protocol

Inflammation-related colon tumors were induced using azoxymethane (AOM) and dextran sulfate sodium (DSS)<sup>[27-29]</sup>. AOM is a colon-specific carcinogen that can be combined with DSS, a mucosal-irritant agent, to mimic inflammation-associated colon carcinogenesis<sup>[29,30]</sup>. The animals were subjected to a single intraperitoneal (*ip*) injection of AOM (A5486; Sigma Aldrich, St. Louis, MO, United States) with a concentration of 12.5 mg/kg. One week after AOM administration, the mice were fed with water containing 3% DSS salt, 36000-50000 Da (02160110; MP Biomedicals, Santa Ana, CA, United States), for 1 wk. All of the mice received solid food and water *ad libitum*, with regular water given after the week of DSS intake.

### Endoluminal ultrasonic biomicroscopy system

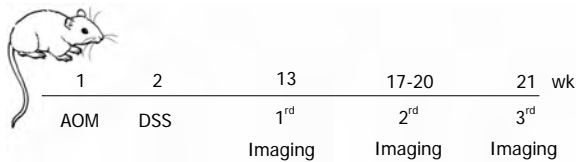
Briefly, images were generated by employing a 3.6-F diameter 40 MHz mini-probe catheter (Atlantis® SR Pro Coronary Imaging Catheter; Boston Scientific Corporation, Natick, MA, United States) mechanically driven by a motordrive unit (MD5; Boston Scientific Corporation, Natick, MA, United States). The ultrasonic transducer rotates 360° around its axis, providing cross-sectional ultrasound images of the colon wall. More details concerning the eUBM instrumentation are described in Alves *et al*<sup>[21]</sup>.

### Simultaneous eUBM and colonoscopic image acquisition

Colonoscopy was used simultaneously with eUBM and served to guide the mini-probe through the colon. The ultrasound mini-probe catheter was inserted into the accessory channel of a pediatric flexible bronchofiberscope (FB120P; Fujinon, Tokyo, Japan), allowing simultaneous acquisition of colonoscopy and eUBM images. The bronchofiberscope has a total length of 920 mm and outer diameters of 2.8 and 2.7 mm for the flexible and distal-end portions, respectively.

To ensure that the colonoscopy and eUBM techniques acquired simultaneous images from the same region, the ultrasonic transducer, at the mini-probe imaging core tip, was positioned outside of the distal end accessory channel extremity, while still as close as possible to the bronchofiberscope extremity. The mini-probe telescoping shaft section was used to advance and retract the imaging core, placing it in the correct position.

During image acquisition, the mice were anesthetized with isoflurane (Cristália; São Paulo, Brazil) at 1.5% in 1.5 L/min oxygen, using a laboratory animal anesthe-



**Figure 1** Schematic overview of the azoxymethane and dextran sulfate sodium model and subsequent image acquisition. A single azoxymethane (AOM) *ip* injection was given to 6-wk-old mice (week 1). One week later (week 2), 3% dextran sulfate sodium (DSS) administration was given in the drinking water for 7 d, followed by regular water. The first endoluminal ultrasonic biomicroscopy (eUBM) and colonoscopic images were acquired at week 13, the second acquisition was from weeks 17-20, and the third acquisition was at week 21.

sia system (EZ-7000; Euthanex, Palmer, PA, United States). The animals were kept in a supine position over a stainless steel heated surgical waterbed at 37 °C using the T/Pump System (Gaymar, Orchard Park, NY, United States). Before the examination, an enema was performed with 1 mL of water to remove feces. Subsequently, the flexible bronchofiberscope containing the ultrasound mini-probe catheter was introduced into the descending colon. Both eUBM and colonoscopy images were captured simultaneously and stored when a lesion was detected by colonoscopy or when the eUBM image revealed a modified colon wall anatomy. During the procedure, the colon was irrigated with water that was injected through a flush port of the mini-probe catheter and that acted as the ultrasound coupling medium between the transducer and the colon wall.

### Study design

The sequential evaluation of colonic lesions by simultaneous *in vivo* eUBM and colonoscopic imaging started at 13 wk after AOM administration and was performed at three different time-points, according to Figure 1: the first one at week 13, the second one between weeks 17 and 20 and the last one at week 21.

Colon lesion images acquired at the first time-point for each mouse were compared with subsequent eUBM/colonoscopic images of the same sites obtained in the following acquisitions. After the last eUBM examination, the images of each lesion were separated for subsequent comparison with histopathology.

### Histological analysis

Once the acquisition of the last image for each animal was completed, each anesthetized mouse was euthanized by cervical dislocation. The distal colon was excised, cleaned and fixed in 4% formaldehyde for 16 h before paraffin embedding. The paraffin-embedded tissues were cross-sectioned (5 µm) stepwise transversally to the colon longitudinal axis and stained with hematoxylin and eosin. All stained sections were analyzed by light microscopy and compared with the ultrasonic images, whose frames were obtained from the same lesions observed with the eUBM and/or colonoscopy.

**Table 1** Simultaneous endoluminal ultrasonic biomicroscopy and colonoscopy image acquisition on colon tumor-bearing mice

Mouse number	Weeks after AOM administration		
	1 <sup>st</sup> eUBM	2 <sup>nd</sup> eUBM	3 <sup>rd</sup> eUBM
1	13	-	-
2	13	-	-
3	13	18	-
4	13	20	-
5	13	20	-
6	13	20	-
7	13	20	-
8	13	17	21
9	13	17	21
10	13	17	21

eUBM: Endoluminal ultrasonic biomicroscopy; AOM: Azoxymethane.

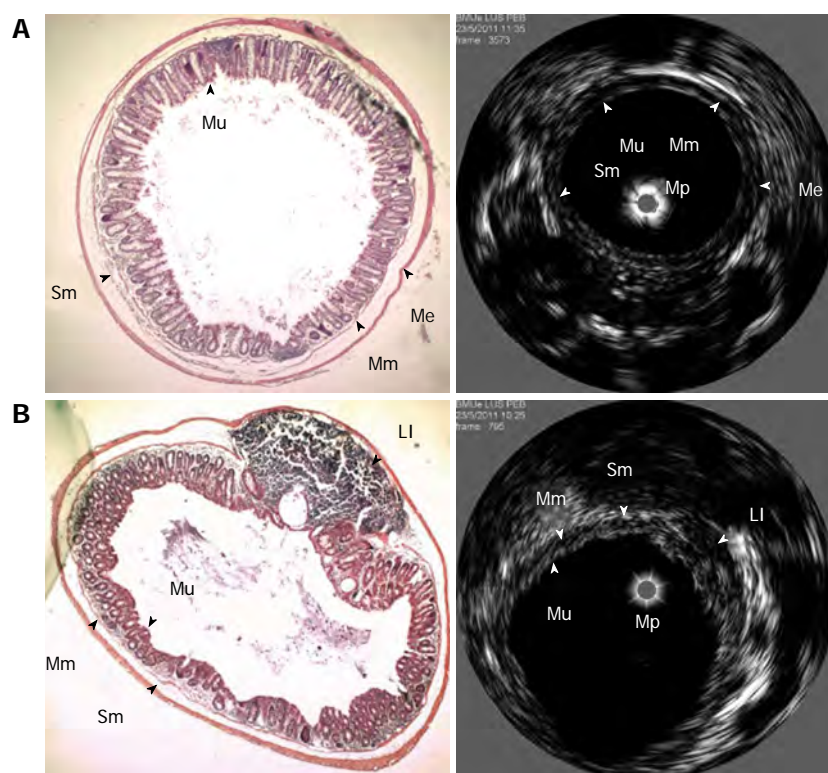
## RESULTS

The time-points for image acquisition of each animal are presented in Table 1. All 10 mice had eUBM and colonoscopic images acquired at week 13. Two animals died immediately after the first imaging acquisition and, consequently, only eight mice were subjected to the second eUBM/colonoscopy imaging acquisition. Due to the advanced stage of colonic tumorigenesis, five animals died after the second time-point image acquisition, and thus, only three were subjected to the third eUBM/colonoscopy imaging acquisition.

An example of interrelated eUBM and histological images of a healthy section from the mouse colon is presented in Figure 2A. The mucosal layer is seen as a hyperechoic circular layer (the first hyperechoic layer moving away from the mini-probe axis), followed by a hypoechoic layer representing the muscularis mucosae. The submucosa corresponds to a hyperechoic layer, followed by the muscularis externa, the second hypoechoic layer. At the center of the lumen is the ultrasound mini-probe, represented by a gray circle. An eUBM image of a colonic lymphoid infiltrate, represented by a hypoechoic region between the mucosa and muscularis externa layers, is presented in Figure 2B with the corresponding histological image.

An example of an eUBM image of a pedunculated tumor, whose size increased during the 6 wk between the first and second time-points, is presented in Figure 3A. At the first eUBM exam, a small elevation in the mucosa layer is seen, indicating an early adenoma. At this time, colonoscopy was unable to visualize the lesion. Six weeks later, eUBM showed that the adenoma had increased in size, and the lesion was then observed in the colonoscopic image. Figure 3B presents an eUBM image of a pedunculated adenoma, whose size remained virtually unchanged between the first and third image acquisitions. The adenoma was also visualized in all of the colonoscopy sections. Finally, a sequence of three eUBM images of a lesion that decreased in size during the observation period is depicted in Figure 3C. This lesion was identified at the first eUBM exam (Figure





**Figure 2** Correlation between endoluminal ultrasonic biomicroscopy and histological images. A: Endoluminal ultrasonic biomicroscopy (eUBM) (right) and the corresponding hematoxylin and eosin-stained histological section (left,  $\times 40$  magnification) obtained from a healthy region of a mouse colon. The eUBM image displays two hyperechoic layers: mucosa (Mu) and submucosa (Sm) and two hypoechoic layers: muscularis mucosae (Mm) and muscularis externa (Me). The ultrasound catheter mini-probe (Mp) is at the center of the lumen; B: eUBM (right) and the corresponding hematoxylin and eosin-stained histological section (left,  $\times 40$  magnification) obtained from a mouse colon containing a lymphoid infiltrate in the colonic wall. The eUBM image displays the mucosa (Mu), muscularis mucosae (Mm) and submucosa layer (Sm). The lymphoid infiltrate (LI) lesion is seen as a hypoechoic region underneath the mucosa. The ultrasound catheter mini-probe (Mp) is at the center. All layers identified in the ultrasound images are well correlated with the histological images from the same site.

3C-a) as a mucosal hyperplasia with ulceration at the top. The ulceration was also visualized by colonoscopy. Four weeks later, at the second eUBM exam (Figure 3C-b), the mucosal hyperplasia had decreased, and a hypoechoic area underneath the mucosa was observed, indicating the emergence of an inflammatory infiltrate. At this point, colonoscopy showed no alterations in this colonic section. At the last eUBM exam (Figure 3C-c), both the mucosal hyperplasia and lymphoid infiltrate had almost completely disappeared. Histological analysis of the same section confirmed the presence of the remaining diminutive lymphoid infiltrate section (Figure 3C-d).

Colonic lesions detected by either the last eUBM or colonoscopy, and confirmed by *post mortem* histology, are indicated in Table 2. Altogether, eUBM identified all of the lesions (tumors, lymphoid infiltrate and mucosal thickening), while colonoscopy identified just 76% of them. Colonoscopy identified all colonic tumors but failed to diagnose lymphoid infiltrates and increased mucosal thickness and failed to differentiate lymphoid infiltrates from small adenomas.

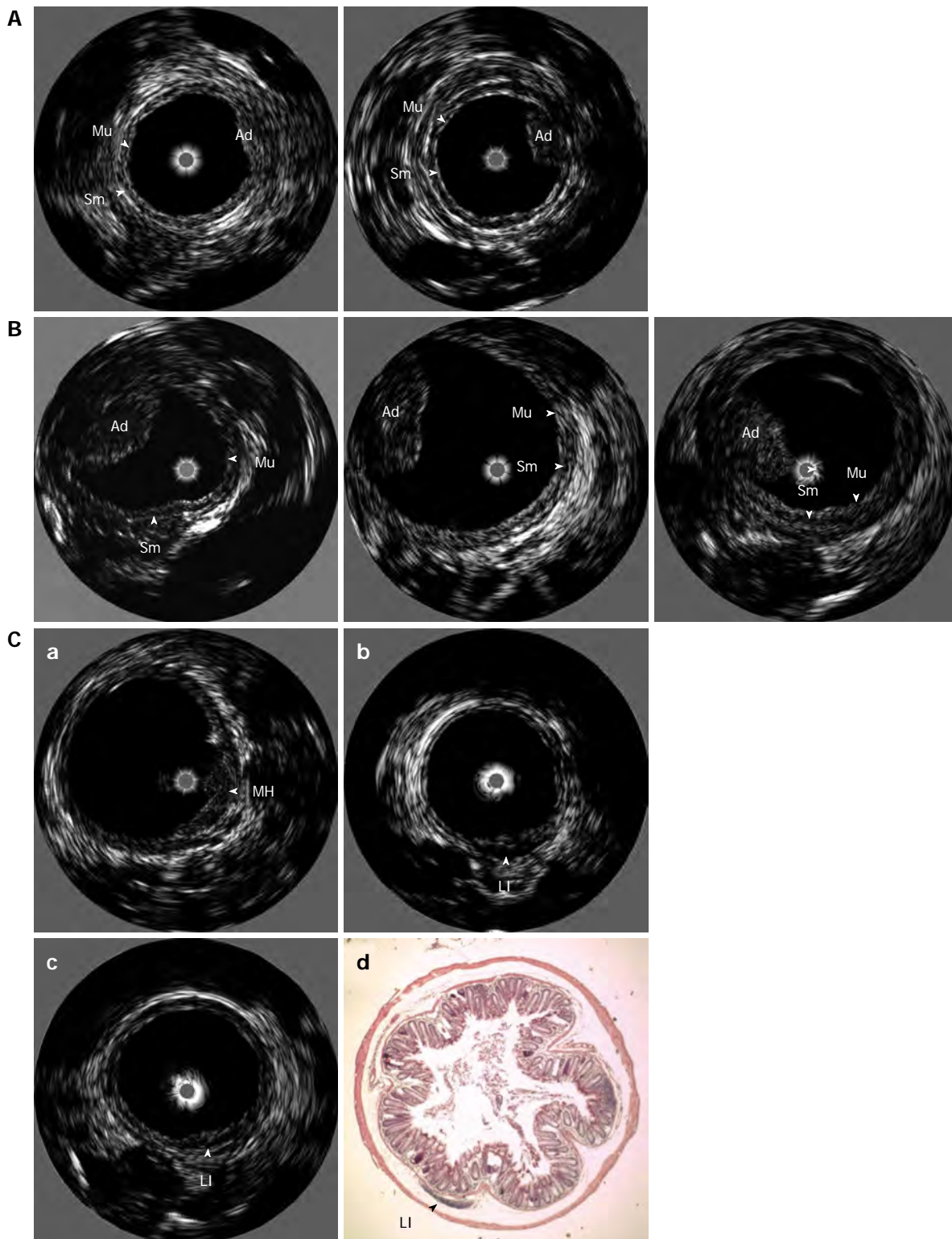
Additionally, the lesion progression outcomes, based on eUBM image analysis, are presented in Table 2. During the observation period, most of the lesions (approximately 67%) increased in size, approximately 14% remained unchanged and 19% regressed.

## DISCUSSION

This report describes the use of a eUBM imaging system for the detection and follow-up of mouse colonic lesions. The simultaneous use of eUBM with colonoscopy was able to detect, diagnose and analyze the progression of tumoral and non-tumoral lesions in a CRC mouse model. Our group has previously demonstrated that two UBM systems, one operating at 45 MHz and the other at 40 MHz, could diagnose mouse colonic lesions *in vitro*<sup>[20]</sup> and *in vivo*<sup>[21]</sup>, respectively. Here, we have demonstrated that a variety of colon lesions can be detected by eUBM in a minimally invasive way. In contrast to histopathological analysis, eUBM can be employed to make repeated measures on the same animal, facilitating the investigation of pathological processes and therapies.

Similar to the previous work, the ultrasound images obtained with eUBM also allowed for the visualization of normal colonic layers: the mucosa, muscularis mucosae, submucosa and muscularis externa (Figure 2A), as well as colon alterations, such as lymphoid infiltrates, ulcerations and tumors (Figure 2B and Figure 3). Confirming our previous findings, lymphoid infiltrates appear as hypoechoic regions underneath a hyperechoic layer representing the mucosa. Colon tumors appear as hyperechoic masses above the mucosa layer. This characteriza-





**Figure 3** Endoluminal ultrasonic biomicroscopy images. **A:** Show increase in tumor volume. Endoluminal ultrasonic biomicroscopy (eUBM) colon images acquired at the first (left) and second (right) time-points from azoxymethane (AOM)-dextran sulfate sodium (DSS)-treated mice. The volume of the pedunculated adenoma (Ad) increased between the first and second eUBM examinations; **B:** Show no alteration in tumor volume. eUBM colon images acquired at the first (left), second (middle) and third (right) time-points from AOM-DSS-treated mice. The lesion observed is a pedunculated tumor. Images show that the tumor volume is unchanged during the observation period; **C:** Show reduction in lesion size. eUBM colon images acquired at the first (a), second (b) and third (c) time-points from AOM-DSS-treated mice. The lesion observed at the first eUBM image is a mucosa hyperplasia (MH) with ulceration at the top. In the subsequent eUBM image, the MH has decreased, and a lymphoid infiltrate (LI) has appeared in the submucosa layer. In the third and last eUBM image, MH and LI have almost completely disappeared, which is confirmed by histological analysis (d,  $\times 40$  magnification). Mu: Mucosa; Sm: Submucosa.

tion is of great importance because it could be used to distinguish small adenomatous polyps from lymphoid

**Table 2** Lesion progression observed by longitudinal endoluminal ultrasonic biomicroscopy and colonoscopic imaging

Animals	Animal lesion	Lesion detection						Lesion progression						
		eUBM			Colonoscopy			Size			Lesion type			
		N°	Yes	No	Yes	No	Obs	↑	↓	=	Tu	LI	MT	
1	L1-1	1	✓		✓			✓				✓	✓	
2	L1-2	1	✓		✓		✓	✓				✓		
3	L1-3	2	✓		✓			✓				✓		
	L2-3		✓		✓			✓				✓		
4	L1-4	2	✓		✓			✓				✓		
	L2-4		✓		✓					✓			✓	
	L3-4		✓		✓			✓					✓	
	L4-4		✓					✓						✓
5	L1-5	2	✓		✓			✓				✓		
	L2-5		✓				✓	✓					✓	
6	L1-6	2	✓		✓			✓				✓		
	L2-6		✓		✓			✓					✓	
	L3-6		✓		✓						✓	✓		
	L4-6		✓			✓					✓		✓	
7	L1-7	2	✓			✓			✓				✓	
	L2-7		✓				✓	✓				✓		
8	L1-8	3	✓		✓				✓			✓		
	L2-8		✓		✓			✓				✓		
	L3-8		✓			✓				✓			✓	
9	L1-9	3	✓						✓				✓	✓
10	L1-10	3	✓		✓			✓				✓		

Tu: Tumor; LI: Lymphoid infiltrate; MT: Mucosal thickening; eUBM: Endoluminal ultrasonic biomicroscopy; Obs: impossible to analyze due to colonic hemorrhage or feces. ↑: Increased lesion size; ↓: Decreased lesion size; =: No alteration.

hyperplasias, both seen by colonoscopy as mucosal elevations.

The correct detection and diagnosis of colonic neoplasias during a colonoscopy is essential for CRC prevention. The ranges for adenoma detection rates during a routine colonoscopy could vary up to 37% among endoscopists<sup>[3]</sup>, increasing the chances to misdiagnose CRC. Most postcolonoscopy cancers have a small macroscopic appearance<sup>[31,32]</sup> and in these cases, the simultaneous use of eUBM with colonoscopy could aid in accurately detecting submucosal invasion in colonic lesions.

The small elevation in the mucosa layer observed with eUBM and registered in Figure 3A was not detected by colonoscopy. Perhaps, this fact was due to the poor bronchofiberscope image quality and could be overcome with high-resolution scopes designed specifically for work with rat and mouse models of colonic diseases<sup>[33]</sup>. These high-resolution scopes are usually rigid telescopes and a working channel is formed in a space between an operating sheath and the telescope external wall. Although the bronchofiberscope used in the present work is unable to produce high-resolution images, it has the advantage of being flexible. According to the authors' experience, this facility of the bronchofiberscope is important to position the eUBM mini-probe tip close to a lesion, which improves the lesion visualization, or at the center of the colon lumen in order to generate circular eUBM images of the colon.

According to the results obtained with this longitudinal evaluation of inflammation-associated colon tumor

progression, most of the lesions increased in size, mimicking human cancer development. All tumoral lesions were diagnosed at the first analysis (13 wk after AOM administration), even when the size was very diminutive. The diameter of the smallest detected tumoral lesion was 0.45 mm, and eUBM was able to identify even smaller structures, such as mucosal elevations with a height of 0.1 mm. Of all the lesions detected by eUBM, approximately 15% of them (four lesions) showed a reduction in size. Of these lesions, two were lymphoid infiltrates, whose size reduction indicated inflammation resolution; one was an increase in mucosal thickness that regressed; and the last one was a small adenoma whose tumoral mass decreased. Besides pedunculated and depressed lesions, we have also detected flat lesions in animal models of CRC and current work is being conducted using *p53* knockout mice, which develop flat lesions with a higher incidence than wild type mice<sup>[34]</sup>, to evaluate the eUBM sensitivity.

The data presented here suggest that the use of high-resolution endoluminal ultrasound is a valuable tool to evaluate the progression of colonic lesions. eUBM detected alterations in mouse colonic lesion morphology and in adenoma volume throughout the examination period. Longitudinal high-resolution ultrasound measurements could be helpful in the monitoring of therapeutic efficacy of chemotherapeutic drugs *in vivo*. Additionally, this technique allows for the study of lesion progression in animal models, providing detailed insights into the biology of tumor development.

The potential of the eUBM technique to differentiate malignant from non-malignant lesions is yet to be implemented. Nowadays, the technique of narrow band imaging (NBI) has the capacity to diagnose colon lesion malignancy in real time based in mucosal and superficial vascular structures imaging enhancement<sup>[35]</sup>. However, eUBM has the potential to detect lesion penetration depth through submucosal layers. Both methodologies have their advantages and limitations and could be performed simultaneously to complement each other.

Another advantage of longitudinal eUBM imaging is the possibility to use ultrasound contrast agents to target specific molecules involved in tumor development, such as the angiogenic promoter vascular endothelial growth factor, providing a minimally invasive tool for molecular diagnosis. This new modality of molecular imaging is now being tested in preclinical models with successful results in the characterization of tumor response to anti-angiogenic treatment<sup>[36-38]</sup>.

In summary, the simultaneous use of eUBM with colonoscopy enhances the ability to correctly diagnose and follow-up colonic lesions, offering rapid imaging acquisition and distinct advantages because high-resolution transmural imaging of the bowel wall improves lesion detection and cost-effectiveness.

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## COMMENTS

### Background

Colonoscopy is the recommended screening method for colorectal cancer screening and follow-up, but it fails to detect some small or nonpolypoid lesions. Therefore, the development of other imaging methods that complement the results of colonoscopy is extremely important. The authors have previously show that endoluminal ultrasonic biomicroscopy (eUBM) associated to colonoscopy improves the detection and diagnose of inflammatory and tumoral colonic lesions in animal models. Here the authors analyze the capacity of eUBM to evaluate the progression of chemically-induced colonic lesions in mice.

### Research frontiers

The use of eUBM to diagnose mucosal and submucosal colorectal lesions and to guide lesion resection has already been proposed as a safe and effective clinical technique. However, its significant role in the routine diagnosis of colonic lesions has not yet been established. The use of animal models contributes in the development and evaluation of new diagnostic tools before they are completely clinically applied.

### Innovations and breakthroughs

A step forward of previous work done by our group, which now includes the longitudinal study of lesion progression.

### Applications

The current results suggest that the use of eUBM simultaneously to colonoscopy enhances the ability to correctly diagnose and follow up colonic lesions. In addition to its potential clinical application, eUBM can aid investigators to study colonic tumorigenesis processes and to evaluate novel therapeutic agents for colorectal cancer.

### Terminology

eUBM, also known as high frequency endoscopic ultrasonography is a relatively new technique in which an ultrasound probe is inserted into the accessory channel of a regular endoscope. eUBM is an useful modality for transmural assessment of the bowel wall.

### Peer review

The authors describe the evaluation of the potential use of colonoscopy and eUBM to track the progression of mouse colonic lesions. This is a clinically very interesting study.

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## Effects of disease severity and necrosis on pancreatic dysfunction after acute pancreatitis

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### Abstract

**AIM:** To evaluate the effects of disease severity and necrosis on organ dysfunctions in acute pancreatitis (AP).

**METHODS:** One hundred and nine patients treated as AP between March 2003 and September 2007 with at least 6 mo follow-up were included. Patients were classified according to severity of the disease, necrosis ratio and localization. Subjective clinical evaluation and fecal pancreatic elastase- I (FPE- I) were used for exocrine dysfunction evaluation, and oral glucose tolerance test was completed for endocrine dysfunction. The correlation of disease severity, necrosis ratio and localization with exocrine and endocrine dysfunction were investigated.

**RESULTS:** There were 58 male and 51 female patients, and mean age was  $56.5 \pm 15.7$ . Of the patients, 35.8% had severe AP (SAP) and 27.5% had pancreatic necrosis. Exocrine dysfunction was identified in 13.7% of the patients [17.9% were in SAP, 11.4% were in mild AP (MAP)] and 34.7% of all of the patients had endocrine dysfunction (56.4% in SAP and 23.2% in MAP). In patients with SAP and necrotizing AP (NAP),

FPE- I levels were lower than the others ( $P < 0.05$  and  $0.001$  respectively) and in patients having pancreatic head necrosis or near total necrosis, FPE-1 levels were lower than  $200 \mu\text{g/g}$  stool. Forty percent of the patients who had undergone necrosectomy developed exocrine dysfunction. Endocrine dysfunction was more significant in patients with SAP and NAP ( $P < 0.001$ ). All of the patients in the necrosectomy group had endocrine dysfunction.

**CONCLUSION:** Patients with SAP, NAP, pancreatic head necrosis and necrosectomy should be followed for pancreatic functions.

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**Key words:** Acute pancreatitis; Exocrine dysfunction; Endocrine dysfunction; Pancreas function test; Pancreatic necrosis

**Core tip:** The aim of this study was to evaluate the effects of disease severity and necrosis on organ dysfunctions in acute pancreatitis (AP). Exocrine and endocrine dysfunctions were investigated according to disease severity and necrosis ratio after acute pancreatitis. Exocrine dysfunction was identified in 13.7% of the patients [17.9% were in severe AP (SAP), 11.4% were in mild AP (MAP) and 34.7% of all of the patients had endocrine dysfunction (56.4% in SAP and 23.2% in MAP)]. Forty percent of the patients who had undergone necrosectomy developed exocrine dysfunction. Endocrine dysfunction was more significant in patients with SAP and NAP. All of the patients in the necrosectomy group had endocrine dysfunction. Patients with SAP, NAP, pancreatic head necrosis and necrosectomy should be followed for pancreatic functions.

Garip G, Sarandöl E, Kaya E. Effects of disease severity and necrosis on pancreatic dysfunction after acute pancreatitis. *World J Gastroenterol* 2013; 19(44): 8065-8070 Available from: URL:

## INTRODUCTION

Eighty percent of the pancreatic mass is devoted to exocrine function, and the remaining part is responsible for endocrine function, which is crucial to the maintenance of homeostasis of the body<sup>[1]</sup>. Clinically, the severity of acute pancreatitis (AP) varies significantly. Some patients experience a mild form (mild AP, MAP) of the disease (80%-90% of all cases), which is a self-limiting condition with patients recovering within 3-4 d after onset of the disease. Serious insult occurs in 20%-30% of the cases in the first week after AP attack and mortality can be 30% in the severe form. Pancreatic necrosis develops in 20% of all cases<sup>[2,3]</sup>. Both the presence and extent of the necrosis affects the clinical course of the disease. Necrosis larger than 50% of the pancreatic mass significantly increases the local and systemic complication rates<sup>[4]</sup>. There are contradictory results from evaluations of exocrine and endocrine dysfunction in mild and severe cases<sup>[5,6]</sup>. In 1984 at the Marseille symposium, it was accepted that pancreatic injury is temporary and endocrine and exocrine functions recover during the following month<sup>[7]</sup>. But there are some contradictory reports claiming that pancreatic injury is persistent<sup>[8-10]</sup>. Currently, it is accepted that pancreatic function recovers in the absence of pancreatic necrosis and if necrosectomy is not performed<sup>[11]</sup>.

Previous studies have demonstrated that severity of AP, extent of the pancreatic necrosis and the cause of pancreatitis are closely related to the magnitude of pancreatic dysfunction<sup>[12,13]</sup>. After severe acute pancreatitis (SAP), Bozkurt *et al*<sup>[14]</sup> and Boreham *et al*<sup>[15]</sup> reported 85% and 86% pancreatic exocrine dysfunction, respectively. Boreham also reported 13% pancreatic exocrine dysfunction after mild cases. The etiologic factor is also correlated with the level of pancreatic injury; acute pancreatitis due to alcohol consumption may cause pancreatic dysfunction<sup>[9]</sup>. While it is mostly  $\beta$  cell injury that induces endocrine dysfunction, insulin resistance may also contribute to glucose intolerance<sup>[16-18]</sup>. Endocrine dysfunction is reported in 15%-35% of the cases<sup>[12,14,18]</sup>.

There is no consensus about the frequency and severity of endocrine and exocrine dysfunction due to acute pancreatitis. Also, experts do not agree on the necessity of enzyme supplementation following the discharge of these patients<sup>[15]</sup>. Our study aimed to clarify the relationship between pancreatic dysfunction, the severity of the disease and the extent of the pancreatic necrosis.

## MATERIALS AND METHODS

This study was undertaken in the Uludag University Department of Surgery-Bursa, Turkey. From March 2003 to October 2007, 216 consecutive patients with AP were evaluated in our center. This study was approved by the

Institutional Review Board of Uludag University (September 11<sup>th</sup> 2007, No: 2007-14/64). Patients who died ( $n = 16$ ) or with less than 6 mo of follow-up after onset of the disease ( $n = 11$ ) were excluded. All of the patients were invited to the hospital to participate the study by phone or mail. Fifty-five patients could not be contacted due to change of address and 25 patients declined to participate. The remaining 109 patients were included the study, and written informed consent was obtained from each subject.

The data of the patients who were treated for AP were recorded prospectively in previously prepared forms. Diagnosis of AP and determination of its etiology were based on clinical evaluation, serum and urine amylase (higher than three times the upper level of normal was considered diagnostic), liver function tests, serum triglycerides, calcium, alkaline phosphatase and abdominal ultrasound (US) at the admission. Within 72 h following admission, contrast enhanced abdominal computed tomography (CECT) was performed. Patients with gallstones on US were assumed to be cases of biliary pancreatitis; patients consuming large amounts of alcohol (but not having chronic pancreatitis) were considered as having alcoholic pancreatitis. In patients with high levels of serum fat (triglyceride level more than 1000 mg/dL), hyperlipidemia was accepted as the etiological factor. Patients with undetermined etiology were considered to be idiopathic cases. For prognostic evaluation and classification of the severity of the disease, the Acute and Physiology and Chronic Health Evaluation II scoring system (APACHE II) was used. Patients with APACHE II  $\geq 8$  were accepted as severe AP (SAP). While patients who had  $< 8$  score were accepted as mild AP (MAP) and treated conservatively (fluid resuscitation only), patients who had SAP were treated with aggressive fluid resuscitation, nutritional support (enteral or parenteral) and antibiotic prophylaxis. If the patient's clinical status deteriorated and CECT findings revealed infected necrosis or if fine needle aspiration cytology demonstrated infection, they were treated surgically. The Beger procedure (open necrosectomy and closed continuous lavage) plus feeding jejunostomy was our treatment of choice. In some of the cases, more conservative procedures (*i.e.*, percutaneous drainage) had to be undertaken due to the patient's condition.

Following designation of the participating patients and receiving informed consent, specific questions were asked of the patients to evaluate the clinical findings of the pancreatic exocrine insufficiency. The findings were recorded on the Subjective Clinical Evaluation (SCE) form (Table 1). If a patient answered "yes" to even only one of the questions on the SCE form, the test was accepted as positive. Exocrine pancreatic function was also evaluated using the fecal pancreatic elastaz-1 (FPE-1) test in a random stool sample. Patients who were having pancreatic enzyme supplements were instructed to taper their enzyme supplementation 1 mo before the FPE-1 test. Stool samples were collected from the patients and

**Table 1 Subjective clinical evaluation test**

Age/sex:
- Did DM develop after AP?
(a) yes (b) no
- Any abdominal pain, discomfort, steatorrhea, weakness, weight loss, lack of appetite after AP?
(a) yes (b) no
- Did you use pancreatic enzyme supplementation? If so, how long time did you use it?
(a) yes (b) no

AP: Acute pancreatitis; DM: Diabetes mellitus.

stored at -20 °C. FPE-1 level was measured by a commercially available Enzyme-Linked Immunosorbent Assay kit (Bioserv Diagnostics, BS-86-01, Rostock, Germany 2007) according to manufacturer's instructions. Stool elastase 1 concentration higher than 200 µg/g stool indicated normal pancreatic function, whereas concentration of 100 to 200 µg/g stool indicated mild to moderate pancreatic insufficiency, and concentrations below 100 µg/g stool were qualified as pointing to severe pancreatic insufficiency<sup>[12]</sup>.

In all of the patients without diagnosed insulin-dependent diabetes mellitus (DM), endocrine pancreatic function was assessed by oral glucose tolerance test (OGTT). Measurement of glucose concentration was made on patients who had fasted overnight and discontinued drugs and food that could have affected the results. After taking blood samples for fasting blood glucose measurement during the OGTT, the patients drank 75 g glucose dissolved in 300 mL, and their blood glucose concentration was measured at 30, 60, 90 and 120 min. Basal blood glucose levels < 120 mg/dL and between 126-200 mg/dL at 120 min were accepted as impaired glucose tolerance. Basal glucose levels > 126 mg/dL and > 200 mg/dL at 120 min were accepted as DM.

The association between pancreatic dysfunction (either SCE or FPE-1 level) and disease severity and necrosis was investigated.

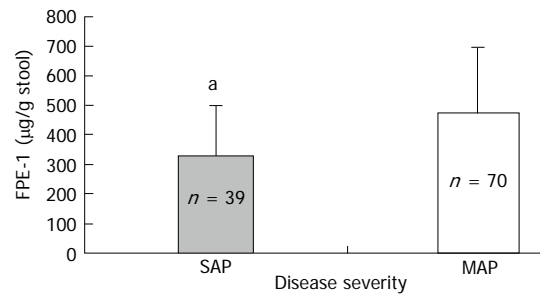
### Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows (Chicago, IL). Non-parametric tests were used to analyze the data. When comparing more than 3 groups, the Kruskal-Wallis test was used. Comparison between 2 groups was made with Mann-Whitney *U* test. The  $\chi^2$  test was used to compare categorical variables. A *P* value of < 0.05 was considered significant.

## RESULTS

### Patient demographics

A total of 109 patients with a mean follow-up of 32 mo (range: 6-48 mo) was included in this study. Fifty-eight of the subjects were male (53.2%), and 51 were female (46.8%). The mean age of the patients was  $56.5 \pm 15.7$  (range: 19-89) years. The etiologies were biliary (66%),



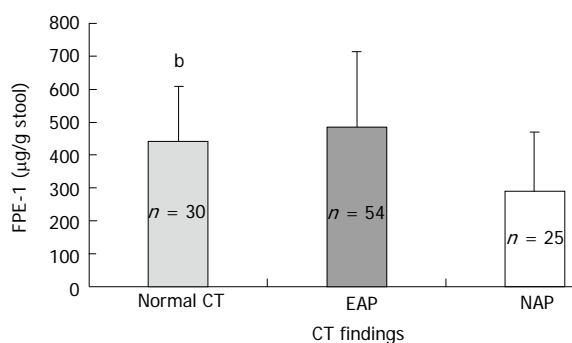
**Figure 1 Disease severity and fecal pancreatic elastase- I level.** Fecal pancreatic elastase- I (FPE-1) level was lower in severe acute pancreatitis (SAP) than mild acute pancreatitis (MAP) ( $P < 0.05$ ). FPE levels are  $330.9 \pm 170.6$  µg/g and  $475.2 \pm 223$  µg/g stool in SAP and MAP, respectively.

idiopathic (15.5%), alcohol (8.2%), hyperlipidemia (4.6%), endoscopic retrograde cholangio-pancreatography related (2.7%) and drug-related (2.7%). According to the APACHE II scoring system, 35.8% of the patients were SAP, and the remaining were MAP. Necrosis was found in 27.5% of the patients, and there was only pancreatic edema in 49.6% of the patients on CECT examination. Almost 23% of the CECT findings were noted to be normal. While necrosis was found in 59% of the SAP cases, it was found in 10% of MAP. On the other hand, of the patients who had necrosis according to CECT findings, 76.6% had SAP, and the remaining had MAP according to APACHE II criteria. Twenty-six percent of the patients who had pancreatic edema on CECT scan had SAP.

Five patients were operated on due to infected pancreatic necrosis, and a Beger procedure plus feeding jejunostomy was performed. Cystoenterostomy was performed in 17 cases due to a pancreatic pseudocyst. Percutaneous drainage was performed in 7 cases for pancreatic and peripancreatic abscesses. The remaining patients were treated medically. Before starting the study, 50.4% of the patients were taken pancreatic enzyme supplementation.

### Exocrine dysfunction after acute pancreatitis

Exocrine dysfunction was detected in 13.7% of the patients according to both subjective clinical evaluation and the FPE-1 test. It was found in 17.9% with SAP and in 11.4% with MAP. Four patients in the SAP group had severe exocrine dysfunction according to FPE-1 measurement, and 11 patients had moderate exocrine dysfunction (7 of them in the MAP and 4 in the SAP group). Disease severity and necrosis were not associated with subjective clinical evaluation (Table 2). FPE-1 was lower in the SAP than MAP group and was lower in patients with necrotizing AP (NAP) than those without necrosis (Figures 1 and 2). FPE-1 was lower in cases with pancreatic head or near total necrosis than patients with necrosis at other localizations (Figure 3). This was under the critical level of FPE-1 (200 mg/g stool). There was no significant correlation between FPE- I level and subjective clinical evaluation.



**Figure 2** Fecal pancreatic elastase- I levels and contrast enhanced abdominal computed tomography findings. Fecal pancreatic elastase- I (FPE-1) level was significantly lower in necrotizing acute pancreatitis (NAP) than the patients without pancreatic necrosis ( $^bP < 0.001$ ). FPE- I levels as follows;  $292.43 \pm 178.78$ ,  $487 \pm 226.21$ , and  $443.91 \pm 167.83$  µg/g stool in NAP, edematous acute pancreatitis (EAP) and normal contrast enhanced abdominal computed tomography (CT) groups, respectively.

**Table 2** The association between disease severity and contrast enhanced computed tomography findings and exocrine dysfunction *n* (%)

Disease severity and CT findings	Exocrine dysfunction	P value
SAP	7/39 (17.9)	NS
MAP	8/70 (11.4)	NS
NAP	8/30 (26.6)	NS
EAP	5/54 (9.2)	NS
Normal CT	2/25 (8.0)	

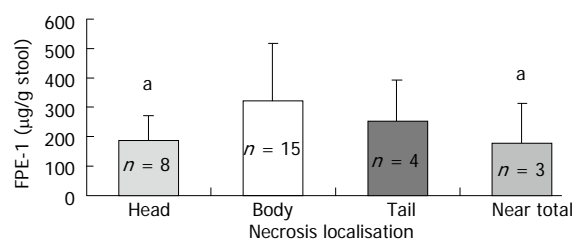
CT: Computed tomography; SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; NAP: Necrotizing acute pancreatitis; EAP: Edematous acute pancreatitis; NS: Not significant.

### Endocrine dysfunction after acute pancreatitis

DM was detected in 11.9% of the cases before the AP attack. DM and impaired glucose tolerance were detected in 30.2% and 4.5% of the remaining cases, respectively, according to the OGTT test. Therefore, endocrine dysfunction was noted to be present in 34.7% of the cases (56.4% with SAP and 23.2% with MAP). According to CECT findings, patients with necrosis had more severe endocrine dysfunction than patients without necrosis (endocrine dysfunction rate was 66.6% in NAP, 27.8% in Edematous AP (EAP) and 12% in normal CECT) (Figure 4).

## DISCUSSION

AP is a mediator disease caused by proinflammatory cytokine release and has a clinical picture ranging from mild disease to multiorgan failure and sepsis. The clinical picture is serious in 15%-20% of patients; complications can develop and mortality can be seen in these patients. Pancreatic necrosis develops in 20%-30% of the patients<sup>[3]</sup>. In this series, SAP was diagnosed in 35.8% and NAP was determined in 27.5% of the AP cases. The fact that SAP and NAP rates in our series are higher than those in the literature might be due to our hospital being the tertiary



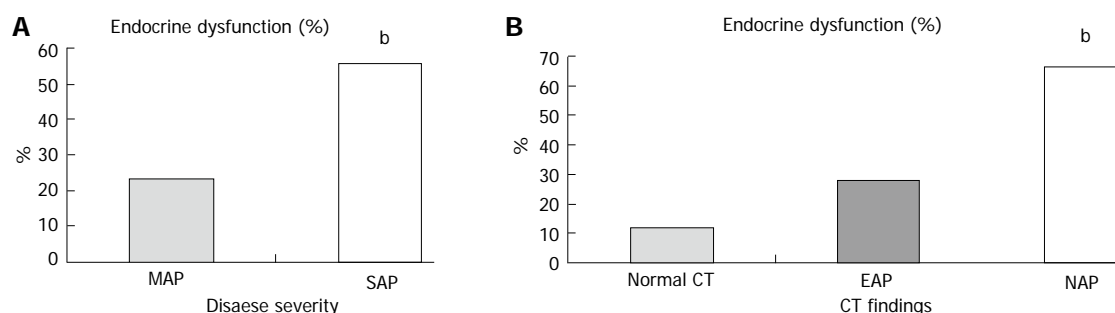
**Figure 3** Necrosis localization and fecal pancreatic elastase- I levels. Fecal pancreatic elastase- I (FPE-1) level was lower in head and near total necrosis of the pancreas than body or tail necrosis ( $^aP < 0.05$ ). FPE-1 levels as follows;  $189.18 \pm 82.7$ ,  $324.86 \pm 194$ ,  $258.06 \pm 134.63$  and  $181.12 \pm 134.25$  µg/g stool in head, body, tail and near total necrosis groups, respectively.

care referral center.

Currently, there is no consensus on whether or not pancreatic functions recover and to what extent after an AP attack. Full pancreatic functional recovery has been reported in some studies<sup>[6,19,20]</sup>, whereas others have reported that both endocrine and exocrine insufficiency might develop<sup>[8-10]</sup>. The research is not conclusive or sufficient to answer the question. The results of previous studies remain conflicting because of very small patient numbers and non-homogenous etiologies that affect pancreatic functions. Using ineffective and non-standardized tests can also lead to some mistakes and controversies<sup>[11]</sup>. In the current study, we used subjective clinical evaluation and the FPE-1 test, which has a relatively high sensitivity for exocrine function. Pancreatic elastase is a specific protease of humans and it undergoes minimal breakdown during intestinal transit. Strong parallelism between stool FPE-1 and amylase, lipase and trypsin in pancreatic juice has been reported<sup>[21,22]</sup>. The sensitivity of the FPE- I test is 90% in SAP and 60%-70% in mild disease<sup>[23]</sup>. Although the test is easy and used widely in many reference laboratories, it is not an ideal test. But, FPE-1 test is more reliable than the direct tests which are more expensive, invasive and time-consuming.

Long term results after AP, in terms of pancreatic functions are heterogeneous. The pancreatic dysfunction rate after AP ranges between 11%-85% in SAP and 13%-55% in MAP<sup>[12,15,16,24]</sup>. Bozkurt *et al*<sup>[14]</sup> reported the results of their relatively small series. They observed mild-moderate exocrine dysfunction rates of 74% and 81% at 1 and 18 mo follow-up, respectively. Severe exocrine dysfunction following AP was noted in 26% at 1 mo and 6% at 18 mo. On the other hand, Ibars *et al*<sup>[11]</sup> observed normal pancreatic exocrine function after AP in their study of the same size. In our study, the exocrine dysfunction rate was found to be 13.7% among the patients. This rate was higher in the NAP group and lower in edematous cases and among patients with normal CECT findings. The duration between the AP attack and FPE-1 test of patients having exocrine dysfunction was relatively long (most of them longer than 24 mo). The relation between pancreatic exocrine dysfunction and pancreatic necrosis and its localization is not clear. Although no relation was found between necrosis and subjective clinical evalua-





**Figure 4 Acute pancreatitis and endocrine dysfunction.** Endocrine dysfunction was much higher in severe acute pancreatitis than mild acute pancreatitis (A) and also much higher in necrotizing acute pancreatitis than without necrosis (B) ( $P < 0.001$ ). SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; NAP: Necrotizing acute Pancreatitis; EAP: Edematous acute pancreatitis.

tion, the FPE-1 level was low in SAP, NAP and patients with pancreatic head necrosis. FPE-1 was noted to be under the critical threshold ( $< 200 \mu\text{g/g}$  stool) in patients having head necrosis. Kemppainen *et al.*<sup>[25]</sup> reported that in patients with pancreatic head and body necrosis, the complication rate was much higher than the others and they attributed this to proximal obstruction.

As seen in some of our cases, the occurrence of exocrine dysfunction or enzyme insufficiency after AP may be independent of organ necrosis and severity of disease. We can only speculate about the cause of this finding. It is not ethical to biopsy the pancreas after AP. On the other hand, measuring tissue microcirculation and organ perfusion is not practical. We can speculate that fibrosis and the loss of functional units might occur during the healing period under cytokine cascade after AP attack. It has been reported that pancreatic exocrine dysfunction is relatively more common in the long term, especially after NAP, and in patients with necrosectomy<sup>[15,26]</sup>. Exocrine dysfunction was observed in 26.6% of NAP cases and 40% of patients who had necrosectomy in our study. We did not analyze the FPE-1 level in different time periods. Therefore, from our data, we cannot draw conclusions about the course of enzyme insufficiency over the long term.

We have very limited data about pancreatic enzyme supplementation and its dosage and duration after AP. Approximately 50% of our cases were on enzyme supplementation before starting the study. Interestingly, only 12.8% of these patients had exocrine dysfunction after one month tapering of enzyme supplementation. On the other hand, only 3 of 15 patients with exocrine dysfunction had been taking enzyme supplementation. Therefore these results showed that enzyme supplementation regimes should be questioned.

The reported rate of endocrine dysfunction after AP is 15%-35%<sup>[18,27]</sup>. The rate in the present study was relatively high (34.7%). In previous studies, endocrine dysfunction has been reported to occur in up to 50%-70% of cases of necrosis or necrosectomy<sup>[5,17,28]</sup>. In our study, endocrine dysfunction was related to disease severity and presence of necrosis but was not related to the necrosis ratio and localization. This finding can be also explained with pancreatic fibrosis occurring as a result of inflam-

mation, leading to exocrine dysfunction. In addition to beta cell loss, increased insulin resistance is thought to be another causal factor for endocrine dysfunction after AP<sup>[17]</sup>. This factor was demonstrated in an experimental study<sup>[29]</sup>. In contrast to exocrine dysfunction, endocrine dysfunction was not related to pancreatic head necrosis. This also can be explained by the fact that islet cells are disseminated homogenously through the organ. Therefore islet cells localized in uninjured regions of the organ can compensate. Endocrine dysfunction can occur without necrosis if the whole pancreas is affected by fibrosis.

Necrosis is a risk factor for pancreatic dysfunction; however, pancreatic dysfunction can occur without necrosis over the long term. It is not easy to detect pancreatic dysfunction, especially exocrine dysfunction. Reasons for this include: (1) There is no ideal test to detect exocrine dysfunction; (2) Pancreatic dysfunction can be explained by apoptosis in patients who did not have necrosis; (3) Ultrastructural changes and fibrosis due to the healing process in the ductal system can cause dysfunction; and (4) Recurrent attacks might be a source of morphologic changes and dysfunction.

In conclusion, long-term quality of life after AP should be evaluated in SAP, NAP, patients having necrosectomy and patients with pancreatic head necrosis.

## COMMENTS

### Background

Eighty percent of the pancreatic mass is devoted to exocrine function, and the remaining part is responsible for endocrine function, which is crucial to the maintenance of homeostasis of the body. This study aimed to clarify the relationship between pancreatic dysfunction, the severity of the disease and the extent of the pancreatic necrosis.

### Research frontiers

To evaluate the effects of disease severity and necrosis on organ dysfunctions in acute pancreatitis.

### Innovations and breakthroughs

Necrosis is a risk factor for pancreatic dysfunction; however, pancreatic dysfunction can occur without necrosis over the long term. It is not easy to detect pancreatic dysfunction, especially exocrine dysfunction.

### Peer review

In this study the authors evaluate the effects of disease severity and necrosis on organ dysfunction in acute pancreatitis. Fecal pancreatic Elastaz-1 and oral glucose tolerance test are used to measure exocrine and endocrine insufficiency. This is an interesting manuscript dealing with an area of investigation

poorly explored in the past.

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## Shugan-decoction relieves visceral hyperalgesia and reduces TRPV1 and SP colon expression

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### Abstract

**AIM:** To evaluate the therapeutic effect of Shugan-decoction (SGD) on visceral hyperalgesia and colon gene expressions using a rat model.

**METHODS:** Ninety-six adult male Wistar rats were randomized into six equal groups for assessment of SGD effects on psychological stress-induced changes using the classic water avoidance stress (WAS) test. Untreated model rats were exposed to chronic (1 h/d for 10 d consecutive) WAS conditions; experimental treatment model rats were administered with intragastric SGD at 1 h before WAS on consecutive days 4-10 (low-dose: 0.1 g/mL; mid-dose: 0.2 g/mL; high-dose: 0.4 g/mL); control treatment model rats were similarly administered

with the irritable bowel syndrome drug, dicetel (0.0042 g/mL); untreated normal control rats received no drug and were not subjected to the WAS test. At the end of the 10-d WAS testing period, a semi-quantitative measurement of visceral sensitivity was made by assessing the abdominal withdrawal reflex (AWR) to colorectal balloon-induced distension (at 5 mmHg increments) to determine the pain pressure threshold (PPT, evidenced by pain behavior). Subsequently, the animals were sacrificed and colonic tissues collected for assessment of changes in expressions of proteins related to visceral hypersensitivity (transient receptor potential vanilloid 1, TRPV1) and sustained visceral hyperalgesia (substance P, SP) by immunohistochemistry and real-time polymerase chain reaction. Inter-group differences were assessed by paired *t* test or repeated measures analysis of variance.

**RESULTS:** The WAS test successfully induced visceral hypersensitivity, as evidenced by a significantly reduced AWR pressure in the untreated model group as compared to the untreated normal control group ( $190.4 \pm 3.48$  mmHg *vs*  $224.0 \pm 4.99$  mmHg,  $P < 0.001$ ). SGD treatments at mid-dose and high-dose and the dicetel treatment significantly increased the WAS-reduced PPT ( $212.5 \pm 2.54$ ,  $216.5 \pm 3.50$  and  $217.7 \pm 2.83$  mmHg respectively, all  $P < 0.001$ ); however, the low-dose SGD treatment produced no significant effect on the WAS-reduced PPT ( $198.3 \pm 1.78$  mmHg,  $P > 0.05$ ). These trends corresponded to the differential expressions observed for both TRPV1 protein (mid-dose:  $1.64 \pm 0.08$  and high-dose:  $1.69 \pm 0.12$  *vs* untreated model:  $3.65 \pm 0.32$ ,  $P < 0.001$ ) and mRNA ( $0.44 \pm 0.16$  and  $0.15 \pm 0.03$  *vs*  $1.39 \pm 0.15$ ,  $P < 0.001$ ) and SP protein ( $0.99 \pm 0.20$  and  $1.03 \pm 0.23$  *vs*  $2.03 \pm 0.12$ ,  $P < 0.01$ ) and mRNA ( $1.64 \pm 0.19$  and  $1.32 \pm 0.14$  *vs*  $2.60 \pm 0.33$ ,  $P < 0.05$ ). These differential expressions of TRPV1 and SP related to mid- and high-dose SGD treatments were statistically similar to the changes induced by dicetel treatment. No signs of overt damage to the rat system were observed for any of the SGD dosages.

**CONCLUSION:** Shugan-decoction can reduce chronic stress-induced visceral hypersensitivity in rats, and the regulatory mechanism may involve mediating the expressions of TRPV1 and SP in colon tissues.

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**Key words:** Shugan-decoction; Visceral hypersensitivity; Sustained visceral hyperalgesia; Water avoidance stress; Transient receptor potential vanilloid 1; Substance P

**Core tip:** The classical rat model of chronic stress induction *via* water avoidance stress (WAS) test was used to investigate the therapeutic effect of the Shugan-decoction (SGD) on visceral hypersensitivity of the gastrointestinal tract and its underlying molecular mechanisms. The study design reflected the therapeutic potential of SGD for treating the stress-related gut aspects of irritable bowel syndrome (IBS) in humans. Mid- and high-dose SGD treatments significantly increased the WAS-reduced pressure thresholds, similarly to those induced by the IBS drug dicetel. The SGD treatments also restored WAS-related changes in transient receptor potential vanilloid 1 and substance P expression in the colon.

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## INTRODUCTION

In recent decades, irritable bowel syndrome (IBS) has emerged as a highly prevalent functional gastrointestinal disorder that is strongly associated with high levels of stress in daily life. The spectrum of IBS symptoms, ranging from discomfort associated with altered bowel habits to recurrent abdominal pain, is non-life threatening, but can severely impact an individual's general wellbeing and severely disrupt daily life. Despite the extensive laboratory- and clinical-based investigations that have been carried out to determine the underlying etiology and pathogenesis of IBS, no precise causative factors have been identified for the onset and progression of this disease. Patients present with an absence of IBS-specific structural and biochemical abnormalities<sup>[1,2]</sup>, but have higher incidences of psychological stress (both acute and chronic), visceral sensory abnormalities, gastrointestinal motility disorders, and gastrointestinal infections.

The theory of increased visceral sensitivity as a feature of IBS has been addressed by numerous studies. Indeed, IBS patients have been reported to show an enhanced sensitivity to colon and rectal balloon dilatation<sup>[3]</sup>. The mechanisms underlying such visceral hypersensitivity

remain unknown, but are likely multifactorial and complex<sup>[4,5]</sup>. Under normal physiological conditions, visceral sensitivity is mediated by a variety of neuron-localized ion channels, such as the transient receptor potential (TRP) non-selective cation channels, that also function in the formation and regulation of hyperalgesia.

The transient receptor potential vanilloid 1 (TRPV1) TRP family member plays a key role in modulation of the sensation of pain and thermal hyperalgesia<sup>[6]</sup> and is widely expressed throughout the gastrointestinal tract<sup>[7]</sup>. In the colon, substance P (SP)-mediated phosphorylation activates TRPV1, thereby enhancing the probability of channel gating promoting development of visceral hypersensitivity<sup>[8]</sup>. In this manner, SP itself acts as an important regulator of sustained visceral hyperalgesia, and has been characterized as an etiological factor of the repeated stress rat model system<sup>[9]</sup>.

Clinical observations of IBS patients have indicated that remarkably aggravated disease symptoms occur during times of increased emotional and mental stress<sup>[10]</sup>. In traditional Chinese medicine (TCM), these disrupted states correspond to liver-depression and spleen-deficiency. Thus, therapies that soothe the liver and strengthen the spleen are applied to IBS patients. One such therapy is the TCM compound Shugan-decoction (SGD), which, when administered orally, has been shown to significantly improve the clinical symptoms of IBS<sup>[11]</sup>. In this study, a rat model of stress-induced visceral hypersensitivity was employed to investigate the efficacy profile and therapeutic mechanism of SGD in IBS-like conditions.

## MATERIALS AND METHODS

### Animals

Ninety-six Wistar rats (150 ± 20 g adult males) were obtained from the Experimental Animal Center of Shanghai University of TCM (China) for analysis. The animals were housed under a 12/12 light cycle, with standard temperature (21-23 °C) and humidity (50% ± 5%) and *ad libitum* access to standard rat chow and tap water. All consecutive daily experimental procedures were conducted between 8:00-11:00 AM to minimize confounding due to diurnal variations.

The study was designed according to the guidelines of ethical treatment in research published by the Committee of International Association for the Study of Pain and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research at the Shanghai University of TCM. All protocols were carried out with the aim of minimizing or eliminating discomfort to the animals.

### Experimental compounds

The constituent ingredients of SGD (white atractylodes rhizome, white peony root, dried old orange peel, Ledebouriella root and *Radix bupleuri*) were purchased as crude herbs from the Yanghetang Pharmacy (Shanghai, China). The aqueous extract of SGD was made by the Herbal Chemistry Lab at the Shanghai University of TCM, using



the following steps: decoction of the crude herbs twice, combination of the two filtrate samples, decompression recovery to obtain the final aqueous extract product. The standard IBS pharmaceutical drug dicetel (pinaverium bromide; 50 mg tablets) was obtained from Solvay Pharma (Suresnes, France).

### **Water avoidance stress test**

Repeated water avoidance stress (WAS) was conducted as previously described to induce chronic psychological stress with gastric disruption<sup>[12]</sup>. Briefly, rats were placed on a clear glass platform (10 × 8 × 8 cm) in the middle of a plexiglass tank (45 × 25 × 25 cm) filled with water at 25 °C (to fill the tank up to 1 cm below the top of the platform), and remained on the platform for 1 h. The WAS procedure was repeated once daily for 10 consecutive days.

### **Treatment and control groups**

Untreated model rats ( $n = 16$ ) were exposed to chronic (1 h/d for 10 d consecutive) WAS conditions. Experimental treatment model rats ( $n = 16$  each dosage group) were administered with intragastric SGD at 1 h before WAS on consecutive days 4–10 (low-dose: 0.1 g/mL; mid-dose: 0.2 g/mL; high-dose: 0.4 g/mL). Control treatment model rats ( $n = 16$ ) were similarly administered the IBS drug, dicetel (0.0042 g/mL). Untreated normal control rats ( $n = 16$ ) received no drug and were not subjected to the WAS test.

### **Measurement of fecal pellet output**

To estimate distal colonic motility, fecal pellet output was measured as previously described<sup>[9]</sup>. Briefly, fecal pellets found in the WAS tank were counted at the end of each 1 h WAS test. For the untreated normal control rats, the amount of fecal pellets left in the home cage were counted over a 60 min period of time. Data are presented as mean ± SE ( $n = 16$ ).

### **Colorectal distension and semi-quantitative measurement of pressure pain threshold**

At the end of the 10-d WAS testing period, a semi-quantitative measurement of visceral sensitivity was made in each group ( $n = 8$  each group) by assessing the abdominal withdrawal reflex to colorectal balloon-induced distension to determine the pressure threshold (evidenced by pain behavior)<sup>[10]</sup>. Briefly, rats were lightly sedated with halothane and a deflated latex balloon (4–5 cm diameter at full inflation) was inserted intra-anally with its end 1 cm proximal to the anus into the descending colon and rectum. Animals were then placed into a small lucite cubicle (20 × 8 × 8 cm) and allowed to wake up and adapt for 30 min prior to initiation of colorectal distension (CRD). The CRD was performed by progressive inflation of the colorectally-inserted balloon at 5 mmHg increments, and stopped when the animal exhibited pain behavior. The pressure pain threshold (PPT) value was recorded as the mmHg pressure that evoked contraction of the animal's abdominal muscles following

balloon-mediated CRD delivered for 30 s duration at 4 min intervals. All the measurements were observed by two investigators (Shi HL and Qian W) working independently and blinded to the animals' grouping.

### **Sacrifice and colon tissue collection**

All rats were sacrificed by cervical dislocation immediately after visceral sensitivity measurements were completed so that the descending colon (2 cm above the anus, which had not undergone CRD) could be removed by dissection. The tissue sample was then divided into two parts: one was fixed with 10% formalin [for subsequent immunohistochemical (IHC) analysis] and the other was snap-frozen and stored at -80 °C [for subsequent real-time polymerase chain reaction (PCR) analysis].

### **IHC analysis**

The IHC analysis of TRPV1 and SP protein expression in colon tissues was performed using the EnVision + System two-step horseradish peroxidase staining technique (Dako-Cytomation, Glostrup, Denmark) with targeted polyclonal rabbit anti-human primary antibodies (1:100 dilutions; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States). Negative controls were run with the primary antibodies omitted from the procedure. Positive detection was indicated by visualization of a brown stain in the cytoplasm. Three randomly selected × 200 magnification fields were evaluated using a BH2 microscope (Olympus, Tokyo, Japan) equipped with a Nikon 4500 digital camera (Tokyo, Japan). The computer-aided image analysis system by Qiu Wei Inc. (Shanghai, China) assessed the area and optical density (OD) of TRPV1 and SP-positive cells in each field. The IHC index was calculated as the average integral optical density: [(positive area × OD)/total area]. Data are presented as mean ± SE ( $n = 6$ ).

### **Real-time PCR**

Total RNA was extracted from the thawed colon tissue samples using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, United States) and reverse transcribed to cDNA by using the Prime-Script™ Reagent Kit (Takara, Tokyo, Japan), according to the manufacturers' instructions. The following primer sets (forward and reverse, respectively) were used for gene-specific amplifications: TRPV1 (GenBank accession No. NM\_031982): 5'-CCACACAAGTGCCGGGGGTC-3' and 5'-CCAGGTCGCCCATGCCGATG-3'; SP (GenBank accession No. NM\_053844): 5'-CTTCCTGGACGCGATGGGCTG-3' and 5'-TGGAATCCTGGCAGGCCCTT-3'; GAPDH (normalizing control; primers were synthesized by Dawei Biotechnology Co., Shijiazhuang, China): 5'-GCCACAGCACTCCATCGAC-3' and 5'-GTCTCCGATCTGGAAAACGC-3'. The real-time PCR was carried out with Synergy Brands Green I dye (Qiagen GmbH, Hilden, Germany) using a Prism 7500 System (Applied Biosystems Inc., Foster City, CA, United States) under the following conditions: 40 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, followed by a single final extension cycle of 72 °C for 7 min.

Samples were run in triplicate and the normalized values were averaged. Data are presented as mean  $\pm$  SE ( $n = 6$ ).

### Statistical analysis

All statistical analyses were carried out with the GraphPad Prism v5.0 software (GraphPad Software Inc, La Jolla, CA, United States). Inter-group differences were assessed by a paired  $t$  test or repeated measures analysis of variance. A  $P$  value of  $< 0.05$  was set as the threshold for statistical significance.

## RESULTS

### WAS-induced visceral hypersensitivity and hyperalgesia are relieved by SGD

The WAS test successfully induced visceral hypersensitivity, as evidenced by a sustained significant increase in fecal pellet output (distal colonic motility) from the untreated model group as compared with the untreated normal control group not subject to the WAS test (day 3:  $8.69 \pm 0.60$  *vs*  $2.31 \pm 0.66$  and day 10:  $8.56 \pm 0.63$  *vs*  $0.56 \pm 0.29$ , both  $P < 0.001$ ). After 7 d of SGD treatment, significant relief of the WAS-stimulated increase in fecal output was achieved by the mid-dose (day 3:  $8.38 \pm 0.77$  *vs* day 10:  $4.31 \pm 0.42$ ,  $P < 0.001$ ) and high-dose (day 3:  $8.19 \pm 0.62$  *vs* day 10:  $3.63 \pm 0.39$ ,  $P < 0.001$ ). Although the extent of relief in these groups was similar to that achieved with the dicetel control treatment (day 3:  $8.75 \pm 0.53$  *vs* day 10:  $4.00 \pm 0.35$ ,  $P < 0.001$  for all *vs* corresponding mid- and high-dose SGD values), none of the treatments reduced fecal output to untreated normal control group levels by day 10. The low-dose SGD treatment produced no significant effect on WAS-stimulated fecal output increase (day 3:  $8.94 \pm 0.84$  and day 10:  $6.88 \pm 0.51$ ; both  $P < 0.001$  *vs* untreated normal control group;  $P > 0.05$  for day 3 *vs* day 10).

The same WAS-induced and SGD-relieved trends were seen for visceral hyperalgesia. The untreated model group showed significantly lower PPT than the untreated normal control group ( $190.40 \pm 3.48$  mmHg *vs*  $224.00 \pm 4.99$  mmHg,  $P < 0.001$ ), which was relieved by the mid- and high-dose SGD treatments ( $212.50 \pm 2.54$  mmHg and  $216.50 \pm 3.50$  mmHg) to a similar extent achieved with dicetel control treatment ( $217.70 \pm 2.83$  mmHg) (all  $P < 0.001$  *vs* untreated model group). Again, the low-dose SGD treatment produced no significant effect on the WAS-reduced pressure threshold ( $198.30 \pm 1.78$  mmHg,  $P > 0.05$  *vs* untreated normal control group and  $P < 0.001$  *vs* untreated model group).

### WAS-reduced expression of colon-expressed genes related to visceral hypersensitivity (TRPV1) and hyperalgesia (SP) was relieved by SGD treatment

IHC detection of TRPV1 and SP in colon tissues of untreated normal control rats showed that their expressions were mainly localized to the mucosa and submucosa (Figure 1). The untreated model group showed significantly higher AOID levels than the untreated normal

controls for both TRPV1 ( $3.65 \pm 0.32$  *vs*  $0.86 \pm 0.11$ ,  $P < 0.001$ ) and SP ( $2.03 \pm 0.12$  *vs*  $0.64 \pm 0.11$ ,  $P < 0.001$ ). These WAS-stimulated increases in protein levels were significantly reduced by the SGD treatments at mid-dose (TRPV1:  $1.64 \pm 0.08$  and SP:  $0.99 \pm 0.20$ ) and high-dose (TRPV1:  $1.69 \pm 0.12$  and SP:  $1.03 \pm 0.23$ ) compared with the untreated model group (TRPV1:  $P < 0.001$  and SP:  $P < 0.01$ ). Furthermore, the extent of reduction was similar to that achieved with dicetel control treatment (TRPV1:  $1.46 \pm 1.60$  and SP:  $0.76 \pm 0.11$ ; both  $P < 0.001$  *vs* untreated model group). The low-dose SGD treatment produced no significant effect on the WAS-stimulated increases in TRPV1 ( $3.48 \pm 0.33$ ,  $P < 0.001$  *vs* untreated model group) or SP ( $1.69 \pm 0.22$ ,  $P < 0.01$  *vs* untreated model group).

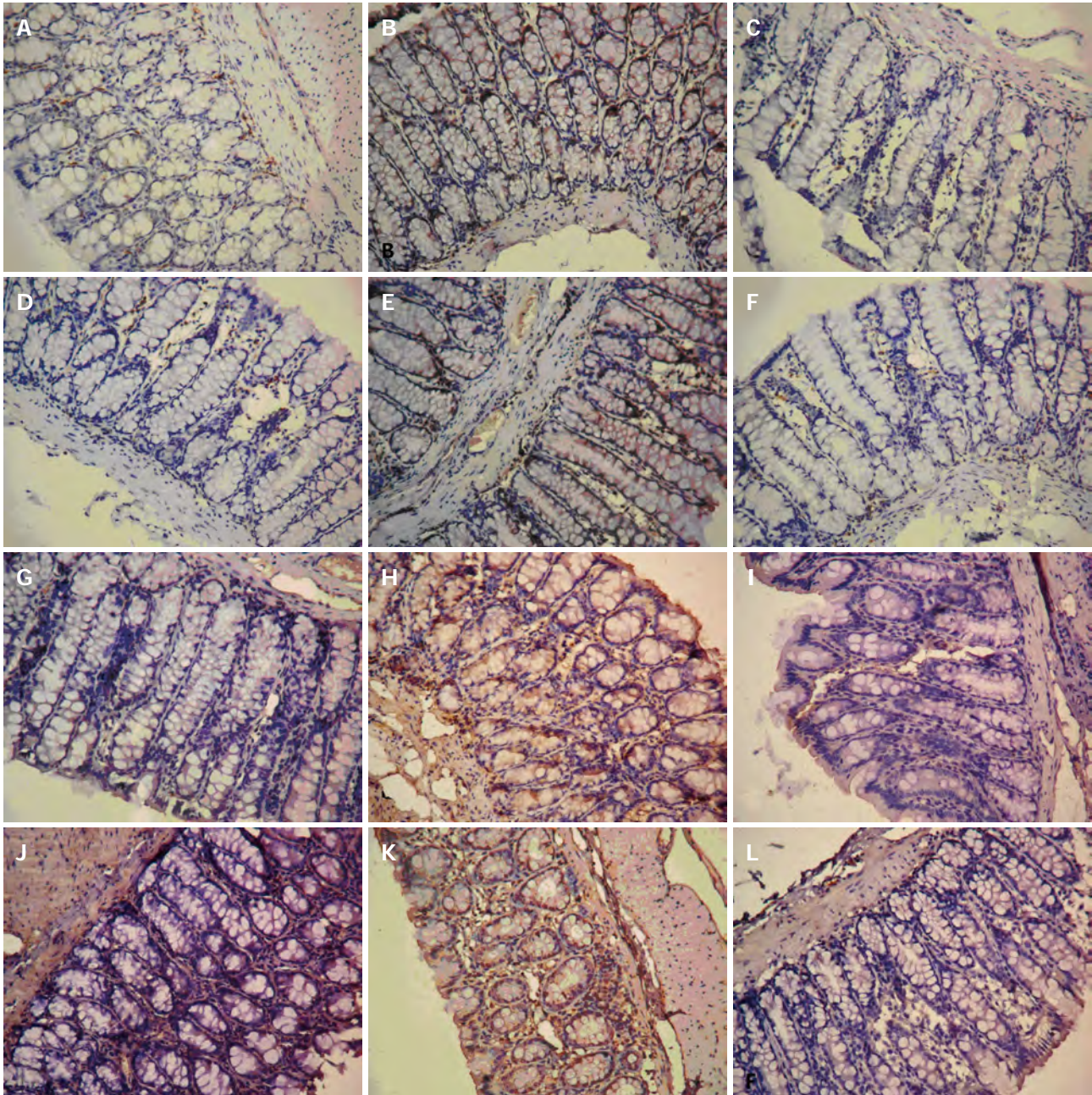
The same WAS-induced and SGD-relieved trends were seen for the gene expressions of TRPV1 and SP. The untreated model group showed significantly higher relative expressions of both genes compared with the untreated normal control group (TRPV1:  $1.39 \pm 0.15$  *vs*  $0.14 \pm 0.03$  and SP:  $2.60 \pm 0.33$  *vs*  $0.70 \pm 0.12$ , both  $P < 0.001$ ). The mid- and high-dose SGD treatments significantly reduced the WAS-increased mRNA expression of TRPV1 ( $0.44 \pm 0.16$  and  $0.15 \pm 0.03$ , both  $P < 0.001$  *vs* untreated model group) and SP ( $1.64 \pm 0.19$  and  $1.32 \pm 0.14$ , both  $P < 0.05$  *vs* untreated model group), with the SP levels being uniquely reduced by mid-dose SGD to levels similar to those of the untreated normal controls ( $P < 0.05$ ). Furthermore, the trends in SGD-mediated relief were similar to those observed with the dicetel control treatment (TRPV1:  $0.22 \pm 0.02$ ,  $P < 0.001$  *vs* untreated model group and SP:  $1.35 \pm 0.13$ ,  $P < 0.01$  *vs* untreated model group). Again, the low-dose SGD treatment produced no significant effect on the WAS-stimulated increase in mRNA expression of TRPV1 ( $0.99 \pm 0.16$ ) and SP ( $2.34 \pm 0.19$ ) (both  $P < 0.001$  *vs* untreated normal control group).

## DISCUSSION

In the present study, the well-established animal model of chronic water avoidance stress was used to stimulate the gastrointestinal tract hypersensitivity that is characteristic of human IBS. The WAS-induced physical manifestations (*i.e.*, increased fecal output and lower PPT) were accompanied by differential expression patterns of genes/proteins related to visceral hypersensitivity (TRPV1) and hyperalgesia (SP) in colon tissues. In addition, the model was used to evaluate the therapeutic efficacy of SGD, as a TCM alternative to dicetel, the pharmacologic agent most commonly used to treat IBS in humans. The findings indicated SGD was able to relieve the WAS-induced visceral hypersensitivity and hyperalgesia, as well as restore the perturbed TRPV1 and SP expressions.

Visceral pain, related to CRD and visceral hypersensitivity, is a hallmark feature of IBS and is often the factor precipitating a patient's presentation to the clinic<sup>[13-16]</sup>. However, the underlying molecular mechanisms of the





**Figure 1** Reductions in the expressions of substance P and transient receptor potential vanilloid 1 protein by water avoidance stress in the colon are relieved by SGJGD treatment. A-F: Substance P protein; G-L: Transient receptor potential vanilloid 1. IHC-detected colon tissues ( $\times 200$ ) from: untreated normal group (A, G); untreated model group (B, H); high-dose Shugan-decoction (SGD) model group (C, I); mid-dose SGD model group (D, J); low-dose SGD model group (E, K); dicetel control model group (F, L).

IBS pain response are poorly understood, which has inhibited development of effective pain management strategies<sup>[17,18]</sup>. The demonstration of TRPV1 as a contributor to WAS-induced colonic hypersensitivity, suggests its potential as a target of molecular therapies that may not only reduce the overactive distal colonic motility, but also relieve the associated lower PPT. Indeed, when TRPV1 was knocked-out in mice, the visceral sensitivity to CRD was significantly reduced<sup>[19]</sup>, and enhanced TRPV1 expression has been observed in a variety of gastrointestinal diseases<sup>[20,21]</sup>, including human cases of IBS<sup>[22]</sup>.

An increased amount of TRPV1-expressing nerve fibers have been reported in IBS-affected tissues from

human patients<sup>[22]</sup>, and may represent a physiological link between increased TRPV1 transcription and the pain response in IBS<sup>[23,24]</sup>. In addition, inflammatory factors are known agonists of TRPV1 channels<sup>[25]</sup> and might explain the common feature of low-grade inflammation in IBS. Considering a previous finding that development of fecal urgency and rectal hypersensitivity correlated with increased immunoreactivity to TRPV1 within the gastrointestinal tract<sup>[20]</sup>, it is possible that therapeutic antagonism of TRPV1 channels may result in antihyperalgesic effects without hypoalgesic activity, and might be beneficial in the treatment of IBS visceral pain<sup>[26]</sup>.

The current study's finding of chronic WAS-induced



changes in SP colon expression agree with other recent studies using the same model system that have implicated this neuropeptide in the maintenance of visceral hyperalgesia<sup>[9,27]</sup>. As a critical neurotransmitter of injurious signals, SP effectively links the gut nervous system to the immune system, stimulating a wide range of effector cells in the stomach and intestine to facilitate proper gastrointestinal motility, sensibility, secretion and absorption. The mechanism by which SP mediates visceral hypersensitivity may involve a myriad of cellular processes and signaling cascades, including promotion of the mast cell degranulation response, the release of histamines, leukotrienes, prostaglandins and bradykinin, all of which can cause inflammatory reactions leading to neuropathic pain<sup>[28]</sup>.

The clinical observations of increased SP expression in the intestinal mucosa of IBS patients<sup>[29,30]</sup>, coupled with the previous demonstration of SP's ability to activate TRPV1 *via* phosphorylation, thereby enhancing the probability of channel gating<sup>[9]</sup>, suggested that SP might be a vital mediator of chronic stress-induced visceral hyperalgesia through the modulation of TRPV1 channels. When TRPV1 channels are activated, a large Ca<sup>2+</sup> influx can lead to cellular depolarization<sup>[31]</sup>, with neurons releasing an array of neurotransmitters to trigger the downstream response of visceral hypersensitivity.

Dicetel is the most commonly applied pharmacotherapy of IBS, yet it is associated with a wide range of side effects, such as itching, rash, nausea and dry mouth. In addition, its widespread adoption in clinical practices worldwide has been hampered by its high monetary cost. In the current study, SGD treatment led to decreased expression of the WAS-stimulated TRPV1 and SP proteins and mRNAs in the hypersensitive colon, and increased the pain threshold of the rats. Thus, SGD appears to be an effective alternative to the pharmacologic agent dicetel for treating IBS by affecting the transcription and translation (and presumably secretion) of TRPV1 and SP in the colon.

In conclusion, the TCM SGD is an effective agent for reducing WAS-induced expressions of TRPV1 and SP in rat colons, thereby reducing visceral hypersensitivity and hyperalgesia. However, the chronic WAS testing (10 consecutive days) used in this study caused no overt damage to the colon's histological structure (data not shown), which may be a limitation in the study's findings, because human IBS is accompanied by significant structural changes (likely associated with the inflammatory component of IBS). Nonetheless, the present findings indicate an underlying mechanism of stress-induced disruption of distal colon motility and pain, which may represent useful targets for molecular based therapies to treat the pain and sensitivity symptoms of abdominal diseases, such as IBS.

## COMMENTS

### Background

Visceral hypersensitivity has been proposed as a significant contributor to the pathophysiology of irritable bowel syndrome (IBS). Activation of the transient

receptor potential vanilloid 1 (TRPV1) channel on neurons, by such effector molecules as the neurotransmitter substance P (SP), increases the probability of channel gating and promotes the formation of visceral hypersensitivity. Therefore, SP-mediated activation of TRPV1 might play a role in the visceral hypersensitivity and hyperalgesia induced by chronic stress conditions, as in IBS. The traditional Chinese medicine (TCM) compound Shugan-decoction (SGD) has been shown to significantly improve the clinical symptoms of IBS patients; however, the therapeutic mechanism of SGD remains unknown.

### Research frontiers

The molecular mechanisms underlying IBS remain to be fully elucidated, and may represent useful targets of therapies to relieve not only the symptoms associated with visceral hypersensitivity (increased distal colonic motility), but also those related to visceral hyperalgesia (abdominal pain, possibly related to an overactive inflammatory response). In this study, the classical rat model of chronic stress inducement *via* the water avoidance stress (WAS) test was used to investigate the underlying molecular mechanisms of visceral hypersensitivity and hyperalgesia in the gastrointestinal tract and to evaluate the related therapeutic effect of SGD for treating the stress-related gut aspects of IBS in humans. Mid- and high-dose SGD treatments significantly increased the WAS-reduced pressure thresholds and restored WAS-related changes in TRPV-1 and SP expression in the colon, suggesting this TCM compound as a feasible alternative to the pharmacological agent dicetel.

### Innovations and breakthroughs

This study provided novel insights into the molecular mechanisms underlying the observations of SGD-mediated improvements in the clinical symptoms of IBS. Specifically, SGD was demonstrated to reduce WAS-induced perturbations in TRPV1 and SP expressions in the colon that accompany visceral hypersensitivity and hyperalgesia.

### Applications

The finding that SGD may reduce WAS-induced visceral hypersensitivity and hyperalgesia through regulation of the colonic expressions of TRPV1 and SP confirm this TCM compound as a useful prescription for the treatment of abdominal pain in IBS.

### Terminology

Shugan-decoction is made according to the classic Tongxieyao Fang recipe and is reported to soothe the liver soothing and strengthen the spleen. Irritable bowel syndrome is a functional gastrointestinal disorder that is associated with high levels of stress in daily life, and manifests as altered bowel habits and recurrent abdominal pain. The water avoidance stress test is a well-established technique for inducing chronic psychological stress with gastric disruption in a rat model system. TRPV1 is widely expressed on neurons throughout the gastrointestinal tract and modulates visceral sensitivity and hyperalgesia. SP is a neurotransmitter that activates TRPV1 and regulates visceral hyperalgesia.

### Peer review

The authors investigated the therapeutic potential and underlying molecular mechanisms of the TCM compound SGD, in comparison to the common IBS pharmacologic agent dicetel, to relieve stress-induced visceral hypersensitivity and hyperalgesia. This is an interesting manuscript, and the general design is acceptable.

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## Clinical characteristics and prognosis of young patients with colorectal cancer in Eastern China

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### Abstract

**AIM:** To explore the clinical characteristics and prognosis of young patients with colorectal cancer patients in Eastern China.

**METHODS:** A total of 1335 patients with colorectal cancer treated from December 1985 to December 2005 at the Second Affiliated Hospital of Zhejiang University School of Medicine were studied retrospectively. The patients were divided into two groups, a younger group (aged  $\leq 30$  years) and an older group (aged  $> 30$

years), and comparison was made in the clinical characteristics and prognosis between the two groups. Chi-square test was used for data analysis of all categorical variables, and overall survival (OS) was calculated by the Kaplan-Meier method. A multivariate analysis was performed using the Cox model.

**RESULTS:** There were 42 (3.1%) and 1293 (96.9%) cases in the younger group and older group, respectively. Univariate analysis showed that the 5- and 10-year OS in the younger group were 33.9% and 26.1%, respectively, and those in the older group were 60.1% and 52.2%, respectively. Younger group had poor survival ( $\chi^2 = 14.146$ ,  $P = 0.000$ ). Multivariate analysis revealed that age was not a dependent factor for prognosis (OR = 0.866, 95%CI: 0.592-1.269,  $P = 0.461$ ). Stratified analysis indicated that in stage III and IV disease, the 5- and 10-year OS were 24.6% and 14.8% in the younger group, and 40.4% and 33.3% in the older group, respectively, with a significant difference between the two groups ( $\chi^2 = 5.101$ ,  $P = 0.024$ ). In the subgroup of radical surgery, the 5- and 10-year OS were 44.3% and 34.2% in the younger group, and 69.6% and 60.5% in the older group, with a difference being significant between the two groups ( $\chi^2 = 7.830$ ,  $P = 0.005$ ).

**CONCLUSION:** Compared with older patients, the younger patients have lower survival, especially in the subgroups of stage III and IV disease and radical surgery.

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**Key words:** Colorectal cancer; Young; Clinicopathologic feature; Prognosis; Radical surgery

**Core tip:** We firstly described the clinical characteristics and prognosis of young patients with colorectal cancer in Eastern China. The incidence rate of colorectal

cancer in young patients was higher than that in other reports. Younger patients with colorectal cancer had more poorly differentiated and advanced tumors, and worse prognosis, especially patients with stage III and IV disease.

Fu JF, Huang YQ, Yang J, Yi CH, Chen HL, Zheng S. Clinical characteristics and prognosis of young patients with colorectal cancer in Eastern China. *World J Gastroenterol* 2013; 19(44): 8078-8084 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8078.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8078>

## INTRODUCTION

As a kind of common cancer, colorectal cancer severely threatens the health of people. Colorectal cancer is the fourth common cancer and the second leading cause of cancer death in the world<sup>[1]</sup>. The majority of patients are affected in their 50s to 70s, but the age at diagnosis is getting younger<sup>[2]</sup>. The annual percentage of colorectal cancer in young people is increasing<sup>[2]</sup>. There has been an increasing number of reports about young colorectal cancer patients in recent years. The outcomes of young colorectal cancer patients varied widely among different regions<sup>[2-4]</sup>. The incidence rate of colorectal cancer in young patients has also been increasing in recent years in China<sup>[5]</sup>. Nearly all reports showed that young colorectal cancer patients had specific clinicopathologic characteristics, including poor histological feature, and more mucinous tumors, signet ring cell tumors, and advanced tumors<sup>[6-8]</sup>. However, the relationship between the age and survival was not confirmed. Some reports documented that young colorectal cancer patients had worse survival compared with the older counterparts<sup>[2,7-9]</sup>. But the others indicated opposite results<sup>[10-15]</sup>. There are still controversies about the definition of the age of young population. This study was to retrospectively analyze the data of patients with colorectal cancer who received surgery at our center over the past 30 years. Based on the distribution of the age, the population with colorectal cancer aged < 30 years was considered as a special subgroup in our center. Therefore, the young population was defined as those aged ≤ 30 years in our study. This study was designed to explore the clinicopathologic characteristics and prognosis of young colorectal cancer patients in Eastern China.

## MATERIALS AND METHODS

A total of 1335 consecutive patients with colorectal cancer (aged 19-92 years, mean 58 ± 13.3 years) treated from December 1985 to December 2005 at the Second Affiliated Hospital of Zhejiang University School of Medicine, located in Eastern China, were studied retrospectively. The patients were divided into two groups, a younger

group (42 cases, aged ≤ 30 years, average age, 26.0 ± 3.5 years) and an older group (1293 cases, aged > 30 years, average age 58.0 ± 12.3 years). The criteria for inclusion were as follows: (1) patients with pathologically confirmed colorectal cancer; and (2) patients who underwent operations, including palliative surgeries. Patients with anal cancer or non-adenomas were excluded. Following the approval by the ethics committee of the hospital, the data including age, gender, tumor location, histological grade, approach of surgery, tumor infiltration, number of metastatic lymph nodes, distant metastasis and survival were obtained. Follow-up was made every 3 mo for 2 years, 6 mo for 5 years, then every one year. The follow-up proceeded through telephone calls or mail correspondence. The events of relapse and death in all patients were recorded.

The deadline of follow-up was November 2011. The follow-up lasted 0-302 mo (median, 57.0 ± 68.1 mo). Finally, 1335 patients who had complete data were analyzed; 267 patients (20.0%) were lost to follow-up, with 5 patients (11.9%) in the younger group and 262 patients (20.3%) in the older group. There was no significant difference in the percentage of lost patients between two groups ( $P = 0.183$ ). The lost patients were taken as censors when the survival was analyzed. Twenty-nine patients died of colorectal cancer in the younger group and 604 patients died in the older group, including 51 patients who died due to other causes. They were considered as censors when cancer-related survival was calculated. All 1335 cases were included when we analyzed the clinicopathologic difference between the two groups.

The tumor was staged according to the 7<sup>th</sup> pathologic TNM staging system of AJCC<sup>[16]</sup>. Tumor location was described in detail as the cecum, ascending colon, liver flexure colon, transverse colon, descending colon, sigmoid, sigmoidectal junction and rectum. Overall survival was calculated from the time of operation to death. Cancer-related survival was from the time of operation to the date of death because of the colorectal cancer. Causes of non-special cancer-related death included benign disease, accident, and secondary cancer. Radical surgery was classified as a procedure for no residual tumor left behind microscopically at resection margins. Palliative surgery was defined as a procedure for the residual tumor left macroscopically, which also included bypass or ileostomy. All palliative surgeries were considered as non-radical surgery.

### Statistical analysis

Data of all categorical variables are summarized using frequencies and percentages. The data were analyzed with  $\chi^2$  test. Overall survival was calculated according to the Kaplan-Meier method. Survival rates were compared by the log-rank test. A multivariate analysis was performed using the Cox model. When a  $P$ -value was less than 0.05, the difference was considered significant. SPSS 16.0 statistical software was used for data analysis.

## RESULTS

### Clinicopathologic characteristics

The patient age ranged from 19 to 92 years, with a median of  $58 \pm 13.3$  years. There were 42 (3.1%) and 1293 (96.9%) cases in the younger group and older group, respectively. The ratio of male to female was 1.3:1 in both groups.

The rectum was the frequent location in colorectal cancer, with a slightly higher rate in the younger group than in the older group (59.5% *vs* 49.3%,  $P > 0.05$ ). Compared with the older group, significantly more patients in the younger group had mucinous tumor (33.3% *vs* 13.8%,  $P = 0.000$ ), signet ring cell cancer (7.1% *vs* 1.7%,  $P = 0.010$ ) and poorly differentiated tumor (59.5% *vs* 15.7%,  $P = 0.000$ ).

As for tumor infiltration, no tumor *in situ* (Tis) was found in the younger group, but 17 (13.1%) patients in older group were diagnosed with tumor *in situ* (Tis). Interestingly, there was no significant difference between the two groups in the tumor infiltration ( $P = 0.264$ ). The percentages of patients with lymph node metastasis ( $\geq 4$  lymph nodes), distance metastasis, stage IV and stage I disease and radical surgery were 35.7%, 28.6%, 31.0%, 2.4% and 66.7%, respectively, in the younger group, and 14.2%, 15.4%, 15.5%, 30.2% and 83.7%, respectively, in the older group, with significant differences between the two groups ( $P = 0.021, 0.021, 0.007, 0.008$  and  $0.008$ , respectively) (Table 1).

### Overall survival

Univariate analysis showed that there was a significant difference in total overall survival between the two groups ( $\chi^2 = 14.146$ ,  $P = 0.000$ ) (Figure 1, Table 2). Multivariate analysis revealed that age was not an independent factor for the prognosis of colorectal cancer (OR = 0.866, 95%CI: 0.592-1.269,  $P = 0.461$ ). TNM stage III/IV, the approach of palliative surgery, rectal cancer, mucinous cancer and poorly differentiated tumor were independent factors for worse prognosis (Table 2).

As for stage I and II disease, the 10-year overall survival and median survival time had not reached until the deadline in the younger group, which might be due to the small sample size of the study. There was no significant difference in the 10-year overall survival and median survival time between the two groups ( $\chi^2 = 0.016$ ,  $P = 0.899$ ) (Figure 2A, Table 3). Fifty-one patients died of other diseases in the older group. In order to diminish the influence of the non-cancer death, the cases in the subgroup of stage I and II disease were analyzed; as a result, there was also no difference in cancer-related survival between the two groups ( $\chi^2 = 0.356$ ,  $P = 0.551$ ) (Figure 2B). For stage III and IV disease, the outcome was worse in the younger group than in the older group ( $\chi^2 = 5.101$ ,  $P = 0.024$ ) (Figure 3, Table 3). For the subgroup of radical surgery, in the older group, the median survival time had not reached until the deadline. There was a significant difference in median survival time between the two groups ( $\chi^2 = 7.830$ ,  $P = 0.005$ ) (Figure 4A, Table 3). In the non-

**Table 1 Clinical and pathologic characteristics of colorectal cancer in the younger group and older group  $n$  (%)**

Variable	Younger group ( $n = 42$ ) ( $\leq 30$ yr)	Older group ( $n = 1293$ ) ( $> 30$ yr)	P value
Gender			NS
Male	24 (57.1)	738 (57.1)	
Female	18 (42.9)	555 (42.9)	
Location of tumor			
Cecum	1 (2.4)	69 (5.3)	
Ascending colon	1 (2.4)	154 (11.9)	
Hepatic flexure	1 (2.4)	86 (6.7)	
Transverse colon	2 (4.8)	52 (4.0)	
Splenic flexure	1 (2.4)	29 (2.2)	
Descending colon	6 (14.3)	49 (3.8)	
Sigmoid	5 (11.9)	201 (15.5)	
Rectosigmoid junction	0 (0)	16 (1.2)	
Rectum	25 (59.5)	637 (49.3)	0.191 <sup>1</sup>
Histology			
Mucinous cancer	14 (33.3)	179 (13.8)	0.000 <sup>2</sup>
Signet ring cell cancer	3 (7.1)	22 (1.7)	0.010 <sup>3</sup>
Papillary adenocarcinoma	5 (11.9)	237 (18.3)	
Tubular adenocarcinoma	17 (40.5)	732 (56.6)	
Undifferentiated adenocarcinoma	1 (2.4)	6 (0.5)	
Adenosquamous cancer	0 (0)	2 (0.2)	
Adenocarcinoma (unclassified)	2 (4.8)	115 (8.9)	
Differentiation			0.000
Well	2 (9.5)	276 (21.3)	
Moderate	14 (33.3)	630 (48.7)	
Poor	25 (28.6)	203 (15.7)	
Undifferentiated	1 (2.4)	184 (14.2)	
Stage T			0.264
Tis	0 (0)	17 (1.3)	
T1	1 (2.4)	41 (3.2)	
T2	4 (9.5)	232 (17.9)	
T3	16 (38.1)	526 (40.7)	
T4	21 (50.0)	477 (36.9)	
Number of metastatic lymph nodes			0.001
0	11 (26.2)	669 (51.7)	
1-3	10 (23.8)	337 (26.1)	
> 4	15 (35.7)	184 (14.2)	
Nx	6 (14.3)	103 (8.0)	
Distant metastasis			0.021
M0	30 (71.4)	1094 (84.6)	
M1	12 (28.6)	99 (7.6)	
AJCC stage			0.001
0	0 (0)	17 (1.3)	
I	1 (2.4)	221 (17.1)	0.008 <sup>4</sup>
II	9 (21.4)	427 (33.0)	
III	19 (45.2)	428 (33.1)	
IV	13 (31.0)	200 (15.5)	0.007 <sup>5</sup>
Approach of surgery			0.028
Radical surgery	28 (66.7)	1084 (83.8)	
Palliative surgery	10 (23.8)	151 (11.7)	
Unresectable	4 (9.5)	58 (4.5)	

<sup>1</sup>Rectum *vs* other sites of tumor; <sup>2</sup>Mucinous cancer *vs* other histological types; <sup>3</sup>Signet ring cell cancer *vs* other histological types; <sup>4</sup>Stage I *vs* other stages; <sup>5</sup>Stage IV *vs* other stages. Tis: Tumor *in situ*; NS: Non-significance.

radical surgery subgroup, there was no significant difference in median survival time between the two groups ( $\chi^2 = 0.112$ ,  $P = 0.737$ ) (Figure 4B, Table 3).

## DISCUSSION

A total of 1335 patients with colorectal cancer were ana-



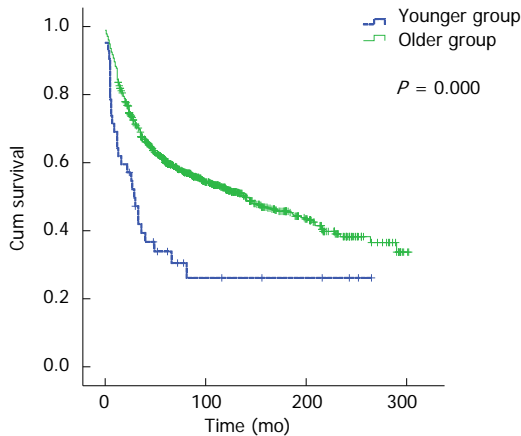


Figure 1 Overall survival of patients in the younger group ( $\leq 30$  years) and older group ( $> 30$  years). The younger group had worse prognosis than the older group ( $P = 0.000$ ).

**Table 2 Multivariate analysis (Cox proportional hazard model) of prognostic factors for 1335 patients with colorectal cancer**

Variable	OR	95%CI	P value
Stage (III + IV / I + II)	2.196	1.827-2.639	0.000
Approach of surgery (non-radical/radical)	4.496	3.718-5.437	0.000
Age ( $> 30$ yr/ $\leq 30$ yr)	0.866	0.592-1.269	0.461
Gender (male/female)	0.997	0.852-1.167	0.970
Tumor location (rectum/colon)	1.270	1.084-1.488	0.003
Differentiation (moderate + well/low)	0.802	0.650-0.990	0.041
Histology (others/mucinous)	0.791	0.632-0.990	0.041

lyzed retrospectively in this study, including 42 (3.1%) patients in the younger group (aged  $\leq 30$  years). In other studies, the incidence rate was less than 1% and 3% if young patients with colorectal cancer were defined as those aged  $\leq 30$  years<sup>[17-19]</sup> and  $\leq 40$  years<sup>[4,15]</sup>, respectively. The incidence rate in this study was higher than in other regions, suggesting an obvious regional difference. In this study, Eastern China refers to Yangtze River delta region where people enjoy a similar lifestyle and economic status. Consequently, the epidemiological characteristics of colorectal cancer in this region are similar. Therefore, data from our center could represent the features of this tumor in Eastern China. There might be statistical biases about the incidence rate of colorectal cancer in young patients because the data were collected retrospectively by a single medical center.

### Gender

There was no significant difference in gender ratio between the two groups. The percentage of female patients is becoming higher with the trend of younger age in gastric cancer. This phenomenon was not seen in colorectal cancer. Estrogen was considered to be related with gastric cancer in younger patients<sup>[20]</sup>. It is not clear whether estrogen was related to the occurrence of colorectal cancer in young people<sup>[21,22]</sup>. On the other hand, this study indicated that female patients with colorectal cancer had bet-

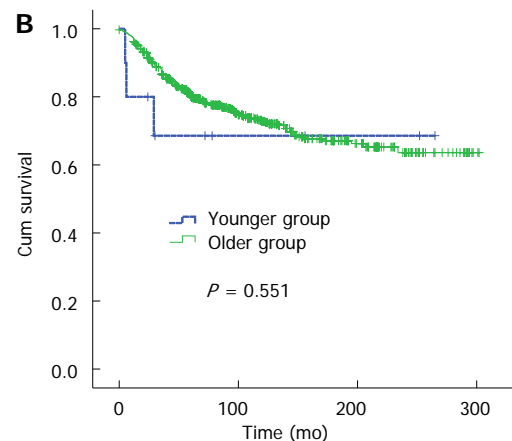
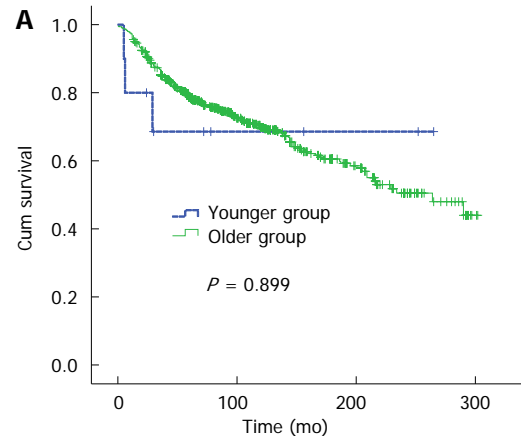


Figure 2 Overall survival of younger patients ( $\leq 30$  years) and older patients ( $> 30$  years) in stage I and II tumor subgroup. A: Overall survival was totally similar between the two groups ( $P = 0.899$ ); B: Cancer-related survival was similar between the two groups ( $P = 0.551$ ).

ter outcome than male patients, but with no significant difference (OR = 0.969,  $P = 0.708$ ) in survival as shown by the multivariate analysis.

### Tumor location

In this study, the rectum and sigmoid were common sites of the tumor in both groups. The proportion of rectal cancer was higher in the younger group (59.5%) than in older group (49.3%), but without significant difference ( $P = 0.191$ ). Some reports indicated that the rate of rectal cancer in younger population was higher than in older one<sup>[2]</sup>. That might be related to the epidemics of colorectal cancer that rectal cancer is more common than colonic cancer in China. Eating habit and lifestyle might contribute more to the occurrence of colorectal cancer than age.

### Pathological characteristics

In this study, mucinous tumor and signet ring cell cancer were more common in the younger group than in the older group. A majority of patients in the younger group had poor histologic grade compared with the older group. Studies on gastric cancer also indicated that there were more poorly differentiated cancers in younger

**Table 3** Survival of subgroup patients by stratified analysis with stage and approach of surgery

	Age (yr)	n	5-yr OS	10-yr OS	Median survival time (mo, 95%CI)
Total <sup>1</sup>	≤ 30	42	33.90%	26.10%	29.0 (18.0-40.0)
	> 30	1293	60.10%	52.20%	140.0 (111.6-168.4)
Stage I and II	≤ 30	10	68.60%	/ <sup>4</sup>	/ <sup>4</sup>
	> 30	665	78.60%	69.80%	264.0 (203.5-324.5)
Stage III and IV <sup>2</sup>	≤ 30	32	24.60%	14.80%	22 (2.6-41.4)
	> 30	628	40.40%	33.30%	35 (27.9-42.1)
Radical surgery <sup>3</sup>	≤ 30	28	44.30%	34.20%	40.0 (10.1-69.9)
	> 30	1082	69.60%	60.50%	/ <sup>5</sup>
Non-radical surgery	≤ 30	14	14.30%	0%	6 (2.3-9.7)
	> 30	211	11.80%	0%	11 (9.2-12.8)

<sup>1</sup> $\chi^2 = 14.146$ ,  $P = 0.000$ ; <sup>2</sup> $\chi^2 = 5.101$ ,  $P = 0.024$ ; <sup>3</sup> $\chi^2 = 7.830$ ,  $P = 0.005$ ; <sup>4</sup>The sample was too small to analyze; <sup>5</sup>The median survival time was not reached.

population than in older population, especially signet ring cell cancer<sup>[23]</sup>. It was not clear about the age impact on the occurrence of gastrointestinal cancer.

### Stage

Compared with the older population, the percentage of patients with stage IV disease increased and that of patients with stage I disease decreased in the younger group. As for the infiltration of tumor and nodal metastasis, the patients in the younger group presented with more aggressive findings. Some studies found that 66.0% of younger patients with colorectal cancer were diagnosed with stage III or IV disease, which was obviously lower (32.0%) in the older patients<sup>[24]</sup>. This may result from the poor differentiation and high aggressiveness of tumors which were often diagnosed in younger patients with colorectal cancer. Besides, younger patients with colorectal cancer often had delayed diagnosis, but the older ones would be diagnosed earlier through screening program.

### Overall survival

Univariate analysis revealed that the patients in the younger group had poorer survival than those in the older group. The impact of young age on the prognosis of colorectal cancer is not confirmed. Some studies showed that young patients with colorectal cancer had more mucinous cancer and signet ring cancer, poorer histologic grade, later stage and worse prognosis<sup>[2,7-9]</sup>. But results were contradictory from other studies which indicated that young age had no impact on the prognosis<sup>[10-15]</sup>. In our study, young colorectal cancer patients had worse prognosis, while multivariate analysis indicated that age was not an independent factor for prognosis. Furthermore, multivariate analysis also showed that disease stage and approach of surgery were strongly related to the prognosis. Worse prognosis might result from stage III and IV disease and non-radical surgery. Therefore, stratified analyses with these two factors were carried out.

The result of stratified analysis with stage indicated that younger patients had poor prognosis, and univariate analysis showed that younger patients presented with mainly stage III and IV disease. The reasons might be that young patients had more poorly differentiated tumor, and

mucinous carcinoma and signet ring cell cancer, which were more aggressive in the same stage. As for patients with stage I and II disease, age exerted no effect on the survival. In this study, there were more patients in the older group who did not die of colorectal cancer. In order to exclude the influence of the non-cancer special death, the cancer-related survival in patients with stage I and II disease was analyzed. The result showed no significant difference in cancer-related survival in stage I and II tumor between the two groups. The study of Quah *et al.*<sup>[25]</sup> considered that patients with an earlier stage disease had better survival in younger group than older group; young patients were more tolerable to surgery and aggressive adjuvant chemotherapy and radiotherapy<sup>[26]</sup>. And the study of McMillan *et al.*<sup>[27]</sup> indicated that in the older group, non-special cancer factors were major causes of death.

The stratified analysis with approach of surgery revealed that patients had poorer prognosis in the younger group than in the older group with radical surgery, but there was no significant difference between the two groups without radical surgery. In stratified analysis with stage, patients with stage I and II disease had similar prognosis between the two groups. Stage I and II tumors were often considered to be resectable. For patients with resectable stage III and stage IV tumors, younger age strongly contributed to poor survival. For patients who received operation without adjuvant chemotherapy in the 1980s and 1990s, the value of postoperative adjuvant therapy should be highlighted for patients with resectable stage III or more advanced colorectal cancer<sup>[28]</sup>.

The current study had some limitations. The clinical data did not include the signs and symptoms of colorectal cancer patients. It was impossible to identify the alarming symptoms for younger patients. Family histories were not described, which were routinely detected in young population as the other studies<sup>[29]</sup>. The percentage of lost patients was 20%, which might influence the result of survival. In China, there are several medical centers owning elaborate clinical data, but few centers carried out the systemic follow-up. The data of 10-year follow-up are rare.

In summary, compared with older patients, the younger ones have specific clinicopathologic characteristics that

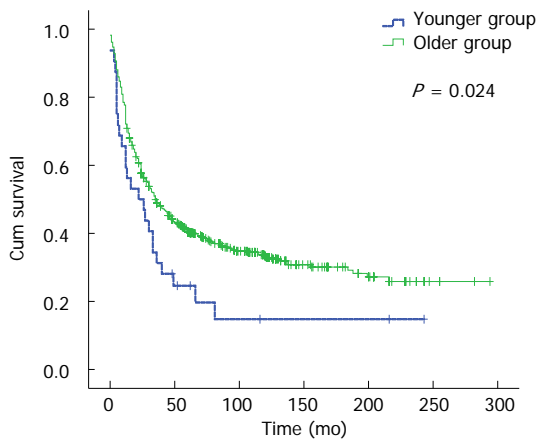


Figure 3 Overall survival of younger patients ( $\leq 30$  years) and older patients ( $> 30$  years) in stage III and IV tumor subgroup. The younger group had worse prognosis than the older group ( $P = 0.024$ ).

are worthy to be explored and managed differentially. Younger patients with colorectal cancer tend to be diagnosed at later stage. For younger patients who have poor survival, especially those with stage III and IV disease and treated by radical surgery, more aggressive adjuvant therapies are recommended.

## COMMENTS

### Background

The incidence rate of colorectal cancer has been increasing in recent years. The onset age of colorectal cancer is getting younger. Should the young colorectal cancer patients be treated as a heterogeneous group? It is important to explore the phenotype of young patients with colorectal cancer.

### Research frontiers

Age is an independent prognostic factor for many cancers such as breast cancer, thyroid cancer and gastric cancer. Young patients have more triple negative breast cancers and worse prognosis. Lymph node-positive thyroid cancers are commonly diagnosed in adolescent patients, who have satisfactory prognoses. Young patients with gastric cancer in early stage have better prognosis than old ones, while their prognoses are worse in advanced gastric cancer. It is unknown about the age impact on the prognosis of colorectal cancer. Some studies showed that young patients with colorectal cancer had more mucinous cancer and signet ring cancer, poorer histologic grade, later stage and worse prognosis. But results were contradictory in other studies which indicated that the young age had no impact on prognosis.

### Innovations and breakthroughs

The authors described systematically for the first time the clinical characteristics and prognosis of young colorectal cancer patients in Eastern China. The incidence rate of young colorectal cancer was higher than in other reports. Colorectal cancer in younger patients was characterized by poorer differentiation and advanced stage. Young colorectal cancer patients had worse prognosis, especially those with stage III and IV disease, rather than stage I and II disease.

### Applications

Relapse risk of postoperative stage II colonic cancer is a crucial factor for decision-making in postoperative treatment. This study showed that age was not an independent risk factor for stage II colorectal cancer. On the other hand, young colorectal cancer patients with stage III and IV disease had worse prognosis, and more aggressive adjuvant therapy is recommended for these patients.

### Terminology

Young patients with colorectal cancer: Onset age of colorectal cancer was less than or equal to 30 years. Eastern China refers to Yangtze River delta region where people have similar lifestyle and economic conditions. Epidemiologi-

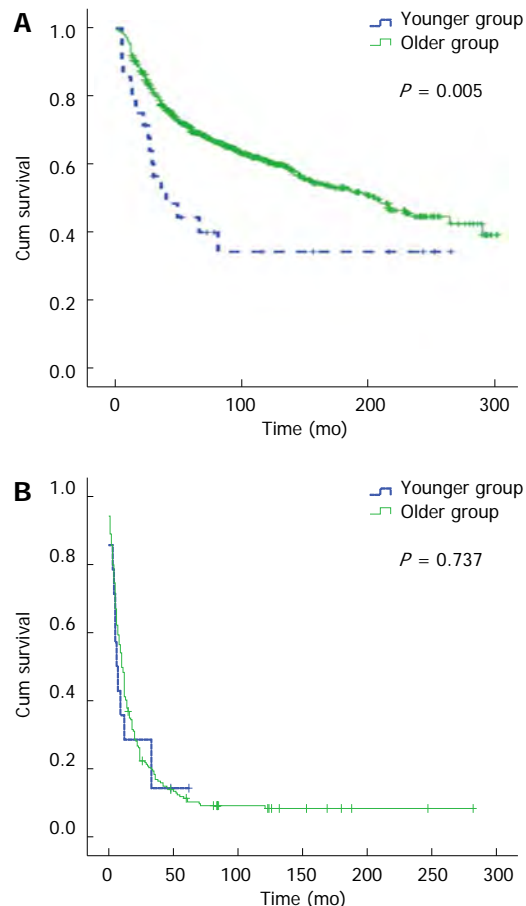


Figure 4 Overall survival of younger patients ( $\leq 30$  years) and older patients ( $> 30$  years) based on approach of surgery. A: The younger group had worse prognosis than the older group ( $P = 0.005$ ) undergoing radical surgery; B: There was no difference between the two groups ( $P = 0.737$ ) treated by non-radical surgery.

cal characteristics of colorectal cancer in this region are also similar.

### Peer review

The study described the detailed clinicopathologic characteristics of 1335 cases of colorectal cancer in Eastern China and analyzed the significance of prognosis by many statistical methods. The major goal of authors was to analyze the difference between younger patients ( $\leq 30$  year-old) and older patients ( $> 30$  year-old). The information enclosed in this manuscript is very plentiful and clear, and the authors applied many different statistical methods to perform the analysis. Although the results are not novel and methodology was orthodox, it is worth reporting the present results.

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## Clinical effects and complications of TIPS for portal hypertension due to cirrhosis: A single center

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### Abstract

**AIM:** To determine the clinical effects and complications of transjugular intrahepatic portosystemic shunt (TIPS) for portal hypertension due to cirrhosis.

**METHODS:** Two hundred and eighty patients with portal hypertension due to cirrhosis who underwent TIPS were retrospectively evaluated. Portal trunk pressure was measured before and after surgery. The changes in hemodynamics and the condition of the stent were assessed by ultrasound and the esophageal and fundic veins observed endoscopically.

**RESULTS:** The success rate of TIPS was 99.3%. The portal trunk pressure was  $26.8 \pm 3.6$  cmH<sub>2</sub>O after surgery and  $46.5 \pm 3.4$  cmH<sub>2</sub>O before surgery ( $P < 0.01$ ).

The velocity of blood flow in the portal vein increased. The internal diameters of the portal and splenic veins were reduced. The short-term hemostasis rate was 100%. Esophageal varices disappeared completely in 68% of patients and were obviously reduced in 32%. Varices of the stomach fundus disappeared completely in 80% and were obviously reduced in 20% of patients. Ascites disappeared in 62%, were markedly reduced in 24%, but were still apparent in 14% of patients. The total effective rate of ascites reduction was 86%. Hydrothorax completely disappeared in 100% of patients. The incidence of post-operative stent stenosis was 24% at 12 mo and 34% at 24 mo. The incidence of post-operative hepatic encephalopathy was 12% at 3 mo, 17% at 6 mo and 19% at 12 mo. The incidence of post-operative recurrent hemorrhage was 9% at 12 mo, 19% at 24 mo and 35% at 36 mo. The cumulative survival rate was 86% at 12 mo, 81% at 24 mo, 75% at 36 mo, 57% at 48 mo and 45% at 60 mo.

**CONCLUSION:** TIPS can effectively lower portal hypertension due to cirrhosis. It is significantly effective for hemorrhage of the digestive tract due to rupture of esophageal and fundic veins and for ascites and hydrothorax caused by portal hypertension.

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**Key words:** Transjugular intrahepatic portosystemic shunt; Cirrhosis; Portal hypertension; Therapeutic effect; Complication

**Core tip:** This study identified the clinical effects and complications of transjugular intrahepatic portosystemic shunt (TIPS) for portal hypertension due to cirrhosis in 280 patients who underwent this procedure at our centre between January 2005 and December 2009. TIPS can effectively lower portal hypertension due to cirrhosis. It is significantly effective for hemorrhage of the

digestive tract due to rupture of esophageal and fundic veins and for ascites and hydrothorax caused by portal hypertension.

Qin JP, Jiang MD, Tang W, Wu XL, Yao X, Zeng WZ, Xu H, He QW, Gu M. Clinical effects and complications of TIPS for portal hypertension due to cirrhosis: A single center. *World J Gastroenterol* 2013; 19(44): 8085-8092 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8085.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8085>

## INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is an effective procedure for portal hypertension due to cirrhosis and related complications. At the end of the 1980s, Rösch *et al*<sup>[1]</sup> and Rössle *et al*<sup>[2]</sup> first reported the use of Palmaz, a self-expanding stent. Since then Palmaz had been gradually applied and disseminated in clinical practice. In our centre, TIPS was used, in the initial stage, mainly for the treatment of patients intolerant of surgery, patients with recurrent hemorrhage despite medication and in patients with refractory ascites. As this procedure was developed and improved, it was also used in the treatment of recurrent hemorrhage of the digestive tract due to cirrhosis, hemorrhage after endoscopic ligation and sclerosing therapy, hemorrhage after surgery, portal thrombosis, ascites and hydrothorax due to portal hypertension, hepatorenal syndrome, and emergency hemorrhage. In this study, the significant clinical effects and complications of TIPS are discussed in 280 patients who underwent this procedure at our centre between January 2005 and December 2009.

## MATERIALS AND METHODS

### Patients

The clinical data on the outcome of TIPS in 280 patients between January 2005 and December 2009 were retrospectively analyzed. These 280 patients with portal hypertension due to cirrhosis met the criteria of the American Hepatological Association<sup>[3,4]</sup> for the clinical application of TIPS.

### TIPS procedure

Patients with cirrhotic portal hypertension underwent routine abdominal enhanced computed tomography (CT) scanning and hepatic portal vein CT three-dimensional reconstruction prior to TIPS. During TIPS, after paracentesis from the right hepatic vein or hepatic segment of the inferior vena cava to the branch of the portal vein, direct portography was carried out, then balloon dilatation, followed by stent placement. Portal venous pressure was measured before and after stent placement. Spring wire loops, a gelatine sponge and sclerosing agent were used for blockage of the collateral circulation of esophageal and fundic varices. The stents used were Zilver stents.

Specifications of the stents: ZIV 6-80-8 or 10-8.0 (Cook Corporation, Bloomington, IN). Specifications of the balloon: ATB 5-35-8-6.0 or 4.0 (Cook Corporation). Specifications of the spring wire loop: MWCE-35-3-3, 4, 5, 8, 10 (Cook Corporation). Generally, the puncture path was dilated with a balloon of 8 mm inside diameter, and a stent of 8 or 10 mm inside diameter was then positioned.

### Postoperative management

Anticoagulant therapy was administered in addition to routine expectant treatment. Heparin sodium 12500 IU was administered by intravenous drip 24-h for 7 d 24 h after surgery, and then oral sodium warfarin tablets for 1 year. Prothrombin time (PT) was maintained for 17-20 s.

### Follow-up

All patients were followed up 1 wk and 1 mo after surgery, followed by every 3 mo for 12 mo and then every 6 mo after 12 mo. Each follow-up visit included ultrasonography, liver and renal function tests, blood ammonia, routine blood examination and blood coagulation tests, and symptoms and signs of portal hypertension. Gastroscopy was performed in each patient from the month 1 to the month 3 after surgery and direct portography from the month 9 to the month 12.

### Statistical analysis

All measurement data are presented as mean  $\pm$  SD. The data before and after surgery were analyzed using the *t* test. *P* values  $< 0.05$  were considered statistically significant. Stent stenosis, hepatic encephalopathy, recurrent hemorrhage and survival were analyzed by the Kaplan-Meier method.

## RESULTS

### Clinical data

All 280 patients had portal hypertension. Of these patients, 220 had severe esophageal varices, 60 had severe esophageal and moderate-severe fundic varices, 42 had a large amount of ascites, 31 had a moderate amount of ascites and 40 had intractable ascites which was complicated by a large right hydrothorax in 4. Table 1 shows the patients' sex, causes of portal hypertension and Child-Pugh grading. Differences between the patients' sex, age, causes and Child-Pugh grading and their survival rate, incidence of rebleeding, incidence of hepatic encephalopathy and incidence of stent stenosis were not statistically significant ( $P > 0.05$ ).

### TIPS procedure

All 280 patients underwent puncture of the right internal jugular vein. Of these patients, 200 underwent puncture of the right hepatic vein, 80 puncture of the inferior vena cava near the liver, 198 underwent puncture of the right branch of the portal vein and 80 puncture of the left branch and 2 had severe hemorrhage in the abdominal cavity during this procedure (1 died, and the other

**Table 1 Clinical data on transjugular intrahepatic portosystemic shunt in 280 patients**

Clinical factor	No. of patients
Sex	
Male	223
Female	57
Age (yr, mean $\pm$ SD)	48.2 $\pm$ 13.7
Procedure	
Elective	260
Emergency	20
Indication for TIPS	
Hemorrhage of upper digestive tract	265
Hepatorenal syndrome	15
Cause	
Cirrhosis after hepatitis B virus infection	168
Cirrhosis after hepatitis C virus infection	10
Hepatitis B virus infection complicated by schistosomiasis	16
Hepatitis B virus infection complicated by alcoholic cirrhosis	54
Alcoholic cirrhosis	24
Unexplained cirrhosis	8
Child-Pugh grading	
A	60
B	184
C	36

TIPS: Transjugular intrahepatic portosystemic shunt.

**Table 2 Changes in dynamics and diameters of blood vessels before and after transjugular intrahepatic portosystemic shunt ( $n = 278$ , mean  $\pm$  SD)**

	Preoperative	Postoperative	<i>P</i> value
Portal venous pressure (cmH <sub>2</sub> O)	46.5 $\pm$ 3.4	26.8 $\pm$ 3.6	< 0.001
Portal venous internal diameter (cm)	1.68 $\pm$ 0.15	1.32 $\pm$ 0.11	0.007
Splenic venous internal diameter (cm)	1.31 $\pm$ 0.05	1.12 $\pm$ 0.03	0.009
Blood velocity in the portal vein (cm/s)	15.2 $\pm$ 4.7	49.3 $\pm$ 18.5	< 0.001
Blood velocity in the shunt pathway (cm/s)		154.0 $\pm$ 32.6	

All parameters shown in Table 1 were significantly different before and after surgery ( $P < 0.01$ ).

survived after emergency treatment). The success rate of surgery was 99.3% and the incidence of lethal complications was 0.7%. Embolism caused a collateral circulation in esophageal and fundal varices.

### Influence of TIPS on liver hemodynamics

Following the establishment of a portosystemic shunt pathway, liver hemodynamics changed. Portal pressure decreased after surgery, the internal diameters of the portal and splenic veins decreased and the blood velocity in the trunk of the portal vein increased (Table 2).

### Liver function before and after TIPS

Liver function was slightly altered after TIPS. No marked changes in alanine aminotransferase (ALT), total bilirubin,

**Table 3 Changes in liver function and prothrombin time before and after transjugular intrahepatic portosystemic shunt ( $n = 278$ , mean  $\pm$  SD)**

TIPS	ALT (IU/L)	TBIL ( $\mu$ mol/L)	Alb (g/L)	PT (s)
1 wk before TIPS	40.78 $\pm$ 5.41	29.33 $\pm$ 5.97	32.49 $\pm$ 5.14	13.43 $\pm$ 1.44
1 mo after TIPS	42.26 $\pm$ 2.32	28.45 $\pm$ 8.71	33.25 $\pm$ 4.18	17.73 $\pm$ 1.83 <sup>a</sup>
<i>P</i> value	0.679	0.813	0.716	0.036

<sup>a</sup> $P < 0.05$  vs the preoperative data. ALT: Alanine aminotransferase; TBIL: Total bilirubin; Alb: Albumin; PT: Prothrombin time; TIPS: Transjugular intrahepatic portosystemic shunt.

and albumin (Alb) before and after surgery were observed. Routine anticoagulant therapy was given postoperatively. PT increased significantly after surgery (Table 3).

### Clinical effects of TIPS

The short-term hemostasis rate was 100% when TIPS was used in the treatment of emergency hemorrhage and recurrent hemorrhage unresponsive to medication, endoscopy or surgery. Ascites disappeared completely in 62% of patients, decreased obviously in 24% and remained in 14%. The total effective rate was 86%. Hydrothorax completely disappeared in 100% of patients. Fifteen patients who had hepatorenal syndrome became responsive to diuretic therapy. Ascites completely disappeared in 7 patients and was obviously reduced in 8 after 7-14 d of observation.

### Complications of TIPS

Complications occurred during surgery and both short- and long-term postoperative complications were observed (Table 4). The most serious complication was abdominal cavity hemorrhage, which frequently endangered the patient's life. Short-term severe complications after surgery were hepatic failure, septicemia and abdominal cavity hemorrhage. Intermediate and long-term complications were stent stenosis and hepatic encephalopathy.

### Follow-up

All the 278 patients who underwent TIPS were followed up. Hemorrhage, stent function, hepatic encephalopathy and survival were observed during the follow-up. The incidence of recurring hemorrhage was 9% in 12 mo, 19% in 24 mo and 35% in 36 mo (Figure 1A). The incidence of stent stenosis was 24% in 12 mo and 34% in 24 mo postoperatively (Figure 1B). The incidence of hepatic encephalopathy was 14% in 3 mo, 17% in 6 mo and 19% in 12 mo (Figure 1C). The cumulative survival rate was 86% in 12 mo, 81% in 24 mo, 75% in 36 mo, 57% in 48 mo and 45% in 60 mo (Figure 2). In our center, 3 patients died 1 mo after TIPS, of whom 2 died of hepatic failure and 1 of septicemia.

## DISCUSSION

TIPS is an effective method of treating portal hyperten-

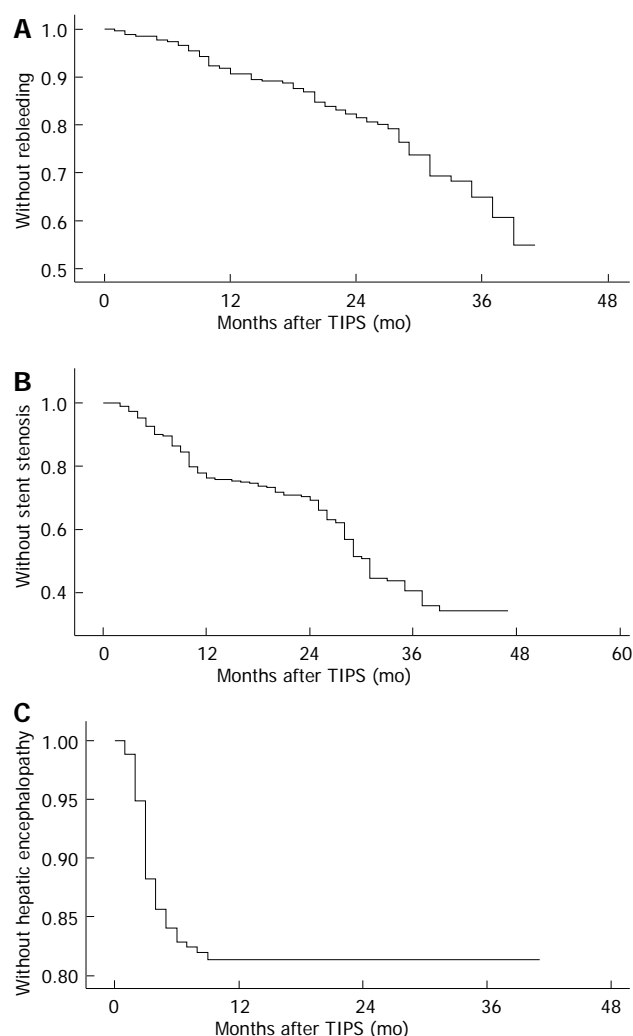
**Table 4** Complications of transjugular intrahepatic portosystemic shunt (*n* = 280) *n* (%)

Complication	
Intraoperative	
Abdominal cavity hemorrhage	2 (0.7)
Puncture of biliary tract	10 (3.6)
Puncture of gallbladder	5 (1.8)
Puncture of hepatic artery	8 (2.9)
Puncture of hepatic capsule	18 (6.4)
Heterotopic embolism	4 (1.4)
Displacement of stent	6 (2.1)
Short-term after TIPS (1 mo)	
Abdominal cavity hemorrhage	2 (0.7)
Hepatic failure	20 (7.2)
Hemorrhagic ascites	7 (2.5)
Hemorrhage of digestive tract	4 (1.4)
Septicemia	3 (1.1)
Hemolysis	8 (2.9)
Hyperglycemia	4 (1.4)
Hemobilia	2 (0.7)
Subcapsular hematoma of liver	3 (1.1)
Puffiness of face	2 (0.7)
Long-term after TIPS (> 1 mo) cumulative incidence	
Stent abnormality	
12 mo	24%
24 mo	34%
Hepatic encephalopathy	
3 mo	14%
6 mo	18%
12 mo	19%

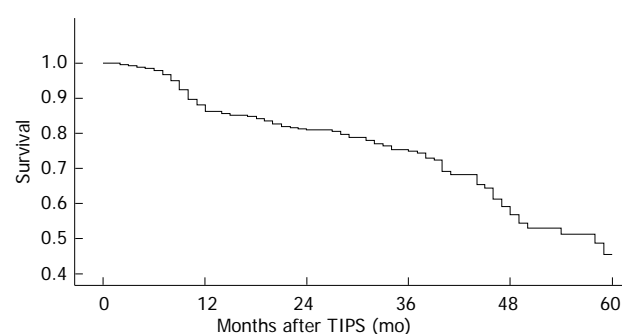
TIPS: Transjugular intrahepatic portosystemic shunt.

sion due to cirrhosis and its complications. Because it is characterized as safe, micro-traumatic, effective and easily repetitive, it has been used more and more widely in clinical practice. Hepatic transplantation has not yet been popularized in China, therefore TIPS is effective for treating portal hypertension due to cirrhosis and its complications, particularly hemorrhage of the digestive tract, and is effective for treating refractory ascites and hydrothorax caused by portal hypertension. It is used mainly in the treatment of approximately 15%-20% of patients with refractory ascites and hemorrhage due to varices that are not responsive to medication or endoscopy. TIPS is used for 99% of cases with these two conditions<sup>[5,6]</sup>. In addition, TIPS is used for the treatment of hepatic hydrothorax, hepatorenal syndrome, hepatopulmonary syndrome and Budd-Chiari syndrome<sup>[7]</sup>. In our study, TIPS was also successfully adopted in emergency and portal thrombosis.

In our study, the success rate of TIPS was 99.3%. Once the shunt pathway was established, the portal vein pressure fell from  $46.5 \pm 3.4$  cmH<sub>2</sub>O before surgery ( $P < 0.01$ ) to  $26.8 \pm 3.6$  cmH<sub>2</sub>O after surgery. The instant rate of hemostasis was 100%. These results are consistent with literature reports<sup>[8-10]</sup> which show that the short-term effective rate of TIPS is 90%-97.4% and the rate of emergency hemorrhage control is 90%-100%. In the present study, the total effective rate of TIPS for ascites was 86% and the rate of elimination of hydrothorax was



**Figure 1** Incidence of recurring hemorrhage (A), stent stenosis (B) and hepatic encephalopathy (C) after transjugular intrahepatic portosystemic shunt.



**Figure 2** Cumulative survival rate after transjugular intrahepatic portosystemic shunt.

100%. These findings are similar to the reported<sup>[9]</sup> effective rate of 50%-92% for refractory ascites and elimination of ascites in 70%-75% of patients. According to the literature reports<sup>[11]</sup>: 82% of patients who underwent TIPS had significantly reduced hydrothorax and in 71% hydrothorax was eliminated, however, patients over 60 did not respond well to TIPS. In our study, hydrotho-



rax was eliminated in 4 cases. However, the number of cases was small and the therapeutic effects remain to be determined. Fifteen patients with hepatorenal syndrome became responsive to diuretic therapy after TIPS and their renal functions were obviously improved. Ascites was eliminated in 7 of these patients and was improved in 8. Eight patients were alive after a one-year of follow-up (53%). According to the literature reports<sup>[12]</sup>, renal function was remarkably improved by TIPS in patients with hepatorenal syndrome and the survival rate was 48% after a one-year follow-up, however, only 10% of the patients who did not undergo TIPS lived for three months. These findings suggest that TIPS is an effective method of treating hepatorenal syndrome.

Of the 280 patients in the present study, 2 had abdominal cavity hemorrhage after TIPS. One patient died and the other survived after portal vein repair. Hemorrhage was due to dilation of the sacculus near the bifurcation of the portal vein. The incidence of this severe complication was 0.7%. It is the most severe complication of TIPS in that a patient immediately suffers from hemorrhagic shock and dies. Therefore, the operator should pay close attention to this complication. As reported<sup>[13]</sup>, the incidence of lethal complications related to the procedure was 0.6%-4.2%. The most critical complications after TIPS were worsening of liver function and hepatic encephalopathy. Both were related to a decrease in blood perfusion in the liver due to the establishment of the shunt pathway<sup>[14]</sup>. In our study, various degrees of hepatic injury occurred in all 280 patients after TIPS, however liver function was gradually restored after approximately 1 mo in most patients. The changes in bilirubin, ALT and Alb were not significantly different. This may be related to our patients having mainly Child-Pugh B and A liver function, few patients with Child-Pugh C liver function (36 patients) and dilation of the path using a balloon of 8 mm inside diameter. The incidence of hepatic failure 1 mo postoperatively was 7.2% (20/278) in our patients, which occurred mainly in emergency and Child-Pugh C TIPS patients. This may be related to poor liver reserve function in some patients and hypoperfusion of the liver due to the artificial shunt and the short supply of hepatic nutrients.

In order to reduce and avoid severe complications of TIPS, the operator is required to be familiar with the anatomy of the portal system. As reported<sup>[15,16]</sup>, the bifurcation of the portal vein is in the liver in about 25.8% of patients, outside the liver in about 48.4% and in the hepatic capsule in about 25.8%. In patients with cirrhosis, the cleavage of the liver is widened and the right trunk and the left horizontal trunk are outside the parenchyma of the liver with bare inferior walls, suggesting that puncture of the bifurcation and peri-bifurcation region is very dangerous. Therefore, the puncture point should be located 2 cm above the bifurcation of the portal vein to reduce or avoid the risk of hemorrhage due to portal vein rupture. In addition, the blood coagulation mechanism is poor in some patients, especially if they have ascites.

Hemorrhage will occur if the hepatic capsule is ruptured, and is not easy to stop. Of our patients, 2 (0.7%) had postoperative abdominal cavity hemorrhage and 7 (2.5%) bloody ascites. The bleeding stopped after management.

The intermediate and long-term complications of TIPS are stent abnormality and hepatic encephalopathy. It is reported<sup>[10,17]</sup> that the rate of stent abnormality (inclusive of stenosis and obstruction) is 17%-50% 6 mo after TIPS and 23%-87% 12 mo after TIPS. The application of a Viatorr stent has improved the condition.

The current criteria<sup>[18-20]</sup> for the evaluation of stent abnormalities (mainly stenosis) are: (1) the velocity of blood flow is over 200 cm/s or less than 50 cm/s in the shunt path, or the diameter of the shunt path is less than 50%; (2) the velocity of blood flow is less than 20 cm/s in the portal vein; (3) the portosystemic pressure gradient is more than or equal to 16 cmH<sub>2</sub>O; (4) portal hypertension recurs, *i.e.*, esophagofundic hemorrhage due to varicose vein or ascites not responsive to low salt diet therapy and routine diuretic therapy. Once the stent abnormality is detected by ultrasound, direct portography and repair should be carried out.

The cumulative rate of stent stenosis is 24% in 12 mo and 34% in 24 mo. The currently used Viatorr stent-graft was first adopted in Europe at the end of 1999 and granted approval by the FDA in 2004. The technical success rate is 100%. The first and second patency rates in one year were 76%-84% and 98%-100%<sup>[21-23]</sup>, respectively. In our study, the patency rate in one year was 76%, which was similar to the first patency rate in the report. Explanations for this rate are as follows: puncture was through the inferior vena cava near the liver (80 cases), avoiding stenosis induced by puncture of the liver vein; the shunt was straight and short apart from the left portal branch; attention was paid to the appliance of the puncture path and care was taken care to avoid angulating the stent; and anticoagulant therapy was given after the procedure, which lasted 1 year. PT was maintained for 17-20 s. It is now accepted that stent stenosis<sup>[24,25]</sup> is related to pseudo-endometrial hyperplasia, the mechanism of which is still unclear but leads to active proliferation of myofibroblasts and the accumulation of extracellular matrix containing collagen. The Viatorr stent-graft has not yet been extensively used, therefore, the prevention of stent stenosis is very important. In our centre a pathological study is now being carried out.

Hepatic encephalopathy is another complication of TIPS. In our study, the incidence of hepatic encephalopathy was 14% in 3 mo and 18% in 6 mo after TIPS. According to the literature<sup>[8,10,21,23,26]</sup>, the incidence of hepatic encephalopathy was 33%-55% after TIPS and 13%-26% after therapeutic endoscopy. International reports<sup>[27,28]</sup> showed that there was no significant difference in the occurrence of hepatic encephalopathy between the bare and Viatorr stents 10 mm in diameter, and the incidence was 20%-30%. The incidence of hepatic encephalopathy was 5%-10% when Viatorr stents of 8 mm in diameter were used, which supported shunting without hepatic

encephalopathy. In our study, the incidence of hepatic encephalopathy was lower than that reported and similar to that of the Viatorr stent and endoscope, which may be related to the selection of patients, puncture paths, stent diameters, etiological treatment and postoperative management. Of the 280 patients, most were graded as Child-Pugh A and B (244/280) with better liver function potential. Cirrhosis was induced mainly by HBV (238/280) and antiviral therapy was given before and after surgery. The patients' general physical condition was improved before surgery as far as possible. A stent with an appropriate diameter was carefully selected to avoid over shunting. Generally, we chose a balloon of 8 mm inside diameter and a stent of 8 mm or 10 mm inside diameter. For all patients, protein intake was limited 1 wk after surgery, bowel movement was regulated and enema with vinegar ordered to prevent intestinal infection. The mechanism of hepatic encephalopathy<sup>[29]</sup> involves multiple factors, but is mainly related to a decrease in blood flow and enhancement of the biological availability of enteric toxins.

The rate of recurrent hemorrhage was 9% in 1 year and 19% in 2 years after TIPS in our study. From previous reports<sup>[10,17,21,30]</sup> the rate was 15% in 1 year and 21% in 2 years after TIPS; and was 48% in 1 year and 52% in 2 years after gastroscopic treatment; and was less than 10% with the Viatorr stent. It was believed that the rate of recurrent hemorrhage was higher in the gastroscope group than in the TIPS group; and was lower in the Viatorr stent group than in the bare stent group. In our study, the rate of recurrent hemorrhage was similar to that of the Viatorr stent. Recurrent hemorrhage was related to stent abnormality. Any cause of stent stenosis or obstruction could lead to portal hypertension again, and the obstructed collateral circulation might reopen or a new collateral circulation could appear, resulting in hemorrhage from esophageal or fundic varices. Once the varicose vein ruptures, recurrent hemorrhage occurs. The maintenance of stent function is important in avoiding this situation. In our research, the rate of stent stenosis was low and the rate of recurrent hemorrhage was also low. In addition, the collateral vein with esophageal and fundic varices due to intraoperative embolism could significantly reduce or delay the occurrence of rebleeding.

Gastroscopy was performed in our patients. The results showed that 68% of patients had complete relief of esophageal varices and 32% had obvious relief. Approximately 80% of patients had complete relief of stomach fundic varices and 20% had obvious relief. This confirmed the effectiveness of TIPS and was an important procedure for recurrent hemorrhage. In our patients, hyperglycemia, puffiness of the face and other rare complications occurred in addition to the complications reported. Hyperglycemia may be explained by the metabolic disorder of glucose in the liver and insulin injection is indicated. The cause of puffiness of the face is unclear, but it gradually disappeared following diuretic therapy.

The cumulative survival rate was 86% at 1 year and 81% at 2 years after TIPS in our patients, which was simi-

lar to previously published reports<sup>[8,9]</sup> that is 64%-87% at 1 year and 56%-71% at 2 years after TIPS. Survival is related to liver function reserve. The survival rate was lower in patients graded as Child-Pugh C than in patients graded as A and B. Our patients were mainly Child-Pugh B, and few patients had Child-Pugh A and B. Statistical analysis showed that the survival rate of the patients was not significantly correlated with their Child-Pugh grading of liver function, which requires further study. There were 15 patients with hepatorenal syndrome in our study, with a death rate of 47% 1 year after TIPS. Three patients died 1 mo postoperatively, of whom 2 died of hepatic failure and 1 of hematosepsis. Our patients mainly developed cirrhosis after hepatitis B virus infection. The etiological treatment is critical in that we found that antiviral therapy and moderate shunting prolonged the survival of patients, especially of those graded as Child-Pugh A and B. These findings remain to be confirmed by future multicenter, randomized and controlled trials.

TIPS is characterized by its effectiveness and few complications. However, stent stenosis and hepatic encephalopathy are still leading factors affecting the intermediate and long-term therapeutic effects. Even though these problems can be solved to a considerable degree by the use of the Viatorr stent, this procedure is not popular in China, and the mechanism of stent stenosis remains to be studied further. Therefore the determination of the therapeutic effects and the association of the shunt pathway with encephalopathy requires further research.

## COMMENTS

### Background

Esophageal and fundic varicose hemorrhage is a critical complication of portal hypertension due to cirrhosis, and often endangers the patient's life. The clinical effects of routine treatment on hepatic thoracoabdominal ascites and hepatorenal syndrome are not good. Transjugular intrahepatic portosystemic shunt (TIPS) is an ideal method of treating these complications.

### Research frontiers

TIPS is one of the most difficult operations in vascular interventional therapy at the present time. A shunt path must be established between the branches of the hepatic veins and portal vein, and at the same time a collateral circulation by embolization in esophageal and fundic varices is necessary to achieve a partial shunt and cutout. TIPS has progressively become the method of choice for treating portal hypertension due to cirrhosis and its complications.

### Innovations and breakthroughs

By comparison with the results in the literature, in this study, TIPS improved patient outcome. Portal vein puncture was guided by replacing routine trans-superior mesenteric indirect portal venography with hepatic enhanced computed tomography (CT) scanning and hepatic portal vein CT three-dimensional graphic reconstruction. More cases were treated as the technique was developed. The patients were followed up over a long period, and satisfactory clinical effects were achieved.

### Applications

As the authors were unable to carry out hepatic transplantation, the complications of cirrhosis were mainly managed clinically, especially varicose hemorrhage and intractable thoracoabdominal ascites. The effects of the presently used drugs, endoscopes and surgical management are not ideal, however, treatment by TIPS has achieved satisfactory results. With the constant expansion of indications, TIPS will be used more extensively.

### Terminology

TIPS involves the establishment of a shunt path in the liver parenchyma between the two puncture points after paracentesis from the right hepatic vein or

hepatic segment of inferior vena cava to the branch of the portal vein, shunting of the portal venous blood flow and lower portal venous pressure, and at the same time causes a collateral circulation by embolization of esophageal and fundic varices and blockage of hemorrhagic blood vessels.

### Peer review

This study comprehensively and systematically evaluated the clinical effects and complications of TIPS for portal hypertension due to cirrhosis, and described how to improve the therapeutic effects of TIPS and the experience in reducing its complications. The improved TIPS is of great clinical significance and favors clinical dissemination.

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## “Metroticket” predictor for assessing liver transplantation to treat hepatocellular carcinoma: A single-center analysis in mainland China

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### Abstract

**AIM:** To validate the “Metroticket” predictor using a large cohort of liver transplantation (LT) patients with hepatocellular carcinoma (HCC) in China.

**METHODS:** In total, 230 cases of LT for HCC treatment at our center, from July 2000 to August 2008, were included in the present study. The predicted 1-, 3- and 5-year post-LT survival rates were calculated using the Metroticket model (<http://89.96.76.14/metroticket/calculator/>). The predicted and observed long-term survival rates were then compared and analyzed.

**RESULTS:** The predicted survival rates for all 230 cases, as calculated by the Metroticket model, were 64.7% and 56.2% at 3 and 5 years, respectively, and the observed survival rates for these patients were 71.3% and 57.8%, respectively. For the 23 cases with macrovascular invasion, the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%. For the 42 cases with microvascular invasion but an absence of macrovascular invasion,

the predicted 5-year survival rate was 44.9%, and the observed 5-year survival rate was 50%. For the remaining 165 patients without any vascular invasion, the predicted 5-year survival rate was 65.8%, and the observed 5-year survival rate was 66.7%.

**CONCLUSION:** The Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

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**Key words:** Metroticket; Model; Survival; Hepatocellular carcinoma; Liver transplantation

**Core tip:** The aim of our study was to validate the “Metroticket” predictor using a large cohort of liver transplantation (LT) patients with hepatocellular carcinoma (HCC). The predicted survival rates for all 230 cases, as calculated by the Metroticket model, were 64.7% and 56.2% at 3 and 5 years, respectively, and the observed survival rates for these patients were 71.3% and 62.2%, respectively. For the 23 cases with macrovascular invasion, the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%. The Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

Lei JY, Wang WT, Yan LN. “Metroticket” predictor for assessing liver transplantation to treat hepatocellular carcinoma: A single-center analysis in mainland China. *World J Gastroenterol* 2013; 19(44): 8093-8098 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8093.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8093>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer globally<sup>[1]</sup>, and this burden is heavier in China, which accounts for nearly 55% of all cases worldwide<sup>[2]</sup>. Despite the prevalence of using the hepatitis B vaccine in recent years, HCC is also the fifth most common malignancy in males and the sixth most common in females in China<sup>[3]</sup>. Liver transplantation (LT), resection and radiofrequency ablation (RFA) were once the only three potential curative treatments for early HCC<sup>[4]</sup>. LT was theoretically the best therapeutic option for HCC patients due to the procedure's overall eradication of the remnant liver with cirrhosis compared with resection and RFA<sup>[5,6]</sup>. Despite its thoroughness, LT was not suitable for all HCC cases: in that time, the very low survival rate after LT in HCC patients was mainly due to advanced HCC<sup>[7]</sup>. The Milan criteria, which were proposed in 1996 by Mazzaferro *et al.*<sup>[8]</sup>, resulted in excellent survival, with a 5-year survival rate of 61.1% compared with the previously observed 5-year survival rate of 25.3% in 1987. Thereafter, dozens of inclusion criteria were introduced for HCC-related LT<sup>[9-13]</sup>. However, these criteria were only inclusion criteria and could not be used to predict the results of LT, and especially the survival and recurrence rates.

In recent years, many groups have found certain risk factors that predict survival and recurrence after LT in HCC patients<sup>[14-18]</sup>. However, only few researchers have found risk factors for HCC recurrence after LT and built predictive models, such as the Metroticket<sup>[19]</sup>, Alpha-feto-protein (AFP)<sup>[20]</sup> and Markov<sup>[21]</sup> models. Derived from the largest collection of pathological data from patients with HCC (1556 overall and 1112 exceeding the Milan criteria), the Metroticket model offers individualized survival predictions based on a continuum of tumor size and number, whereby each patient is assigned an individual prognosis for 3- and 5-year survival<sup>[22]</sup>. The Metroticket model has been validated in several studies<sup>[6,21,22]</sup>. However, no analysis has been performed on the effectiveness of this predictive model using data from China with a large cohort of HCC cases, where nearly 55% of all cases worldwide<sup>[2]</sup> occurred and 24801 cases of LT were performed. Thus, in the present study, we aimed to prove the prognostic accuracy of the Metroticket model using single-center data from mainland China.

## MATERIALS AND METHODS

Our study used data from a retrospective database on LT in HCC patients that was developed at our center between August 2000 and August 2008 (230 consecutive patients). All of the data from these patients, including baseline demographic data, preoperative laboratory and radiological data, intraoperative data, postoperative recovery data and long-term outcomes, were retrospectively analyzed. All of these data were collected from the China Liver Transplant Registry System. Demographic data in-

cluded age, gender, height, weight and body mass index (BMI). Preoperative liver function data included underlying liver disease and liver function (Child score and MELD score). Tumor characteristics included the tumor number, diameter and differentiation. Intraoperative data included the graft type (DDLT/LDLT), operative time, blood loss and rate of transfusion. Postoperative data included mortality, complications (classified using the Clavien system), hospital stay days and overall cost. Long-term outcomes were mainly the overall survival rate.

The diagnosis of HCC was confirmed preoperatively in all patients if the patient simultaneously fulfilled the following three criteria: radiological evidence of HCC (helical triple-phase computed tomography or magnetic resonance imaging scans in arterial, portal venous and delayed venous phases; blush with washout; and a pseudo-capsule), serology positive for hepatitis B or C and levels of AFP > 400 ng/mL. If the patient lacked one of these features, biopsy (histology or cytology) was performed to prove HCC. For each patient in the present study, a "Metroticket"-predicted survival score was calculated using the online calculator (<http://89.96.76.14/metroticket/calculator/>). All of the imaging data were based on pre-transplant radiological measurements obtained within 15 d pre-LT. The Metroticket calculator only incorporates tumors greater than 10 mm in diameter and no more than 10 nodules. We also divided all of the patients into subgroups according to the presence of micro- and macrovascular invasion. Thus, the main analysis was a comparison between the Metroticket model-predicted and observed survival rates, and the subgroup analysis also compared the Metroticket model-predicted and observed survival rates in the presence and absence of macrovascular invasion.

All of the deceased donors were brain-dead donors at our hospital, and no prisoners served as donors at our center. All of the liver donations were voluntary and altruistic. Written consent was given by the donors or their families. For all of these procedures, authorization was obtained from the donors' families, the ethics committee and the Red Cross Society of China. The surgical procedure and postoperative antiviral and immunosuppression protocols have been previously reported<sup>[23-25]</sup>.

Descriptive statistics are expressed as proportion for categorical variables, and mean  $\pm$  SD or median and range were used for continuous variables. The predicted survival rates at 3 and 5 years were calculated using the Metroticket online calculator for each patient, and the mean sum of the individual scores was calculated and compared with our observed survival rates at 3 and 5 years. Overall survival was defined as the time interval between LT and death from any cause. Survival rates were estimated using the Kaplan-Meier method, whereas statistical significance between survival curves was tested by the log-rank test. Statistical tests were considered to be significant when the corresponding *P*-value was less than 5%. Statistical analyses were performed using the SPSS package (SPSS 17.0, Inc., Chicago, IL).

**Table 1** Complications of recipients, as classified by the Clavien system *n* (%)

	LT to treat HCC <i>n</i> = 230
Grade I: Treated conservatively without any drugs	22 (9.6)
Pleural effusion	8
Wound infection	8
Bile leak	6
Grade II: Treated with medication	14 (6.1)
Pneumonia	2
Ascites	2
Bile leak	2
Acute or chronic rejection	6
Hepatic artery thrombosis	2
Grade IIIa: Intervention using local anesthesia	25 (10.9)
Hydrothorax	11
Bile leak	6
Ileus	2
Upper gastrointestinal bleeding	3
Intra-abdominal abscess	3
Grade IIIb: Intervention using general anesthesia	17 (7.4)
Intra-abdominal Bleeding	6
Biliary obstruction	3
Intra-abdominal abscess	4
Portal venous thrombosis	2
Hepatic artery thrombosis	2
Grade IVa: Single-organ dysfunction	6 (2.6)
Small-for-size syndrome	2
Renal dysfunction	2
Respiratory failure	2
Grade IVb: Multi-organ dysfunction	2 (0.9)
Grade V: Death	22 (9.6)
Respiratory failure	3
Graft-vs-host disease	1
Cardiopulmonary arrest	2
Liver failure	4
Septic shock	3
Bleeding	3
Rejection	5

LT: Liver transplantation; HCC: Hepatocellular carcinoma.

## RESULTS

The baseline demographics of all patients showed that there were many more male patients (210 cases) than female ones (20 cases). The patients' mean age was  $46.1 \pm 10.3$  years, mean height was  $165.2 \pm 9.1$  cm, mean weight was  $67.3 \pm 8.8$  kg and mean BMI was  $23.2 \pm 2.2$  kg/m<sup>2</sup>. Underlying liver disease showed that most of these patients (215 cases) were diagnosed with HBV infection. Two patients had HCV, and 13 patients did not have hepatitis B or C. There were 100 patients who were HBV-DNA positive ( $> 1.00E + 03$  copies/mL). The preoperative liver function reflected by the MELD score of these patients was  $11.1 \pm 5.5$  and 129 patients had Child-Pugh A, 66 patients had Child-Pugh B, and 36 patients had Child-Pugh C.

The preoperative imaging scan indicated that the mean diameter of all targets was  $8.6 \pm 5.0$  cm and that the mean target number was  $3.1 \pm 2.9$  for these HCC patients. In total, 26 new tumor targets were found in the explanted liver in 14 patients, and the diameter of these new targets ranged from 0.6 to 2.4 cm. The mean

preoperative AFP level was 1838.2 ng/mL:  $< 400$  ng/mL in 97 patients, 400-800 ng/mL in 12 patients, 800-1200 ng/mL in 19 patients, and  $> 1200$  ng/mL in 102 patients. Explanted tumor histopathologic grading indicated 78 patients with good differentiation, 78 patients with moderate differentiation and 74 patients with poor differentiation.

The intraoperative and postoperative data showed that 177 patients had accepted whole-graft LT and that 53 cases had accepted living-donor LT at our center. The mean graft to recipient weight ratio was 0.81 for the 53 DDLT cases. The mean operative time was  $7.8 \pm 2.1$  h, mean blood loss was  $874.5 \pm 422.5$  mL, and mean length of hospital stay was  $33.2 \pm 12.3$  d. Table 1 shows the postoperative complications for all cases. All of these postoperative complications were classified using the Clavien system. The overall complication rate was 47%, the serious (more than grade III) complication rate was 22.5%, and the mortality rate was 9.6% in the hospital.

For all 230 patients, the predicted survival rates calculated by the Metroticket model based on preoperative imaging data were 64.7% and 56.2% at 3 and 5 years, respectively, and the observed survival rates for these patients were 71.3% and 57.8%, respectively. The actuarial 3- and 5-year survival rates were 71.7% (95%CI: 62.3%-77.0%) and 64.8% (53.5%-68.4%), respectively. The Metroticket predictions of the 3- and 5-year survival rates both fell within the 95%CI of the actuarial survival. For the subgroup patients (23 cases) with macrovascular invasion, the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%. For the subgroup patients (42 cases) with microvascular invasion but an absence of macrovascular invasion (as proven by pathological examination), the predicted 5-year survival rate was 44.9%, and the observed 5-year survival rate was 50%. For the patients (165 cases) without macro- or microvascular invasion, the predicted 5-year survival rate was 65.8%, and the observed 5-year survival rate was 66.7%. The most common recurrence site was the liver (78.6%), followed by intra-abdominal metastasis (22.1%), lung metastasis (20.2%), bone metastasis (13.2%) and brain metastasis (4.6%).

## DISCUSSION

For HCC patients, LT is one of the most effective treatments. However, there are still continual pressure on limited donor resources, especially in China, and debate about what should be considered as an acceptable minimum survival outcome<sup>[22]</sup>. Since the first introduction of the Milan criteria for HCC-related LT in 1996, proposed by Mazzaferro *et al*<sup>[8]</sup>, more than one decade of excellent outcomes of LT for HCC treatment was achieved with these restrictive selection criteria. However, many groups worldwide have suggested expanding the Milan criteria due to comparable survival and recurrence outcomes<sup>[9-13]</sup>. Groups everywhere have suggested adding different types of risk factors for recurrence to the inclusion criteria: for example, Toso *et al*<sup>[26]</sup> proposed a total volume

of 115 cm<sup>[3,11]</sup>, and Zheng *et al.*<sup>[27]</sup> proposed the AFP level and histological grade. However, most of the criteria only considered the tumor diameter or number alone<sup>[10]</sup>. The Metroticket calculator was the first to combine the tumor number with the size of the largest nodule and is a model designed to predict 3- and 5-year overall survival after transplantation on the basis of the characteristics of the HCC (the size of the largest nodule, the number of nodules and the presence or absence of vascular invasion) in a given patient. This model changes the paradigm from "one size fits all" to an individual prognosis for each patient<sup>[22]</sup>. Our key finding is that the Metroticket calculator is an accurate predictor of post-transplant survival for patients with an absence of macrovascular or microvascular invasion, but not for patients with macrovascular invasion.

The Metroticket model was built in 2009 based on data from Europe. These HCC cases were caused by alcoholic or hepatitis C virus-related liver cirrhosis. Raj *et al.*<sup>[22]</sup> tried to evaluate the veracity of this model, but the study cohort was relatively small (82 cases), as mentioned as a weakness in the report, and only 40 cases included HBV. Compared with the small sample size and low rate of HBV cases in Raj's study, our study included 230 cases of HCC-related LT, and nearly all of our cases (93.5%, 215 cases) were HBV cases. Thus, our study may be more reliable and convincing. The Metroticket calculator was derived from explants' pathological data, but many reports<sup>[21,22,28]</sup> have proven the model's validity based on pre-transplant radiological criteria. Therefore, this model can be applied prospectively to patient selection.

Compared with other inclusion criteria, such as the Milan, Up-to-Seven and UCSF criteria, the Metroticket model provides a continuous range of survival probabilities rather than a dichotomous "in or out" basis for patient selection<sup>[22]</sup>. The upper limit of the tumor number is 10, and there is no upper limit for tumor diameter; the calculated tumor diameter is the largest one. Most importantly, the model also considers the presence of vascular invasion, which is a very strong risk factor for HCC recurrence after LT. This model considered all of these risk factors when it was built and thus may provide a reliable prediction of outcome for a patient who plans to accept LT for HCC treatment. However, there are certain limitations, as mentioned in Raj's study<sup>[22]</sup>. The diagnosis of microvascular invasion requires biopsy, with a risk of needle-tract seeding<sup>[29]</sup> and bleeding and false negatives<sup>[30,31]</sup>. Several other risk factors are AFP levels<sup>[32,33]</sup>, the neutrophil-to-lymphocyte ratio<sup>[15,34]</sup> and the serum C-reactive protein<sup>[35]</sup> and gene<sup>[36]</sup>, and all of these reported risk factors and biomarkers are available before transplantation and can be routinely used to predict recurrence and survival after HCC-related LT.

In the present study, we first examined the effectiveness of the Metroticket model in a subgroup of patients with macrovascular invasion. Our results showed that in subgroup patients with macrovascular invasion, the observed 5-year survival rate was only 8.7%, which was

much lower than the predicted 5-year survival rate of 43.5%. It is known that vascular invasion is an independent risk factor for HCC recurrence after LT, especially in the presence of macrovascular invasion<sup>[16]</sup>. However, there are still certain differences between the effects of macro- and microvascular invasion on HCC recurrence. As mentioned in other studies, macrovascular but not microvascular invasion is a risk factor for HCC recurrence<sup>[37,38]</sup>. In the present study, we found that the Metroticket model can be used to predict the outcome of microvascular invasion cases but not macrovascular invasion cases. However, the Metroticket calculator website does not make a distinction between micro and macrovascular invasion. Based on our results, we believe that the Metroticket calculator needs revision on the topic of vascular invasion.

Certain potential limitations of this study are related to our single-center data analysis. The need for a 5-year follow-up limited the size of our study, as we could only include patients (230 cases) who received transplants before 2009. The retrospective nature of our study also limited the reliability. In future work, multiple-center, randomized control trials and a larger number of studies may be needed.

In conclusion, with accurately predicted 3- and 5-year survival rates, the Metroticket model should be introduced as a useful tool for selecting HCC patients for LT based on preoperative imaging examinations. However, macrovascular invasion should be considered as a contraindication to use of the Metroticket model.

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## COMMENTS

### Background

Liver transplantation was theoretically the best therapeutic option for hepatocellular carcinoma (HCC) patients due to the procedure's overall eradication of the remnant liver with cirrhosis compared with resection and radiofrequency ablation. Dozens of inclusion criteria were introduced for HCC-related liver transplantation (LT). However, these criteria were only inclusion criteria and could not be used to predict the results of LT, and especially the survival and recurrence rates. Recent years, many groups have found certain risk factors that predict survival and recurrence after LT in HCC patients. However, only few researchers have found risk factors for HCC recurrence after LT and built predictive models, such as the Metroticket. The Metroticket model offers individualized survival predictions based on a continuum of tumor size and number.

### Research frontiers

The Metroticket model has been validated in several studies. However, no analysis has been performed on the effectiveness of this predictive model using data from China. Thus, in the present study, this study aimed to prove the prognostic accuracy of the Metroticket model using single-center data from mainland China.

### Innovations and breakthroughs

The Metroticket model was introduced several years ago, but there is still no consensus about its effectiveness. 230 cases of LT for HCC treatment at our center were included in the present study. The predicted 1-, 3- and 5-year post-LT survival rates were calculated using the Metroticket model (<http://89.96.76.14/metroticket/calculator/>). The predicted and observed long-term survival rates



were then compared and analyzed. Due to the similar predicted and observed long-term survival rates, the Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

### Applications

The Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

### Terminology

Liver transplantation is a surgical method to cure end-stage liver disease, removing the liver with disease and implanting one or part of new liver from the donor.

### Peer review

This is an interesting study to evaluate the effectiveness of the Metroticket model.

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## Decreased histone H2B monoubiquitination in malignant gastric carcinoma

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detect the differential levels of uH2B, H3K4-2me and H3K4-3me modifications in GC specimens from chemo/radiotherapy-naïve patients who underwent potentially curative surgical resection ( $n = 159$ ) and in a random sampling of non-tumor gastric epithelium specimens (normal controls,  $n = 20$ ). The immunohistochemistry (IHC)-detected modifications were classified as negative, low-level, or high-level using a dual-rated (staining intensity and percentage of positively-stained cells) semi-quantitative method. The relationships between uH2B modification levels and clinicopathological parameters of GC were assessed by a Wilcoxon rank sum test (pairwise comparisons) and the Kruskal-Wallis H test (multiple comparisons). The correlation between uH2B modification and survival was estimated by Kaplan-Meier analysis, and the role of uH2B as an independent prognostic factor for survival was assessed by multivariate Cox regression analysis.

**RESULTS:** The presence and level of H3K4-2me and H3K4-3me IHC staining was similar between the normal controls and GC specimens. In contrast, the level of uH2B was significantly lower in the malignant gastric tissues (*vs* normal control tissues) and decreased along with increases in dedifferentiation (well differentiated > moderately differentiated > poorly differentiated). The level of uH2B correlated with tumor differentiation ( $P < 0.001$ ), Lauren's diffuse- and intestinal-type classification ( $P < 0.001$ ), lymph node metastasis ( $P = 0.049$ ) and tumor-node-metastasis stage ( $P = 0.005$ ). Patients with uH2B+ staining had higher 5-year survival rates than patients with uH2B-staining ( $52.692 \pm 2.452$  *vs*  $23.739 \pm 5.207$ ,  $P < 0.001$ ). The uH2B level was an independent prognostic factor for cancer-specific survival (95%CI: 0.237-0.677,  $P = 0.001$ ).

**CONCLUSION:** uH2B displays differential IHC staining patterns corresponding to progressive stages of GC. uH2B may contribute to tumorigenesis and could be a potential therapeutic target.

### Abstract

**AIM:** To investigate H2B monoubiquitination (uH2B) and H3K4 di- and tri-methylation (H3K4-2me, H3K4-3me) levels and their clinical significance in gastric cancer (GC).

**METHODS:** Immunohistochemistry (IGC) was used to

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**Key words:** Gastric cancer; Epigenetics; Histone modification; H2B monoubiquitination; Nuclear immunostaining

**Core tip:** The abundant H2B monoubiquitination (uH2B) modification detected by immunohistochemistry (IHC) in normal human gastric epithelium is decreased in malignant gastric cancer specimens, and the decreasing trend is correlated with decreased tumor differentiation, Lauren's classification intestinal-type, presence of lymph node metastasis, and TNM stage. Positive uH2B staining is associated with higher 5-year survival. Multivariate analysis identified uH2B modification level as an independent prognostic factor for gastric cancer-specific survival. Collectively, these findings indicate the clinical significance of IHC-detected uH2B differential staining patterns as a potential prognostic biomarker in early stage gastric cancer.

Wang ZJ, Yang JL, Wang YP, Lou JY, Chen J, Liu C, Guo LD. Decreased histone H2B monoubiquitination in malignant gastric carcinoma. *World J Gastroenterol* 2013; 19(44): 8099-8107 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8099.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8099>

## INTRODUCTION

Focused public health efforts to increase awareness of gastric cancer (GC) and implementation of screening programs to detect malignancy in asymptomatic patients have led to a decline in the overall mortality of this disease worldwide. Asian countries continue to report the highest incidence rates of GC and these cases have worse prognosis. The low overall 5-year survival rate of GC cases in China (about 40%)<sup>[1,2]</sup> highlights the particular burden facing these nations' healthcare systems and the impact on the overall social and economic well-being of their citizens.

The aggressive nature of GC remains a particular challenge to clinical management of this malignancy, and surgical resection of the affected tissues is the only effective treatment, with chemo/radiotherapy providing some benefit as adjuvant treatment. However, the efficacy of GC surgery is reliant upon the disease stage at which it is applied. Delays associated with incorrect or mis-diagnosis of the generally non-specific clinical symptoms in early stage GC (when the tumor is localized and has low risk of metastasis) can completely preclude surgery. Indeed, it has been reported that > 30% of GC patients in China are diagnosed at malignancy stages that are too far advanced for resection to be a feasible (benefit: risk) option<sup>[3]</sup>.

One way to improve timely diagnosis in GC patients is to develop more accurate and sensitive methods of screening. Biomarkers, such as epigenetic modifications,

are good candidates for such tests as they are detectable in serum samples and may reflect not only the presence of disease, but also its prognosis (when differential levels correspond to progressive stages of tumor pathology). In addition, diagnostic and prognostic biomarkers represent putative molecular targets of therapeutic strategies and may be exploited to develop more effective, less invasive and more individualized therapies against these aggressive tumors.

Several forms of epigenetic modifications exist, and their various alterations to the chromatin structure affect gene expression and have been implicated in pathological processes underlying a multitude of disease conditions, including tumorigenesis<sup>[4,5]</sup>. In particular, the post-translational modifications (PTMs) of histones, including acetylation, methylation, phosphorylation and ubiquitination, function as regulators of DNA-associated signaling networks required for normal physiological processes<sup>[6]</sup>, such as cell growth, cycling, and movement - all important features of human cancer<sup>[6-9]</sup>.

Compared to the other histone modifications, ubiquitination is less well studied and its specific roles in many types of tumors remain to be precisely defined. Focused research efforts involving monoubiquitination of lysine 120 on histone H2B (uH2B), however, have begun to elucidate its regulatory mechanism and its downstream effects under normal physiological conditions. Upon catalyzation by ubiquitin-conjugating enzyme (Rad6) and ubiquitin-protein ligase (RNF20)<sup>[10,11]</sup>, uH2B acts to promote or suppress gene transcription<sup>[12,13]</sup>. Intriguingly, recruitment of RNF20 to gene promoter regions, mediated by transactivators such as Gal4 or p53, has been shown to be required for full induction of transcription of genes related to cancer, such as p21 and MDM2. Furthermore, de-regulation of uH2B has been suggested as an etiology of cancer development<sup>[14,15]</sup>.

The current study was designed to investigate the potential roles of three forms of histone modification, uHB and di- and tri-methylation at H3 lysine 4 (H3K4-2me and H3K4-3me, respectively), in gastric carcinoma and in relation to its clinicopathological features. Detecting cancer type- and stage-specific differential immunohistochemistry (IHC)-staining patterns of histone modifications may represent a useful biomarker-based prognostic method and provide novel insights into potentially manipulable targets of anti-GC molecular therapies.

## MATERIALS AND METHODS

### Clinical samples

One-hundred-and-fifty-nine formalin-fixed, paraffin-embedded GC tissue specimens obtained from gastrectomy or upper-gastrointestinal endoscopy performed at the Department of Gastrointestinal Surgery and Digestive Endoscopy Center of West China Hospital between January 2006 to January 2007 were selected for analysis. The GC specimens included 23 well-differentiated, 55



moderately-differentiated and 81 poorly-differentiated tumors. According to the Lauren classification system, 60 were intestinal-type and 59 were diffuse-type GC. According to staging by the tumor-node-metastasis (TNM) system, 15 were at stage I, 20 were at stage II, 99 were at stage III and 25 were at stage IV.

According to the medical records, all GC specimens were obtained during potentially curative surgical resection, and none of the patients had received preoperative chemotherapy or radiotherapy. Follow-up data was available for all patients until December 2012 or until death.

In addition, 20 non-tumor gastric mucosa specimens, including sections from normal and inflammatory epithelium, were randomly selected for use as normal controls.

### ***IHC staining of uH2B, H3K4-2me, and H3K4-3me***

The GC and normal control specimens (5  $\mu$ m) were deparaffinized and incubated with 0.3% hydrogen peroxide in 28% methanol for 30 min to quench the endogenous peroxidase activity. Following EDTA/high-pressure antigen retrieval, the sections were exposed to 1% bovine serum albumin for 20 min to block non-specific binding sites and then to primary antibodies against uH2B, H3K4-2me and H3K4-3me (Cat. No. 05-1312, 05-1338, and 05-1339 respectively; Millipore, Billerica, MA, United States) for 30 min. An additional 15 min post-antibody blocking step was carried out before exposure to the PowerVision+ poly-horseradish peroxidase (HRP)-anti-mouse/rabbit IgG secondary antibodies (Leica Biosystems, Newcastle, United Kingdom) for 30 min and HRP antibody (VECTASTAIN<sup>®</sup>; Vector Laboratories Inc., Burlingame, CA, United States) for 30 min. Immunoreactivity was visualized upon exposure to the DAB chromogen. The processed tissue sections were then counterstained with hematoxylin, dehydrated and mounted.

### ***Dual-rated semiquantitative analysis of IHC staining levels***

The degree of uH2B, H3K4-2me and H3K4-3me immunostaining in each specimen was assessed by two investigators (Yang JL and Wang YP) working independently, as described below. The two sets of results were compared and in the case of disagreement, the section was re-examined by both investigators simultaneously with discussion to achieve a consensus score.

For each processed specimen, three high-power ( $\times 200$ ) magnification fields encompassing an average of 1000 cells (range: 800-1200) were selected (BX51 microscope; Olympus, Tokyo, Japan) and image obtained (FAST 1394 camera with accompanying QCapture suite software; QImaging, Surrey, BC, Canada) to capture an overall representation of different staining densities. An immunoreactivity score (IRS) for each of the three modifications detected was calculated as the product of staining intensity (SI) multiplied by percentage of positively-stained cells (PP)<sup>[16]</sup>. SI was defined according to a four-point gradient scale, where no staining = 0, weak-coloring (light yellow) = 1, moderate-coloring (bright yellow) = 2,

and strong-coloring (brown) = 3. PP was defined according to a four-point positive/negative scale, where 0-9% positive cells = 0, 10%-25% positive cells = 1, 26%-50% positive cells = 2, 51%-75% positive cells = 3, and > 75% positive cells = 4.

The triplicate IRS scores for each of the three detected modifications were averaged for each specimen and used to classify the degree of uH2B, H3K4-2me and H3K4-3me immunostaining as follows: no modification: 0; low-level modification; 1-5; high-level modification:  $\geq 6$ .

### ***Statistical analysis***

All statistical analyses were performed by the SPSS software suite, version 13.0 (SPSS Inc., Chicago, IL, United States). The relationships between uH2B modification levels and clinicopathological parameters of GC were examined by a Wilcoxon rank sum test (for pairwise comparisons) and the Kruskal-Wallis *H* test (for multiple comparisons). The correlation between uH2B modification and survival was estimated by Kaplan-Meier analysis. The role of uH2B as an independent prognostic factor for survival was assessed by multivariate Cox regression analysis. The threshold for statistical significance was set as  $P < 0.05$ .

## **RESULTS**

### ***uH2B, and not H3K4-2me or H3K4-3me, shows differential IHC staining in GC associated with extent of tumor differentiation***

The IHC staining patterns of H3K4-2me and H3K4-3me were similar between the GC and normal control tissues, with the nuclear staining distributed evenly, regardless cancer status or tumor differentiation (Figure 1). In contrast, the uH2B staining patterns and IRS scores were remarkably different between the GC and the normal control tissues, as well as between the different classes of tumor differentiation (Figure 2). All 20 non-tumor mucosa specimens showed high-level uH2B modification ( $\geq 6$  IRS). The amount of GC specimens with high-level uH2B modification decreased in conjunction with increasing level of tumor dedifferentiation, with IRS scores  $\geq 6$  seen in 65.2% (15/23) of well-differentiated GC tumors, 47.2% (26/55) of moderately-differentiated GC tumors, and 2.4% (2/81) of poorly-differentiated GC tumors. Moreover, this trend of decreased uH2B with increased degree of differentiation was statistically significant ( $P < 0.001$ , Table 1), suggesting that uH2B may play a role in maintenance of tumor differentiation.

### ***Differential uH2B IHC staining correlates with Lauren classification of the histological type of tumor***

When the GC specimens were divided by the Lauren classification, significantly more of the intestinal-type samples showed positive uH2B staining than the diffuse-type samples [90.0% (54/60) *vs* 71.2% (42/59),  $P <$

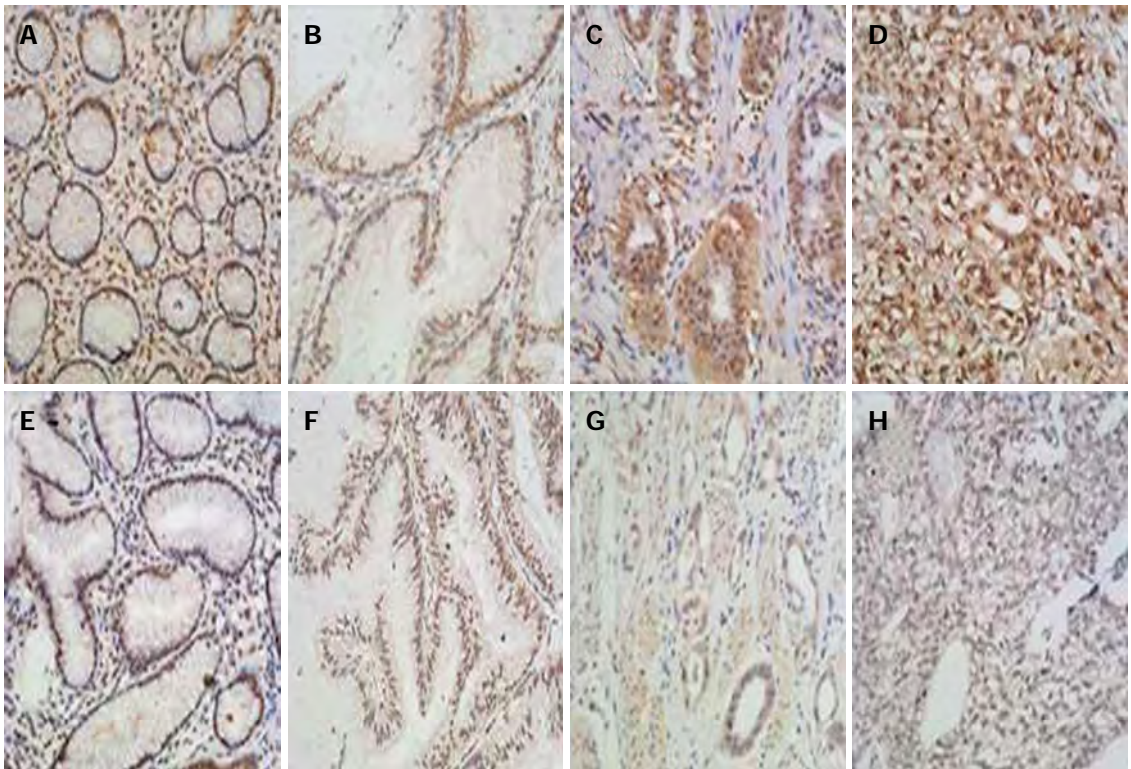


Figure 1 Immunohistochemical nuclear staining of H3K4-2me and H3K4-3me. H3K4-2me (A-D) and H3K4-3me (E-H) in normal gastric mucosa (A, E), well-differentiated gastric cancer (GC) tumor (B, F), moderately-differentiated GC tumor (C, G), and poorly-differentiated GC tumor (D, H). Regardless of the GC differentiation status, H3K4-2me and H3K4-3me displayed high-level nuclear signals, as visualized by immunohistochemistry. Magnification: × 200.

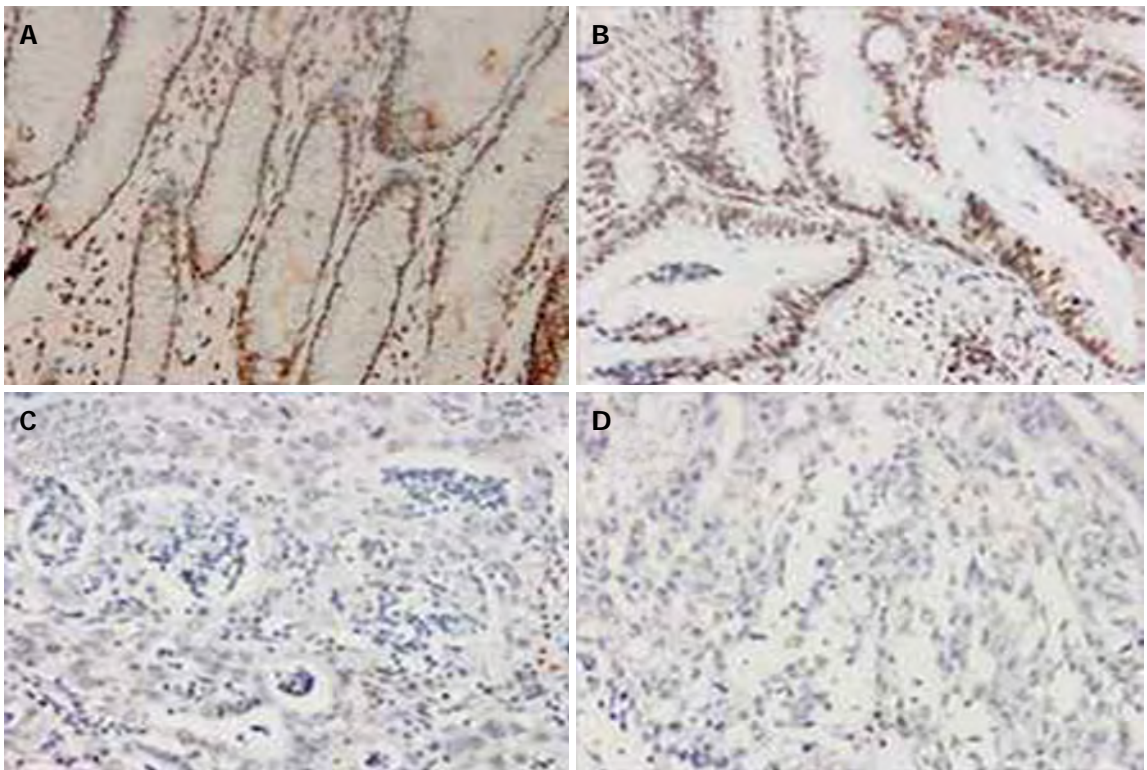
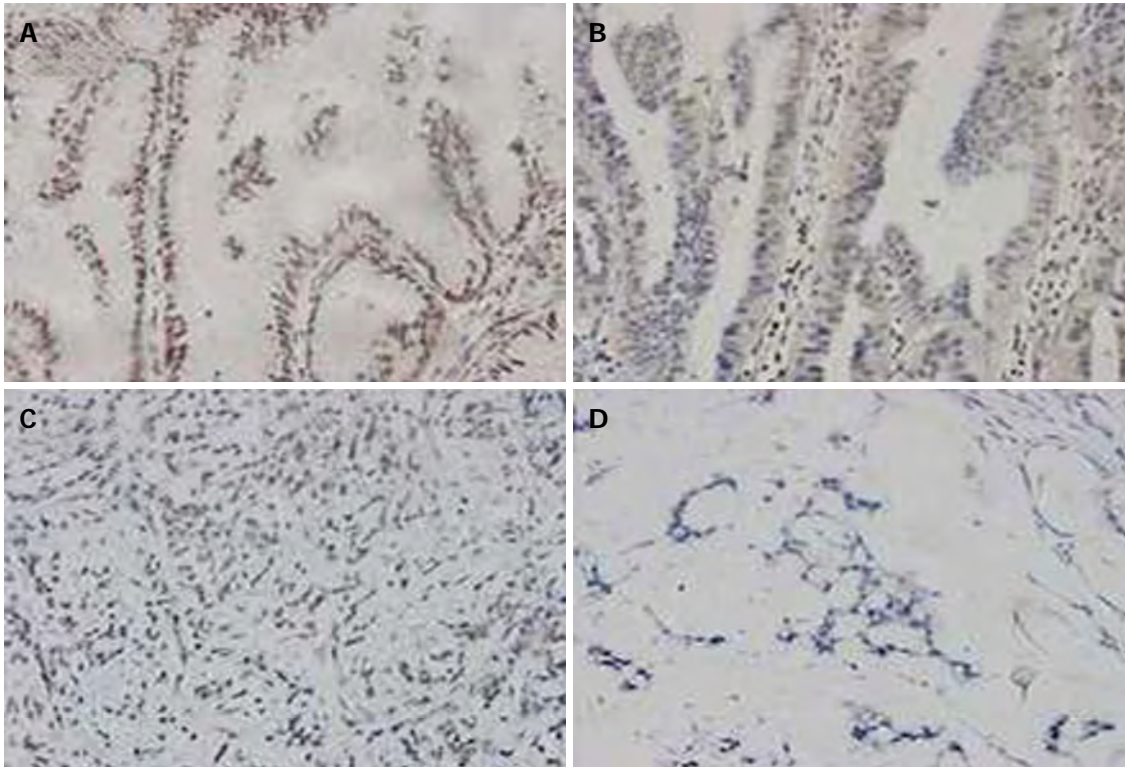


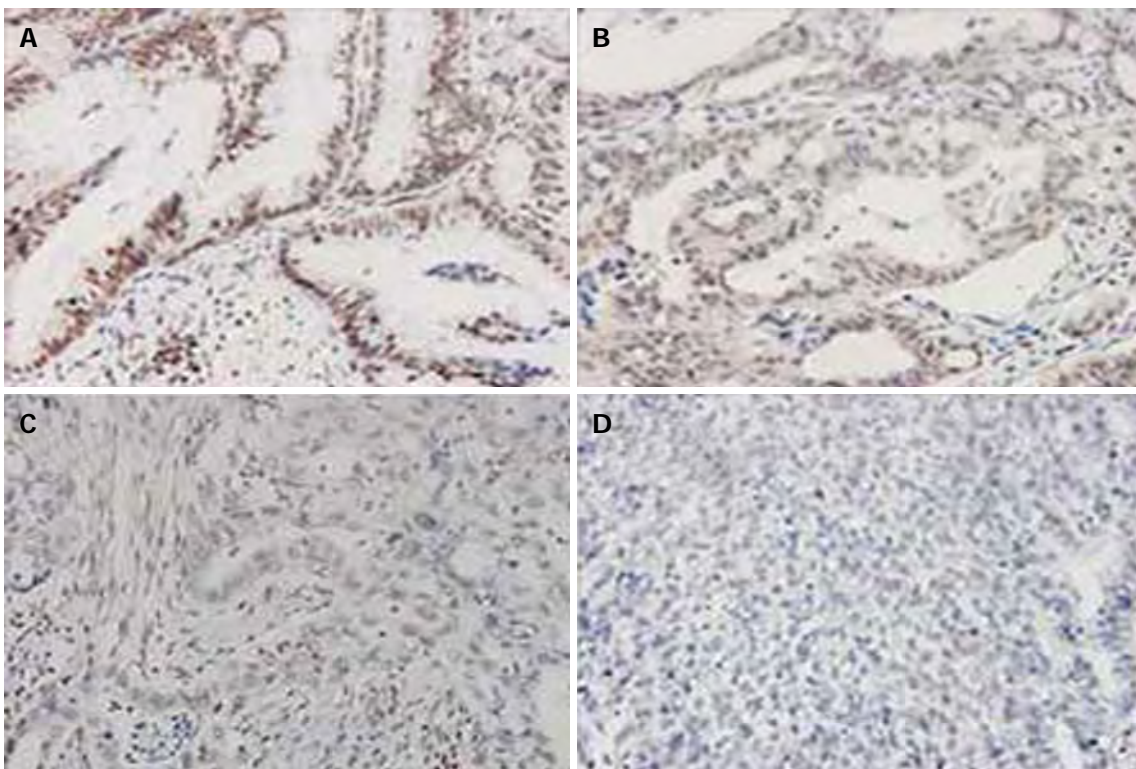
Figure 2 Immunohistochemical detection of staining patterns of uH2B in gastric cancer at various stages of differentiation. A: Normal gastric mucosa shows high-level staining (brown); B: Well-differentiated gastric cancer (GC) shows high-level staining; C: Moderately-differentiated GC shows low-level staining; D: Poorly-differentiated GC shows negative staining. Magnification: × 200.

0.05; Figure 3]. In addition, significantly more of the intestinal-type tumors showed high-level modification



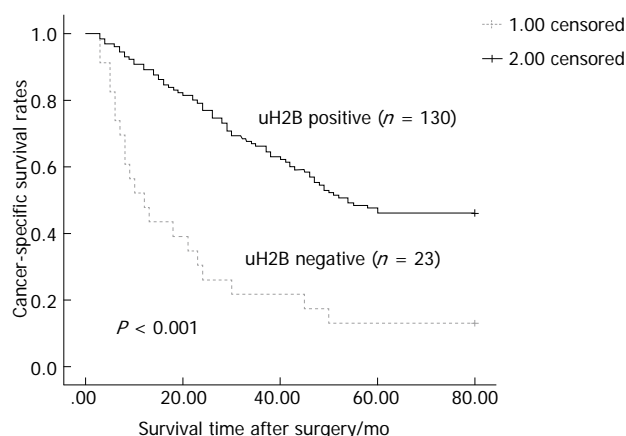


**Figure 3** Level of nuclear staining of uH2B according to Lauren classification of tumor type. A, B: Intestinal-type tumors showing (A) high-level staining (brown) of well-differentiated tumors and (B) fewer uH2B<sup>+</sup> cells and moderate staining (yellow) of moderately-differentiated tumors; C, D: Diffuse-type tumors showing (C) low-level staining (light yellow) and few uH2B<sup>+</sup> cells of poorly-differentiated tumors and (D) negative staining in poorly-differentiated tumors. Magnification:  $\times 200$ .



**Figure 4** Immunohistochemical detection of uH2B staining at different TNM stages. A: Stage I (well-differentiated) gastric cancer (GC) tumor shows high-level staining; B: Stage II (moderately-differentiated) GC tumor shows fewer uH2B<sup>+</sup> cells and moderate staining (yellow); C: Stage III (poorly-differentiated) GC tumor shows few uH2B<sup>+</sup> cells and low-level staining; D: Stage IV (dedifferentiated) GC tumor shows no uH2B<sup>+</sup> cells and negative staining. Magnification:  $\times 200$ .

[55.0% (33/60) *vs* low-level modification: 35.0% (21/60),  $P < 0.05$ ; Figure 3], and this pattern was significantly dif-



**Figure 5** Kaplan-Meier curves of cancer-specific survival for gastric cancer patients based on uH2B<sup>+</sup> and uH2B<sup>-</sup> status, as detected by immunohistochemistry. The 5-year survival rate of patients with positive uH2B staining ( $n = 130$ ) was significantly higher than that of patients with negative uH2B staining ( $n = 23$ ).

**Table 1** Immunohistochemical detection of uH2B modification levels and gastric cancer clinicopathological parameters

Parameter	n	IHC Staining Level			u/H <sup>1</sup>	P value
		Negative	Low	High		
Age (yr)					3059.000	0.969
< 60	93	10	62	21		
≥ 60	66	15	29	22		
Sex					2728.000	0.373
Male	100	13	59	28		
Female	59	12	32	15		
Tumor differentiation					40.376	< 0.001
Well	23	1	7	15		
Moderate	55	7	22	26		
Poor	81	17	62	2		
Lauren classification					933.000	< 0.001
Intestinal	60	6	21	33		
Diffuse	59	17	35	7		
Lymph node metastasis					2330.000	0.049
Absent	53	2	35	16		
Present	106	23	56	27		
TNM stage					12.896	0.005
I	15	1	6	8		
II	20	2	15	3		
III	99	14	55	30		
IV	25	8	15	2		

<sup>1</sup>u represents the test statistic of the Kruskal-Wallis H test and H represents the Wilcoxon ranks sum test. IHC: Immunohistochemistry.

ferent from that seen in the diffuse-type tumors [*vs* high-level modification in diffuse-type: 11.9% (7/59),  $P < 0.001$ ; Table 1].

#### Differential uH2B IHC staining correlates with TNM stage and lymph node metastasis

When the GC specimens were divided by TNM stages, a statistically significant trend in differential uH2B modification level was observed. As shown in Figure 4, the frequency of high-level uH2B modification was 53.3% (8/15) in stage I tumors, 15.0% (3/20) in stage II tumors, 30.3% (30/99) in stage III tumors, and 8.0% (2/25)

**Table 2** Multivariate analysis for prognostic factors in gastric cancer-specific survival of patients

Variable	Comparison	RR	95% CI	P value
Age (yr)	< 60 <i>vs</i> ≥ 60	0.761	0.493-1.174	0.217
Sex	male <i>vs</i> female	0.961	0.618-1.494	0.858
Tumor differentiation	Well <i>vs</i> moderate, poor	0.497	0.301-0.819	0.006
Lymph node metastasis	Present <i>vs</i> absent	3.274	1.728-6.201	< 0.001
TNM stage	I <i>vs</i> II, III, IV	1.695	1.112-2.583	0.014
uH2B modification	IHC stain positive <i>vs</i> negative	0.400	0.237-0.677	0.001

in stage IV tumors. The difference in frequency of high-level uH2B modification detected in stage I and stage IV tumors reached statistical significance ( $P = 0.005$ ; Table 1).

In addition, GC cases with lymph node metastasis showed a significantly lower frequency of high-level uH2B modification [25.4% (27/106) *vs* no lymph node metastasis: 30.2% (16/53),  $P = 0.049$ ; Table 1].

#### Prognostic significance of uH2B modification in GC

Of the 159 GC patients treated with surgical resection, 96 (60.4%) died from GC-related causes during the follow-up period and six died from non-GC causes. When the overall patient population was divided by presence of uH2B staining, GC-related deaths were found to have occurred in a significantly higher proportion of patients with negative uH2B staining than those with positive uH2B staining [88.0% (22/25) *vs* 55.2% (74/134),  $P < 0.05$ ]. The cumulative 5-year cancer-specific survival rate was 43.4%. Moreover, the 5-year survival rate of patients with positive uH2B staining was significantly higher than that of patients with negative uH2B staining ( $52.69 \pm 2.45$  *vs*  $23.74 \pm 5.21$ ,  $P < 0.001$ ; Figure 5).

According to Cox multivariate regression, uH2B modification level is an independent prognostic factor for cancer-specific survival of GC patients. The risk of death in patients with negative uH2B staining was 2.5-times (1:0.4) that of patients with positive uH2B modification (RR = 0.40, 95%CI: 0.237-0.677,  $P = 0.001$ ; Table 2).

## DISCUSSION

In this study, immunohistochemical detection of human GC samples was performed as a semi-quantitative approach to measure the H2B monoubiquitination at lysine 121 and investigate its potential clinical significance with regards to diagnosis (GC *vs* control tissues) and prognosis (progressive stages of GC tumorigenesis). To the best of our knowledge, this study provides the first evidence of correlation between uH2B modification level and clinicopathological and prognostic features of human GC, including tumor differentiation, Lauren's classification, lymph node metastasis, and TNM stage but not with sex or age (data not shown).

The modification of H2B at lysine 121, shown by



the percentage of positivity and intensity of immunohistochemical detection, was significantly less robust in tumors of lower differentiation level. The degree of differentiation is considered to be strongly associated with the malignancy of cancer; therefore, this result indicates that reduced uH2B may be correlated with a worse prognosis. The more frequent and intense staining of uH2B observed in intestinal-type tumors in the current study, compared to the diffuse-type tumors, suggests that loss of uH2B may contribute to GC tumor progression. From a histological perspective, the composition of intestinal-type GC tumors includes a remarkable amount of ductal structures, displaying a better differentiation than the diffuse-type GC tumors that may be related to the better prognosis of the former tumor type<sup>[17,18]</sup>. Finally, the current observation of lower uH2B modification level in higher grade TNM stages, which tend to be more aggressive and invasive towards the inner tissues and more metastatic, suggest that this modification may be a useful predictive biomarker of the invasive potential of a GC specimen.

The collected results of the current study indicate that the progressive stages of GC are accompanied by differential uH2B modification levels that are detectable by IHC. Prenzel *et al.*<sup>[19]</sup> reported a similar finding for human specimens of breast cancer. Specifically, the abundant uH2B signals detected by IHC in normal mammary epithelium and benign breast tumors were absent in most malignant and metastatic breast tumors. Urasaki *et al.*<sup>[20]</sup> also demonstrated drastically reduced uH2B modification levels in breast, colon and lung cancer cells, as compared to the abundant expression in matched normal control tissues. Thus, loss of uH2B may lead to progression and metastasis of tumors, in general.

The mechanisms underlying tumor-related decreases in uH2B remain unknown. Besides the known Rad6 and RNF20 regulatory enzymes<sup>[10,11]</sup>, other de/ubiquitinating enzymes are likely to be involved in the dynamic process of uH2B promotion of tumorigenesis. For example, the ubiquitin-specific protease 22 (USP22), a member of the recently identified polycomb/cancer stem cell signature<sup>[21]</sup>, has been shown to deubiquitinate H2Aub1 or H2Bub1 *in vitro*, suggesting functions in epigenetic regulation, cancer progression and transcription activation<sup>[22,23]</sup>. If USP22 plays a role in the cancer-related differential uH2B modifications, then corresponding changes in USP22 expression may be detected.

In fact, studies of USP22 expression level in cancer have demonstrated significant upregulation in malignant tumors (compared to the normal low or moderate levels in non-cancerous skeletal, heart, muscle, liver and lung tissues)<sup>[24]</sup> and significant correlations to tumor relapse, invasion depth, pathological stage and lymph node metastasis<sup>[25]</sup>. Moreover, a study of primary GC showed that upregulated USP22 protein expression was related to lymph node metastasis<sup>[26]</sup>. Future investigations may elucidate a role for USP22 in the de-regulation of uH2B, particularly in GC.

Another potential regulator of tumor-related decreases in uH2B is the ubiquitin-specific peptidase 49 (USP49) complex, which specifically deubiquitinates histone H2B *in vivo* to enhance the stability of a nucleosome spanning the affected exons<sup>[27]</sup>. Again, further research is required to determine whether USP49 acts as a histone H2B-specific deubiquitinase to promote tumorigenesis.

While the current study provided clear evidence of decreased uH2B in malignant and poorly-differentiated human GC specimens, the physiological significance of the loss of this histone code remains unclear. The critical roles of H3K4-2me and H3K4-3me modifications in gene expression and cellular viability are well established; however, the requirement for uH2B in these processes remains controversial. Genome-wide studies have shown that uH2B is associated mostly with actively transcribed genes in mammals, suggesting that H2B is universally required for gene transcription. However, loss of the H2B-specific RNF20/40 enzyme produced only moderate effects on a fraction of the transcriptome and no overall effects on cell viability<sup>[28]</sup>. This apparent differential function of H3K4 modifications and the uH2B modification is in line with the observations of the current study of GC tissues, whereby the uH2B level showed a unique gradual decrease from the benign to malignant stages.

In conclusion, the compelling evidence of uH2B not being required for viability of malignant cells in gastric carcinoma provided by the current study indicates that the decrease of uH2B might be an early event in GC and could be a counteracting factor against carcinogenesis of GC.

## COMMENTS

### Background

Gastric cancer (GC) remains a significant healthcare burden worldwide, with high mortality rates in countries with the highest incidences, such as China. Surgery is currently the only effective treatment, but must be applied in the early stages when the tumor is less aggressive. Unfortunately, the asymptomatic nature of early stage GC leads to missed diagnosis and precludes early surgical management. There is an urgent need to identify sensitive biomarkers that accurately diagnose GC at the early stage, as well as indicate prognosis for a particular patient. Such factors may also represent novel targets of molecular therapies, helping to overcome the limitations and risks associated with surgical resection.

### Research frontiers

The physiological and pathological roles of monoubiquitination on lysine 120 of histone H2B (uH2B) remain to be fully elucidated. As a general transcriptional regulator, de-regulation of uH2B may contribute to tumorigenesis and cancer progression by promoting expression of cancer-associated genes or suppressing expression of anti-tumor genes. Investigating the differential level of uH2B in specific cancer types, such as GC, will provide insights into its clinicopathological and prognostic significance.

### Innovations and breakthroughs

In this study, immunohistochemical (IHC) analysis of human GC specimens was used to detect the tumor-related levels of uH2B modification. Application of a dual-rated semi-quantitative method to score the IHC results allowed for statistical correlation analysis of uH2B modification level and tumor-related features. The GC-related decrease in uH2B modification level was positively correlated with extent of tumor dedifferentiation, intestinal-type tumors, occurrence of lymph node metastasis, worse TNM stage and lower 5-year survival rate. IHC-detected differential uH2B

modification may be developed as clinically useful prognostic biomarker in early GC.

### Applications

The uH2B differential staining pattern detected by IHC that accompanies progressive stages of GC not only indicated a potentially important role for this histone modification in carcinogenesis, but also suggested its potential as a target of molecular therapy.

### Terminology

uH2B, the monoubiquitination on lysine 120 of histone H2B, is a general transcriptional regulator, and its deregulation has been implicated in various cancers. IHC is a well-established laboratory detection method that exploits the antibody-antigen binding reaction to identify and visualize the location and level of a target protein in tissues or individual cells.

### Peer review

This paper is an interesting article regarding the role of histone modification events in the development of GC. The results of the paper show that decrease of uH2B might be an early event in GC, which could be a counteracting factor against carcinogenesis of gastric cancer. Furthermore, histone modification plays important roles in understanding the pathogenesis of gastric carcinoma, and could be a potential therapeutic target in the future

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## siRNA-targeted inhibition of growth hormone receptor in human colon cancer SW480 cells

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### Abstract

**AIM:** To determine the effects of RNAi-mediated inhibition of the growth hormone receptor (*GHR*) gene on tumors and colon cancer cells *in vivo*.

**METHODS:** Construction of a eukaryotic vector for human *GHR* expression, the pcDNA<sup>TM</sup>6.2-GW/EmGFP-small interfering RNAs (siRNAs)-*GHR* plasmid, was used to inhibit *GHR* expression. Thirty-six BALB/c nude mice were randomly divided into groups and treated with normal saline (NS), recombinant plasmid (*G<sub>2</sub>*), growth hormone (GH), 5-fluorouracil (FU), *G<sub>2</sub>*+FU or *G<sub>2</sub>*+FU+GH. Each nude mouse was subcutaneously inoculated with  $1 \times 10^7$  human colon cancer SW480 cells; the nude mice were weighed before inoculation and on the 2<sup>nd</sup>, 5<sup>th</sup>, 8<sup>th</sup>, 11<sup>th</sup>, 14<sup>th</sup> and 17<sup>th</sup> day after inoculation. All nude mice were sacrificed after 17 d. Each subcutaneous tumor was removed and studied. Tumor volume was measured on the 5<sup>th</sup>, 8<sup>th</sup>, 11<sup>th</sup>, 14<sup>th</sup> and 17<sup>th</sup> day after inoculation. The expression of *GHR* protein in the tumor tissue was detected by Western blotting analy-

sis, and the differences in *GHR* mRNA expression in the tumor tissue were detected by real-time quantitative reverse transcription-polymerase chain reaction.

**RESULTS:** Compared to the control group, the weights of the inoculated nude mice on the 17<sup>th</sup> day after inoculation were: *G<sub>2</sub>*:  $21.60 \pm 0.71$  g, GH:  $21.64 \pm 0.45$  g, FU:  $18.94 \pm 0.47$  g, FU+*G<sub>2</sub>*:  $19.40 \pm 0.60$  g, *G<sub>2</sub>*+FU+GH:  $21.04 \pm 0.78$  g *vs* NS:  $20.68 \pm 0.66$  g,  $P < 0.05$ ; the tumor volumes after the subcutaneous inoculation were: *G<sub>2</sub>*:  $9.71 \pm 3.82$  mm<sup>3</sup>, FU:  $11.54 \pm 2.42$  mm<sup>3</sup>, FU+*G<sub>2</sub>*:  $11.42 \pm 1.11$  mm<sup>3</sup>, *G<sub>2</sub>*+FU+GH:  $10.47 \pm 1.02$  mm<sup>3</sup> *vs* NS:  $116.81 \pm 10.61$  mm<sup>3</sup>,  $P < 0.05$ . Compared to the GH group, the tumor volumes were significantly decreased in the experimental groups. The *GHR* protein expression (*G<sub>2</sub>*:  $0.39 \pm 0.02$ , FU:  $0.40 \pm 0.02$ , FU+*G<sub>2</sub>*:  $0.38 \pm 0.01$ , *G<sub>2</sub>*+FU+GH:  $0.39 \pm 0.01$  *vs* NS:  $0.94 \pm 0.02$ ,  $P < 0.05$ ) and the *GHR* mRNA expression (*G<sub>2</sub>*:  $14.12 \pm 0.10$ , FU:  $15.15 \pm 0.44$ , FU+*G<sub>2</sub>*:  $16.46 \pm 0.27$ , *G<sub>2</sub>*+FU+GH:  $15.37 \pm 0.57$  *vs* NS:  $12.63 \pm 0.14$ ,  $P < 0.05$ ) were significantly decreased and increased, respectively, in the experimental groups.

**CONCLUSION:** Inhibition of *GHR* in human colon cancer SW480 cells resulted in anti-tumor effects in nude mice.

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**Key words:** Growth hormone receptor; Small interfering RNAs; Colon cancer; Gene therapy; Signaling pathway

**Core tip:** Human growth hormone receptor (*GHR*) is highly expressed in colon cancer tissues. GH/*GHR* plays an important role in colon cancer emergence and development. After specific binding of GH to *GHR* in tumor tissues, the JAK-STAT signaling pathway is activated, resulting in improved cell growth and proliferation. small interfering RNAs (siRNAs)-targeted inhibition of the human *GHR* gene was used to investigate



its impact on the emergence and development of colon cancer and to determine how human colon cancer cells respond to GHR suppression. The siRNA-containing plasmid could suppress GHR expression in colon cancer cells and exhibited anti-tumor effects in nude mice.

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## INTRODUCTION

As shown previously by our team, human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. Additionally, less differentiated tumor tissues have higher levels of GHR expression. During tumor development, the expression of GHR demonstrates an upward tendency<sup>[1,2]</sup>. Some researchers<sup>[3-6]</sup> believe that the expression of GHR in tumor tissue is linked with the vegetative state of the tumor and that GH and GHR play important roles in the emergence and development of colon cancer. After specific binding of GH to GHR in tumor tissues, the JAK-STAT signal transduction pathway is activated, resulting in improved cell growth and proliferation<sup>[7-10]</sup>. Signal transduction therapy is a commonly used chemotherapy strategy, and currently, treatment often involves the use of small interfering RNAs (siRNAs) that target different signal transduction pathways<sup>[11-13]</sup>.

In this study, siRNA targeting the human *GHR* gene was used to investigate the impact that GHR has on the emergence and development of colon cancer and to determine how human colon cancer cells respond to the suppression of GHR expression.

## MATERIALS AND METHODS

### Experimental animals and cell lines

Thirty-six 8-wk old, female BALB/c nude mice, weighing between 20 and 22 g, were purchased from Vital River Laboratories (VRL) with license No. SCXK (Jing) 2006-0009. The mice were kept in the SPF environment of the animal experiment center in Kunming Medical University. The human colon cancer cell line SW480 was obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science.

### Laboratory reagents

The HQ high purity plasmid extraction kit was purchased from Invitrogen (Invitrogen, Carlsbad city, California, United States). The BCA protein concentration kit (Tiangen Biology and Chemistry) and the molecular mass albumin standard were purchased from Tiangen Biology and Chemistry (Fermentas Company). The mouse monoclonal anti-human GHR antibody was obtained

from R and D Company (MAB1210), and the goat secondary anti-mouse IgG-HRP antibody was purchased from Abmart Company. RNase H was obtained from Invitrogen, and the Golden Taq PCR kit was purchased from Tiangen Biology and Chemistry. SYBR Green-Real Master Mix was purchased from Tiangen Biology and Chemistry, and all primers used in the study were obtained from Invitrogen.

### Preparation of cell suspension

Colon cancer SW480 cells were cultivated in RPMI 1640 nutrient solution supplemented with 10% fetal calf serum (FCS),  $10.0 \times 10^3$  U/L penicillin, and 100 mg/L streptomycin in a 37 °C incubator with 50 mL/L CO<sub>2</sub>. This is an adherent cell line. Cells in the exponential growth phase were harvested using 0.25% trypsin, and the cells were resuspended using a machine. The cells were then centrifuged at 2000 rpm for 5 min. Then, the supernatant was removed, and the cells were resuspended in physiological saline at a concentration of  $1 \times 10^7$  cells/mL. Trypan blue staining was used to ensure that cell viability was above 95%; after resuspension the cells were stored in an ice bath.

### Construction of siRNA and eukaryotic expression vectors

siRNA oligonucleotides were designed against the mRNA sequence of human *GHR* found in GenBank, which had a total length of 4414 bp (Accession No.: X06562, GI: 31737). We used the RNAi Designer website (<http://bio-info.clontech.com/rnaidesigner>) to design the siRNA that targeted the hGHR mRNA (527-547: GTCAGTTTA-ACTGGGATTCAT). Using the BLAST search program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search the EST database, we found that the siRNA was not homologous to another gene and would be an effective siRNA sequence. The complementary single strand primer was incubated at 94 °C in annealing buffer solution for 3 min, and the oligonucleotides were annealed at 37 °C for 1 h. The annealed oligonucleotides were then phosphorylated at 37 °C for 30 min with T4 DNA-PNK. The oligonucleotides were then ligated into the linearized (BamHI/HindIII) pcDNA<sup>TM</sup> 6.2-GW/EmGFP-GHR-siRNA plasmid using T4 DNA ligase. The final product was transformed in competent *E. coli* DH5 $\alpha$  cells, and the transformants were spread on transformation plates containing 50  $\mu$ g/mL spectinomycin dihydrochloride (Sigma, Catalog No. S4014). The plates were kept in a 37 °C incubator overnight, and three co-nobium clones were picked from each plate and subcultured. The plasmids were extracted using plasmid extraction kits, and the plasmids were confirmed by restriction enzyme digestion with *Eco*R I, *Sac* I and *Sal* I. The transformation liquid was also sequenced to ensure that recombination had not occurred in the insert fragments during the cloning process. Finally, the pcDNA<sup>TM</sup>6.2-GW/EmGFP-GHR-siRNA plasmids were constructed successfully and are referred to as G2 throughout the paper. Additionally, the plasmids were extracted and diluted in

DMEM as a precaution.

### Groups and method of drug distribution

We subcutaneously injected  $1 \times 10^7$  human colon cancer SW480 cells into BALB/c nude mice. On the first day after the injection, the mice were divided into six groups and administered the indicated drug and dose. (1) Normal saline (NS): 10  $\mu$ L of NS was injected into the abdominal cavity of each mouse; (2) Plasmid (G<sub>2</sub>): 10  $\mu$ g of the G<sub>2</sub> eukaryotic expression plasmid was injected subcutaneously into each mouse; (3) Growth hormone (GH): 2 IU/kg rhGH, a physiological dose of GH, was hypodermically injected into each mouse; (4) 5-fluorouracil (FU): 20 mg/kg 5-FU was injected into the abdominal cavity of each mouse; (5) 5-FU+plasmid (FU+G<sub>2</sub>): 20 mg/kg 5-FU and 10  $\mu$ g of the G<sub>2</sub> plasmid were injected into the abdominal cavity of each mouse; (6) 5-FU+GH+plasmid (FU+GH+G<sub>2</sub>): 20 mg/kg 5-FU, 10  $\mu$ g of the G<sub>2</sub> plasmid, and 2 IU/kg rhGH were injected into the abdominal cavity of each mouse; and (7) Each of the above groups was treated every 5 d for three rounds of treatment.

### Observation index

**Observation of weight and tumor volume of nude mice:** The weight of the nude mice was recorded before injection of the SW480 cells and on the 2<sup>nd</sup>, 5<sup>th</sup>, 8<sup>th</sup>, 11<sup>th</sup>, 14<sup>th</sup> and 17<sup>th</sup> day after injection. The length and the minimum diameter of the tumors were recorded, and the tumor volume was calculated on the 5<sup>th</sup>, 8<sup>th</sup>, 11<sup>th</sup>, 14<sup>th</sup> and 17<sup>th</sup> day after hypodermic injection of human colon cancer SW480 cells. The following equation was used to calculate the tumor volume:  $V = (A \times B^2)/2$ , where A represents the major diameter and B represents the minimum diameter.

### Expression of GHR in tumor tissues as detected by Western blotting analysis

A sample of each tumor was removed from the nude mice and cut into pieces. Cleanser lysate solution containing PMSF (400  $\mu$ L) was added to the tumor sample in a homogenizer. After the cells were lysed for 30 min, the homogenate was centrifuged at 12000 rpm for 5 min at 4 °C. The supernatant was removed, placed in 0.5 mL centrifuge tubes, and stored at -20 °C. Then, 20  $\mu$ L of the lysate sample was separated and analyzed using SDS-PAGE; the proteins were electrotransferred onto nitrocellulose membranes and detected using a chemiluminescent detection system. Beta-actin was used as a loading control. The images were analyzed using the BandScan5.0 program. The ratio of GHR expression to beta-actin expression was analyzed using the integral optical density value (RV) of the band in the same sample. The results are shown as the expression of GHR relative to beta-actin, and the results were measured as, mean  $\pm$  SD.

### Expression of GHR mRNA in tumor tissue was detected by quantitative reverse transcription-polymerase chain reaction

Twenty microliters of the lysate sample was centrifuged

at 5000 rpm for 10 min. Prechilled PBS was used to wash the cells, and total RNA was extracted using the Trizol reagent in a one-step method. For first strand synthesis, 1  $\mu$ g of total RNA was combined with 1  $\mu$ L of 0.5  $\mu$ g/ $\mu$ L oligo primer and 12  $\mu$ L of deionized water. The mixture was then incubated at 70 °C for 5 min. Then, the samples were quenched in a bath of ice water and centrifuged at 5000 rpm for 4 s. Next, 4  $\mu$ L of 5  $\times$  reaction buffer, 1  $\mu$ L of 20 U/ $\mu$ L ribonuclease, and 2  $\mu$ L of 10  $\mu$ mol/ $\mu$ L dNTPs were added, and the mixture was incubated at 37 °C for 5 min. Then, 1  $\mu$ L of 200 U/ $\mu$ L reverse transcriptase was added, and the reaction was incubated at 42 °C for 60 min, followed by a 10 min incubation at 70 °C. After the reaction, the cDNA was incubated at 0 °C and was stored at -20 °C. Real time PCR with the SYBR-Green fluorochrome was used to detect the expression of GHR mRNA. For this reaction, 10  $\mu$ L of cDNA, 2  $\mu$ L of both forward and reverse primers, 10  $\mu$ L of buffer solution, 4  $\mu$ L of ddH<sub>2</sub>O, and 1  $\mu$ L of the ROX fluorochrome were incubated at 95 °C for 10 min, followed by 35 cycles of 94 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s; a final extension time of 5 min at 72 °C ended the reaction. GAPDH was used as a negative control. The following primers were used in this experiment: GHR forward: GCAGCTATCCTTAGCAGAGCAC; GHR reverse: AAGTCTCTCGCTCAGGTGAACG; GAPDH forward: GGTCTCCTCTGACTTCAACA; and GAPDH reverse: GAGGGTCTCTCTTCTTCT. The levels of GHR mRNA in the transfection group and the control group were determined by quantitative PCR, and the  $\Delta$ CT value of the GHR mRNA was determined between the two groups. It was demonstrated that a higher  $\Delta$ CT value represented a larger inhibition of GHR mRNA.

### Statistical analysis

Data were expressed as the mean  $\pm$  SD. Univariate or multivariate data were analyzed using variance analysis and pairwise comparison *t* test by SPSS 18.0 statistical software package. Statistical significance was considered at  $P \leq 0.05$ .

## RESULTS

### Weight changes in the tumor-bearing mice

At the end of the experiment, all 36 tumor-bearing mice survived from inoculation 2 to 17 d, except for the NS group. After injection, there was a statistically significant difference ( $P < 0.05$ ) in the weight of the mice compared with their weight before injection. The weight of the GH group increased after inoculation with SW480 cells, while the other groups decreased in weight ( $P < 0.05$ ). The weight of the G<sub>2</sub>, FU, and G<sub>2</sub>+FU groups noticeably decreased ( $P < 0.05$ ) compared with the NS group. The weight of the FU and G<sub>2</sub>+FU groups decreased compared with the G<sub>2</sub> group; however, this change was not significant. After the addition of GH, the weight of the G<sub>2</sub>+GH+FU group increased compared with the FU group in the same period ( $P < 0.05$ ; Table 1).

**Table 1** Weight changes of tumor-bearing mice ( $n = 6$ ; mean  $\pm$  SD)

Time	Weight (g, mean $\pm$ SD)					
	NS	G <sub>2</sub>	GH	FU	FU+G <sub>2</sub>	G <sub>2</sub> +FU+GH
Pre-operation	20.69 $\pm$ 0.67	21.92 $\pm$ 0.70	20.67 $\pm$ 0.57	21.93 $\pm$ 0.58	21.86 $\pm$ 0.73	21.25 $\pm$ 0.79
Inoculation 2 d	20.64 $\pm$ 0.60	21.86 $\pm$ 0.79	20.82 $\pm$ 0.56	20.89 $\pm$ 0.66 <sup>1</sup>	20.09 $\pm$ 0.55 <sup>1,3</sup>	20.41 $\pm$ 0.85 <sup>1,3</sup>
Inoculation 5 d	20.65 $\pm$ 0.49	21.56 $\pm$ 0.81	20.96 $\pm$ 0.54 <sup>1</sup>	20.94 $\pm$ 0.67 <sup>1</sup>	19.92 $\pm$ 0.58 <sup>1,3,5</sup>	20.42 $\pm$ 0.76 <sup>1</sup>
Inoculation 8 d	20.65 $\pm$ 0.65	21.53 $\pm$ 0.56 <sup>1</sup>	21.18 $\pm$ 0.44 <sup>1</sup>	20.41 $\pm$ 0.73 <sup>1,3</sup>	19.92 $\pm$ 0.52 <sup>1,2,3,5</sup>	20.53 $\pm$ 0.70 <sup>1,3</sup>
Inoculation 11 d	20.67 $\pm$ 0.63	21.53 $\pm$ 0.73 <sup>1</sup>	21.27 $\pm$ 0.53 <sup>1,4</sup>	19.86 $\pm$ 0.57 <sup>1,3</sup>	20.06 $\pm$ 0.52 <sup>1,2,3,5</sup>	20.63 $\pm$ 0.81 <sup>1,5</sup>
Inoculation 14 d	20.61 $\pm$ 0.62	21.60 $\pm$ 0.68 <sup>1,2</sup>	21.51 $\pm$ 0.44 <sup>1,4</sup>	9.46 $\pm$ 0.52 <sup>1,2,3</sup>	19.86 $\pm$ 0.92 <sup>1,2</sup>	20.86 $\pm$ 0.72 <sup>1,4</sup>
Inoculation 17 d	20.68 $\pm$ 0.66	21.60 $\pm$ 0.71 <sup>1</sup>	21.64 $\pm$ 0.45 <sup>1,2,4</sup>	18.94 $\pm$ 0.47 <sup>1,2,3</sup>	19.40 $\pm$ 0.60 <sup>1,2,3</sup>	21.04 $\pm$ 0.78 <sup>1,4</sup>

<sup>1</sup>Compared with pre-operation,  $P < 0.05$ ; <sup>2</sup>Compared with NS group,  $P < 0.05$ ; <sup>3</sup>Compared with G<sub>2</sub> group,  $P < 0.05$ ; <sup>4</sup>Compared with FU group,  $P < 0.05$ ;<sup>5</sup>Compared with GH group,  $P < 0.05$ . NS: Normal saline; G<sub>2</sub>: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.**Table 2** Tumor volume changes in tumor-bearing mice ( $n = 6$ , mean  $\pm$  SD)

Time	Subcutaneous tumor volume (mm <sup>3</sup> , mean $\pm$ SD)					
	NS	G <sub>2</sub>	GH	FU	FU+G <sub>2</sub>	G <sub>2</sub> +FU+GH
Inoculation 5 d	7.72 $\pm$ 1.61	7.93 $\pm$ 1.74	8.11 $\pm$ 1.65	7.42 $\pm$ 1.51	6.51 $\pm$ 1.20	7.33 $\pm$ 1.32
Inoculation 8 d	20.19 $\pm$ 4.91 <sup>2,3</sup>	13.44 $\pm$ 4.12 <sup>1,3</sup>	33.28 $\pm$ 3.24 <sup>1,2,3</sup>	17.51 $\pm$ 5.75 <sup>3</sup>	15.12 $\pm$ 5.01 <sup>3</sup>	15.44 $\pm$ 4.23
Inoculation 11 d	106.02 $\pm$ 6.61 <sup>2,3</sup>	21.12 $\pm$ 4.04 <sup>1,3</sup>	151.90 $\pm$ 8.31 <sup>1,2,3</sup>	21.00 $\pm$ 5.07 <sup>1,3</sup>	19.22 $\pm$ 4.33 <sup>1,3</sup>	22.97 $\pm$ 4.95 <sup>1,2,3</sup>
Inoculation 14 d	133.41 $\pm$ 6.43 <sup>2,3</sup>	20.00 $\pm$ 4.75 <sup>1,3</sup>	178.93 $\pm$ 3.11 <sup>1,2,3</sup>	16.23 $\pm$ 6.51 <sup>1,3</sup>	11.55 $\pm$ 4.11 <sup>1,2,3</sup>	12.12 $\pm$ 3.11 <sup>1,2,3</sup>
Inoculation 17 d	116.81 $\pm$ 0.61 <sup>2,3</sup>	9.71 $\pm$ 3.82 <sup>1,3</sup>	149.01 $\pm$ 3.02 <sup>1,2,3</sup>	11.54 $\pm$ 2.42 <sup>1,3</sup>	11.42 $\pm$ 1.11 <sup>1,3</sup>	10.47 $\pm$ 1.02 <sup>1,3</sup>

<sup>1</sup>Compared with NS group,  $P < 0.05$ ; <sup>2</sup>Compared with G<sub>2</sub> group,  $P < 0.05$ ; <sup>3</sup>Compared with GH group,  $P < 0.05$ . NS: Normal saline; G<sub>2</sub>: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

### Changes in tumor volume in the tumor-bearing mice of all groups

By the end of the experiment, the tumor volume of the mice in all groups increased compared to the fifth day after inoculation. The tumor volume of the GH group had the most dramatic increase, followed by the NS group. The tumor volumes of the G<sub>2</sub>, FU, G<sub>2</sub>+FU, and G<sub>2</sub>+GH+FU groups only slightly increased. Compared with the NS group in the same period, the tumor volume of the experimental group obviously decreased, whereas that of the GH group significantly increased ( $P < 0.05$ ). Compared with the G<sub>2</sub> group, the G<sub>2</sub>+FU group had a more pronounced tumor inhibition ( $P < 0.05$ ). There was no obvious difference in the tumor volume of the G<sub>2</sub>+GH+FU group compared with the G<sub>2</sub>, FU, and G<sub>2</sub>+FU groups in the same period (Table 2).

### GHR protein expression in the subcutaneous tumors of tumor-bearing mice in all groups

The expression levels of GHR protein in the tumors of the GH (0.94  $\pm$  0.02) and NS (0.94  $\pm$  0.02) mice were significantly higher than the GHR levels in the G<sub>2</sub> (0.39  $\pm$  0.021), FU (0.40  $\pm$  0.02), G<sub>2</sub>+FU (0.38  $\pm$  0.01) and G<sub>2</sub>+FU+GH (0.39  $\pm$  0.01) mice. However, there was no significant difference between the G<sub>2</sub> group and the FU, G<sub>2</sub>+FU, and G<sub>2</sub>+FU+GH groups ( $P > 0.05$ ).

### GHR mRNA expression in the subcutaneous tumors of tumor-bearing mice

Compared with the NS group and the control group that did not have a plasmid, the  $\Delta$ CT value of the G<sub>2</sub>, FU, G<sub>2</sub>+FU, and G<sub>2</sub>+FU+GH groups significantly increased

( $P < 0.05$ ). In the experimental groups, the inhibition ratios of the FU+G<sub>2</sub> and FU+G<sub>2</sub>+GH groups against GHR mRNA were higher than that of the G<sub>2</sub> group ( $P < 0.05$ ; Table 3).

## DISCUSSION

Colon cancer is one of the most common malignant tumors<sup>[14-16]</sup>. Currently, the treatment for colon cancer is surgery combined with radiotherapy and chemotherapy. However, most patients cannot undergo operation or do not respond to chemotherapeutics, leading to the failure of the therapy. Determining the appropriate tumor target spot related to gene and specific therapy has become a hotspot of research in tumor therapy<sup>[17]</sup>. Due to its action as an anabolic agent and mitogen, GH has a wide range of functions in substance metabolism and body fluid equilibrium, which can accelerate the use of nitrogen and improve the synthesis of liver and muscle proteins. The nutritional effect of GH has already been shown in cachexia<sup>[18]</sup>. Because GH can increase the brittleness of chromosomes, which, in turn, can cause malignant transformation of cells, and can increase tumor growth, GH has been excluded as a therapy option for the treatment of tumors<sup>[19,20]</sup>. It has been demonstrated by many epidemiological researchers that patients who receive long term treatment with growth hormone have an increased risk of colon cancer<sup>[3,21]</sup>, and GHR expression in the colon might relate to the occurrence, development and metastasis of these tumors<sup>[1,22]</sup>. The presence of GHR in the local tissue is a prerequisite for GH to play its role, which means that when determining



**Table 3**  $\Delta$ CT value of growth hormone receptor mRNA expression detected by real-time reverse transcription-polymerase chain reaction in all groups

	NS	LP	Negative	GH	G <sub>2</sub>	FU	FU+G <sub>2</sub>	G <sub>2</sub> +FU+GH
$\Delta$ CT	12.63 $\pm$ 0.14	12.63 $\pm$ 0.43	12.67 $\pm$ 0.21	12.71 $\pm$ 0.39	14.12 $\pm$ 0.10 <sup>1</sup>	15.15 $\pm$ 0.44 <sup>1</sup>	16.46 $\pm$ 0.27 <sup>1,2</sup>	15.37 $\pm$ 0.57 <sup>1,2</sup>

<sup>1</sup>Compared with NS group,  $P < 0.05$ ; <sup>2</sup>Compared with G<sub>2</sub> group,  $P < 0.05$ . NS: Normal saline; G<sub>2</sub>: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

whether to use GH as a therapy, it is important to know the expression and distribution of GHR in a specific tumor cell. GHR is highly expressed in colon cancer tissues<sup>[1,2]</sup>, and high GHR expression has been correlated with poor patient prognosis<sup>[23]</sup>.

RNAi refers to complementary double-stranded RNAs (dsRNAs) that bind to specific endogenous mRNAs, resulting in the degradation of those mRNAs and the silencing of gene expression. Aiming at the relevant signal of the auxanodifferentiation of tumor cells for transduction and targeting the interference of the expression of crucial proteins in transduction pathways of cell signaling can inhibit the growth of a tumor specifically and highly efficiently<sup>[24,25]</sup>.

In this research, we constructed plasmids containing siRNAs that targeted the expression of GHR in colon cancer cells, thereby decreasing the expression of GHR in the colon cancer tissues and blocking the GHR-induced signal transduction that promotes tumor cell growth. The results demonstrated that, compared to the control group, the tumor volume and the mRNA and protein expression of GHR in the tumor tissue significantly decreased. Additionally, the combination of GHR silencing and 5-FU treatment had an anti-tumor effect. After the siRNA blocked the expression of GHR in the tumor tissue, the addition of GH could bind to the GHR in the normal tissue. As a result, the weight and nutrition of the nude mice may improve, and GH treatment increases the nude mouse tolerance towards chemotherapy and increases the chemosensitivity of the tumor cells<sup>[9]</sup>. Compared with the other experimental groups, the FU+G<sub>2</sub>+GH group had no significant difference in its reduced tumor volume or decreased expression of GHR protein and mRNA.

Our research showed that the siRNA-containing plasmid influenced the expression of GHR in the colon cancer cells and played an anti-tumor role in the nude mice.

## COMMENTS

### Background

Human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. In addition, less differentiated tumor tissues have higher levels of GHR expression. During tumor development, the expression of GHR increases. Some researchers believe that the expression of GHR in tumor tissue is linked with the vegetative state of the tumor, and GH/GHR plays an important role in the emergence and development of colon cancer. After specific binding between GH and GHR in tumor tissue, the JAK-STAT signal transduction pathway is activated, leading to improved cell growth and proliferation.

### Research frontiers

Signal transduction therapy is a commonly used chemotherapy strategy, and currently, treatment often involves the use of small interfering RNAs (siRNAs) that target different signal transduction pathways. RNAi refers to complemen-

tary double-stranded RNAs that bind endogenous mRNAs, resulting in the specific degradation of that mRNA, which leads to decreased expression of that gene. Aiming at the relevant signal of the auxanodifferentiation of tumor cells and targeting the interference of the expression of crucial proteins in transduction pathways of cell signaling can inhibit the growth of tumor specifically and highly efficiently.

### Innovations and breakthroughs

In this study, siRNA-targeted inhibition of the *GHR* gene was used to investigate GHR's impact on the emergence and development of colon cancer and to determine how human colon cancer cells respond to the suppression of *GHR* gene expression.

### Applications

The results showed that the siRNA-containing plasmid influenced the expression of GHR in the colon cancer cells and played an anti-tumor role in the nude mice.

### Peer review

The manuscript is well designed and had appropriate methodology.

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## Laparoscopic vs open total gastrectomy for gastric cancer: A meta-analysis

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### Abstract

**AIM:** To conduct a meta-analysis comparing laparoscopic total gastrectomy (LTG) with open total gastrectomy (OTG) for the treatment of gastric cancer.

**METHODS:** Major databases such as Medline (PubMed), Embase, Academic Search Premier (EBSCO), Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials (CENTRAL) in The Cochrane Library were searched for studies comparing LTG and OTG from January 1994 to May 2013. Evaluated endpoints were operative, postoperative and on-

cological outcomes. Operative outcomes included operative time and intraoperative blood loss. Postoperative recovery included time to first flatus, time to first oral intake, hospital stay and analgesics use. Postoperative complications comprised morbidity, anastomotic leakage, anastomotic stenosis, ileus, bleeding, abdominal abscess, wound problems and mortality. Oncological outcomes included positive resection margins, number of retrieved lymph nodes, and proximal and distal resection margins. The pooled effect was calculated using either a fixed effects or a random effects model.

**RESULTS:** Fifteen non-randomized comparative studies with 2022 patients were included (LTG - 811, OTG - 1211). Both groups had similar short-term oncological outcomes, analgesic use (WMD -0.09; 95%CI: -2.39-2.20;  $P = 0.94$ ) and mortality (OR = 0.74; 95%CI: 0.24-2.31;  $P = 0.61$ ). However, LTG was associated with a lower intraoperative blood loss (WMD -201.19 mL; 95%CI: -296.50--105.87 mL;  $P < 0.0001$ ) and overall complication rate (OR = 0.73; 95%CI: 0.57-0.92;  $P = 0.009$ ); fewer wound-related complications (OR = 0.39; 95%CI: 0.21-0.72;  $P = 0.002$ ); a quicker recovery of gastrointestinal motility with shorter time to first flatus (WMD -0.82; 95%CI: -1.18--0.45;  $P < 0.0001$ ) and oral intake (WMD -1.30; 95%CI: -1.84--0.75;  $P < 0.00001$ ); and a shorter hospital stay (WMD -3.55; 95%CI: -5.13--1.96;  $P < 0.0001$ ), albeit with a longer operation time (WMD 48.25 min; 95%CI: 31.15-65.35;  $P < 0.00001$ ), as compared with OTG.

**CONCLUSION:** LTG is safe and effective, and may offer some advantages over OTG in the treatment of gastric cancer.

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**Key words:** Gastric cancer; Laparoscopic total gastrectomy; Laparoscopic assisted total gastrectomy; Open

total gastrectomy; Meta-analysis

**Core tip:** Currently, surgical resection is the mainstay treatment for gastric cancer. With technical advances and improved instrumentation, laparoscopic total gastrectomy (LTG) is being used increasingly to treat this malignant disease. However, compared with conventional open total gastrectomy (OTG), the safety and technical feasibility of LTG have not been adequately evaluated. This study clarified that, compared with OTG, LTG has similar short-term oncological outcomes, analgesic use and mortality. Furthermore, LTG was associated with lower intraoperative blood loss and overall complication rate, fewer wound-related complications, quicker recovery of gastrointestinal motility and a shorter hospital stay, albeit with a longer operation time.

Xiong JJ, Nunes QM, Huang W, Tan CL, Ke NW, Xie SM, Ran X, Zhang H, Chen YH, Liu XB. Laparoscopic vs open total gastrectomy for gastric cancer: A meta-analysis. *World J Gastroenterol* 2013; 19(44): 8114-8132 Available from: URL: <http://www.wjnet.com/1007-9327/full/v19/i44/8114.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8114>

## INTRODUCTION

Gastric cancer is one of the most common cancers worldwide and is a leading cause of cancer death<sup>[1]</sup>. Despite improvements in diagnosis and systemic therapy, surgery, in the form of gastrectomy with lymph node dissection, still forms the mainstay of treatment<sup>[2]</sup>. Since it was first described in 1994<sup>[3]</sup>, laparoscopic surgery, and more specifically laparoscopic distal gastrectomy, has been used widely in the far East to treat early gastric cancers and is associated with many advantages over open surgery<sup>[4-7]</sup>. On the other hand, laparoscopic total gastrectomy (LTG) with lymph node dissection, which was reported in 1999<sup>[8]</sup>, is practiced less widely and is more challenging to perform<sup>[9]</sup>. The procedure is associated with a high risk of bleeding and a technically demanding anastomosis, all within a narrow operating field<sup>[9,10]</sup>. However, with technical advances and improved instrumentation, LTG is now being used increasingly to treat gastric cancer<sup>[11-14]</sup>.

A number of studies comparing the short-term or long-term outcomes, of LTG vs conventional open total gastrectomy (OTG) for early and advanced gastric carcinoma have shown it to be feasible, oncologically effective and safe in experienced hands<sup>[14-17]</sup>. LTG offers the potential advantage of being less invasive, causing less surgical trauma with less postoperative pain and a quicker recovery<sup>[18,19]</sup>. However, most studies were too small to adequately evaluate the surgical outcomes of LTG. The aim of the current study was to inform future surgical practice by comparing the technical feasibility, effectiveness, and safety of LTG and OTG in the treatment of

early and advanced gastric cancer, through a systematic review and meta-analysis of published comparative studies.

## MATERIALS AND METHODS

### Literature search

A comprehensive literature search in Medline (PubMed), Embase, Academic Search Premier (EBSCO), Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials (CENTRAL) in The Cochrane Library was carried out for relevant studies, between January 1994 and May 2013, comparing OTG and LTG in the treatment of gastric cancer. The following search terms were used: “gastric cancer; laparoscopic total gastrectomy; laparoscopic assisted total gastrectomy; minimally invasive surgery; open total gastrectomy” along with their synonyms or abbreviations. Reference lists of selected articles were also examined to identify relevant studies that were not identified in the database searches. Investigators and experts in the field of laparoscopic surgery were contacted to ensure that all relevant studies were identified. Only comparative clinical trials with full-text descriptions were included. Final inclusion of articles was determined by consensus; when this failed, a third author adjudicated.

### Inclusion criteria

Studies included: (1) English language articles published in peer-reviewed journals; (2) human studies; (3) studies with at least one of the outcomes mentioned; (4) clear documentation of the operative techniques as “open” or “laparoscopic” or “laparoscopic-assisted”; and (5) where multiple studies came from the same institute and/or authors, either the higher quality study or the more recent publication was included in the analysis.

### Exclusion criteria

Excluded studies: (1) abstracts, letters, editorials, expert opinions, case reports, reviews and studies lacking control groups; (2) studies for benign lesions and gastrointestinal stromal tumor (GIST); (3) studies comparing two laparoscopic surgical approaches or comparing laparoscopic and robot-assisted gastrectomy; (4) studies including only subgroup analyses comparing LTG with OTG; and (5) repeated reports between authors, centers, and the patient community.

### Outcomes of interests

Operative outcomes included operation time and intraoperative blood loss. Oncological outcomes included positive resection margins, number of retrieved lymph nodes, and proximal and distal resection margins. Postoperative recovery outcomes included time to first flatus, time to first oral intake, analgesic use and hospital stay. Outcomes for postoperative complications included overall complication rate, anastomotic leakage, anastomotic stenosis, ileus, bleeding, abdominal abscess, wound-related prob-

lems and mortality.

### Data extraction and quality assessment

Two independent observers using standardized forms extracted the data. The recorded data included study characteristics, quality assessment and perioperative outcomes. The quality of the studies was assessed using the modified Newcastle-Ottawa Scale, with changes made to reflect the needs of this study<sup>[20,21]</sup>. The maximum number of stars in the selection, comparability, and outcome categories were 3, 4, and 2, respectively. Studies achieving 6 or more stars were considered high quality<sup>[22]</sup>.

### Statistical analysis

Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). For continuous variables, treatment effects were expressed as weighted mean difference (WMD) with corresponding 95% confidence interval (CI). For categorical variables, treatment effects were expressed as odds ratio (OR) with corresponding 95%CI. Heterogeneity was evaluated using the  $\chi^2$  test, and a  $P$  value  $< 0.1$  was considered significant;  $I^2$  values were used for the evaluation of statistical heterogeneity<sup>[23]</sup>. A fixed-effects model was initially calculated for all outcomes<sup>[24]</sup>, but if the test rejected the assumption of homogeneity of the studies, then a random-effects analysis was performed<sup>[25]</sup>. Sensitivity analyses were performed by removing individual studies from the data set and analyzing the effect on the overall results, to identify sources of significant heterogeneity. Subgroup analyses were also undertaken by including only high quality studies to present cumulative evidence. Funnel plots based on the operation time were constructed to evaluate potential publication bias<sup>[26]</sup>.

## RESULTS

### Description of trials included in the meta-analysis

The search strategy generated 91 relevant clinical studies, among which 19 full text articles<sup>[9,10,14-18,27-38]</sup> were identified for further investigation. Of these, four studies<sup>[18,31,35,36]</sup> were excluded for various reasons: 1 study<sup>[36]</sup>, based on an administrative database, was used to assess hospital practice performance with regard to the quantity of medical care items and diet provided during hospitalization; another study<sup>[35]</sup> only compared LTG with OTG in a subgroup analysis; and two studies were repeated reports<sup>[18,31]</sup>. Finally, 15 studies<sup>[9,10,14-17,27-30,32-34,37,38]</sup> were identified for inclusion, of which two were prospective non-randomized comparative studies<sup>[14,28]</sup>, the rest being retrospective comparative studies. Figure 1 shows the study selection process in our meta-analysis.

### Study and patient characteristics

Two thousand and twenty-two patients, 811 patients from the LTG group and 1211 patients from the OTG group, were included in the study. Eleven stud-

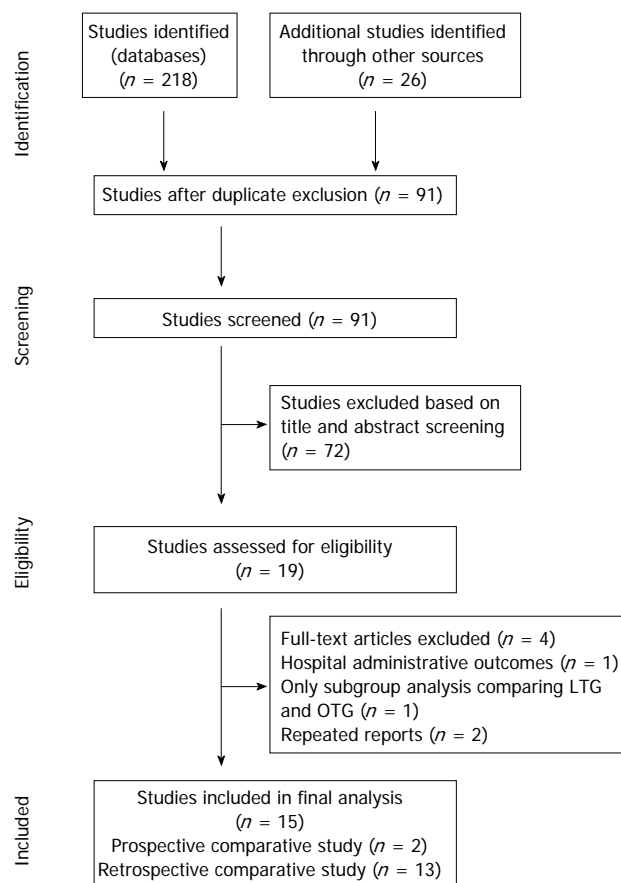


Figure 1 Flow diagram outlining the study selection process according to PRISMA guidelines. OTG: Open total gastrectomy; LTG: Laparoscopic total gastrectomy.

ies<sup>[9,10,14,16,17,28,29,32,34,37,38]</sup> included patients with both early and advanced gastric cancer, while three studies<sup>[15,30,33]</sup> only included patients with early gastric cancer; one study<sup>[27]</sup> only included patients with advanced gastric cancer. In seven studies<sup>[14,17,27,30,32,33,37]</sup>, D2 lymph node dissection was exclusively performed, while D1+ $\beta$  was completed in three studies<sup>[9,15,28]</sup>. The remaining studies<sup>[10,16,29,34,38]</sup> reported D1+ $\alpha/\beta$  and D2 dissections. All the studies were conducted in Asia and Europe, and were published between 2009 and 2013. The sample size ranged from 19 to 448 patients. From the nine studies<sup>[9,15,17,27,28,30,32,37,38]</sup> that reported data on conversion to an open procedure; LTG was converted to an open procedure in five patients in two studies<sup>[17,37]</sup>. The study characteristics (Table 1), quality assessment scoring (Table 2), perioperative outcomes of the included studies (Table 3) and the results of the meta-analysis (Table 4) have been summarized appropriately.

### Operative outcomes

“Operation time” was reported in all studies. The analysis showed that the LTG group had a significantly longer operation time compared with the OTG group (WMD 48.25 min, 95%CI: 31.15-65.35,  $P < 0.00001$ ), albeit with a significant heterogeneity ( $I^2 = 93\%$ ). Data from 12 studies<sup>[9,14-17,27-29,31,33,37,38]</sup> were pooled together to obtain the



Table 1 Study characteristics

Author, year	Country	Study design	Group	No. of patients	Age (yr)	Gender (M/F)	BMI (kg/m <sup>2</sup> )	ASA (1:2:3)	Tumor size (cm)	Tumor stage <sup>1</sup>	Extent of LND	Population
Dulucq <i>et al</i> <sup>[28]</sup> , 2005	France	PCS	LTG	8	75 ± 8	3/5	NA	NA	5.5 ± 2	NA	D1 + β	EGC + AGC
			OTG	11	67 ± 14	5/6	NA	NA	6.1 ± 0.4	NA		
Usui <i>et al</i> <sup>[9]</sup> , 2005	Japan	RCS	LTG	20	66.0 ± 10.4	13/7	21.3 ± 3.1	NA	NA	8/10/2/0/0	D1 + β	EGC + AGC
			OTG	19	66.2 ± 10.2	14/5	22.1 ± 2.4	NA	NA	10/8/1/0/0		
Kim <i>et al</i> <sup>[34]</sup> , 2008	South Korea	RCS	LTG	27	57.3 ± 14.2	16/11	22.6 ± 3.1	NA	NA	NA	D1 + α/β, D2	EGC + AGC
			OTG	33	61.6 ± 9.2	23/10	22.4 ± 2.1	NA	NA	NA		
Mochiki <i>et al</i> <sup>[15]</sup> , 2008	Japan	RCS	LTG	20	66 ± 2.4	16/4	NA	NA	3.6 ± 0.5	NA	D1 + β	EGC
			OTG	18	63 ± 2.2	16/2	NA	NA	5.7 ± 0.8	NA		
Topal <i>et al</i> <sup>[14]</sup> , 2008	Belgium	PCS	LTG	38	68 (37-85)	23/15	24 (17-30)	NA	47 (7-180)	0/17/7/10/4	D2	EGC + AGC
			OTG	22	69 (38-86)	17/5	24 (17-30)	NA	30 (10-180)	0/7/7/6/2		
Kawamura <i>et al</i> <sup>[30]</sup> , 2009	Japan	RCS	LTG	46	64 ± 10.4	10/36	22.8 ± 3.0	15/27/4	NA	NA	D2	EGC
			OTG	35	65.2 ± 10.7	10/25	22.9 ± 2.4	14/15/6	NA	NA		
Sakuramoto <i>et al</i> <sup>[16]</sup> , 2009	Japan	RCS	LTG	30	63.7 ± 9.2	12/18	21.9 ± 2.7	9/20/1	4.0 ± 2.9	0/25/2/3/0	D1 + β, D2	EGC + AGC
			OTG	44	67.2 ± 9.9	10/34	22.5 ± 3.6	8/28/8	6.1 ± 3.7	0/15/17/12/0		
Du <i>et al</i> <sup>[27]</sup> , 2010	China	RCS	LTG	82	60.4 ± 18.5	54/28	22.3 ± 2.6	NA	5.4 ± 1.4	0/3/36/43/0	D2	AGC
			OTG	94	57.8 ± 17.2	61/33	22.5 ± 2.4	NA	5.9 ± 1.6	0/6/31/57/0		
Kim <i>et al</i> <sup>[33]</sup> , 2011	South Korea	RCS	LTG	63	55.9 ± 12.2	43/20	22.7 ± 2.5	45/15/3	3.8 ± 2.1	NA	D2	EGC
			OTG	127	57.3 ± 11.1	81/46	23.0 ± 2.9	86/39/2	3.9 ± 2.7	NA		
Eom <i>et al</i> <sup>[10]</sup> , 2012	South Korea	RCS	LTG	100	54.9 ± 13.5	57/43	22.7 ± 2.8	NA	4.3 ± 2.9	NA	D1 + β, D2	EGC + AGC
			OTG	348	58.7 ± 11.5	254/94	23.8 ± 2.9	NA	4.4 ± 3.0	NA		
Guan <i>et al</i> <sup>[17]</sup> , 2012	China	RCS	LTG	41	60.7 ± 9.1	33/8	NA	NA	NA	0/18/20/3/0	D2	EGC + AGC
			OTG	56	57.8 ± 9.9	40/16	NA	NA	NA	0/25/25/6/0		
Siani <i>et al</i> <sup>[38]</sup> , 2012	Italy	RCS	LTG	25	65 ± 8.5	15/10	NA	NA	NA	0/6/5/14/0	D1 + α/β, D2	EGC + AGC
			OTG	25	66 ± 7.8	18/7	NA	NA	NA	0/4/5/16/0		
Kim <i>et al</i> <sup>[32]</sup> , 2013	South Korea	RCS	LTG	139	58 (30-84)	86/53	23.6 (13.6-32.4)	85/46/8	3.2 (0.2, 15)	NA	D2	EGC + AGC
			OTG	207	56 (31-84)	134/73	24.1 (16.7-35.2)	137/52/18	4.0 (0.3, 22)	NA		
Jeong <i>et al</i> <sup>[29]</sup> , 2013	South Korea	RCS	LTG	122	63.2 ± 11.2	89/33	23.1 ± 3.4	33/80/9	NA	NA	D1 + β, D2	EGC + AGC
			OTG	122	62.6 ± 11.7	93/29	23.5 ± 3.2	43/67/12	NA	NA		
Lee <i>et al</i> <sup>[37]</sup> , 2013	South Korea	RCS	LTG	50	50.6 ± 22.1	32/18	23.2 ± 3.7	34/11/5	NA	0/24/13/9/4	D2	EGC + AGC
			OTG	50	51 ± 22.6	32/18	23 ± 3.4	31/16/3	NA	0/24/13/9/4		

Continuous variables are presented as means ± SD or median and range. <sup>1</sup>Pathological tumor stage (0/ I / II / III / IV). PCS: Prospective comparative study; RCS: Retrospective comparative study; LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy; BMI: Body mass index; NA: Not available; ASA: American Society of Anesthesiologists; LND: Lymph node dissection; EGC: Early gastric cancer; AGC: Advanced gastric cancer; M/F: Male/female.

mean intraoperative blood loss in the two groups. LTG was associated with a significantly lower intraoperative blood loss compared with OTG (WMD -201.19 mL, 95%CI: -296.50--105.87 mL,  $P < 0.0001$ ), with a significant heterogeneity ( $I^2 = 98\%$ ). Forest plots for operative outcomes are shown in Figure 2.

### Postoperative recovery

Twelve studies<sup>[9,16,17,27-30,32-34,37,38]</sup> reported the time to first flatus and eight studies<sup>[9,16,17,27,29,32,33,37]</sup> reported data on oral intake post-surgery. Our analyses showed that patients undergoing LTG had a quicker recovery of intestinal motility compared with the OTG group. The time to first flatus (WMD -0.82, 95%CI: -1.18--0.45,  $P < 0.0001$ ) and the time to first oral intake (WMD -1.30, 95%CI: -1.84--0.75,  $P < 0.0001$ ) were significantly shorter in the LTG group compared with the OTG group. Analysis of the 13 studies<sup>[9,10,15-17,28-30,32-34,37,38]</sup> that reported the duration of hospital stay indicated that LTG was associated with a significantly shorter postoperative hospital stay compared with OTG (WMD -3.55, 95%CI: -5.13--1.96,  $P < 0.0001$ ). However, there was no statistically significant difference between the two groups in the use of analgesics post-surgery (WMD -0.09, 95%CI: -2.39-2.20,  $P = 0.94$ ). Forest plots for postoperative recovery outcomes are shown in Figure 3.

### Postoperative complications

A pooled analysis of 14 studies<sup>[10,14-17,27-30,32-34,37,38]</sup> indicated that the overall complication rate was significantly lower in the LTG group compared with the OTG group (OR = 0.73, 95%CI: 0.57-0.92,  $P = 0.009$ ). Also, the analysis of 13 studies<sup>[10,15-17,27-30,32-34,37,38]</sup> suggested that patients in the LTG group had significantly fewer wound-related complications compared with the OTG group (OR = 0.39, 95%CI: 0.21-0.72,  $P = 0.002$ ). However, there were no significant differences in the rate of anastomotic leak (OR = 1.6, 95%CI: 0.88-2.91,  $P = 0.12$ ), anastomotic stenosis (OR = 1.22, 95%CI: 0.68-2.21,  $P = 0.50$ ), ileus (OR 1.26, 95%CI: 0.69-2.30;  $P = 0.46$ ), bleeding (OR = 1.42, 95%CI: 0.70-2.87;  $P = 0.33$ ), abdominal abscess (OR = 0.53, 95%CI: 0.28-1.03,  $P = 0.06$ ) or mortality (OR = 0.74, 95%CI: 0.24-2.31,  $P = 0.61$ ) between the two groups. Forest plots for postoperative outcomes are shown in Figure 4.

### Oncological outcomes

All included studies reported data on the number of lymph nodes retrieved; there was no significant difference between the two groups (WMD -2.49, 95%CI: -5.18-0.21,  $P = 0.07$ ), albeit with a significant heterogeneity in the result ( $I^2 = 74\%$ ). Five studies<sup>[14,17,27,28,32]</sup> reported

**Table 2** Quality assessment scoring of included studies, according to NOS criterion

Author, year	Selection			Comparability <sup>1</sup>		Outcome assessment		Star Score
	1	2	3	4	5	6	7	
Dulucq <i>et al</i> <sup>[28]</sup> , 2005	*	*	*	*		*	*	*****
Usui <i>et al</i> <sup>[9]</sup> , 2005	*	*	*			*	*	*****
Kim <i>et al</i> <sup>[34]</sup> , 2008	*	*	*			*		*****
Mochiki <i>et al</i> <sup>[15]</sup> , 2008	*	*	*	*		*	*	*****
Topal <i>et al</i> <sup>[14]</sup> , 2008	*	*	*	**	**	*		*****
Kawamura <i>et al</i> <sup>[30]</sup> , 2009	*	*	*	**	*	*	*	*****
Sakuramoto <i>et al</i> <sup>[16]</sup> , 2009	*	*	*	**		*	*	*****
Du <i>et al</i> <sup>[27]</sup> , 2010	*	*	*	**		*	*	*****
Kim <i>et al</i> <sup>[33]</sup> , 2011	*	*	*	**	*	*		*****
Eom <i>et al</i> <sup>[10]</sup> , 2012	*	*	*			*	*	*****
Guan <i>et al</i> <sup>[17]</sup> , 2012	*	*	*		*	*		*****
Siani <i>et al</i> <sup>[38]</sup> , 2012	*	*	*	*	*	*	*	*****
Kim <i>et al</i> <sup>[32]</sup> , 2013	*	*	*	**		*		*****
Jeong <i>et al</i> <sup>[29]</sup> , 2013	*	*	*	**	*	*		*****
Lee <i>et al</i> <sup>[37]</sup> , 2013	*	*	*	**	*	*	*	*****

Based on Newcastle-Ottawa Scale with maximum of \*\*\* for selection, \*\*\*\* for comparability, and \*\* for outcome assessment. <sup>1</sup>Comparability variables are (1) age, (2) sex, (3) body mass index, (4) American Society of Anesthesiologists, (5) comorbidity, (6) tumor size and (7) tumor stage. Group comparable for (1)-(3) or (4)-(7) (if yes, two stars, one star if one of these three characteristics was not reported, even if there were no other differences between the two groups and other characteristics had been controlled; no points were assigned if the two groups differed).

**Table 3** Perioperative outcomes

Author, year	Group	Operation time (min)	Intraoperative blood loss (mL)	No. of resected lymph nodes (n)	Time to first flatus (d)	Time to first oral intake (d)	Hospital stay (d)	Analgesics use (times)	Postoperative complications (%)	In-hospital Mortality (%)
Dulucq <i>et al</i> <sup>[28]</sup> , 2005	LTG	183 ± 48	81 ± 107	24 ± 12	3.6 ± 1.2	NA	16.9 ± 3	NA	0	0
	OTG	165 ± 60	125 ± 95	20 ± 8	4.7 ± 1.2	NA	24 ± 9	NA	18	9
Usui <i>et al</i> <sup>[9]</sup> , 2005	LTG	280.1 ± 45.2	227.5 ± 148.1	28.0 ± 15.1	2.9 ± 0.9	5.7 ± 2.1	15.5 ± 3.9	2.1 ± 1.3	NA	NA
	OTG	266.4 ± 48.2	393.1 ± 173.6	28.9 ± 14.3	4.2 ± 1.4	8.8 ± 1.3	23.2 ± 4.6	3.4 ± 4.4	NA	NA
Kim <i>et al</i> <sup>[34]</sup> , 2008	LTG	527.5 ± 95.7	NA	27.2 ± 15.7	3.6 ± 0.9	NA	16.2 ± 7.1	NA	7.4	0
	OTG	320.9 ± 75.8	NA	37.2 ± 15.7	4.1 ± 1.3	NA	16.0 ± 9.3	NA	24.2	0
Mochiki <i>et al</i> <sup>[15]</sup> , 2008	LTG	254 ± 10	299 ± 50	26 ± 3	NA	NA	19 ± 3	NA	25	0
	OTG	248 ± 12	758 ± 78	35 ± 4	NA	NA	29 ± 3	NA	16.7	0
Topal <i>et al</i> <sup>[14]</sup> , 2008	LTG	187 ± 60	10.0 ± 98.8	NA	NA	NA	NA	NA	39.5	2.6
	OTG	152.5 ± 25	450.0 ± 337.5	NA	NA	NA	NA	NA	40.9	4.5
Kawamura <i>et al</i> <sup>[30]</sup> , 2009	LTG	291.9 ± 59.4	54.9 ± 45.3	48.5 ± 16.3	4.1 ± 1.0	NA	15.5 ± 3.3	6.9 ± 5.6	8.7	0
	OTG	272.1 ± 76.8	304.3 ± 237.3	47.1 ± 21.5	4.3 ± 1.3	NA	18.8 ± 6.3	4.0 ± 3.2	22.9	0
Sakuramoto <i>et al</i> <sup>[16]</sup> , 2009	LTG	313 ± 81	134 ± 98	43.2 ± 17.2	2.4 ± 1.1	4.9 ± 1.1	13.5 ± 2.7	6.8 ± 6.4	16.7	0
	OTG	218 ± 53	407 ± 270	51.2 ± 22.1	3.3 ± 1.0	6.0 ± 2.1	18.2 ± 9.6	11.8 ± 11.0	27.3	0
Du <i>et al</i> <sup>[27]</sup> , 2010	LTG	275 ± 78	156 ± 112	34.2 ± 13.5	3.5 ± 0.8	3.5 ± 0.8	NA	NA	9.8	0
	OTG	212 ± 51	339 ± 162	36.4 ± 19.1	5.3 ± 1.3	5.3 ± 1.3	NA	NA	24.5	2.1
Kim <i>et al</i> <sup>[33]</sup> , 2011	LTG	150.8 ± 31.2	179.7 ± 123.8	38.7 ± 15.7	3.3 ± 0.7	4.3 ± 1.7	8.1 ± 3.8	5.3 ± 4.9	12.7	0
	OTG	131.2 ± 21.6	272.7 ± 209.6	35.6 ± 13.1	3.8 ± 0.8	5.6 ± 4.4	9.6 ± 5.3	3.6 ± 3.9	18.9	0
Eom <i>et al</i> <sup>[10]</sup> , 2012	LTG	283.7 ± 84.1	NA	48.3 ± 16.4	NA	NA	12.6 ± 15.5	NA	27	1
	OTG	198.5 ± 59.7	NA	49.8 ± 18.4	NA	NA	14.3 ± 16.7	NA	23.6	0.9
Guan <i>et al</i> <sup>[17]</sup> , 2012	LTG	235.7 ± 38.5	104.2 ± 42.9	23.1 ± 8.0	3 ± 0.7	2.2 ± 0.9	9.7 ± 2.2	NA	4.9	0
	OTG	211.5 ± 33.2	355.6 ± 51.3	24.2 ± 7.5	3.3 ± 0.4	3.1 ± 0.5	13.6 ± 3.6	NA	5.4	0
Siani <i>et al</i> <sup>[38]</sup> , 2012	LTG	211 ± 23	250 ± 150	35 ± 18	2.1 ± 0.9	NA	10.5 ± 1.5	NA	16	0
	OTG	185 ± 19	495 ± 190	40 ± 16	4.1 ± 1.5	NA	14.5 ± 3.1	NA	4	0
Kim <i>et al</i> <sup>[32]</sup> , 2013	LTG	144 ± 104.3	NA	37 ± 24	3 ± 2	3 ± 12.3	7 ± 19.3	3 ± 24.5	10	0
	OTG	137 ± 105	NA	34 ± 18.8	4 ± 2.3	5 ± 10	8 ± 9	4 ± 9.3	21.7	0
Jeong <i>et al</i> <sup>[29]</sup> , 2013	LTG	289 ± 89	249 ± 204	42 ± 15	2.9 ± 0.8	3.9 ± 4.4	11.8 ± 11.8	NA	23.8	1.6
	OTG	203 ± 78	209 ± 157	46 ± 17	3.0 ± 0.8	3.6 ± 3.3	10.8 ± 7.0	NA	17.2	0.9
Lee <i>et al</i> <sup>[37]</sup> , 2013	LTG	258 ± 54	167.3 ± 135.2	48.4 ± 18.4	4 ± 1.2	5 ± 1.7	9.3 ± 4.2	NA	24	0
	OTG	198 ± 57	178.4 ± 107	54.3 ± 20.5	4.5 ± 1.5	6.1 ± 2.5	11.7 ± 7.3	NA	32	0

LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy; NA: Not available.

data on positive resection margins; in only one study<sup>[14]</sup>, resection margins were found to be positive in one patient each from the LTG and OTG groups and with no significant difference between the two groups (OR = 0.57, 95%CI: 0.03-9.55,  $P = 0.69$ ). There were also no

significant differences in the lengths of the proximal resection margin (WMD -0.26, 95%CI: -0.54-0.01,  $P = 0.06$ ) and distal resection margin (WMD 0.32, 95%CI: -0.05-0.68,  $P = 0.09$ ) between the two groups when data from four studies<sup>[10,27,32,33]</sup> were pooled. Seven studies re-

**Table 4 Results of meta-analysis comparing laparoscopic total gastrectomy vs open total gastrectomy**

Outcome of interest	No. of studies	No. of patients	OR/WMD	95%CI	P value	Heterogeneity P value	I <sup>2</sup>
Operative outcomes							
Operation time (min)	15	2022	48.25	31.15-65.35	< 0.00001	< 0.00001	93%
Intraoperative blood loss (mL)	12	1168	-201.19	-296.50--105.87	< 0.0001	< 0.00001	98%
Postoperative recovery							
Time to first flatus (d)	12	1412	-0.82	-1.18--0.45	< 0.0001	< 0.00001	90%
Time to first oral intake (d)	8	1266	-1.3	-1.84--0.75	< 0.00001	< 0.00001	82%
Hospital stay (d)	13	1786	-3.55	-5.13--1.96	< 0.0001	< 0.00001	86%
Analgesics use (times)	5	730	-0.09	-2.39-2.20	0.94	0.0008	79%
Postoperative complications							
Overall complication	14	1983	0.73	0.57-0.92	0.009	0.08	37%
Anastomotic leakage	14	1983	1.6	0.88-2.91	0.12	0.68	0%
Anastomotic stenosis	13	1923	1.22	0.68-2.21	0.50	0.95	0%
Ileus	13	1923	1.26	0.69-2.30	0.46	0.85	0%
Bleeding	13	1923	1.42	0.70-2.87	0.33	0.26	23%
Abdominal abscess	13	1923	0.53	0.28-1.03	0.06	0.37	8%
Wound problems	13	1923	0.39	0.21-0.72	0.002	0.75	0%
Oncological outcomes							
Positive resection margins	5	698	0.57	0.03-9.55	0.69	-	-
No. of resected lymph nodes	14	1962	-2.49	-5.18-0.21	0.07	< 0.00001	74%
Proximal resection margin (cm)	4	1160	-0.26	-0.54-0.01	0.06	0.65	0%
Distal resection margin (cm)	4	1160	0.32	-0.05-0.68	0.09	0.22	32%

ported data on long-term survival following the two procedures<sup>[10,15,16,27,28,37,38]</sup>. Lee *et al*<sup>[37]</sup> reported no significant difference in the disease-specific survival rate between the LTG and OTG groups at a median follow-up of 50 months; there were also no significant differences reported in the disease-free survival rate (100% vs 90.9%,  $P = 0.5$ ) and the cumulative survival rate (91.5% vs 95.2%,  $P = 0.618$ ) in patients with stage I cancer (TNM) between the LTG and OTG groups. Eom *et al*<sup>[10]</sup> reported no significant difference in the disease-free survival rates between the LTG and OTG groups, after adjustment for five variables (age, tumor size, Lauren classification, depth of invasion and lymph node metastasis). Mochiki *et al*<sup>[15]</sup> reported no significant difference in the cumulative 5-year or disease-specific survival rates between the LTG and OTG groups, while Siani *et al*<sup>[38]</sup> reported 5-year overall and disease free survival rates of 55.7% and 54.2% in the LTG group and 52.9% and 52.1% in the OTG group respectively, with no statistically significant differences. However, as the duration of follow-up varied between studies, it was difficult to compare the survival rates. Forest plots for oncological outcomes are shown in Figure 5.

### Sensitivity and subgroup analysis

Sensitivity analyses were performed by removing individual studies from the data and analyzing the effect on the overall results to identify sources of significant heterogeneity. These exclusions did not alter the results obtained from the cumulative analyses. Subgroup analyses were undertaken for all outcome measures by including only high quality studies. Analysis of the high-quality studies showed that there were no significant differences for any of the outcomes. These are shown in Figure 6.

### Publication bias

The funnel plot based on the operation time is shown in

Figure 7. There was no broad evidence of publication bias, as none of the studies lay outside the 95%CI limits.

## DISCUSSION

Laparoscopic surgery is being used increasingly to treat gastric cancer, and has been shown to have many advantages over open surgery. However, LTG is less widely practiced compared with laparoscopic distal gastrectomy because of the technical challenges it poses and the absence of compelling evidence to substantiate its use<sup>[9]</sup>. Technical advances, better instrumentation and increasing surgical experience in the procedure are aiding its increasing application to treat of early and advanced gastric cancer. The aim of the current study was to inform future surgical practice by comparing the technical feasibility, effectiveness, and safety of LTG with OTG in the treatment of early and advanced gastric cancer, using a systematic review and meta-analysis of published comparative studies.

Our analyses indicated that the operation time was significantly longer in the LTG group than in the OTG group. This may be because LTG is more technically demanding than OTG and may result from the learning curve associated with the procedure<sup>[9,10,35]</sup>. While adequate training in laparoscopic techniques is necessary, it was concluded that an experienced laparoscopic surgeon would not require any more time to perform LTG compared with OTG<sup>[15]</sup>. In one study, the operation time for LTG in the later period was significantly shorter than in the early period; this related to the experience gained by the surgeon over the period of the study<sup>[34]</sup>. Further development in surgical techniques, especially for anastomosis and new instruments, may further decrease the operation time for LTG<sup>[10]</sup>. In our study, LTG was associated with a significantly lower intraoperative blood

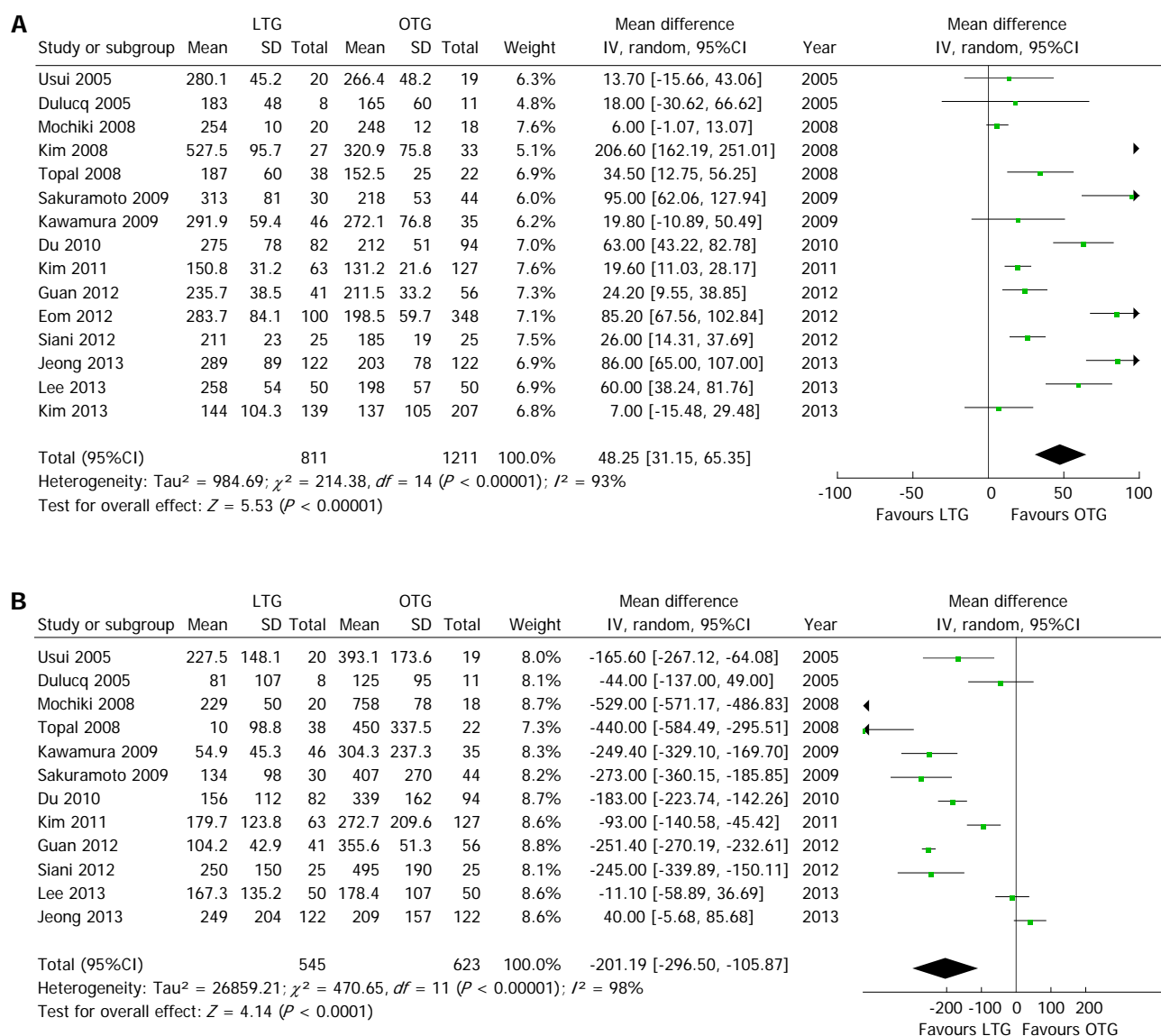
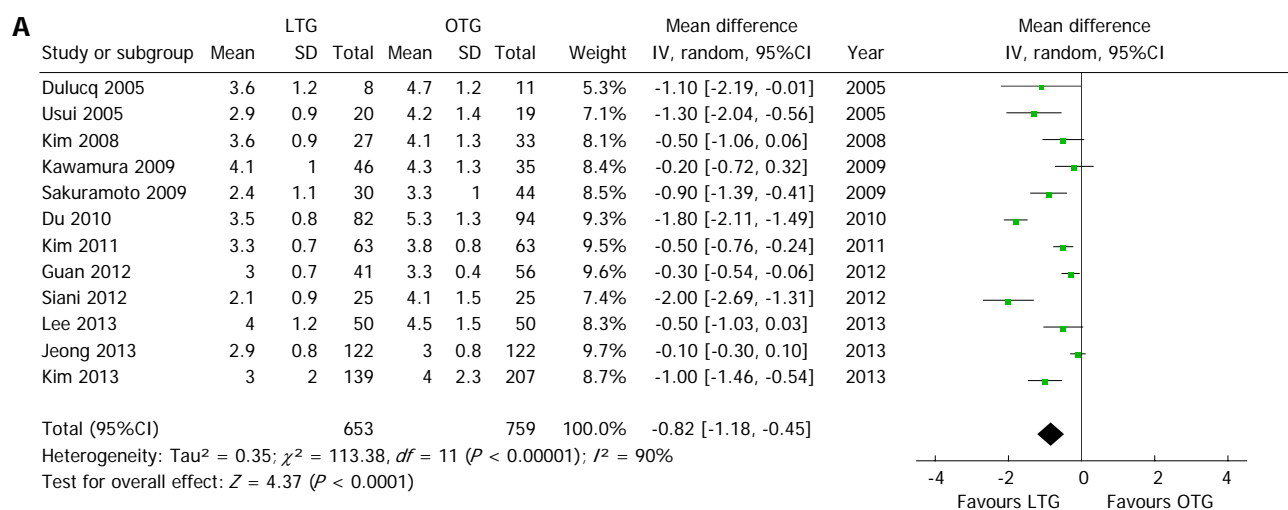


Figure 2 Forest plots illustrating results of operative outcomes in the form of a meta-analysis comparing laparoscopic total gastrectomy vs open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) with 95%CI was calculated using the random effects model. A: Operation time; B: Intraoperative blood loss. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.





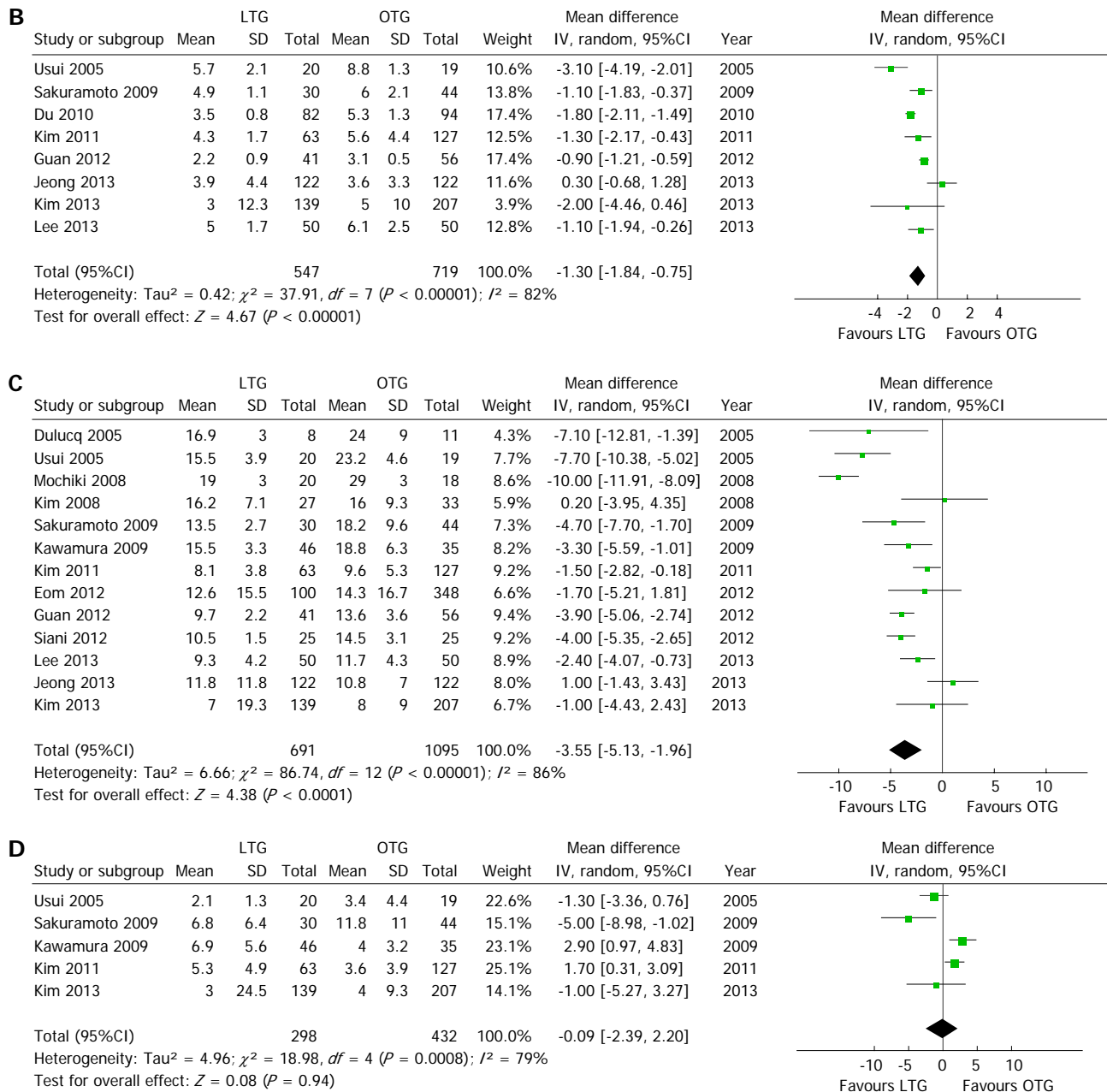


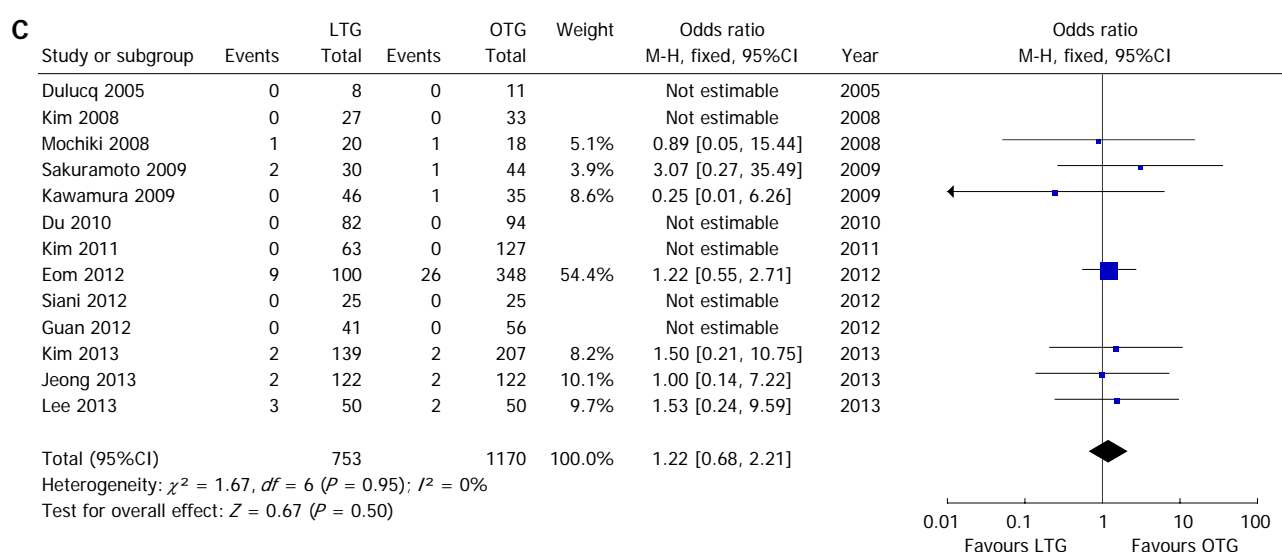
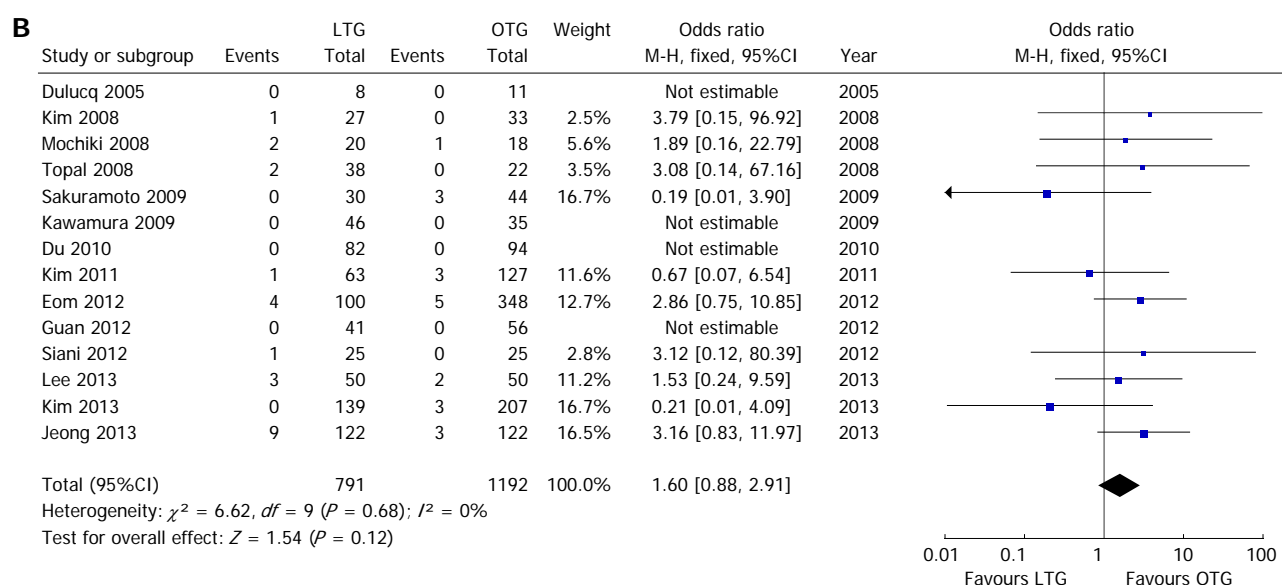
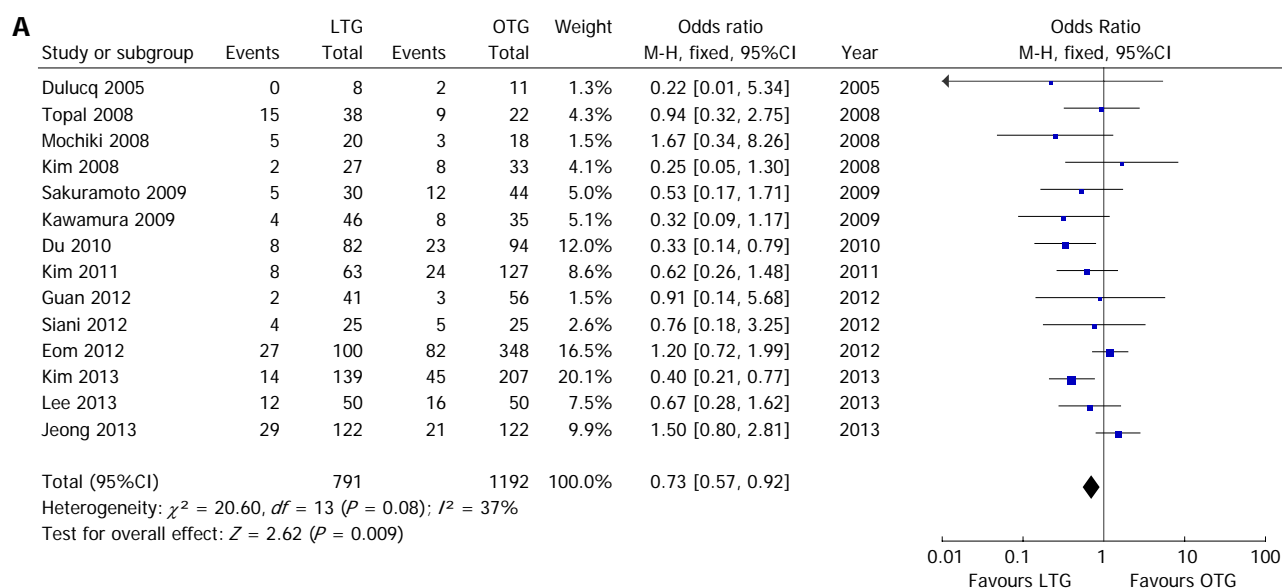
Figure 3 Forest plots illustrating results of postoperative recovery in the form of a meta-analysis comparing laparoscopic total gastrectomy vs open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) with 95%CI was calculated using the random-effects model. A: Time to first flatus; B Time to first oral intake; C: Hospital stay; D: Analgesic use. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

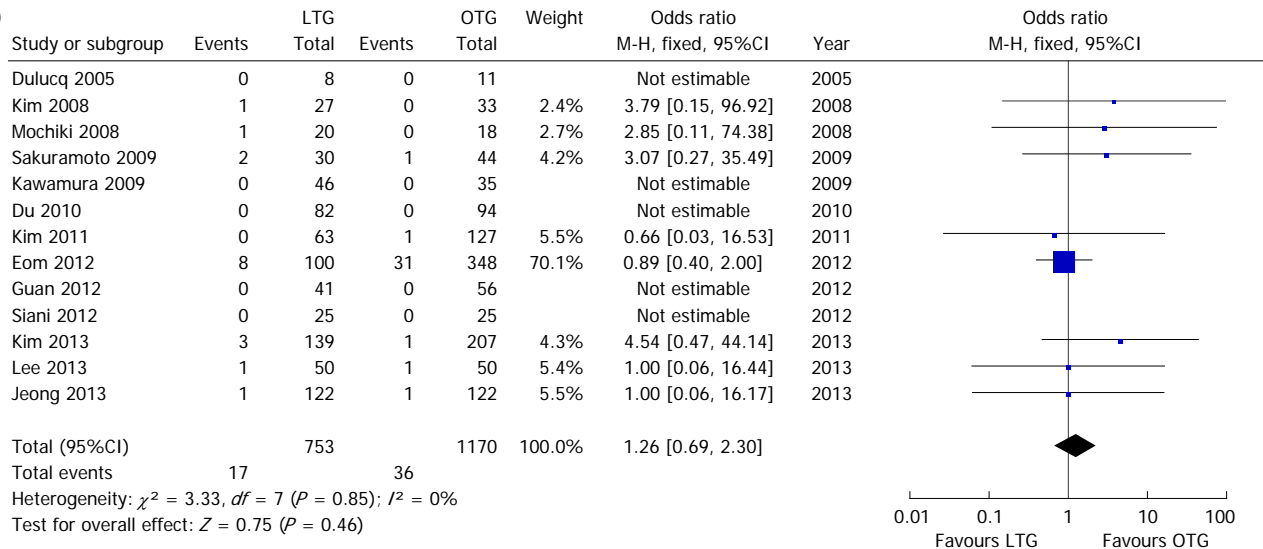
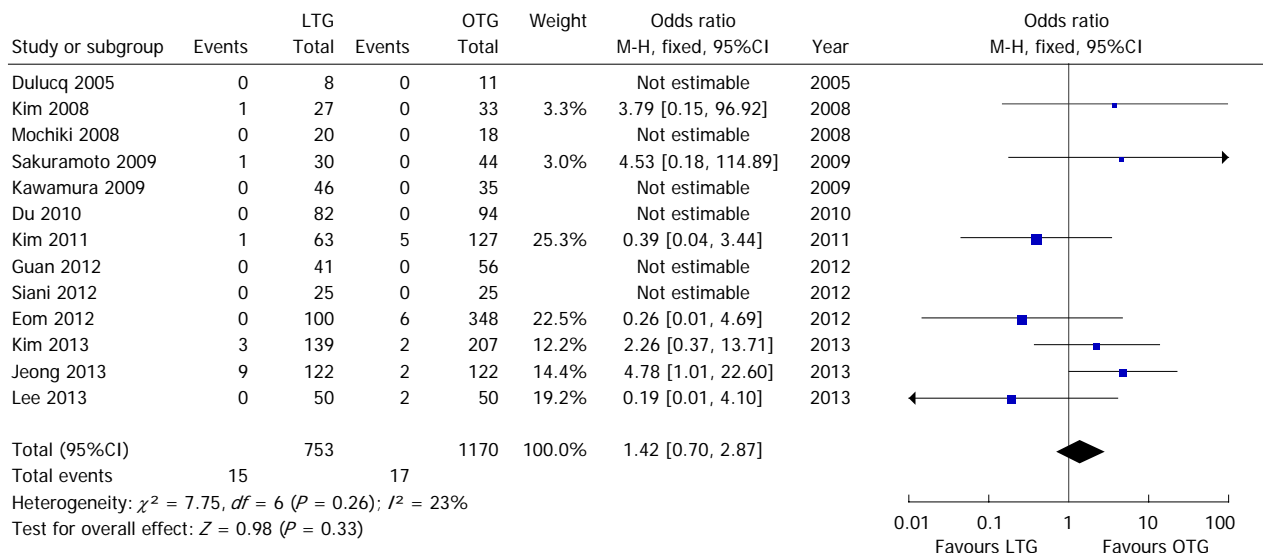
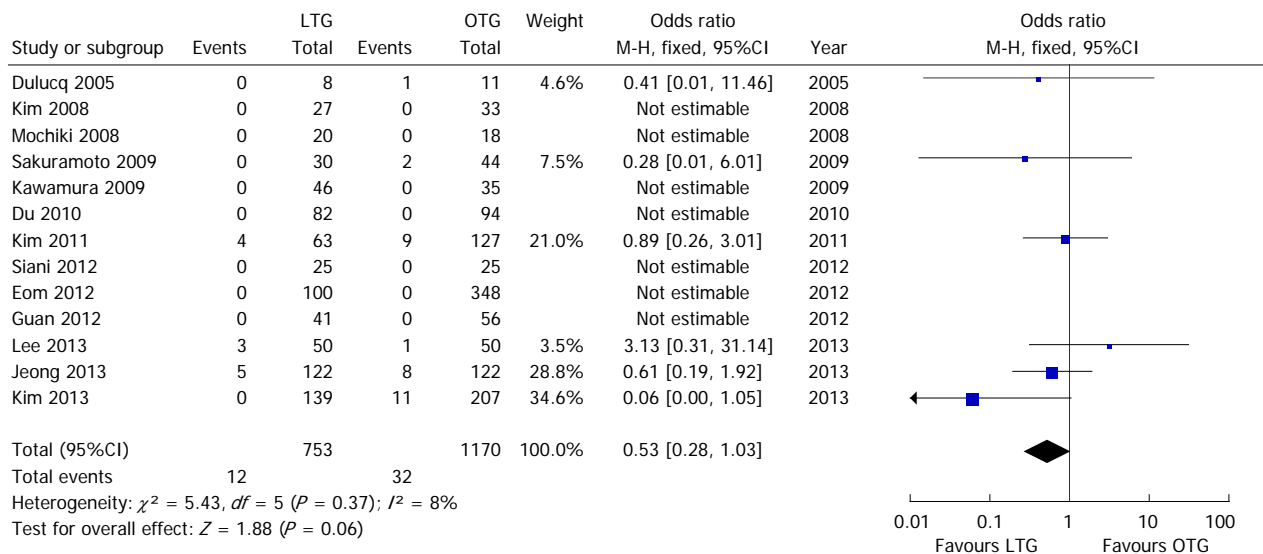
loss, which depends considerably on a surgeon's skill and experience<sup>[34]</sup>.

The times to first flatus and to first oral intake were significantly shorter in the LTG group compared with the OTG group, which suggests that intestinal motility recovered more quickly in the LTG group. Also, the period of hospital stay was significantly shorter in the LTG group. LTG is a less invasive procedure and is associated with less surgical trauma. This results in a reduced inflammatory response and better glucose tolerance, which may aid a quicker recovery<sup>[19,30]</sup>. Pain following LTG subsides earlier when compared to OTG<sup>[18]</sup>. However, our study showed no significant difference in the postoperative use of analgesics between the two groups.

A quicker recovery and shorter hospital stay have important cost and quality of life implications for the wider use of LTG in the treatment of gastric cancer.

Total gastrectomy has often been described as high-risk<sup>[39,40]</sup> and LTG is technically demanding<sup>[9,10]</sup>. Common postoperative complications associated with LTG include anastomotic leak, anastomotic stenosis and luminal bleeding<sup>[37]</sup>. The anastomotic complications could be caused by excessive traction applied on the esophagus and jejunal limb mobilization<sup>[10]</sup> or may reflect the learning curve associated with LTG<sup>[37]</sup>. In our study, the overall complication rate was significantly lower in the LTG group compared with the OTG group. Also, there were significantly fewer wound-related complications in the LTG



**D****E****F**

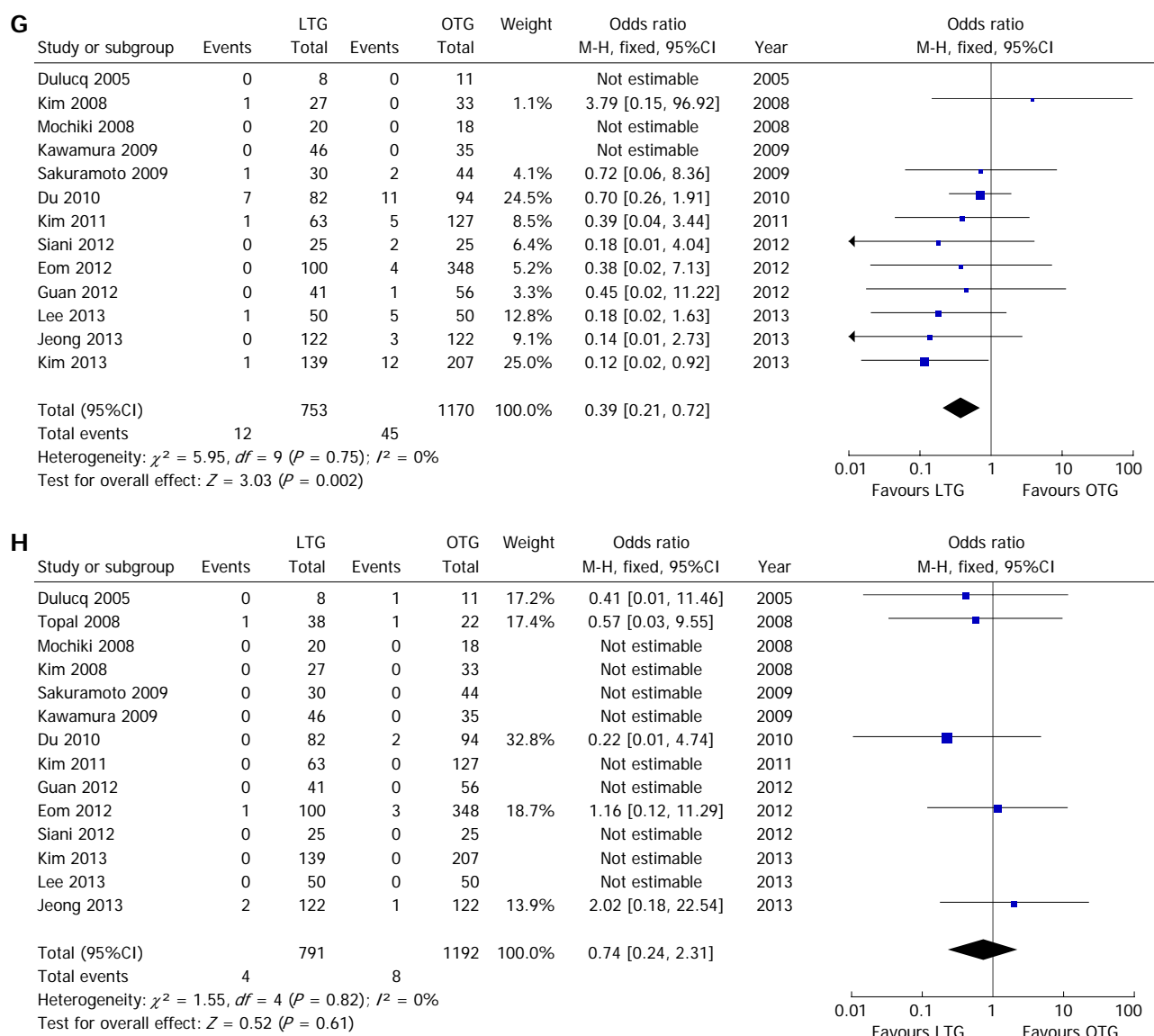
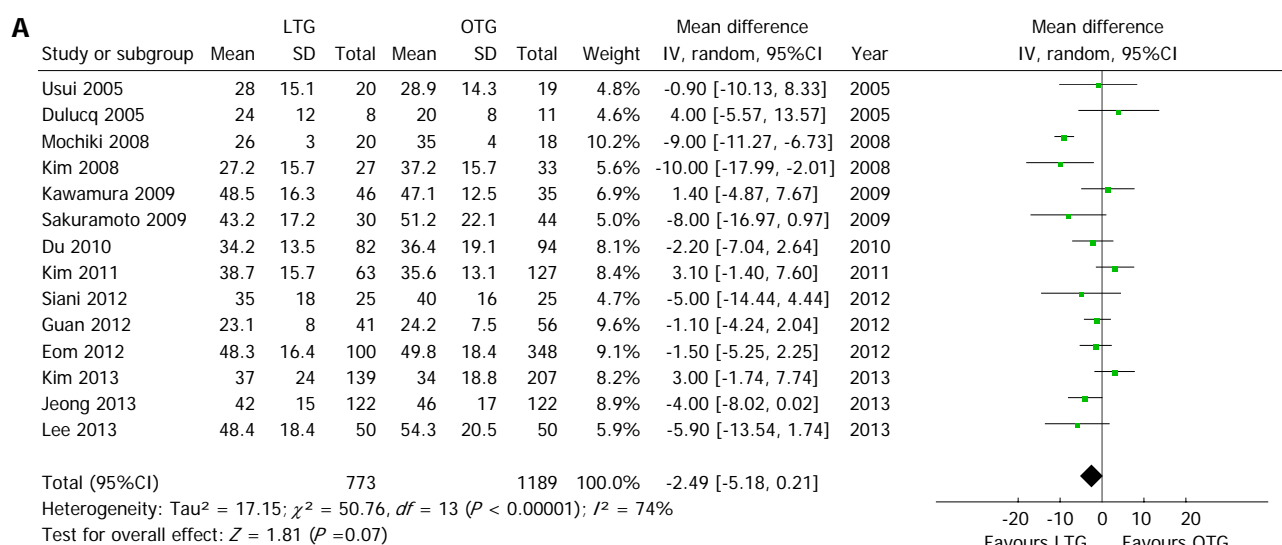


Figure 4 Forest plots illustrating results of postoperative complications in the form of a meta-analysis comparing laparoscopic total gastrectomy vs open total gastrectomy for gastric cancer. Pooled odds ratio (OR) with 95%CI was calculated using the fixed-effects model. A: Overall complication rate; B: Anastomotic leak; C: Anastomotic Stenosis; D: Ileus; E: Bleeding; F: Abdominal abscess; G: Wound-related complications; H: Mortality. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.





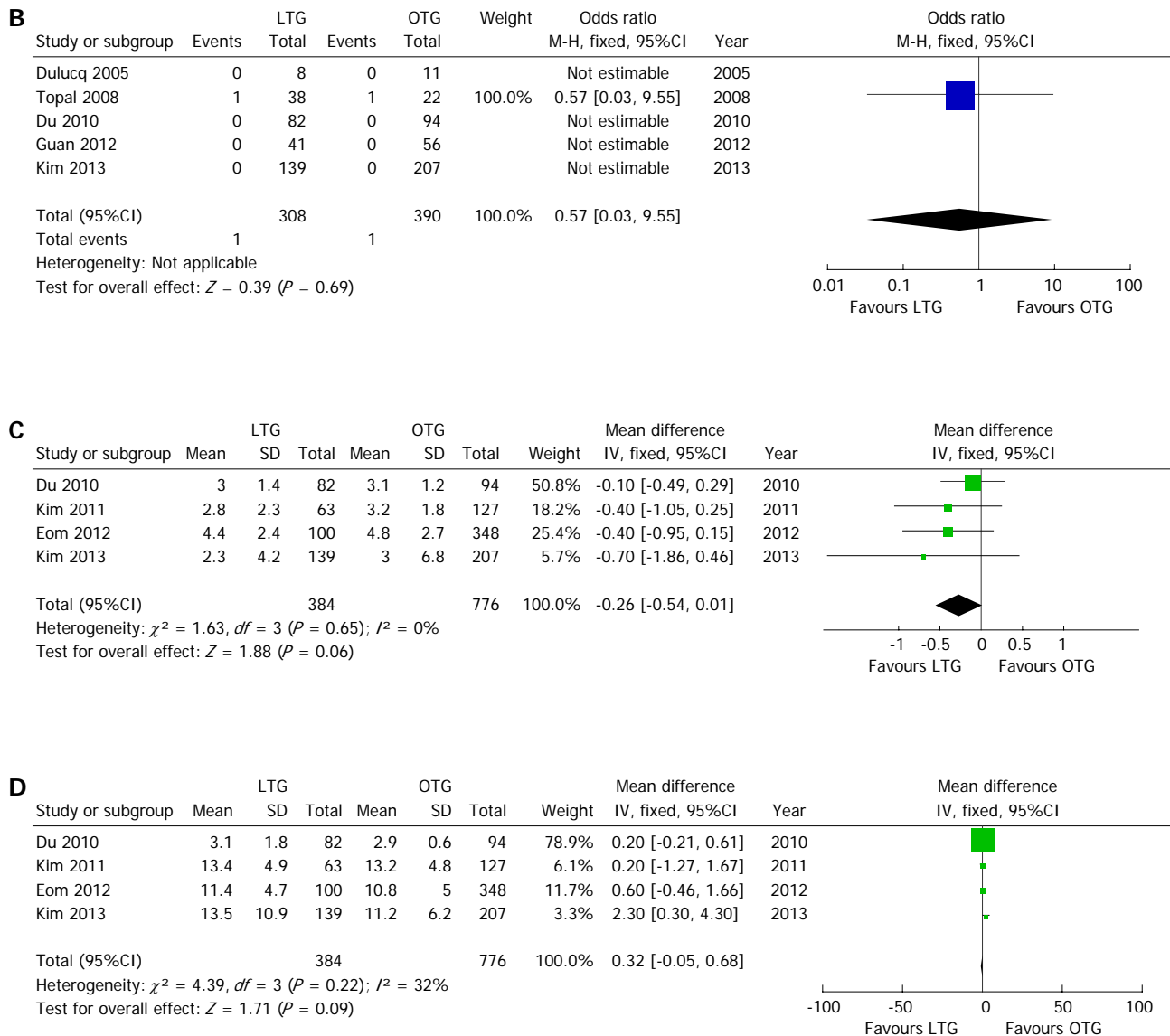
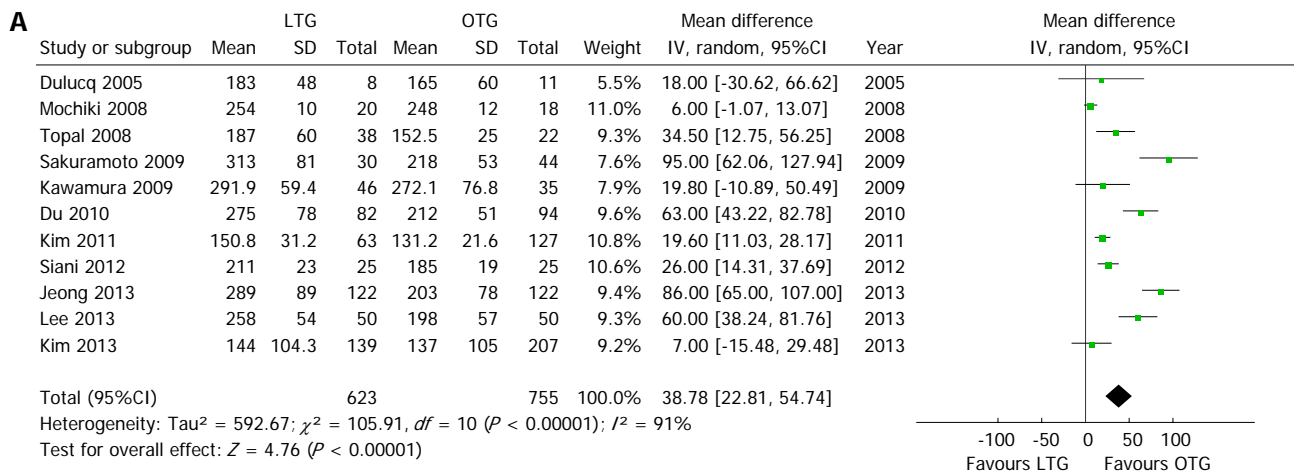
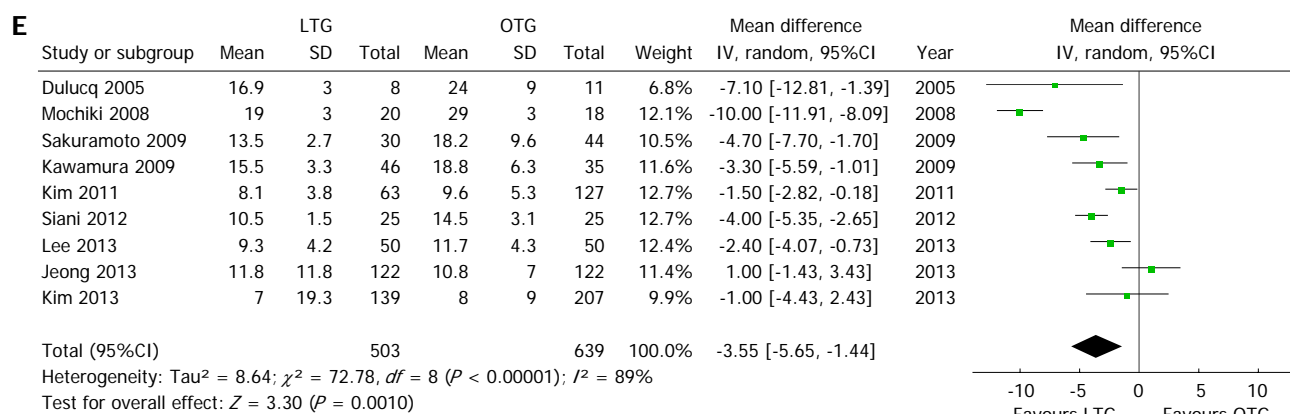
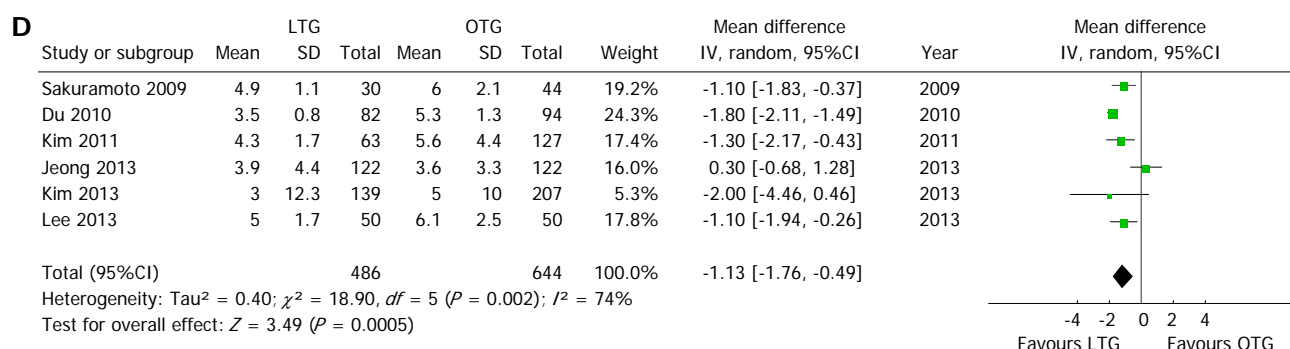
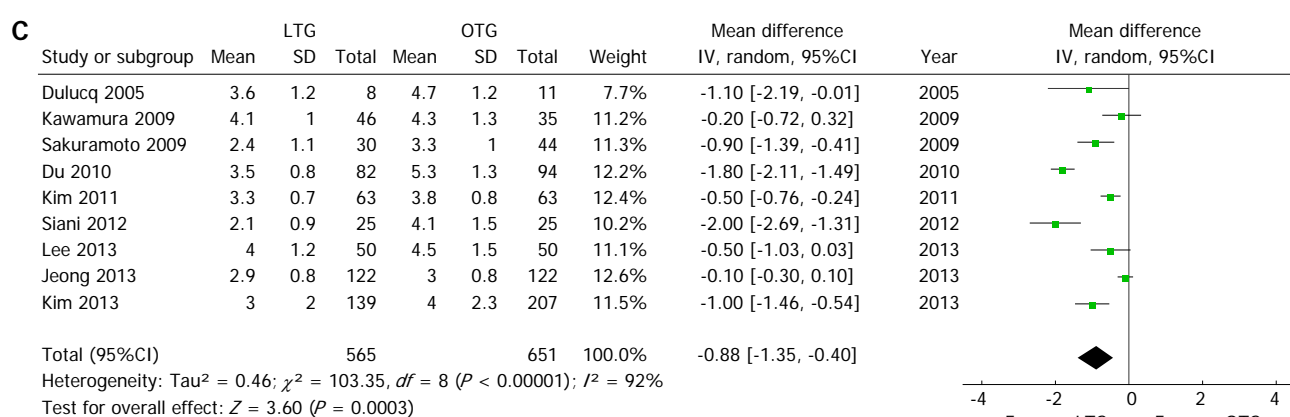
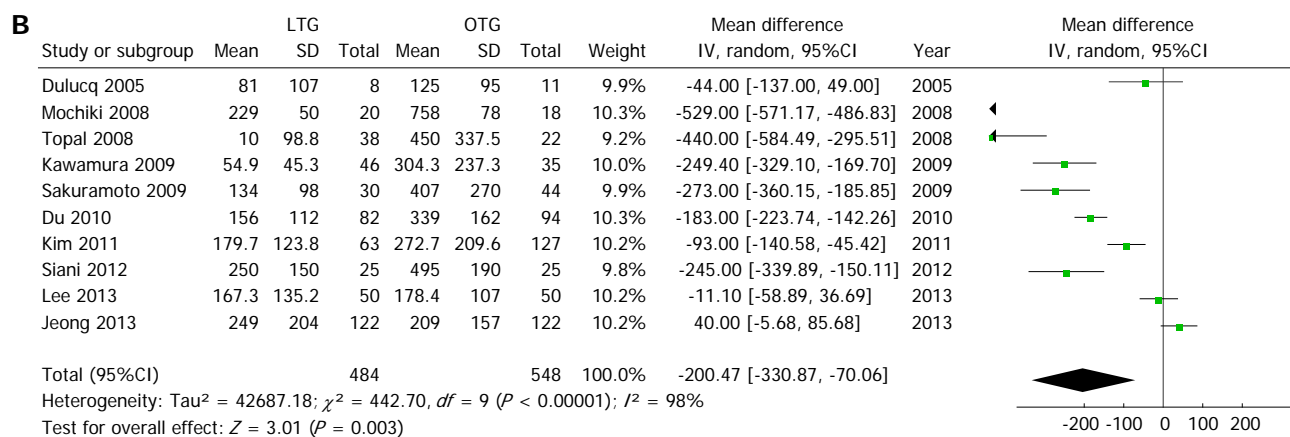
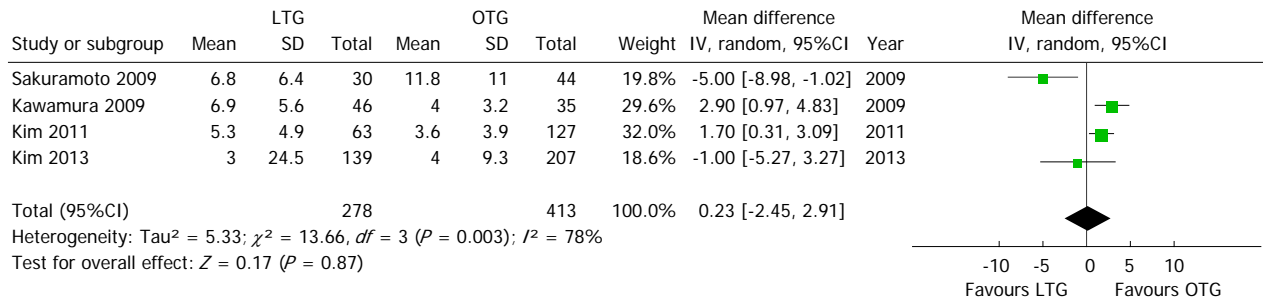
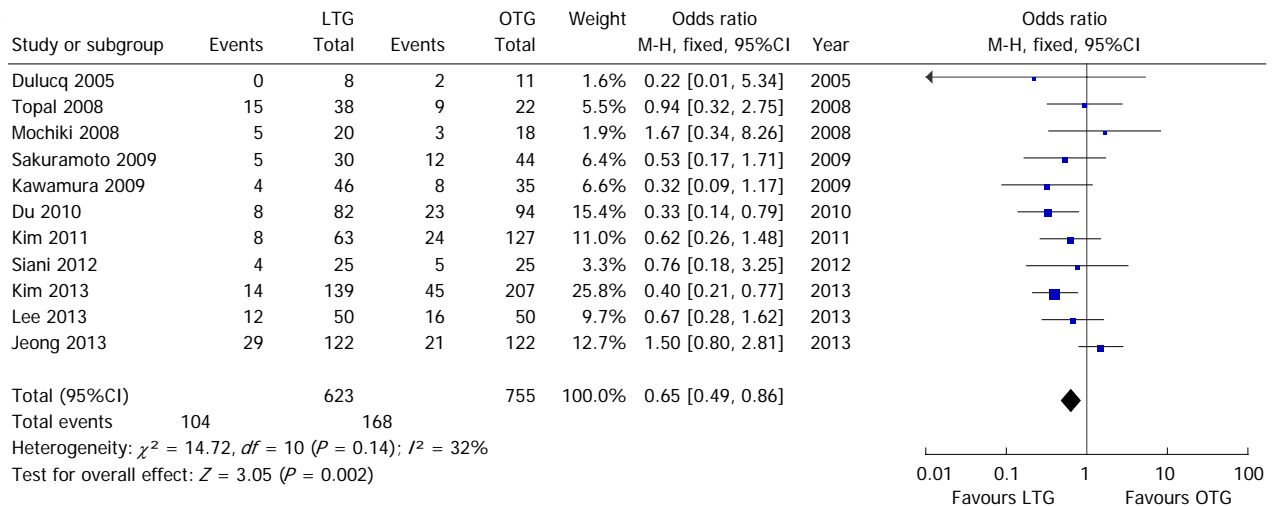
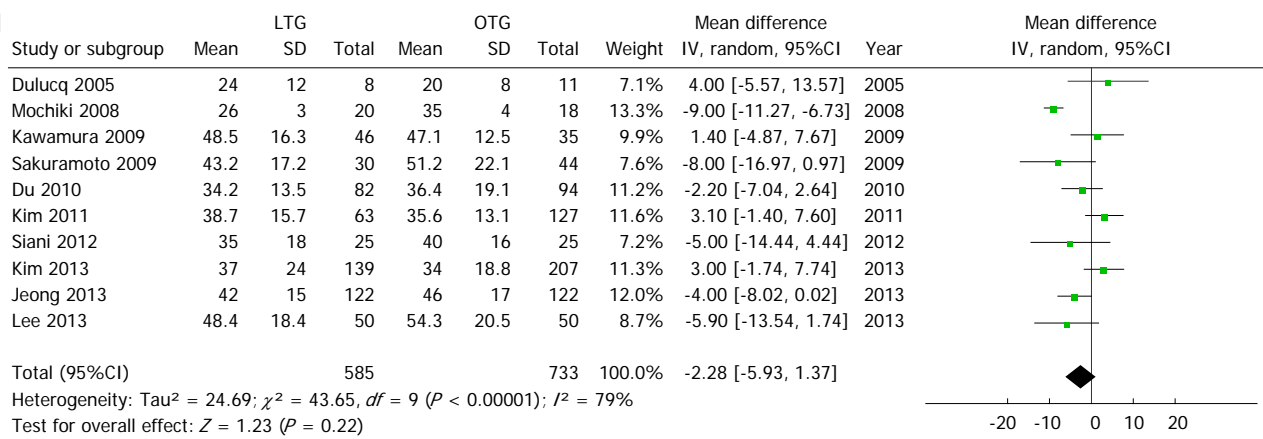
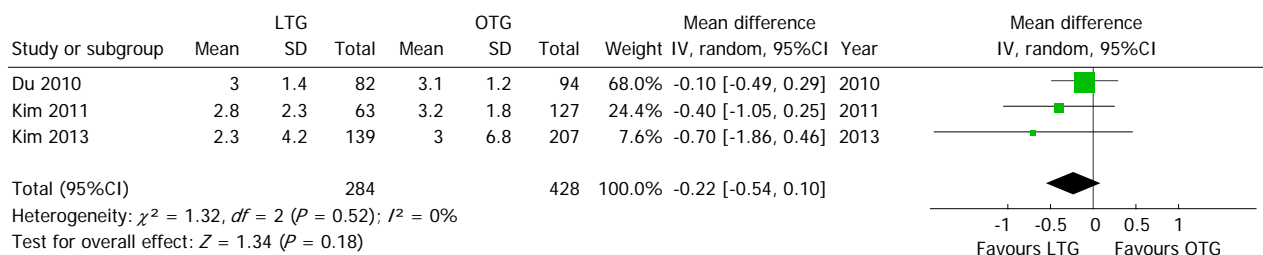
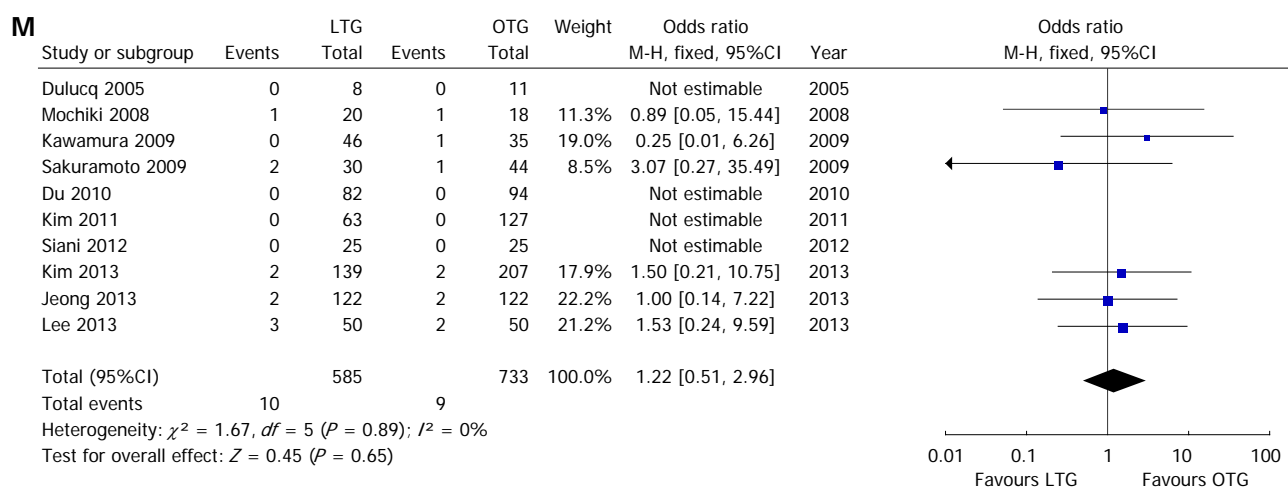
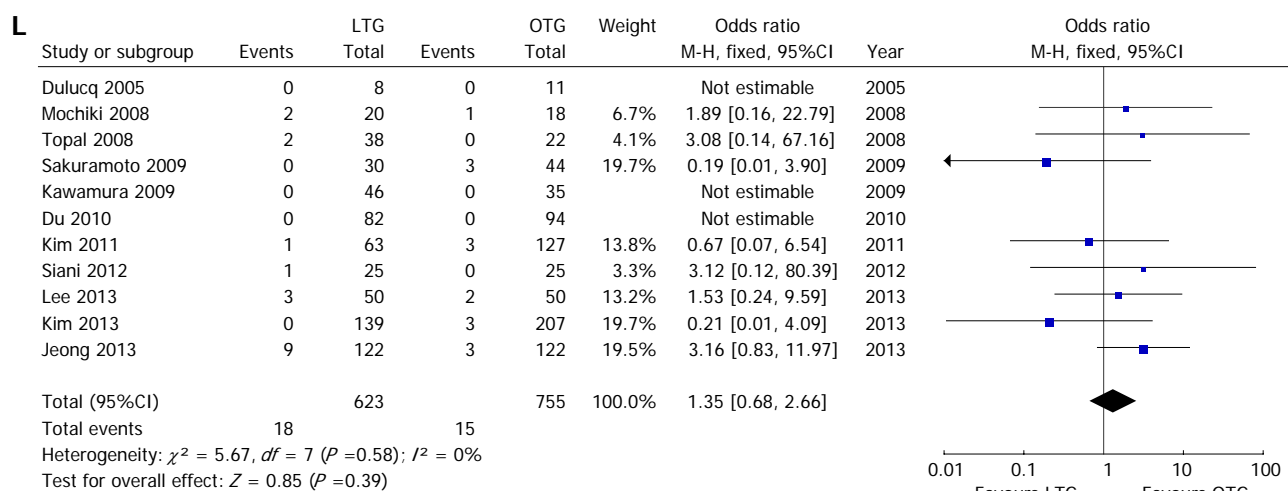
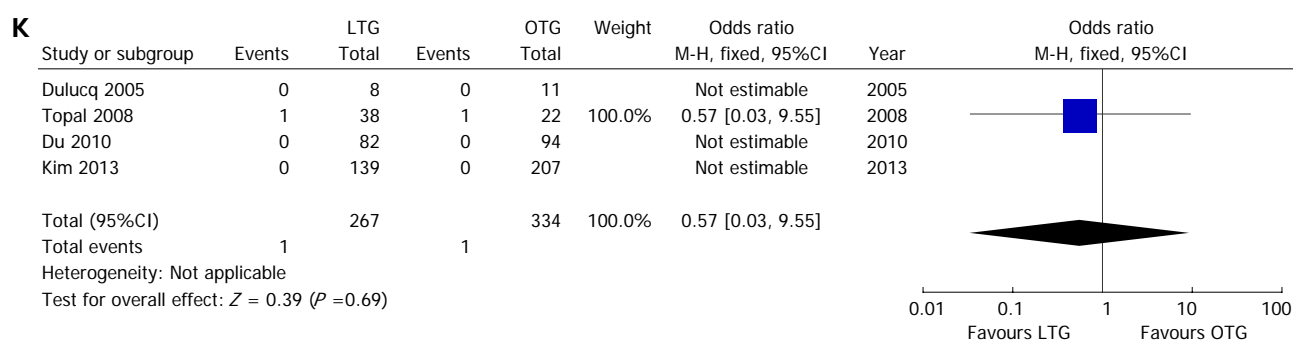
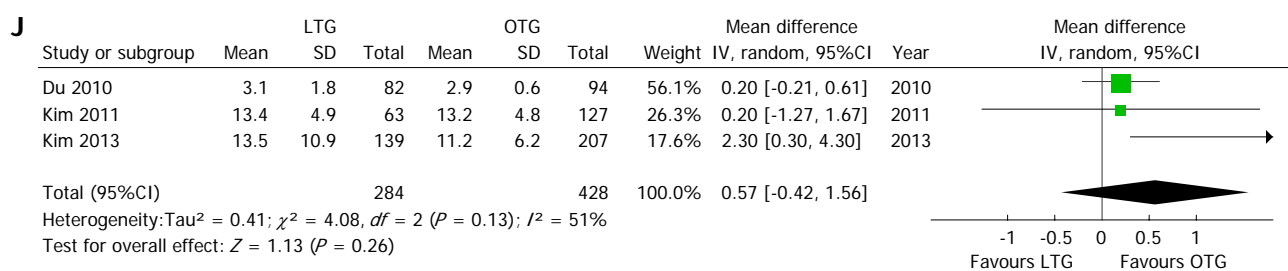


Figure 5 Forest plots illustrating results of oncological outcomes in the form of a meta-analysis comparing laparoscopic total gastrectomy vs open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) or odds ratio (OR) with 95%CI were calculated using the fixed or random-effects model. A: No. of resected lymph nodes; B: Positive resection margins; C: Proximal resection margin; D: Distal resection margin. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

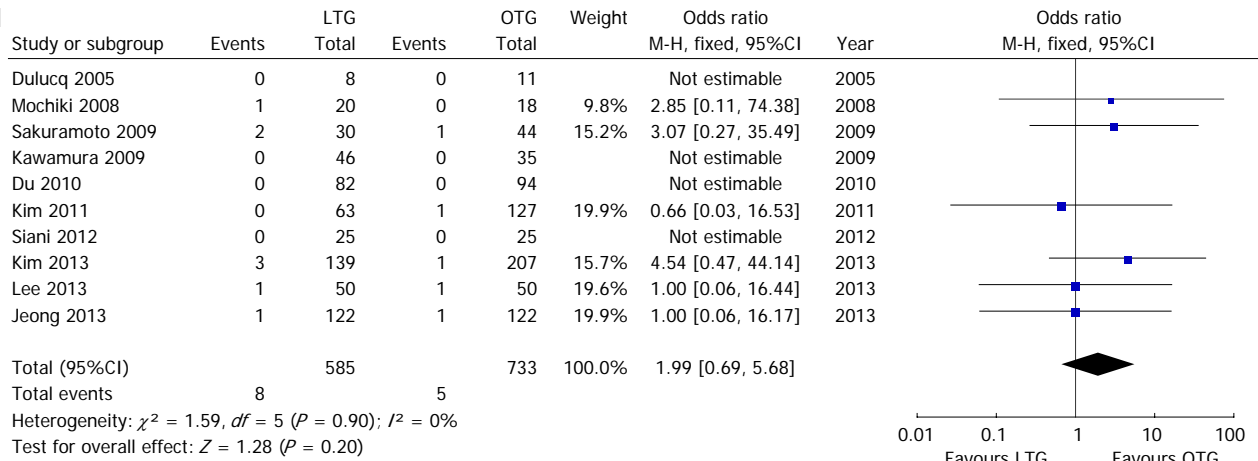
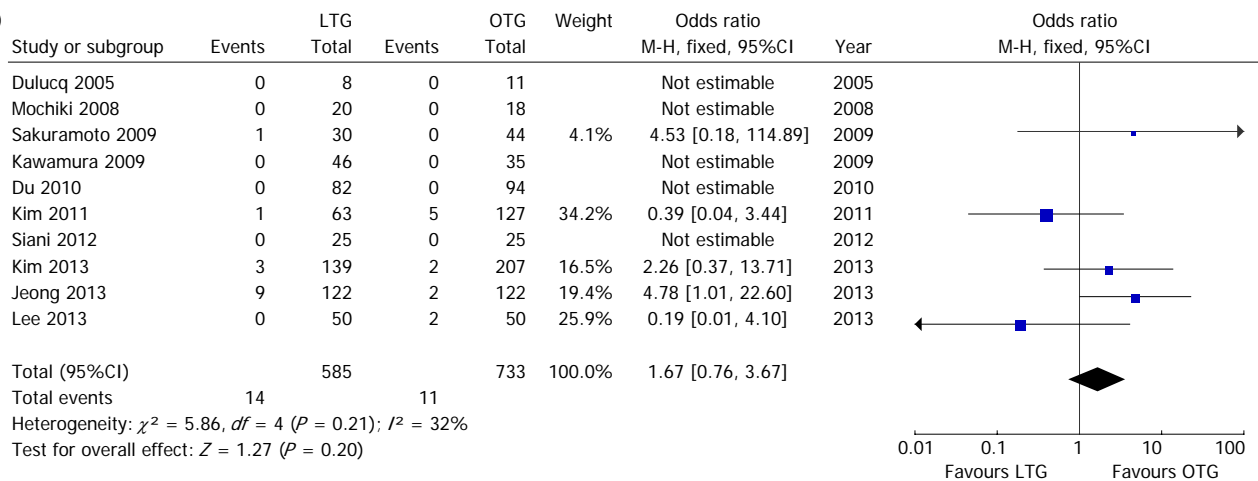
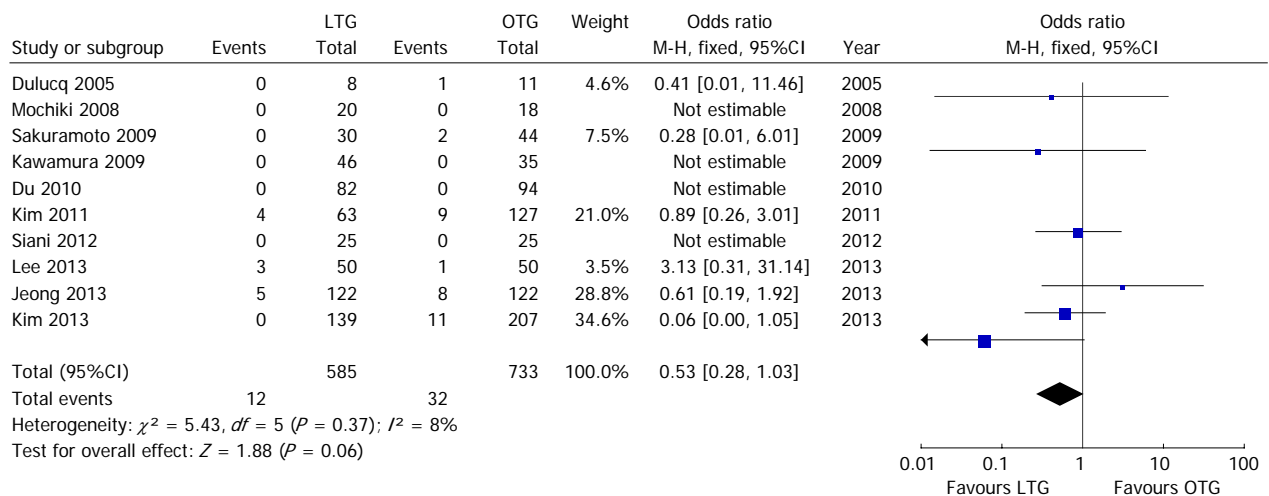


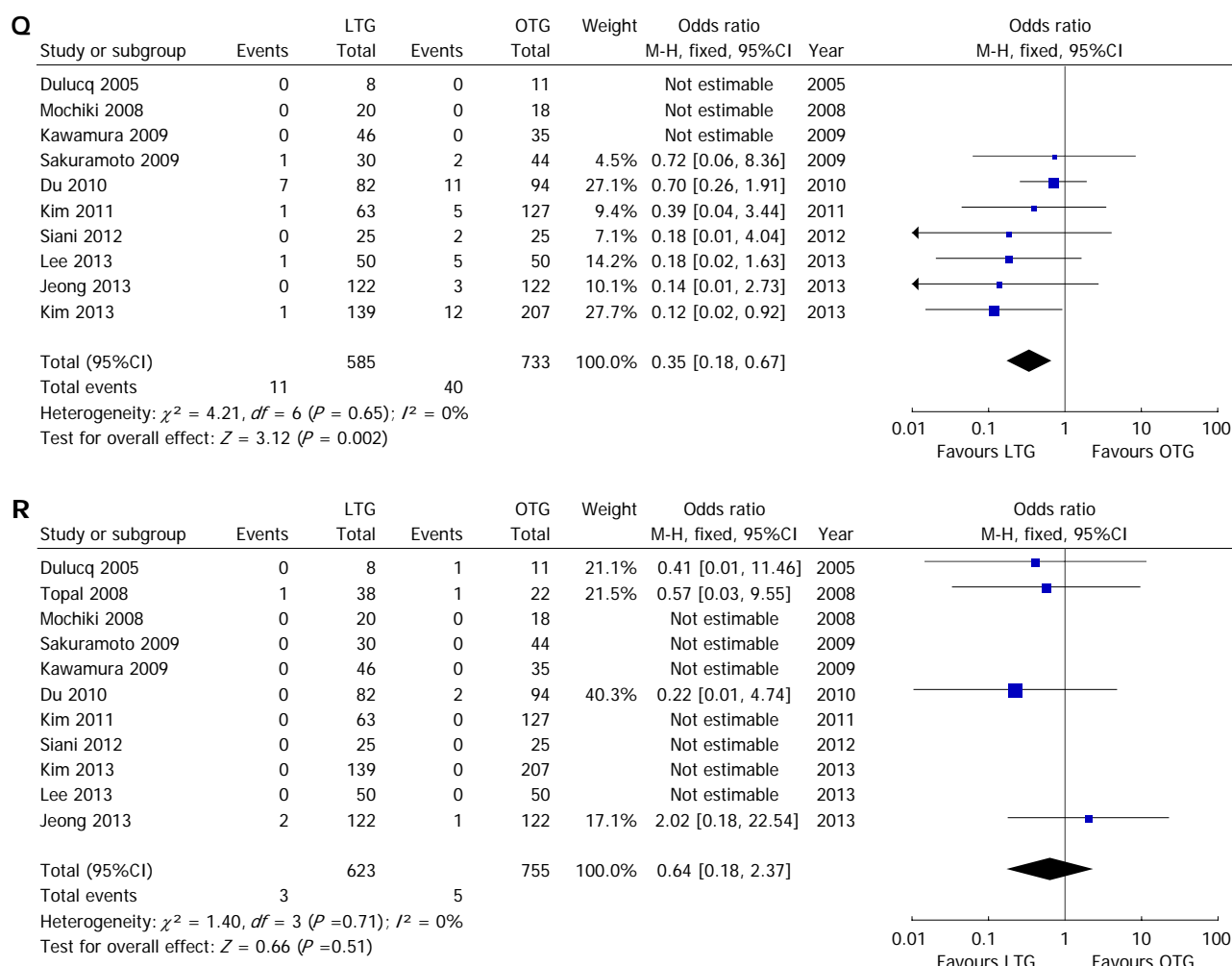


**F****G****H****I**





**N****O****P**



**Figure 6** Forest plots illustrating results of all outcomes in the form of a meta-analysis comparing laparoscopic total gastrectomy vs open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) or odds ratio (OR) with 95%CI were calculated using the fixed or random-effects model. A: Operation time; B: Intraoperative blood loss; C: Time to first flatus; D: Time to first oral intake; E: Hospital stay; F: Analgesics use; G: Postoperative complications; H: No. of resected lymph nodes; I: Proximal resection margin; J: Distal resection margin; K: Positive resection margins; L: Anastomotic leakage; M: Anastomotic Stenosis; N: Ileus; O: Bleeding; P: Abdominal abscess; Q: Wound-related complications; R: Mortality. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

group. However, there were no significant differences in rate of anastomotic leak, anastomotic stenosis, bleeding, abdominal abscess and postoperative mortality in the two groups. These results indicate that LTG is a safe procedure.

While lymph node metastasis is associated with a poor prognosis in gastric cancer, the extent of lymph node dissection required is open to debate. Many surgeons believe that D1+ $\alpha$  or  $\beta$  dissection is adequate for early gastric cancer, and D2 dissection is optimal for advanced gastric cancer, although this remains controversial<sup>[41,42]</sup>. Surgical removal of at least 15 lymph nodes is advocated in gastric cancer<sup>[43]</sup>. The mean number of harvested lymph nodes in all included studies was more than 15. The surgical approach did not appear to influence the lymph node yield; however, LTG with extended lymph node dissection may require further refinement of the operative technique and improved instrumentation, and should be performed with caution by surgeons with adequate experience in laparoscopic gastrectomy<sup>[29]</sup>. Another major concern of laparoscopic resection for gastric cancer is obtaining clear

proximal esophageal and distal duodenal margins<sup>[17]</sup>. Five included studies reported tumor margins, but only one study reported positive resection margins in one patient each in LTG and OTG, respectively; there was no statistically significant difference between the two groups. Our analyses also showed that there was no significant difference in the lengths of the proximal and distal resection margins between the two groups. Seven studies reported data on long-term survival following the two procedures. However, as the duration of follow-up varied between studies, it was difficult to compare them.

Our study has some limitations. Firstly, all the studies included were non-randomized, because of a lack of randomized controlled trials. Secondly, there was significant heterogeneity in the studies with respect to the extent of lymph node dissection, tumor staging and surgical anastomosis techniques. Also, there were differences in the number of patients in the two groups and between studies.

In conclusion, compared with OTG, LTG with regional lymph node dissection for early and advanced gas-

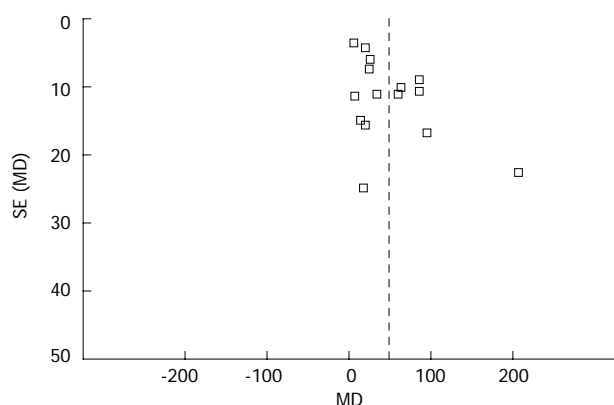


Figure 7 Funnel plot of operation time of all included studies.

tric cancer is safe and effective; with comparable short-term oncological outcomes; lower intraoperative blood loss and overall complication rates; fewer wound-related complications; quicker recovery of gastrointestinal motility and a shorter hospital stay, albeit with a longer operating time. However, there is a need to develop well-designed, adequately powered, prospective, multicenter, randomized controlled trials, investigating LTG with adequate long-term follow-up, before recommending its wider use in surgical practice.

## COMMENTS

### Background

Since laparoscopic total gastrectomy (LTG) was first reported in 1999, it has been used increasingly to treat gastric cancer as result of technical advances and improved instrumentation. However, compared with conventional open total gastrectomy (OTG), the safety and efficacy of LTG is not known.

### Research frontiers

To conduct a meta-analysis comparing the safety and effectiveness of LTG with OTG in patients with gastric cancer; the available perioperative and oncological outcomes were included in this study.

### Innovations and breakthroughs

Based on this meta-analysis, when compared with OTG, LTG for early and advanced gastric cancer is safe and effective; with comparable short-term oncological outcomes; lower intraoperative blood loss and overall complication rates; fewer wound-related complications; quicker recovery of gastrointestinal motility and a shorter hospital stay, albeit with a longer operating time.

### Applications

LTG is safe, effective and offers some advantages over OTG in the treatment of early and advanced gastric cancer. However, well-designed prospective multicenter, randomized controlled trials investigating the advantage of LTG with adequate long-term follow-up need to be performed before recommending its wider use in surgical practice.

### Peer review

In the future, LTG will be rapidly developed in the field of abdominal minimally invasive surgery. This is a well-written study that clarifies some advantages of LTG in the treatment of patients with early and advanced gastric cancer. This study may be interesting for gastrointestinal surgeons worldwide.

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## Effectiveness of interferon-gamma release assays for differentiating intestinal tuberculosis from Crohn's disease: A meta-analysis

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**Author contributions:** Su SB designed the study, searched the databases, extracted the data, analyzed the results, and wrote the manuscript; Chen W helped design the study, searched the databases, and wrote and revised the manuscript; Fan JH formulated the research question, and helped with database searches and analysis; Luo W and Peng P helped design the data abstraction form and served as second reviewers in extracting the data; all authors have read and approved the final manuscript.

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### Abstract

**AIM:** To investigate the clinical usefulness of interferon-gamma release assays (IGRAs) in the differential diagnosis of intestinal tuberculosis (ITB) from Crohn's disease (CD) by meta-analysis.

**METHODS:** A systematic search of English language studies was performed. We searched the following databases: Medline, Embase, Web of Science and the Cochrane Library. The Standards for Reporting Diagnostic Accuracy initiative and Quality Assessment for Studies of Diagnostic Accuracy tool were used to assess the methodological quality of the studies. Sensitivity, specificity, and other measures of the accuracy of IGRAs in the differential diagnosis of ITB from CD were pooled

and analyzed using random-effects models. Receiver operating characteristic curves were applied to summarize overall test performance. Two reviewers independently judged study eligibility while screening the citations.

**RESULTS:** Five studies met the inclusion criteria. The average inter-rater agreement between the two reviewers for items in the quality checklist was 0.95. Analysis of IGRAs for the differential diagnosis of ITB from CD produced summary estimates as follows: sensitivity, 0.74 (95%CI: 0.68-0.80); specificity, 0.87 (95%CI: 0.82-0.90); positive likelihood ratio, 5.98 (95%CI: 3.79-9.43); negative likelihood ratio, 0.28 (95%CI: 0.18-0.43); and diagnostic odds ratio, 26.21 (95%CI: 14.15-48.57). The area under the curve was 0.92. The evaluation of publication bias was not significant ( $P = 0.235$ ).

**CONCLUSION:** Although IGRAs are not sensitive enough, they provide good specificity for the accurate diagnosis of ITB, which may be helpful in the differential diagnosis of ITB from CD.

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**Key words:** Intestinal tuberculosis; Crohn's disease; Interferon-gamma; Meta-analysis

**Core tip:** The misdiagnosis rate between Crohn's disease (CD) and intestinal tuberculosis (ITB) is 50%-70%. Interferon-gamma release assays (IGRAs) have been used mainly to identify latent tuberculosis infection in patients in several areas and countries. However, the clinical usefulness of IGRAs in the differential diagnosis of ITB from CD is unknown. This is the first study to investigate the clinical usefulness of IGRAs in the differential diagnosis of ITB from CD by meta-analysis. IGRAs provided good specificity for ITB,

and should be helpful in the differential diagnosis of ITB from CD.

Chen W, Fan JH, Luo W, Peng P, Su SB. Effectiveness of interferon-gamma release assays for differentiating intestinal tuberculosis from Crohn's disease: A meta-analysis. *World J Gastroenterol* 2013; 19(44): 8133-8140 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8133.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8133>

## INTRODUCTION

Tuberculosis (TB) is a major worldwide cause of morbidity and mortality<sup>[1,2]</sup>. The geography of TB is changing and expanding due to immigration, human immune deficiency virus, immune suppressants, and the development of multidrug-resistant strains of TB<sup>[1-5]</sup>, especially in privileged areas of the world. Intestinal tuberculosis (ITB) is an important extra-pulmonary TB that primarily affects the ileum and colon, causing gastrointestinal symptoms such as diarrhea or abdominal pain. Along with the increased incidence of TB, the incidence of ITB has also increased. Recently, with the emergence of Crohn's disease (CD) in Asian countries<sup>[3,6,7]</sup>, differentiating between ITB and CD is more important than ever. Unfortunately, it is difficult to differentiate ITB from CD due to similar symptoms, and pathologic, radiologic, and endoscopic findings<sup>[4,8]</sup>.

ITB and CD are both chronic granulomatous inflammatory disorders of the intestine<sup>[9,10]</sup>, but have a different pathophysiology, clinical course, and treatment options. ITB could be completely cured if diagnosed early and treated appropriately. CD is not curable and recurs easily. Although several endoscopic and histologic parameters to differentiate these two diseases have been suggested<sup>[11,12]</sup>, a large number of ITB cases are diagnosed by assessing the outcomes of empirical anti-tuberculosis therapy. Moreover, in South Korea, 42%-45% of patients with CD received empirical anti-tuberculosis therapy before they were finally diagnosed with CD<sup>[13,14]</sup>.

A delayed diagnosis of ITB and CD may result in a delay in initiating effective therapy, resulting in a negative economic impact and increased morbidity and mortality. Furthermore, the use of steroids, immune suppressants and biological agents after a presumptive diagnosis of CD, can result in severe and sometimes fatal complications such as systemic dissemination of TB. In recent years, T-cell based interferon-gamma (IFN- $\gamma$ ) release assays (IGRAs) have increasingly been used to replace the traditional tuberculin skin test (TST) as a diagnostic tool for TB. IGRAs have been shown to have superior sensitivity and specificity<sup>[15,16]</sup>. There are two commercially available methods for IGRAs: the QuantiFERON-TB Gold In-Tube (QFT-G-IT) method and the T-SPOT-TB method. QFT-G-IT uses an enzyme-linked immunosorbent assay to measure antigen-specific production

of IFN- $\gamma$  by circulating T-cells in whole blood being challenged with *Mycobacterium tuberculosis* (MTB)-specific antigens. T-SPOT-TB test is a blood IFN- $\gamma$  assay measuring the number of activated T-cells by identifying IFN- $\gamma$  release when stimulated by MTB-specific antigens, including early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). However, whether IGRAs contribute to the differential diagnosis of ITB from CD remains controversial. In the present study, we systematically analyzed and assessed the clinical utility of IGRAs in distinguishing ITB from CD *via* meta-analysis techniques.

## MATERIALS AND METHODS

### Search strategy and study selection

We searched the following databases: Medline (1980-2013), Embase (1980-2013), Web of Science (1990-2013) and the Cochrane Library. An updated search was carried out in March 2013. The following search terms were used: "intestinal tuberculosis", "Crohn's disease", "interferon-gamma/IFN- $\gamma$ ", "sensitivity", "specificity" and "accuracy". We contacted experts in the specialty and searched the reference lists of primary and review articles. Although no language restrictions were imposed initially, our resources only permitted the review of articles published in the English language for the full text review and final analysis. Conference abstracts and letters were excluded due to unavailable data.

A study was included if it provided both sensitivity (true-positive rate) and specificity (false-positive rate) of IGRAs for the differential diagnosis of ITB from CD, or provided IGRAs values in a dot-plot form which allowed the results to be extracted for individual study subjects. Patients of any age diagnosed with ITB underwent smear or culture of MTB and/or histologic observation of ileum and/or colon tissue, as well as clinical diagnosis, such as response to anti-TB therapy. All patients were diagnosed with CD according to the Japanese diagnostic criteria<sup>[17]</sup> or the World Health Organization diagnostic criteria<sup>[18]</sup> based on clinical, endoscopic, radiological and pathological features. In addition, we selected studies which included at least 10 ITB/CD specimens eligible for inclusion in order to reduce selection bias due to a small number of participants. Two reviewers (Chen W and Fan JH) independently judged study eligibility while screening the citations. Disagreements were resolved by consensus.

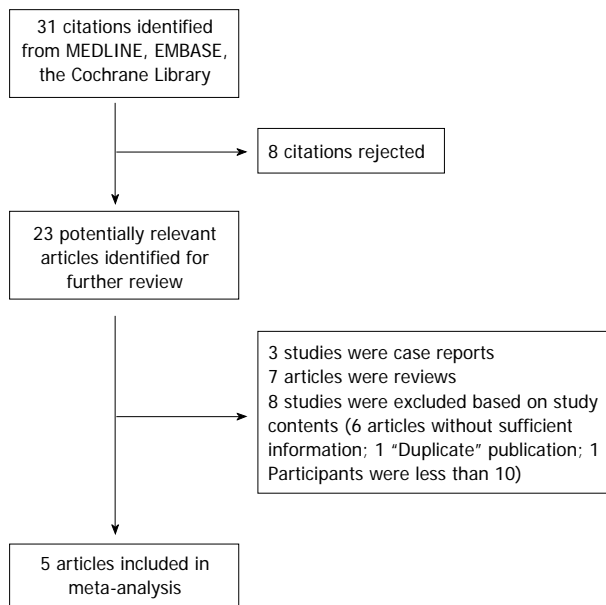
### Data extraction and quality assessment

Two reviewers (Chen W and Fan JH) checked and extracted data independently. The reviewers were blinded to publication details, and disagreements were resolved by consensus. Data retrieved from the reports included participant characteristics, assay methods, sensitivity and specificity data, cutoff values, year of publication, and methodological quality. The value of IGRAs provided in dot plots were measured by placing scalar grids over

**Table 1** Summary of the included studies

Study	Country/Area	Patients (n)	Assay method	Cutoff	Test results				Quality score	
					TP	FP	FN	TN	STARD	QUADAS
Lee <i>et al</i> <sup>[28]</sup>	South Korea	60	T-SPOT-TB	-	12	8	0	40	16	11
Lei <i>et al</i> <sup>[29]</sup>	China	191	T-SPOT-TB	-	36	5	6	62	18	13
Kim <i>et al</i> <sup>[30]</sup>	South Korea	128	QFT-G-IT	0.35 IU/mL	43	6	21	58	17	12
Li <i>et al</i> <sup>[31]</sup>	China	84	T-SPOT-TB	-	16	16	3	49	17	12
Kim <i>et al</i> <sup>[32]</sup>	South Korea	147	QFT-G-IT	0.35 IU/mL	50	7	25	65	18	13

T-SPOT-TB: An enzyme-linked immunosorbent spot assay; QFT-G-IT: Quanti-FERON-TB Gold In-Tube; TP: True-positive; FP: False-positive; FN: False-negative; TN: True-negative; STARD: Standards for reporting diagnostic accuracy; QUADAS: Quality assessment for studies of diagnostic accuracy.

**Figure 1** Flowchart of study selection.

the plots, and analyzed using a receiver operating characteristic (ROC) curve for each study (SPSS; Chicago, IL, United States). A summary of each study, including the numbers of true-positive, false-positive, false-negative and true-negative results, is shown in Table 1.

We assessed the methodological quality of studies using guidelines established by the standards for reporting diagnostic accuracy (STARD)<sup>[19]</sup> initiative and the quality assessment for studies of diagnostic accuracy (QUADAS) tool<sup>[20]</sup>. In addition, the following study design characteristics were retrieved: (1) cross-sectional design (*vs* case-control design); (2) consecutive or random sampling of patients; (3) blind (single or double) interpretation of determination and reference standard results; and (4) prospective data collection. If primary studies did not show data that met the above criteria, we requested the data from the authors. The “unknown” items were treated as “no” if we did not receive a response from the authors.

### Statistical analysis

We used standard methods recommended for meta-analyses of diagnostic test evaluations<sup>[21]</sup>. Analyses were performed using two professional statistical software

programs (STATA, version 11; Stata Corporation, College Station, TX, United States and Meta-DiSc for Windows; XI Cochrane Colloquium; Barcelona, Spain). The following measures of test accuracy were analyzed for each study: sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value (PPV), negative predictive value (NPV) and diagnostic odds ratio (DOR).

The analysis was based on a summary ROC (SROC) curve<sup>[21]</sup>. Sensitivity and specificity as a single test threshold identified for each study were used to plot an SROC curve<sup>[22]</sup>. A random-effects model was adopted to calculate the average sensitivity, specificity, and other measures across studies<sup>[23,24]</sup>.

The term heterogeneity refers to the degree of variability in results across studies, which was used in relation to meta-analyses. We detected statistically significant heterogeneity with the  $\chi^2$  test. To assess the effects of STARD and QUADAS scores on the diagnostic ability of IGRAs, we included them as covariates in the univariate meta-regression analysis (inverse variance weighted). We also analyzed the effects of other covariates on DOR, such as cross-sectional design, consecutive or random sampling of patients, single or double interpretation of determination, reference standard results, and prospective data collection. The relative DOR (RDOR) was calculated according to standard methods to analyze the change in diagnostic precision in the study per unit increase in the covariate<sup>[25,26]</sup>. Since publication bias is of concern for meta-analyses of diagnostic studies, we tested for the potential presence of this bias with funnel plots and the Egger test<sup>[27]</sup>.

## RESULTS

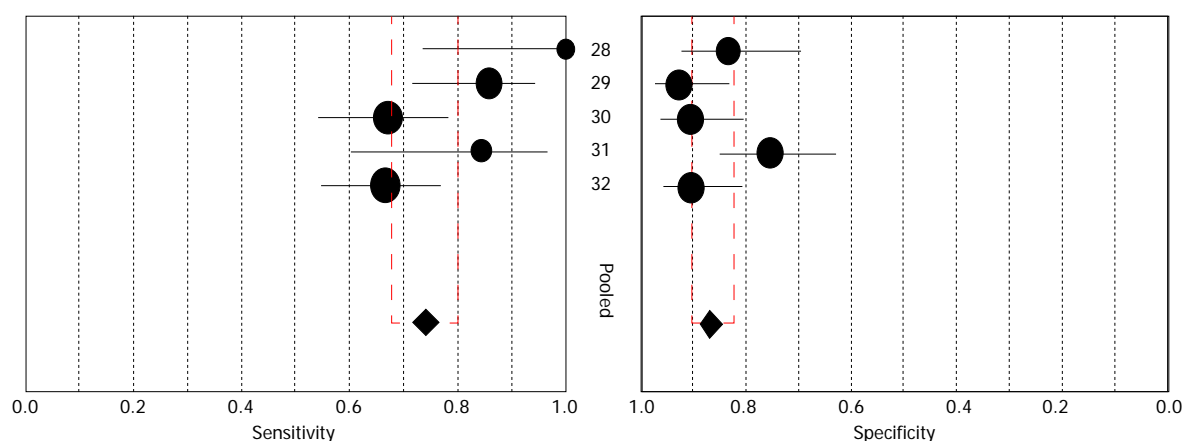
### Selection and summary of studies

Five out of 31 publications reporting IFN- $\gamma$  for the differential diagnosis of ITB from CD were considered to be eligible for inclusion in the analysis<sup>[28-32]</sup>. Of these 31 publications, 8 citations were rejected, 3 studies were case reports, 7 papers were reviews, and 8 studies were excluded based on study contents (Figure 1). A total of 5 studies including 616 patients were available for analysis, and the clinical characteristics of these studies, along with STARD and QUADAS scores, are outlined in Table 1.

**Table 2** Characteristics of the included studies

Ref.	ITB/CD patients (n)	Reference standard	Cross-sectional design	Consecutive or random	Blinded design	Prospective
Lee <i>et al</i> <sup>[28]</sup>	12/44	Bac/His or Clin	Unknown	Yes	Unknown	Yes
Lei <i>et al</i> <sup>[29]</sup>	88/103	Bac/His	Unknown	Yes	No	Yes
Kim <i>et al</i> <sup>[30]</sup>	64/64	Bac/His	No	Yes	No	Yes
Li <i>et al</i> <sup>[31]</sup>	19/65	Bac/His or Clin	Yes	Yes	No	Yes
Kim <i>et al</i> <sup>[32]</sup>	75/72	Bac/His or Clin	No	Yes	No	Yes

ITB: Intestinal tuberculosis; CD: Crohn's disease; Bac: Bacteriology; His: Histology; Clin: Clinical course.



**Figure 2** Forest plot of estimates of sensitivity and specificity for interferon-gamma release assays in the differential diagnosis of intestinal tuberculosis from Crohn's disease. Forest plot shows sensitivity and specificity of interferon-gamma release assays for intestinal tuberculosis diagnosis. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars indicated 95%CI. Numbers indicate the studies included in the meta-analysis, as cited in the reference list. Pooled estimates for interferon-gamma release assays were as follows: sensitivity, 0.74 (95%CI: 0.68-0.80) and specificity, 0.87 (95%CI: 0.82-0.90).

### Quality of reporting and study characteristics

The average inter-rater agreement between the two reviewers for items in the quality checklist was 0.95. All studies were collected from consecutive patients. The average sample size was 112 (range, 60-191) in the included studies. All studies reported that the study design was prospective (Table 2). None of the studies reported blinded interpretation of the IGRAs independent of the reference standard.

### Diagnostic accuracy

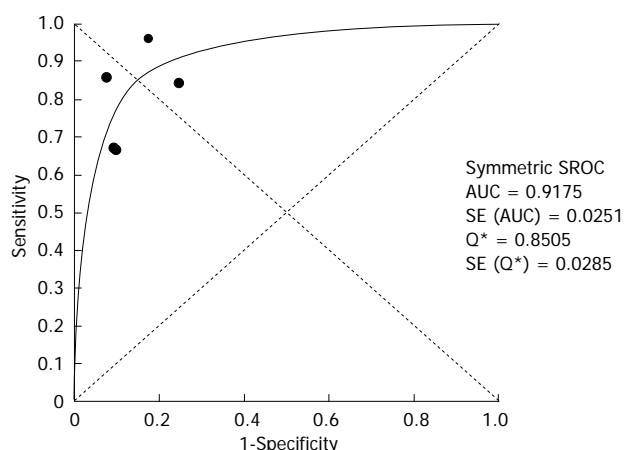
The sensitivity and specificity of IGRAs in the 5 studies for the differential diagnosis of ITB from CD are shown in the forest plot (Figure 2). Sensitivity of IGRAs for ITB diagnosis ranged from 0.54 to 1.00 (mean, 0.74; 95%CI: 0.68-0.80), while specificity ranged from 0.63 to 0.98 (mean, 0.87; 95%CI: 0.82-0.90). We also noted that PLR was 5.98 (95%CI: 3.79-9.43), NLR was 0.28 (95%CI: 0.18-0.43) and DOR was 26.21 (95%CI: 14.15-48.57). The Chi-square values of sensitivity, specificity, PLR, NLR and DOR were 15.22 ( $P = 0.0043$ ), 10.55 ( $P = 0.0322$ ), 9.28 ( $P = 0.0544$ ), 9.74 ( $P = 0.0504$ ) and 4.99 ( $P = 0.2882$ ), respectively, indicating heterogeneity for sensitivity and specificity between studies.

Two methods of IGRAs were used in the included studies in this meta-analysis. One was the T-SPOT-TB test, in which mononuclear cells from blood are used and the number of IFN- $\gamma$  producing cells responding

to antigens such as the ESAT-6 and CFP-10 is reported. The other method of IGRAs was QuantiFERON-TB Gold In-Tube (QFT-G-IT), which measures T-cell INF- $\gamma$  production (expressed as pg/mL or IU/mL) in blood in response to a cocktail of ESAT-6, CFP-10 and TB 7.7. The  $P$  value following a comparison of overall diagnostic values from T-SPOT-TB and QFT-G-IT was 0.3073. It could not be concluded that the overall accuracy of T-SPOT-TB for the differential diagnosis of ITB from CD was superior or inferior to that of QFT-G-IT.

The SROC plot is different from the traditional ROC plot that explores the effect of varying thresholds on sensitivity and specificity in a single study. In a SROC plot, any of the data points represent a separate study. The SROC curve presents a global summary of test performance and shows the tradeoff between sensitivity and specificity. A graph of the SROC curve for IGRA determination showing true-positive rates and false-positive rates from individual studies is shown in Figure 3. As a global measure of test efficacy we used the  $Q$ -value, the intersection point of the SROC curve with a diagonal line from the left upper corner to the right lower corner of the ROC space, which corresponds to the highest common value of sensitivity and specificity for the test. This point represents an overall measure of the discriminatory power of a test. Our data showed that the SROC curve was positioned near the upper left corner and that the maximum joint sensitivity and specificity was 0.87.





**Figure 3** Summary receiver operating characteristic curves for interferon-gamma release assays. Solid circles represent each study included in the meta-analysis. The size of each study is indicated by the size of the solid circle. Summary receiver operating characteristic (SROC) curves summarize the overall diagnostic accuracy.

The area under the curve (AUC) was 0.92. These data indicated that the overall accuracy of IGRAs was not as high as expected.

### Multiple regression analysis

By using the STARD guidelines<sup>[19]</sup>, a quality score for each study was compiled on the basis of title and introduction, methods, results and discussion (Table 1). Quality scoring was also carried out using QUADAS<sup>[20]</sup>, in which a score of 1 indicated a fulfilled criterion, 0 if an unclear criterion, and -1 if the criterion was not achieved. These scores were used in the meta-regression analysis to assess the effect of study quality on the RDOR of IGRAs in the differential diagnosis of ITB from CD. All studies were of high quality (STARD score,  $\geq 13$ ; QUADAS score,  $\geq 10$ ) in this review. The differences in the studies with or without blinding, cross-sectional, consecutive/random and prospective designs did not reach statistical significance ( $P = 0.218$ ), indicating that the study design did not substantially affect the diagnostic accuracy.

### Publication bias

Although the Egger test is widely used to evaluate publication bias, it is not useful if less than 10 studies are included. Based on this meta-analysis, which included five articles, we would consider that there was potential for publication bias.

## DISCUSSION

The misdiagnosis rate between CD and ITB is 50%-70%<sup>[4,5,33,34]</sup>. It is important to differentiate between ITB and CD in order to provide effective and prompt therapies due to the increasing incidence of CD and widespread drug-resistant TB<sup>[8]</sup>. In recent years, methods including TST, MTB culture and acid fast bacilli staining have been used for the detection of TB infection. However, the low sensitivity and specificity and complicated processing of

samples has limited the use of these methods<sup>[35,36]</sup>. New techniques, such as CT enteroclysis, capsule endoscopy, single and double balloon enteroscopy, polymerase chain reaction (PCR) and immunological assays for MTB, have also been used in clinical practice. PCR was associated with high sensitivity, but low specificity<sup>[37,38]</sup>. Endoscopic and histopathological examinations are also conducted to differentiate between the two disorders<sup>[39]</sup>, but specific and precise criteria are lacking. The T-SPOT-TB test, an IGRA, has mainly been used to identify latent tuberculosis infection in patients in several areas and countries including the United States, Europe and Japan. However, the clinical usefulness of IGRAs for the differential diagnosis of ITB from CD is unknown.

In recent studies, the most popular biomarkers proposed for the diagnosis of TB-related disease were adenosine deaminase and  $\text{INF-}\gamma$ <sup>[40,41]</sup>. The levels of both biomarkers were significantly higher in tuberculous peritonitis than in non-tuberculous peritonitis patients. Both showed relatively high sensitivity and specificity in diagnosing tuberculous peritonitis<sup>[42-47]</sup>. However, for distinguishing ITB from CD, the present meta-analysis has shown that the mean sensitivity of IGRAs was 0.74, while the mean specificity was 0.87. The maximum joint sensitivity and specificity was 0.85, while the AUC was 0.92, indicating that overall accuracy was relatively high, but not as high as expected.

The DOR is a single indicator of test accuracy that combines the sensitivity and specificity data into a single number<sup>[48]</sup>. The DOR of a test is the ratio of the odds of positive test results in the patient with disease relative to the odds of positive test results in the patient without disease. The value of DOR ranges from 0 to infinity, and higher values indicate better discriminatory test performance (higher accuracy). A DOR of 1.0 indicates that a test did not discriminate between patients with and those without disease. In the present meta-analysis, the mean DOR was 26.21, indicating that IGRAs may be helpful in the differential diagnosis of ITB from CD.

Since the SROC curve and the DOR are not easy to interpret and use in clinical practice<sup>[49]</sup>, the likelihood ratios are considered to be more clinically meaningful<sup>[49]</sup>. We also determined both PLR and NLR as measures of diagnostic accuracy. Likelihood ratios of  $> 10$  or  $< 0.1$  generate large and often conclusive shifts from pretest to posttest probability (indicating high accuracy). A PLR value of 5.98 suggests that patients with ITB have an approximately six-fold higher chance of being  $\text{INF-}\gamma$  assay-positive compared with CD patients. This six-fold high probability would be considered not high enough to begin or to continue anti-TB treatment in ITB patients, especially in the absence of any malignant evidence (for clinical purposes). On the other hand, NLR was found to be 0.28 in the present meta-analysis. If the  $\text{INF-}\gamma$  assay result was negative, the probability that this patient has ITB is approximately 28%, which is not low enough to rule out ITB from CD. These data suggest that a negative  $\text{INF-}\gamma$  assay result should not be used alone as a justification to deny or to discontinue anti-TB therapy. The

choice of therapeutic strategy should be based on the results of culture of MTB, morphological observation of capsule endoscopy or single/double balloon enteroscopy, and/or histologic observation of peritoneal tissue, as well as other clinical data, such as response to anti-TB therapy.

The PPV is the proportion of patients with positive test results who are correctly diagnosed, while the NPV is the proportion of patients with negative test results who are correctly diagnosed. The pooled results showed that the PPV for IGRAs was 0.74, suggesting that 26% of positive results would actually be false positives. On the other hand, the NPV for IGRAs was 0.87, indicating a false negative rate of 13%. The relatively high NPV suggests that IGRAs would be acceptable for clinical purposes.

An exploration of the reasons for heterogeneity rather than computation of a single summary measure is an important goal of meta-analysis<sup>[50]</sup>. In our meta-analysis, both STARD and QUADAS scores were used in the meta-regression analysis to assess the effect of study quality on RDOR. All the studies were of high quality (STARD score of  $\geq 13$  or QUADAS score of  $\geq 10$ ). We found that there was no statistical heterogeneity for sensitivity, specificity, PLR, NLR, and DOR among the studies, which indicated that the differences in the studies with or without blinding, cross-sectional, consecutive/random and prospective designs did not reach statistical significance, and the study design did not substantially affect diagnostic accuracy.

Our meta-analysis has several limitations. Firstly, the exclusion of conference abstracts, letters to the editors, and non-English-language studies might have led to publication bias. Secondly, misclassification bias may have occurred. ITB is not always diagnosed by either histologic or microbiological examination. Some patients were diagnosed with ITB based on the clinical course. This issue regarding accuracy of diagnosis could cause nonrandom misclassification, leading to biased results. Thirdly, all the articles were from Asia, and this may also have led to publication bias. Finally, the number of studies that met the inclusion criteria was not large enough. Multi-center and large blinded randomized controlled trials using IGRAs for ITB diagnosis should be performed.

In conclusion, evidence from the present meta-analysis showed that although IGRAs are not sensitive enough, they did show good specificity for the diagnosis of ITB, which may be helpful in the differential diagnosis of ITB from CD. IFN- $\gamma$  may be a clinical diagnostic marker for the differential diagnosis of ITB from CD. Currently, the literature focusing on the use of IGRAs in ITB is limited; thus, further large multicenter studies are necessary to substantiate the diagnostic accuracy of IGRAs in patients with ITB or CD.

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## COMMENTS

### Background

The differential diagnosis of intestinal tuberculosis (ITB) from Crohn's disease (CD) is challenging. The misdiagnosis rate between CD and ITB is 50%-70%. T-cell based interferon-gamma release assays (IGRAs) have increasingly been used as a diagnostic tool in the differential diagnosis of ITB from CD. However, whether IGRAs contribute to accurate ITB diagnosis remains controversial.

### Research frontiers

IGRAs have mainly been used to identify latent tuberculosis infection in patients in several areas and countries including the United States, Europe and Japan. However, the clinical usefulness of IGRAs for the differential diagnosis of ITB from CD is unknown.

### Innovations and breakthroughs

This is the first time that the clinical usefulness of IGRAs for the differential diagnosis of ITB from CD has been investigated by meta-analysis.

### Applications

IGRAs provided good specificity for ITB, and should be helpful in the differential diagnosis of ITB from CD. Interferon-gamma may be a clinical diagnostic marker for the differential diagnosis of ITB from CD.

### Terminology

IGRAs: T-cell based interferon-gamma release assays have increasingly been used to replace the traditional tuberculin skin test as a diagnostic tool for tuberculosis. IGRAs have been shown to have superior sensitivity and specificity. ITB: Intestinal tuberculosis is an important extra-pulmonary tuberculosis that primarily affects the ileum and colon, causing gastrointestinal symptoms such as diarrhea or abdominal pain. Standards for reporting diagnostic accuracy and quality assessment for studies of diagnostic accuracy scores: these scores are used in the meta-regression analysis to assess the effect of study quality on relative diagnostic odds ratio.

### Peer review

This study is an interesting meta-analysis comment. It provides a new evidence of IGRAs helping differential diagnosis ITB from CD.

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## Seven synchronous early gastric cancer with 28 lymph nodes metastasis

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the risk of lymph node metastasis, but if their differentiations are poor or if they have lympho-vascular invasion, multiple lymph node metastases could incur even if the depth of invasion is limited to the mucosal layer or the upper portion of the submucosal layer.

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**Key words:** Early gastric cancer; Synchronous; Metastasis; Lymph node; Endoscopy

**Core tip:** Early gastric cancer is often found synchronously in 2 to 3 lesions. However, this case reports on an unprecedented case of 7 lesions of early gastric cancer. Furthermore, this case deserves more attention because 28 out of 48 lymph nodes showed post-operative metastasis, even though there was only 1 invasion to the 1/3 of the submucosal layer and the remaining 6 invading only up to the mucosal layer. This report speaks to the necessity of extra caution in diagnosing multiple synchronous lesions of early gastric cancer with esophagogastro-duodenoscopy.

### Abstract

An 85 year male patient complaining epigastric discomfort was admitted. From the esophagogastroduodenoscopy, three early gastric cancer (EGCa) lesions had been identified and these were diagnosed as adenocarcinoma with poorly differentiated cell type. The patient underwent operation. From the post-operative mapping, however, additional 4 EGCa lesions were found, and the patient was diagnosed with 7 synchronous EGCa. Out of the 7 EGCa lesions, 6 had shown invasion only to the mucosal layer and one had shown invasion into the 1/3 layer of submucosa. In spite of such superficial invasions, 28 of 48 lymph nodes had been identified as metastases. The multiple lesions of EGCa do not increase

Seong H, Kim JI, Lee HJ, Kim HJ, Cho HJ, Kim HK, Cheung DY, Kim DJ, Kim W, Kim TJ. Seven synchronous early gastric cancer with 28 lymph nodes metastasis. *World J Gastroenterol* 2013; 19(44): 8141-8145 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8141.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8141>

### INTRODUCTION

Owing to the recent development of diagnostic technology through esophagogastroduodenoscopy (EGD), the prevalence of early gastric cancer (EGCa) is increasing. Also, reports of multiple synchronous EGCa lesions

are increasing as well. The prevalence of multiple EGCa does not differ between advanced gastric cancer patients as 6%-14%<sup>[1]</sup> and EGCa patients as 8.3%-17%<sup>[2]</sup>. Multiple EGCa show a high level of prevalence in elder patients or in male patients, and also when the cancer is well differentiated or invasion is limited to the mucosal layer<sup>[3]</sup>. And most accessory lesions have been known to occur adjacent to the main lesion with same or even better differentiation<sup>[4]</sup>. It was found that there was no difference in lymph node metastasis when comparing multiple EGCa with single EGCa in general, but if the invasion depth was deep, the possibility of lymph node metastasis was even higher<sup>[3]</sup>. If the indications of endoscopic treatment are expanded, since even the surgical treatment tends to orient towards less invasive methods to preserve the normal part of stomach as much as possible, accurate pretreatment diagnosis is important for the multiple lesions of EGCa. In our case, 7 EGCa had been found in an 85 year old male patient and there were multiple lymph node metastases identified post-operatively even though the cancer had shown invasion into the upper portion of the submucosal layer.

## CASE REPORT

An 85-year-old male patient complaining of epigastric pain for 3 mo was admitted to our hospital. He had no special medical, family and social history, not to mention cancer, and was found nonspecific in his physical examination and initial laboratory finding. From the results of his physical examination, we found that his blood pressure was 135/87 mmHg, pulse rate was 70 times/min, respiratory rate was 20 times/min and body temperature was 36.5 °C. The conjunctivae were not pale and no jaundice was observed from the sclerae. There were no palpable lymph nodes from the neck examination, and the auscultation had a normal respiratory sound from the thorax. There was no palpable mass, no tenderness or no rebound tenderness found from the abdominal examination.

From the complete blood count of the laboratory findings, hemoglobin was 12.4 g/dL, white blood cell count was 5670/mm<sup>3</sup> and the platelet count was 212000/mm<sup>3</sup>, whereas biochemistry examination revealed, fasting blood glucose as 93 mg/dL, urea nitrogen as 14.5 mg/dL, creatinine as 1.19 mg/dL, aspartate aminotransferase as 30 IU/L and alanine aminotransferase as 22 IU/L, total bilirubin was 0.66 mg/dL, direct bilirubin was 0.22 mg/dL, total protein was 6.7 g/dL and albumin 3.56 g/dL, presenting that all the results were in the normal range. Also, tumor markers such as carcinoembryonic antigen and cancer antigen 19-9 were within normal limit as 2.59 ng/mL and 11.13 U/mL.

From the EGD, the whole stomach had atrophic mucosal change from antrum to cardia, open type III atrophic gastritis and had no *Helicobacter pylori* infection in Warthin-Starry silver stain. There were findings of a well demarcated erythematous depressed erosion in the sized

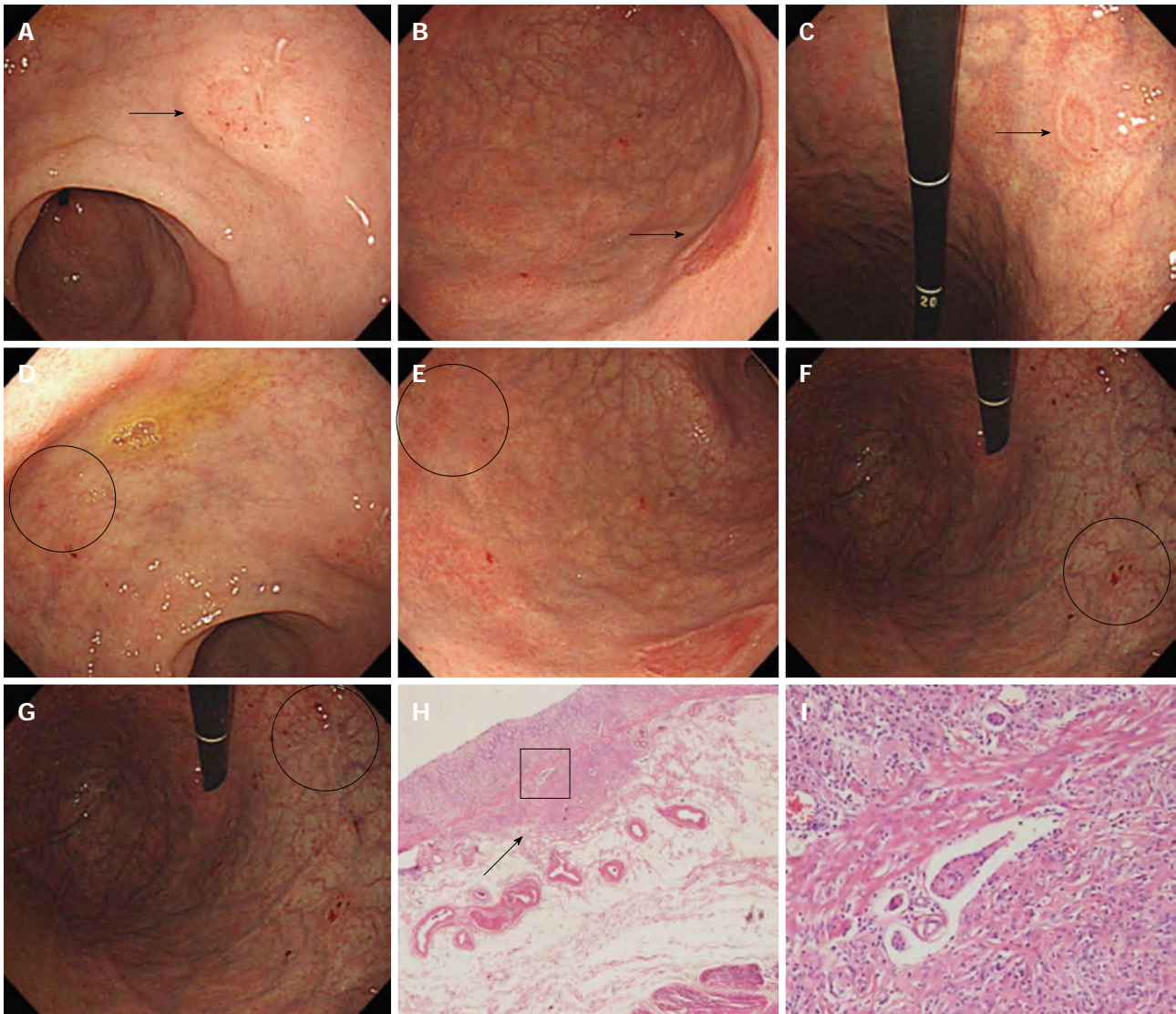
of 8 mm on the posterior wall of proximal antrum (Figure 1A) and a depressed mucosal lesion with a red colored center, pale boundary and clear margin in the size of 15 mm on the posterior wall of lower body (Figure 1B), as well as a depressed erosion in the sized of 7 mm on the posterior wall of lower body (Figure 1C). The lesions were diagnosed as EGCa IIc and the biopsy revealed an adenocarcinoma, poorly differentiated cell type. Although the lesions occurred in multiple regions showing reddish depression with surrounding white rim, the main lesion was very small and in its early stage, suggesting that they are multiple EGCa rather than metastasis. From the abdominal computerized tomography, the locations of lesions could not be identified. Furthermore there was neither any finding of metastasis to neighboring organs such as the liver or pancreas nor any finding of lymph node enlargement in the neighboring areas.

As the patient had shown three lesions of EGCa, poorly differentiated cell type cell type, we did not perform endoscopic submucosal dissection but instead performed subtotal gastrectomy. After the surgery, we also performed a mapping of the subtotal gastrectomy specimen and were able to diagnose additional lesions (Figure 2). The lesions were flat erosions 6 mm in diameter on the anterior wall of proximal antrum (Figure 1D), depressed mucosal lesion 10 mm in diameter on the anterior wall of lower body (Figure 1E), depressed mucosal lesion 7 mm in diameter on the anterior wall of mid body (Figure 1F) and squamous mucosal lesion in the sized 2 mm on the lesser curvature of mid body (Figure 1G). We had reviewed pictures taken during EGD, but could not find any definite lesion and there were no ulcer findings of each lesions (Figure 1).

In histopathological findings, each lesion was identified as adenocarcinoma, poorly differentiated cell type which was the diffuse type and the growth pattern was infiltrative type in accordance with Lauren's classification. All lesions were composed of poorly differentiated adenocarcinoma. There were no lesions with well or moderate differentiated cancer component and the back ground was atrophic gastritis, marked grade (Figure 3). On the invasion depth, the lesion (Figure 3A, C-G) had invaded into the mucosal layer, while the lesion (Figure 3B), which was the largest, had shown invasion into 1/3 of the submucosal layer, SM1, 1000 µm (Figure 1H and I) (Table 1). There was no perineural invasion but vascular and lymphatic invasion were found from the subtotal gastrectomy specimen. The grade of lymphatic invasion was marked (Figure 1I) and out of the 48 resected lymph nodes, 28 lymph nodes had shown metastasis (Figure 3H). Lymph node metastasis was as follow: 1 (4/4), 3 (4/6), 4 (3/10), 5 (5/6), 6 (12/15), 8a (0/7). According to American Joint Committee on Cancer TNM staging classification for gastric cancer, the final pathologic stage was T1bN3bM0.

## DISCUSSION

The diagnosis of EGCa is increasing as the performance



**Figure 1 Endoscopic and histologic findings.** Multiple early gastric cancer lesions were as follow: A: Raised lesion on the posterior wall of the proximal antrum; B: Erythematous depressive lesion; C: Depressed lesion on the posterior wall of the low body; D: III demarcated flat lesion on the anterior wall of the proximal antrum; E, F: III demarcated depressed lesion on the anterior wall of the low body (E), on the anterior wall of the mid body (F); G: III demarcated flat lesion on the lesser curvature of mid body; H: Adenocarcinoma in lesion B showed invasion into 1/3 of the submucosal layer (arrow) ( $\times 40$ ); I: Lymphatic invasion magnified in quadrangle in H ( $\times 100$ ).

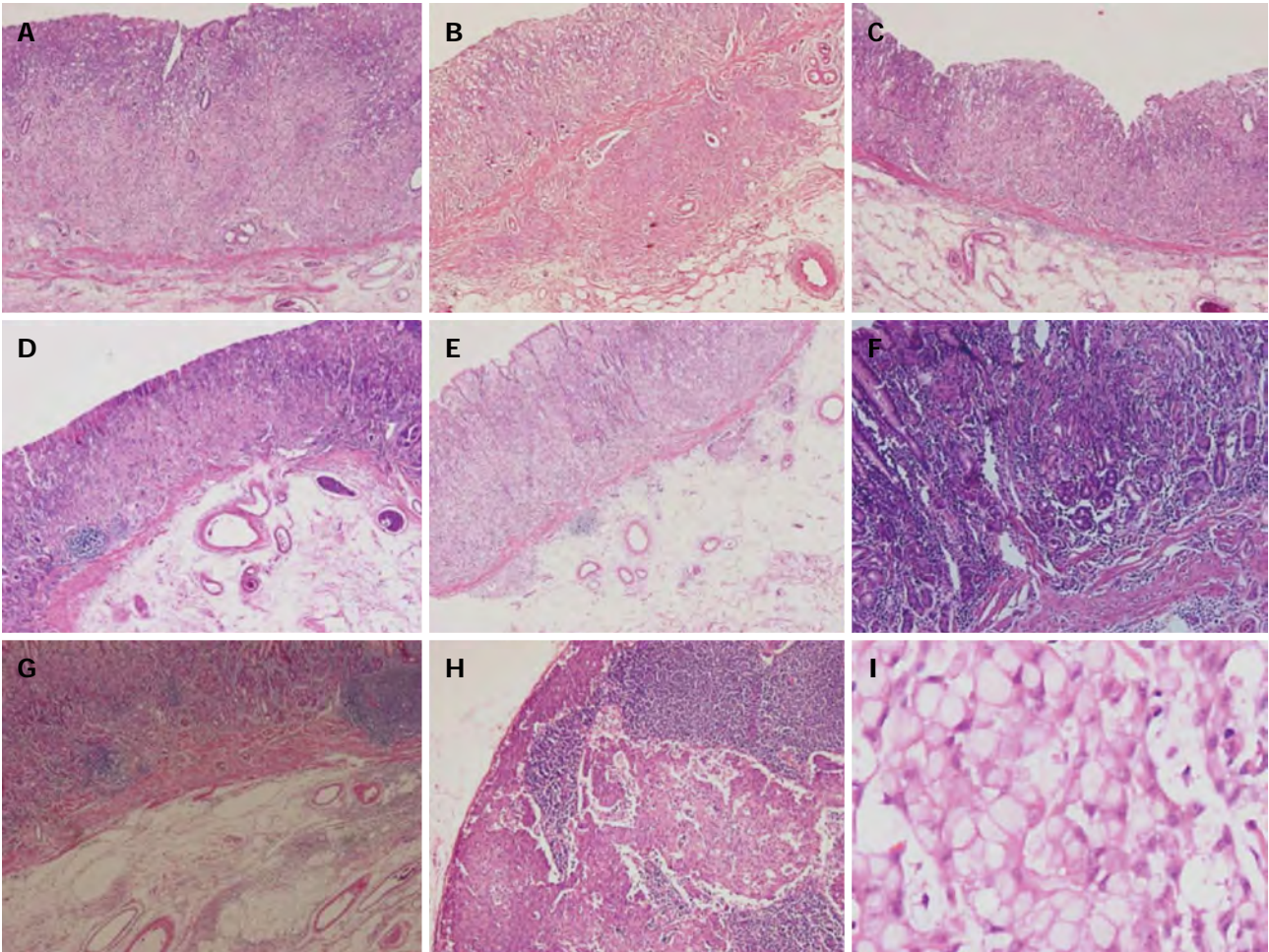


**Figure 2 Gross specimen.** Three lesions (D-F) are located on the anterior wall, and the other three lesions (A-C) are on the posterior wall. One lesion (G) shows flat early gastric cancer type IIb configuration and is centered at the body, lesser curvature.

of upper endoscopy-related equipments advances and as pathological diagnosis techniques became more developed in recent days. In South Korea, health screening EGD is performed biannually to the entire public nationwide and this led to increase in diagnosis of early stage stomach cancer, thereby leading to increase in diagnosis of multiple EGCa. However, in spite of much effort to find multiple EGCa lesions, the rate of lesions unidentified prior to surgical intervention are as high as 20% to 25%<sup>[4]</sup>.

In the past, gastrectomy was the major treatment of gastric cancer even if multiple gastric cancers were not identified, but because the multiple gastric cancers were included in the resected portion of stomach there was no significant difference in prognosis in some cases. However, in recent days, as endoscopic submucosal dissection is being used as treatment of EGCa, the finding of multiple EGCa is considered important. Moreover, as more





**Figure 3 Histopathological findings.** A-G: Adenocarcinoma, poorly differentiated in each early gastric cancer lesions in Figure 1 (× 40); H: Lymph node metastasis after gastrectomy (× 40); I: Signet ring cell type of biopsy specimen in esophagogastrroduodenoscopy (× 100).

Table 1 Cancer type, size and depth of invasion for each lesion of multiple early gastric cancer							
	A	B	C	D	E	F	G
Type	II c	II c	II c	II b	II c	II c	II b
Size (mm <sup>2</sup> )	6 × 6	15 × 12	7 × 7	6 × 5	10 × 8	7 × 7	2 × 2
Depth	M	SM1	M	M	M	M	M

M: Mucosa; SM1: A third layer of the submucosa.

non-invasive procedures are preferred for elderly patients for the treatment of EGCa, the importance of finding multiple lesions became more significant.

According to Moertel *et al.*<sup>[5]</sup>, diagnosis of multiple EGCa requires evidence of pathological malignancy for each lesion, which should be present at independent locations without the possibility of metastasis from the other organs. Multiple EGCa are more prevalent in male or older patients. In many cases, the area of occurrence is located at middle third portion and lower third portion of the stomach, whereas most accessory lesions incur at adjacent locations to the main lesion, usually the distal part<sup>[3,4]</sup>. Most multiple EGCa have shapes of the elevated type or flat type rather than the depressed type, and most

are associated with well differentiated lesions rather than poorly differentiated lesion, and majority of them show invasion only to the mucosal layer<sup>[3,4]</sup>. In addition, when there is adenoma or atrophic gastritis, and when the patient has a family history of stomach cancer, multiple EGCa is more likely to be prevalent<sup>[1,6]</sup>.

The prognosis and 5 year survival rate of multiple EGCa as well as single EGCa are both similarly over 90%<sup>[7]</sup>. The recurrence rate is also similarly 11.2% in both types, and a substantial number of them are considered to be caused by multiple synchronous EGCa which had been overlooked during prior EGD<sup>[8]</sup>. Therefore, it is important to keep in mind the possible existence of synchronous lesion when establishing plans for examination and treatment.

There are arguments that total gastrectomy should be performed for the treatment of multiple EGCa due to the risk of recurrence but subtotal gastrectomy can also be performed as the treatment of multiple EGCa as they mainly occur at the distal part and not much different in prognosis. In consideration of the post-operative quality of life, even if subtotal gastrectomy is performed when possible with accurate diagnosis, the results are not different from the cases performed with total gastrectomy.



Recently, endoscopic submucosal dissection is increasing trend as the treatment method of EGCa, and lymph node metastasis becomes an important factor in deciding endoscopic therapy over surgical treatment. Other important factors for prediction of lymph node metastasis include presence/absence of ulcer lesion, tumor size, and invasion depth. Due to the increased accuracy of pre-operative CT scan and endoscopic ultrasonography, it is easier to find lymph node metastasis, therefore the identification of depth of invasion as major risk factors of lymph node metastasis becomes important<sup>[9]</sup>. In our case, although the depth of invasion is limited to the mucosal layer or the 1/3 part of the submucosa layer, numerous lymph node metastasis had occurred. Such outcomes were considered to be caused not from the presence of multiple lesions but from the lympho-vascular invasion.

This case was finally diagnosed as a very rare case of EGCa with 7 multiple synchronous EGCa in a male patient of old age. In addition, the patient had shown metastasis of 28 lymph nodes out of 48 resected lymph nodes although its depth of invasion was limited to the mucosal and the 1/3 part of the submucosal layer. Thereby, we report this very rare case with literature review in order to inform the importance of accurate diagnosis on multiple EGCa.

## ACKNOWLEDGMENTS

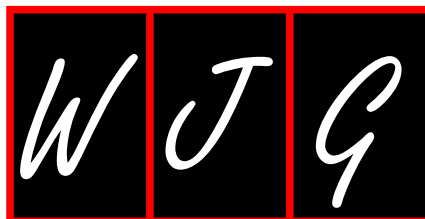
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**L- Editor:** A **E- Editor:** Zhang DN





## Small cell carcinoma of the liver and biliary tract without jaundice

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dice; Liver mass; Bile duct mass; Neuroendocrine tumor

**Core tip:** We report a rare case of small cell carcinoma of the liver and biliary tract. Despite its rarity, liver and bile duct small cell carcinoma should be considered in the differential diagnosis of atypical chest pain because this symptom might indicate the presence of abdominal malignancy. We also explain why previous studies have reported inconsistent immunohistochemical findings in tissues obtained from extrapulmonary small cell carcinomas.

Jo JM, Cho YK, Hyun CL, Han KH, Rhee JY, Kwon JM, Kim WK, Han SH. Small cell carcinoma of the liver and biliary tract without jaundice. *World J Gastroenterol* 2013; 19(44): 8146-8150 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8146.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8146>

### Abstract

An 80-year-old woman presenting with chest pain was found to have a large, lobulated soft tissue mass in the liver and nearby tissues on abdominal computed tomography (CT). The tumor had invaded the common hepatic artery and main portal vein. Jaundice developed 4 wk later, at which point, a pancreas and biliary CT scan revealed a large mass in the right lobe of the liver and a hilar duct obstruction, which was found to be a small cell carcinoma. Despite its rarity, liver and bile duct small cell carcinoma should be considered in the differential diagnosis of atypical chest pain without jaundice.

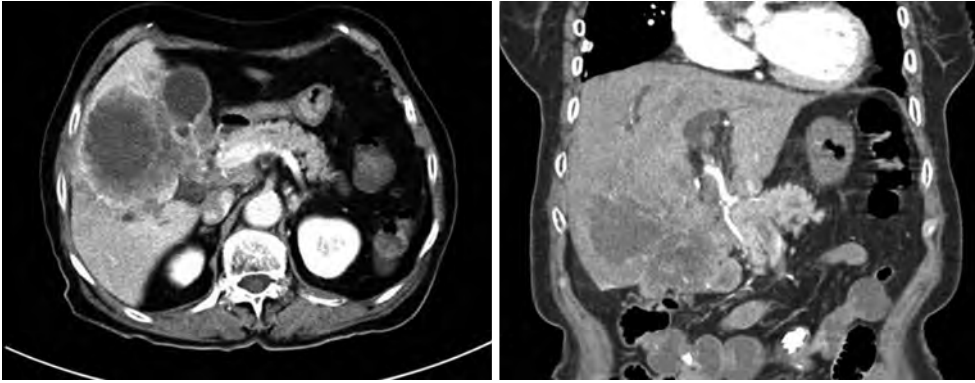
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**Key words:** Extrapulmonary small cell carcinoma; Jaun-

### INTRODUCTION

Most small cell carcinomas occur in the lung, and extrapulmonary small cell carcinoma comprises only 2.5%-4% of all small cell carcinoma cases<sup>[1,2]</sup>. These malignancies are now considered to be distinct clinicopathological entities from small cell lung cancer, and there is little consensus regarding the optimal treatment strategy in such cases. Extrapulmonary small cell carcinomas are rarely found in the trachea, larynx, thymus, esophagus, stomach, small intestine, colon, prostate, gallbladder, skin, breast, and uterine cervix, and they are even rarer in the liver or biliary tract<sup>[3]</sup>. To the best of our knowledge, only 10 cases of primary small cell carcinomas of the liver have been reported in the English literature<sup>[4-10]</sup>.

Here, we report the case of a patient with small cell carcinoma of the liver and biliary tract initially presenting with atypical chest pain without jaundice.



**Figure 1** Findings of initial abdominal computed tomography. Computed tomography showed a large mass located in the liver, common hepatic duct, and common bile duct.

## CASE REPORT

An 80-year-old woman was admitted to our hospital with a 7-d history of anorexia and chest pain. On admission, she was afebrile, her blood pressure and pulse rate were normal, and she appeared well nourished despite the recent anorexia. During the work-up for chest pain, an electrocardiogram showed a normal sinus rhythm and an echocardiogram showed no significant functional abnormality. The scleras were not icteric. The abdomen was mildly distended, with tenderness in the right upper quadrant. Laboratory studies revealed a white blood cell count of  $4500/\text{mm}^3$  (normal range,  $6000\text{--}10000/\text{mm}^3$ ); hemoglobin,  $12.1\text{ g/dL}$  (normal range,  $12\text{--}16\text{ g/dL}$ ); platelet count,  $199000/\text{mm}^3$  (normal range,  $130000\text{--}450000/\text{mm}^3$ ); serum albumin,  $3.8\text{ g/dL}$  (normal range,  $3.0\text{--}5.0\text{ g/dL}$ ); aspartate aminotransferase (AST),  $101\text{ U/L}$  (normal range,  $5\text{--}37\text{ U/L}$ ); alanine aminotransferase (ALT),  $64\text{ U/L}$  (normal range,  $5\text{--}40\text{ U/L}$ ); alkaline phosphatase,  $720\text{ U/L}$  (normal range,  $39\text{--}117\text{ U/L}$ ); gamma-guanosine-5'-triphosphate,  $215\text{ U/L}$  (normal range,  $7\text{--}49\text{ U/L}$ ); total bilirubin,  $0.4\text{ mg/dL}$  (normal range,  $0.2\text{--}1.2\text{ mg/dL}$ ); and proBNP,  $170.6\text{ pg/mL}$  (normal range,  $0\text{--}125\text{ pg/mL}$ ). Coagulation profiles were within normal limits. After confirming the absence of a significant cardiac problem, we performed esophagogastrosocopy and colonoscopy; however, these investigations also yielded no significant findings, except for that of chronic atrophic gastritis. Abdominal computed tomography (CT) revealed a lobulated soft tissue mass measuring  $10.1\text{ cm}$  and located in the liver, common hepatic duct, common bile duct, gall bladder, and hepatoduodenal ligament. The intrahepatic duct was dilated, and the tumor had invaded both the common hepatic artery and main portal vein (Figure 1); multiple enlarged lymph nodes were present near the celiac and common hepatic arteries and in the left gastric and aortocaval spaces. Additional laboratory studies showed that CA19-9 concentration was  $12.57\text{ U/mL}$  (normal range,  $0\text{--}37\text{ U/mL}$ ) and  $\alpha$ -fetoprotein (AFP) concentration was  $2.19\text{ ng/mL}$  (normal range,  $0\text{--}10.9\text{ ng/mL}$ ). Concentrations of carcino-embryonic antigen (CEA) and PIVKAI were not assessed.

These findings led to clinical suspicion of cholangio-

carcinoma, but the patient refused to undergo a biopsy and was discharged. However, four weeks later, the patient visited the hospital with jaundice. At this second visit, laboratory analysis of the patient's blood provided the following results: AST,  $566\text{ U/L}$ ; ALT,  $261\text{ U/L}$ ; alkaline phosphatase,  $6783\text{ U/L}$ ; gamma-GTP,  $476\text{ U/L}$ ; total bilirubin,  $25.8\text{ mg/dL}$ ; and direct bilirubin,  $19.8\text{ mg/dL}$ . A CT scan of the pancreas and biliary duct revealed a large mass in the right lobe of the liver as well as an obstruction of the hilar duct (Figure 2). An ultrasonography-guided gun biopsy was performed, and pathological analysis of the biopsy specimen revealed a tumor consisting of tightly packed nests and diffuse irregularly shaped sheets of cells with areas of necrosis (Figure 3). The tumor cells were of small-to-intermediate size with hyperchromatic, round-to-oval nuclei and scanty, poorly defined cytoplasm. The nuclear chromatin was finely granular, and nucleoli were absent or inconspicuous. Very few cell borders were visible, and there was frequent nuclear molding (Figure 3). The tumor cells were immunoreactive for synaptophysin and CD 56, but negative for hepatocyte-specific antigen (HSA) and thyroid transcription factor (TTF)-1. The Ki-67 index was high (more than 80%), and approximately 5 mitotic cells/10 HPF were observed. Taken together, these findings led us to diagnose small cell carcinoma (Figure 4). On the basis of the World Health Organization 2010 classification for neuroendocrine tumors (NETs)/neuroendocrine carcinomas (NECs), we identified the carcinoma as a grade 3 (G3) NEC (small cell type).

The tumor was unresectable; therefore, palliative chemotherapy was considered to be the best treatment option. However, the patient refused chemotherapy because of her advanced age and poor health. Hence, only supportive care, including percutaneous transhepatic biliary drainage, was provided, and the patient died 8 wk after the diagnosis was confirmed.

## DISCUSSION

Extrapulmonary small cell carcinomas represent only 0.1%–0.4% of all cancer cases<sup>[11,12]</sup>. Most small cell carcinomas arise in the lung or bronchial trees as small cell lung cancers (SCLCs). However, cases of extrapulmonary



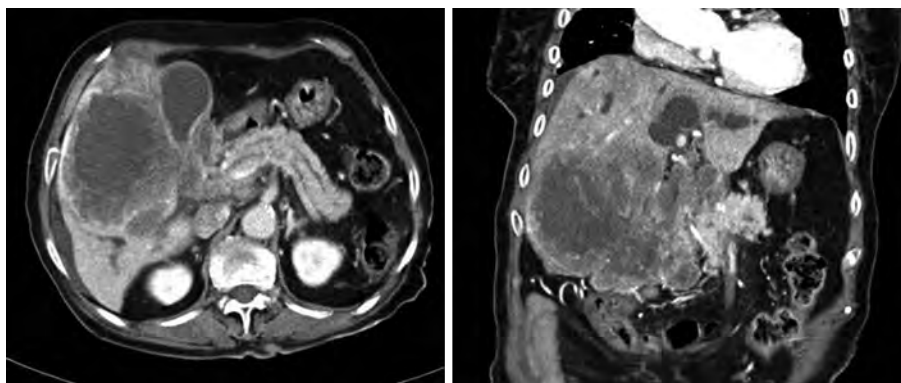


Figure 2 Computed tomography scan obtained before biopsy. One month after the first visit, a computed tomography scan of the pancreas and biliary duct revealed that the large mass in the right lobe of the liver had grown and that there was an obstruction of the hilar duct.

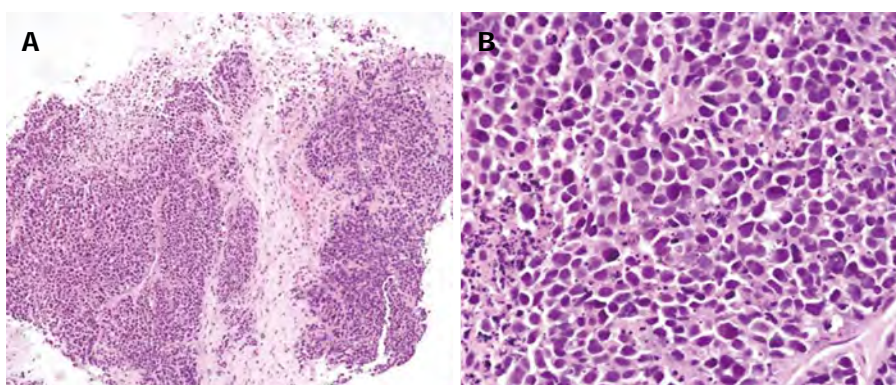


Figure 3 Tumor consisted of tightly packed nests and diffuse, irregularly shaped sheets of cells with necrotic areas. A: The tumor cells were of small-to-intermediate size with hyperchromatic, round-to-oval nuclei and scanty, poorly defined cytoplasm (HE,  $\times 100$ ); B: The nuclear chromatin was finely granular, and nucleoli were absent or inconspicuous. Cell borders were rarely seen, and nuclear molding was common (HE,  $\times 400$ ).

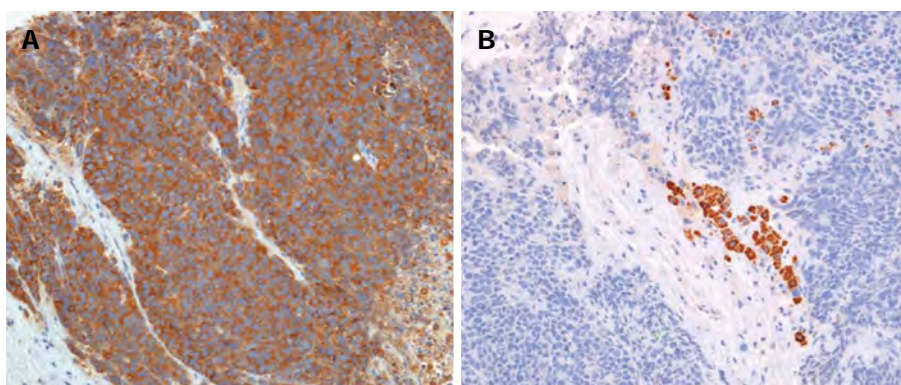


Figure 4 Immunohistochemical findings ( $\times 200$ ). The tumor cells were immunoreactive for synaptophysin (A) and negative for hepatocyte-specific antigen (B).

small cell carcinomas are gradually becoming more common, and there have been reports of small cell carcinomas in both the gall bladder<sup>[13]</sup> and pancreas<sup>[14]</sup>. A few studies have reported small cell NEC of the ampulla of Vater<sup>[15,16]</sup>. However, reports of small cell carcinoma of the liver or common bile duct, as in the present case, are extremely rare.

Differential diagnosis is important to exclude pulmonary small cell carcinoma. In most cases, a finding of occult extrapulmonary small cell carcinoma is subsequently

found to be a distant metastasis from an undetected small cell lung cancer. To exclude this possibility, chest radiography and CT, and/or bronchoscopic examination with appropriate biopsies and sputum cytology are required. In addition, growing evidence suggests that positron emission tomography (PET) is also sufficiently sensitive to rule out SCLC<sup>[17-19]</sup>. In the present case, results of sputum cytology were negative for malignancy, and there was no evidence of lung cancer on chest CT, making bronchoscopy unnecessary.



Extrapulmonary small cell carcinomas shows structural features of both primitive epithelial and neuroendocrine differentiation<sup>[17]</sup>. Neuroendocrine carcinoma arises from embryonic neural crest cells, which migrate to the bronchopulmonary system and gastrointestinal tracts during development. However, these cells do not usually migrate to the liver and bile duct; this may be the reason for the rare occurrence of neuroendocrine tumors of the liver and bile duct<sup>[1,20]</sup>.

Small cell carcinoma can be distinguished from other carcinomas or lymphomas that have cells of a similar size. Under light microscopy, the diameter of small cell carcinoma cells is generally 2 or 3 times greater than that of a small lymphocyte. In addition, small cell carcinoma cells are spindle-like, have a fusiform or polygonal shape, and tend to grow in sheets or ribbon- or rosette-like patterns. Extensive necrosis and high mitotic rates are also typical features that differentiate small cell carcinoma from atypical carcinoid tumors<sup>[5]</sup>.

Furthermore, several distinct immunohistochemical features associated with extrapulmonary small cell carcinoma are potentially important in distinguishing it from hepatic metastasis of small cell lung carcinoma<sup>[21-24]</sup>; unfortunately, the results of previous studies are somewhat inconsistent in this respect. Ryu *et al.*<sup>[4]</sup> reported an 8 cm-sized small cell carcinoma of the liver that was positive for CD56/C-kit/synaptophysin and negative for TTF-1. Zanconati *et al.*<sup>[7]</sup> studied 3 cases and found that all of the tumors were positive for AE1/AE3, CK8, CK18, CK19, and NSE and negative for S-100/CEA, and that 2 of them were AFP positive. Frazier *et al.*<sup>[25]</sup> reported a case of small cell carcinoma of the liver in which the patient underwent hepatic segmentectomy and adjuvant etoposide/cisplatin therapy. The tumor in this case was positive for CD56/NSE/c-kit/synaptophysin/mixed CK/EMA and negative for CK7, CK8, CK19, CK20, AFP, CEA, vimentin, and desmin/TTF-1/AFP. In the present case, the tumor was positive for both CK and synaptophysin, weakly positive for CD56, and negative for HSA. These tumor cells originate from multipotent stem cells that can differentiate into various cell types; this may explain the frequent coexistence of mixed tumors with various immunohistochemical features.

Small cell carcinoma frequently shows distant metastasis and consequently has a poor prognosis. Correspondingly, patients with small cell lung cancer generally have poor long-term survival. However, some cases of good long-term survival have been reported in patients with extrapulmonary small cell carcinoma. Little is known about the survival of patients with small cell carcinoma of the liver; generally, the clinical course of this condition is not well described, and reports of patient survival vary. Zanconati *et al.*<sup>[7]</sup> reported two cases in which the patients died soon after diagnosis, and treatment could not be initiated. In contrast, Sengoz *et al.*<sup>[6]</sup> reported two cases of extrapulmonary small-cell carcinoma of the liver, in which one patient survived for 13 mo after chemotherapy and the other survived 67 mo after receiving right hemihepatectomy. Choi *et al.*<sup>[10]</sup> reported the case of a patient

who survived more than 18 mo after receiving treatment with oral etoposide alone.

It is often not possible to effectively treat small cell carcinoma of the biliary system with surgical resection alone. As an alternative, Hazama *et al.*<sup>[26]</sup> performed surgical resection after neoadjuvant chemotherapy for small cell carcinoma of the common bile duct, and Okamura *et al.*<sup>[27]</sup> suggested that multimodality treatment including neoadjuvant chemotherapy, surgical resection, and adjuvant chemotherapy improves survival of patients with small cell carcinoma of the biliary system. The generally accepted optimal treatment for extrapulmonary small cell carcinoma is appropriate surgical resection followed by adjuvant chemotherapy. However, Levenson asserted that there is no survival benefit from using surgery to treat either small cell lung cancer or extrapulmonary small cell carcinoma. This may be because the most important prognostic factor is the extent of disease at diagnosis, when most patients with extrapulmonary small cell carcinoma already have occult metastasis<sup>[1]</sup>.

In the present case, we could not administer palliative chemotherapy because of the patient's advanced age and very poor performance status. Although there is no established standard treatment for extrapulmonary small cell carcinoma, chemotherapy should be tried if the patient can tolerate it, because this malignancy is often chemosensitive<sup>[28]</sup>. The recommended chemotherapy regimen for extrapulmonary small cell carcinoma is the same as that for small cell lung cancer<sup>[10]</sup>. Furthermore, for patients who are able to undergo surgical resection, platinum-based adjuvant chemotherapy is also advisable, because it can reduce the chance of systemic recurrence<sup>[29-31]</sup>.

In previous reports of primary small cell carcinoma of the liver or bile duct, patients usually presented with jaundice, a palpable mass, or abdominal discomfort as their first symptom. Despite its rarity, liver and bile duct small cell carcinoma should be considered in the differential diagnosis of atypical chest pain as this symptom might indicate the presence of an abdominal malignancy.

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## Malignant paraganglioma of the rectum: The first case report and a review of the literature

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### Abstract

Paragangliomas typically develop in the extra-adrenal sites along the sympathetic and/or the parasympathetic chain. Occasionally, the tumors may arise in some exotic sites, including the head and neck region and the urogenital tract. Paraganglioma presenting as a primary rectal neoplasm has not been well described in the literature. Here, we report the first case of malignant paraganglioma arising in the rectum of a 37-year-old male. He presented to the clinic because of hematochezia with tenesmus. The anorectal digital examination and colonoscopic examination revealed a polypoid mass of the rectum, measuring approximately 4 cm in diameter. The overall morphology and immunophenotype were consistent with a typical paraganglioma. However, the tumor exhibited features suggestive of malignant potential, including local extension into adjacent adipose tissue, nuclear pleomorphism, confluent tumor necrosis, vascular invasion and metastases to regional lymph nodes. In conclusion, we present the first case of rectal malignant paraganglioma. Due to the unexpected occurrence in this region, malignant paraganglioma may be misdiagnosed as other tumors with overlapping features; in particular, a neuroendocrine tumor of epithelial origin. Because of the differences in

treatment, separating paraganglioma from its mimics is imperative. Combination of morphology with judicious immunohistochemical study is helpful in obtaining the correct diagnosis.

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**Key words:** Rectum; Paraganglioma; Malignancy

**Core tip:** We report a rare case of malignant paraganglioma arising in the rectum of a 37-year-old male. To the best of our knowledge, the current case represents the first case of malignant paraganglioma arising in the rectum. Due to the unexpected occurrence in this region, rectal paraganglioma may be misdiagnosed as other common types of tumors with overlapping features; in particular, a neuroendocrine tumor of epithelial origin. Because of the differences in treatment, separating paraganglioma from its mimics is imperative.

Yu L, Wang J. Malignant paraganglioma of the rectum: The first case report and a review of the literature. *World J Gastroenterol* 2013; 19(44): 8151-8155 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8151.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8151>

### INTRODUCTION

Paragangliomas are rare but well-described non-epithelial neuroendocrine tumors that typically develop in the extra-adrenal sites along the sympathetic and/or the parasympathetic chain<sup>[1]</sup>. Occasionally, the tumors may arise in some exotic sites where normal paraganglia are not well documented. The majority of these unusual tumors have been described preferentially in the head and neck region and the urogenital tract<sup>[2]</sup>. With the exception of gangliocytic paraganglioma, paraganglioma is extremely rare in the gastrointestinal tract. Of note, the hitherto reported gas-

trointestinal paragangliomas are exclusively limited to the stomach<sup>[3-7]</sup>. To the best of our knowledge, paraganglioma has not been well described in the rectum. Due to the unexpected occurrence in this region, rectal paraganglioma may be misdiagnosed as other common types of rectal tumors with morphological overlap. Because the treatments vary, separation of rectal paraganglioma from its mimics, in particular neuroendocrine tumors of epithelial origin, is imperative. In this study, we report a case of malignant paraganglioma presenting as a primary rectal neoplasm to broaden the clinical and morphological spectrum.

## CASE REPORT

A 37-year-old male presented to the clinic because of hematochezia. The symptom had lasted for 8 mo and was accompanied intermittently with tenesmus. On anorectal digital examination, a round firm mass was identified on the posterior wall of the rectum. Colonoscopic examination revealed a polypoid mass, measuring approximately 4 cm in diameter. Clinically, the mass was suspected to be a rectal carcinoma. A biopsy was performed and was interpreted as a low-grade neuroendocrine tumor. After the admission, laparoscopic radical rectectomy was performed. The postoperative course was uneventful. The patient received no adjunctive therapy after surgery and is well at 9-mo follow-up.

### Pathologic studies and findings

Hematoxylin and eosin-stained sections were reviewed. Immunohistochemical study was performed on 4-mm thick unstained sections of formalin-fixed paraffin-embedded tissue using the standard EnVision technique. The primary antibodies used in the study included antibodies against Chromogranin A (dilution 1:200), synaptophysin (dilution 1:100), neuron-specific enolase (dilution 1:100), CD56 (dilution 1:50), S100 protein (dilution 1:300), pancytokeratin (dilution 1:100), cytokeratin 8/18 (dilution 1:50), epithelial membrane antigen (dilution 1:200), CD34 (dilution 1:50), Human Melanoma Black 45 (dilution 1:60), alpha smooth muscle actin (dilution 1:400), desmin (dilution 1:500), CD117 (dilution 1:100), and discovered on GIST-1 (DOG1) (dilution 1:100). Heat-induced epitope retrieval was performed using a pressure cooker. Appropriate positive controls were run simultaneously throughout the process.

The resected specimen consisted of a segment of rectum measuring 11 cm in length. A polypoid mass was observed protruding into the intestinal cavity, measuring 4.0 cm × 4.0 cm × 1.5 cm in size. On the cut section, the tumor was red-brownish in color and fleshy in consistency, involving the full thickness of the intestinal wall with local extension into the adjacent adipose tissue.

Histologically, the tumor was composed of sheets or organoid nests of large polygonal cells surrounded by a rich network of delicate arborizing vasculature, generating a characteristic “zellballen” (Figure 1A and B). The polygonal cells contained copious eosinophilic

to amphiphilic granular cytoplasm, with round to oval nuclei and prominent nucleoli. Although focal nuclear pleomorphism was present (Figure 1C), mitotic activity was relatively low (1-2/50 high power fields). Confluent tumor necrosis and vascular invasion were observed (Figure 1D and E). In addition, the tumor cells metastasized to regional lymph nodes (Figure 1F).

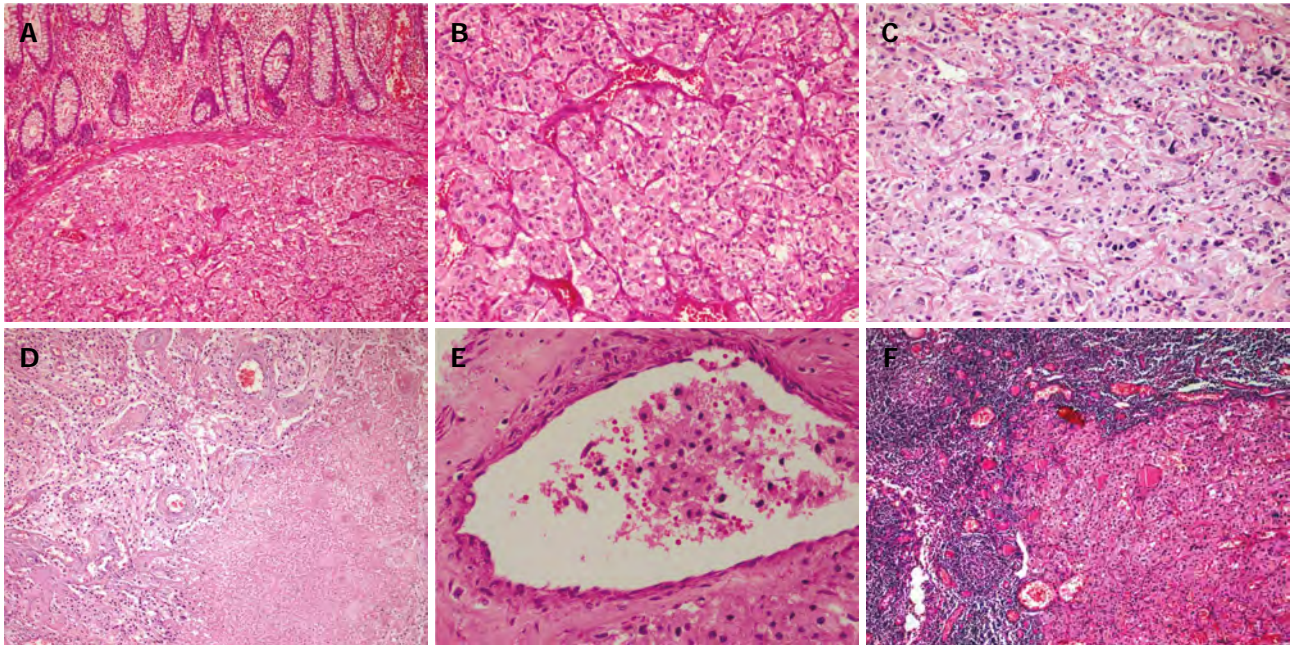
Immunohistochemically, the large polygonal cells exhibited diffuse and strong expression of chromogranin A (Figure 2A), synaptophysin, CD56, neuron-specific enolase and vimentin. In areas with distinct organoid structure, the staining of S100 protein highlighted the presence of slender sustentacular cells located at the periphery of the tumor nests (Figure 2B). However, in areas with more diffuse architecture, the sustentacular cells were difficult to identify. The tumor cells were negative for all of the epithelial, melanocytic, myogenic and Cajal cell markers tested in this study. The Ki67 index was approximately 20% (Figure 2C). The endothelial markers of CD34 and CD31 outlined the rich vascular network (Figure 2D).

## DISCUSSION

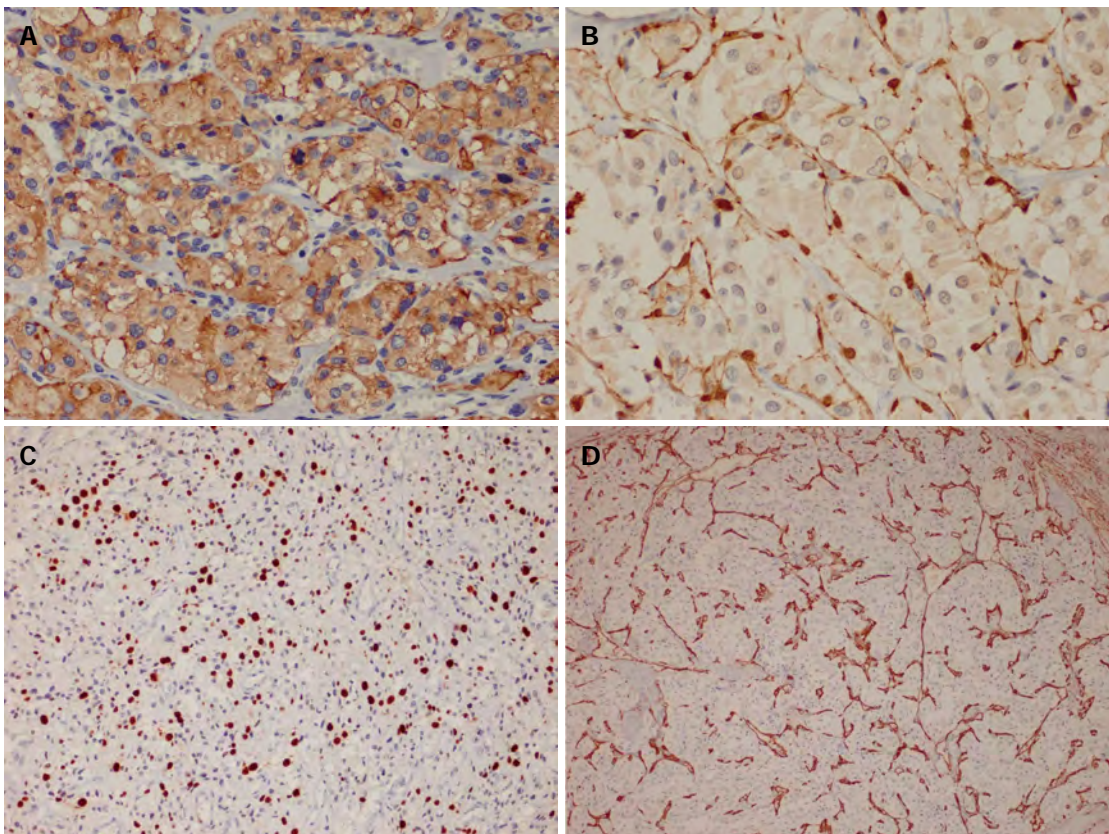
Paragangliomas have been rarely reported in the gastrointestinal tract. Approximately 12 tumors have been described in this region, most of which were located in the stomach, with only one tumor occurring in the rectum<sup>[3-8]</sup>. Of note, the only reported rectal paraganglioma was included in a study focusing on a statistical analysis and was only quoted as an anatomic location. Insufficient data were provided in that case, either clinically or pathologically.

In the current study, we present the clinical and pathological features of a malignant paraganglioma occurring in a 37-year-old male who presented with non-specific symptoms. Clinically, the lesion was suspected as a rectal carcinoma based on anorectal digital findings and rectoscopy examination. Due to the striking organoid structure and strong positivity for neuroendocrine markers, the biopsy specimen was initially interpreted as a low-grade neuroendocrine tumor of epithelial origin, formerly known as a carcinoid tumor. The final diagnosis of paraganglioma was established on the postoperative specimen, which provided enough sections for comprehensive review. The negativity for epithelial marker and presence of slender sustentacular cells allowed the distinction from an epithelial neuroendocrine neoplasm. Other tumors that may enter into the differential diagnosis include neoplasms with perivascular epithelioid cell differentiation (PEComas), alveolar soft part sarcoma (ASPS) and, rarely, gastrointestinal stromal tumor (GIST) of epithelioid subtype. Like paragangliomas, both PEComas and ASPS may exhibit an organoid or nesting pattern surrounded by thin-walled vessels. By immunohistochemistry, PEComas typically express melanocytic and sometimes express myogenic markers, whereas ASPS is characterized by nuclear staining of TFE3 with no expression of neuroendocrine antibodies. The application





**Figure 1** Histological features. A-B: The tumor was composed of sheets or organoid nests of large polygonal cells surrounded by a rich network of delicate arborizing vasculature, generating a characteristic "zellballen" (A: HE, × 100; B: HE, × 400); C: Focal nuclear pleomorphism (HE, × 400); D: Confluent tumor necrosis (HE, × 100); E: Vascular invasion (HE, × 400); F: Metastases of lymph nodes (HE, × 100).



**Figure 2** Immunohistochemistry. A: The tumor cells exhibited diffuse and strong expression of chromogranin A; B: S100 protein highlighted the presence of slender sustentacular cells located at the periphery of the tumor nests; C: The Ki67 index was approximately 20%; D: CD34 outlined the rich vascular network.

of Cajal cell markers, namely CD117 and DOG1, will facilitate the distinction from an epithelioid GIST.

Malignant paraganglioma accounts for 14%-50% of

all paragangliomas in some large series<sup>[9,10]</sup>. Only three cases of primary malignant paraganglioma have been reported in the gastrointestinal tract, all of which occurred



in the stomach<sup>[4,6]</sup>. To the best of our knowledge, rectal malignant paraganglioma has not been described thus far. The current case represents the first case of malignant paraganglioma arising in the rectum.

The diagnosis of malignancy in a paraganglioma is based principally on the aggressive behavior of the tumor. According to the World Health Organization classification of tumors of the endocrine system, the reliable diagnostic criteria of malignant paraganglioma refer to the presence of metastasis or tumor spread in sites normally devoid of chromaffin tissue<sup>[11]</sup>. Although not considered definitive, several morphological features are believed to be correlated with malignant potential. These features include large size of the tumor (> 5 cm), prominent nuclear pleomorphism, increased mitotic activity, presence of confluent tumor necrosis, diffuse growth pattern with a lack of sustentacular cells, extension to adjacent tissues or structures, vascular invasion and high Ki67 index<sup>[11,12]</sup>. It is worth noting that none of these features are able to identify a malignant tumor alone. As an alternative approach, some scoring systems have been proposed in the evaluation of malignant potential. One of the most utilized scoring systems is the "Pheochromocytoma of the Adrenal gland Scales Score (PASS)"<sup>[13]</sup>. A PASS score > 6 is highly suggestive of potential aggressive biological behavior. The current case exhibited local extension into the adjacent adipose tissue, focal nuclear atypia, confluent tumor necrosis, vascular invasion, high Ki67 index and metastases to regional lymph nodes, justifying a diagnosis of malignant paraganglioma.

Recently, an increasing number of studies have focused on identifying molecular markers or biomarkers that can reliably predict malignant potential of the paraganglioma. Several markers, including telomerase, telomerase associated protein, heat shock protein 90, SNAIL and miR-483-5p, also have been found to be closely related to the malignant potential of paraganglioma<sup>[10,14]</sup>. On the other hand, molecular genetic detection is only gradually being applied to paraganglioma. It has been demonstrated that malignant paraganglioma is strongly associated with SDHB mutations<sup>[15]</sup>. Despite these recent advances, it remains difficult to reliably predict the outcome of any given patient.

With regard to the prognosis, the 5-year survival rate in malignant paraganglioma is approximately 30%-50%<sup>[10]</sup>. The majority metastasize to regional lymph nodes, followed by bone, liver and lung<sup>[11,14]</sup>. At present, there is no optimal therapy for malignant paraganglioma. For the past few years, molecular targeted therapy has been increasingly applied in the therapeutic protocol of malignant paraganglioma. Some targeted agents, such as hypoxia inducible factor-1 inhibitors, mammalian target of rapamycin inhibitors (everolimus), and receptor tyrosine kinase inhibitors (sunitinib), have been attempted with promising effectiveness<sup>[14]</sup>. Nevertheless, more clinical trials are needed. The patient in the current study received no adjunctive chemotherapy or radiotherapy. He remains well 9 mo after the surgery and continues to be closely monitored.

In summary, we present the first case of rectal malignant paraganglioma. Because of its rarity in the rectum, malignant paraganglioma may be misdiagnosed as other tumors with overlapping features. Because of the differences in treatment, separation of malignant paraganglioma from its mimics, in particular from neuroendocrine carcinoma, is imperative. Combination of morphology with judicious immunohistochemical study is helpful in obtaining the correct diagnosis.

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## Ileal conduit stomal variceal bleeding managed by endovascular embolization

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Author contributions: Yao DH, Luo XF and Li X designed the report; Luo XF performed the operation; Zhou B performed image collection; and Yao DH wrote the paper.

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### Abstract

Ileal conduit stomal varices are rare, and may result in bleeding. The standard treatment modality for management of this type of hemorrhage has not been established. We present the case of a 70-year-old woman with progressive ileal conduit stomal variceal bleeding which was successfully managed by endovascular embolization *via* the transjugular transhepatic approach. In conclusion, transjugular transhepatic endovascular embolization is a good choice in patients with ileal conduit stomal variceal bleeding who have failed conservative therapy.

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**Key words:** Ectopic variceal bleeding; Ileal conduit; Stomal bleeding; Cirrhosis; Hemostasis; Transjugular transhepatic embolization

**Core tip:** Ileal conduit stomal varices are very rare, and may result in refractory bleeding. We present the case

of a 70-year-old woman with progressive ileal conduit stomal variceal bleeding which was successfully managed by endovascular embolization *via* the transjugular transhepatic approach.

Yao DH, Luo XF, Zhou B, Li X. Ileal conduit stomal variceal bleeding managed by endovascular embolization. *World J Gastroenterol* 2013; 19(44): 8156-8159 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8156.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8156>

### INTRODUCTION

Varices are a common complication of liver cirrhosis with portal hypertension. Typically, they are found in the gastro-esophageal region. Ectopic varices are rare, and can arise along the entire gastrointestinal tract, including the duodenum, jejunum, ileum, colon and rectum, but seldom at the umbilicus, in the peritoneum and stomas<sup>[1,2]</sup>. Ectopic varices can present with hemorrhage, accounting for up to 5% of all variceal bleeding<sup>[1]</sup>. However, due to the difficulty in their diagnosis and treatment, the mortality secondary to their initial bleeding is up to 40%<sup>[3]</sup>. Currently, reports on ectopic variceal bleeding are mostly located in the gut, especially in the duodenum and rectum. Variceal bleeding from a stoma, especially from an ileal conduit stoma, has rarely been reported<sup>[4]</sup>. Here, we present a case of ectopic variceal bleeding from an ileal conduit stoma (Figure 1) which was successfully managed by endovascular embolization *via* the transjugular transhepatic approach.

### CASE REPORT

A 70-year-old woman, who had undergone cystectomy and an ileal conduit due to interstitial cystitis two years before, presented with chronic bleeding from the ileal



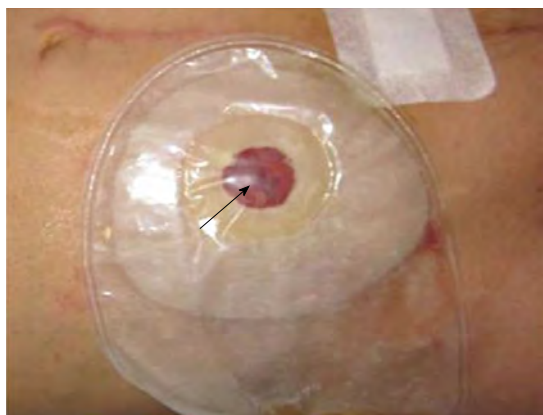


Figure 1 The bluish ectopic varices at the ileal conduit stoma (black arrow).

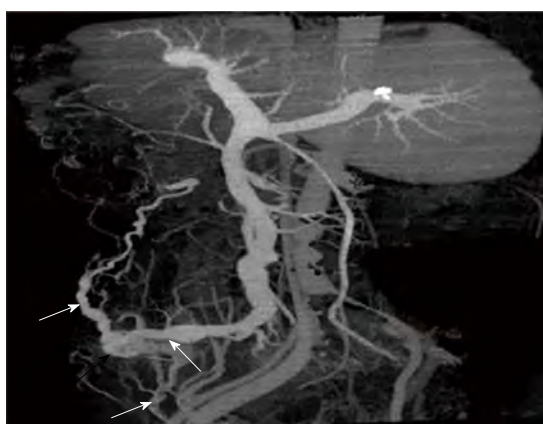


Figure 2 Three-dimensional reconstruction showed the ectopic varices (black arrow) at the ileal conduit stoma fed by the superior mesenteric vein (white arrow) and communicated to the paraumbilical vein (white arrow) and femoral vein (white arrow).

conduit stoma after the operation. The patient also had drug-induced liver cirrhosis and a history of two episodes of hepatic encephalopathy. The bleeding was first considered to be wound bleeding and was treated with homeostatic drugs. However, the hemorrhage persisted, and an enhanced multislice computed tomography (CT) scan and 3-dimensional (3D) reconstruction imaging showed ectopic varices fed by the superior mesenteric vein (SMV) and communicated to the paraumbilical vein and femoral vein at the ileal conduit ostomy (Figure 2). The hemorrhage could be paused by local compression. Two weeks previously, with the bleeding worsening, local compression and vasoactive therapy (Octreotide 50 mg/h) failed to achieve hemostasis. A wound resuture was then performed, however, the result was disappointing. Three days before, massive hemorrhage had occurred and the patient developed hemorrhagic shock (systolic blood pressure 88 mmHg, heart rate 103/min) and became severely anemia (hemoglobin 57 g/L, red blood cell  $2.14 \times 10^{12}/L$ ). The platelets, prothrombin time and international normalized ratio were  $78 \times 10^9/L$ , 18.6 s, and 1.18, respectively. Considering her hepatic function [Child-Pugh B (9 score)], abundant ascites and her history of recurrent hepatic encephalopathy,

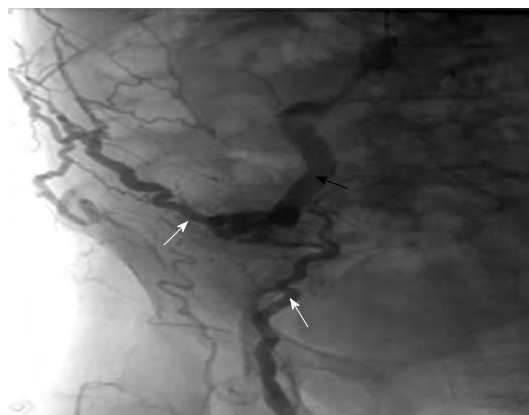


Figure 3 Opacification showed the varices fed by the superior mesenteric vein (black arrow) and communicated to the paraumbilical vein and femoral vein (white arrows).

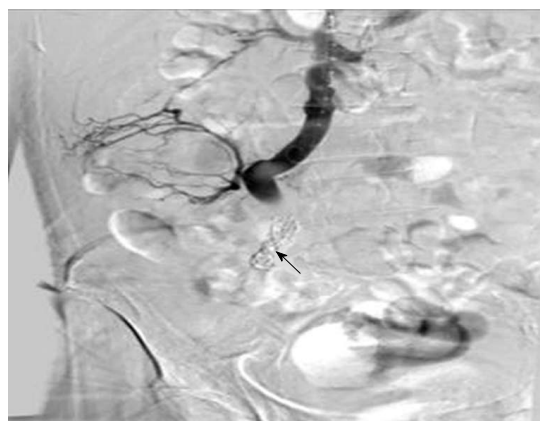


Figure 4 Opacification showed disappearance of the ileal conduit stoma varices and colic (black arrow) in the ectopic varices.

an emergency transjugular transhepatic embolization was planned. Portal venography and peripheral superior mesenteric venography demonstrated varices arising from the SMV with retrograde flow toward the stoma and communicated to the paraumbilical vein and femoral vein (Figure 3). The portal-pressure gradient measured during surgery was 18 cmH<sub>2</sub>O. Ectopic varices embolization with a stainless steel coil was then performed. The portal-pressure gradient measured after the operation was 19 cmH<sub>2</sub>O, and postoperative opacification showed that the varices had disappeared (Figure 4). The patient had no complications following the procedure and received conservative medical therapy with Propranolol after the operation. Follow-up at 4 mo showed no focal bleeding.

## DISCUSSION

Reports on the hemorrhage of ectopic varices are limited, and most are of digestive tract bleeding or umbilical vein bleeding<sup>[5-9]</sup>. Abdominal intestinal ostomy may result in the formation of collateral vessels at the stoma, such as the development of ileostomy stomal varices after ileal conduit ostomy and ileostomy in several case

reports<sup>[4,10,11]</sup>. The patient in this study developed ileal conduit stomal variceal bleeding. Due to persistent hemorrhage, a contrast enhanced multislice CT scan and 3D reconstruction imaging were performed which revealed ectopic varices fed by the SMV and communicated to the paraumbilical vein and femoral vein at the stoma. The correct diagnosis of variceal bleeding with portal hypertension was made.

The standard therapy for ectopic variceal bleeding has not yet been determined, and a recent review<sup>[12]</sup> recommended that management should include medical conservative therapy, endoscopic therapy, interventional therapy, surgical shunt or liver transplantation. Similar to variceal bleeding from the esophageal-gastric region, sclerotherapy or band ligation of the varices are theoretically feasible. However, because of potential necrosis and perforation following sclerotherapy, and a high risk of massive hemorrhage following sloughing of the occluded varices after band ligation, and persistence of portal hypertension, the results of this treatment modality were disappointing<sup>[13]</sup>, especially in stomal variceal bleeding<sup>[11]</sup>. Our patient was treated with ectopic varices suture ligation, however, bleeding did not stop. As transjugular intrahepatic porto-systemic shunt (TIPS) alone or in combination with variceal embolization has demonstrated effectiveness for the hemostasis of ectopic variceal bleeding in patients with portal hypertension in some studies<sup>[14,15]</sup>, it seemed appropriate that our patient should be treated with this modality. However, the current common understanding of TIPS shunt creation for hemostasis is that it increases the incidence of hepatic encephalopathy and damages liver function. Our patient had a history of recurrent hepatic encephalopathy, thus, the TIPS shunt was not applicable in this patient. Surgical shunts have been shown to be effective in preventing hemorrhage recurrence, but are associated with mortality ranging from 1% to 50%, and many patients are not healthy enough to endure the operation<sup>[11]</sup>. Our patient had hemorrhagic shock, therefore it was clearly unwise to choose this treatment modality. Thus, embolization of the ectopic varices was the best choice for this patient. Usually, varices embolization can be managed *via* the percutaneous transhepatic<sup>[16-18]</sup> or transjugular transhepatic route<sup>[4]</sup>. Hemoperitoneum is the most common complication of percutaneous transhepatic embolization<sup>[18]</sup>, other complications include bile leak, liver trauma, and portal thrombosis<sup>[17]</sup>. As the patient had abundant ascites and we had successfully performed thousands of TIPS procedures, endovascular embolization of the stomal varices *via* the transjugular transhepatic route for hemostasis was the appropriate therapy in this case. The varices were occluded after the procedure and no complications occurred during the procedure. No focal bleeding was observed after four months of follow-up.

In conclusion, although rare, when a patient with ileal conduit stoma presents with persistent stomal bleeding, ectopic variceal bleeding from the ileal conduit should be considered. Endovascular embolization *via* the tran-

sjugular transhepatic approach is a reasonable choice in patients with stomal variceal bleeding which failed conservative therapy and local resuture, especially in an emergency situation.

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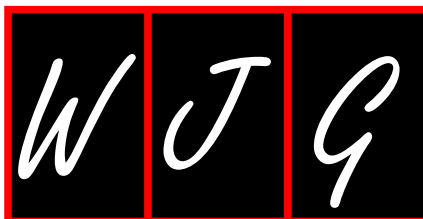
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**L- Editor:** Webster JR **E- Editor:** Wang CH





## Hydroxycitric acid does not promote inflammation or liver toxicity

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### Abstract

Garcinia cambogia extract (GC) with its active component consisting of hydroxycitric acid (HCA) is widely utilized for weight loss. Various HCA salts are available, including calcium, magnesium, potassium and mixtures of these. Experimentally, these salts exhibit different properties with some, but not all, improving glucose tolerance and blood pressure. Recently, obesity-prone C57BL/6J mice were fed a high-fat diet (HFD, 45 kcal% fat) with or without GC (1%, w/w) for 16 wk. The active arm reduced visceral fat, adipocyte size and serum glucose, yet purportedly also exhibited hepatic collagen accumulation, lipid peroxidation and increased mRNA levels of genes related to oxidative stress. The latter findings are at odds with a large body of animal and human studies that have been conducted on the safety and efficacy of HCA. This literature shows HCA to be protective against the liver toxicity associated with ethanol and dexamethasone administration, and to maintain serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase at near normal levels. In both animal and clinical literature, elevated intakes of HCA *per se* have not led to signs of inflammation or hepatotoxicity. The compound has

been found to reduce markers of inflammation in brain, intestines, kidney and serum.

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**Key words:** Garcinia cambogia; Hepatic collagen; Hepatic inflammation; Hepatic oxidative stress; Hydroxycitric acid; Metabolic syndrome; Tumor necrosis factor- $\alpha$ ; Weight loss

**Core tip:** The preponderance of animal and human studies of Garcinia cambogia extract have found it to reduce markers of inflammation in brain, intestines, kidney and serum and to be either protective or neutral in terms of liver health. The limited reports of toxicities thus far have been linked to improperly manufactured materials and/or to peculiarities with the animal models used. The available data indicate that Garcinia cambogia extract/hydroxycitric acid does not cause liver toxicity.

Clouatre DL, Preuss HG. Hydroxycitric acid does not promote inflammation or liver toxicity. *World J Gastroenterol* 2013; 19(44): 8160-8162 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i44/8160.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i44.8160>

### TO THE EDITOR

Kim *et al*<sup>[1]</sup> recently reported that a Garcinia cambogia extract (GC, 1%, w/w) fed to C57BL/6J mice in conjunction with a high-fat diet (HFD, 45 kcal% fat) for 16 wk protected against “HFD-induced obesity by modulating adipose fatty acid synthesis and  $\beta$ -oxidation but induces hepatic fibrosis, inflammation and oxidative stress<sup>[1]</sup>.” A review of this article in light of other published research on (-)-hydroxycitric acid (HCA), the active component in GC, raises a number of questions. The most significant



are these: what was the form of HCA used (not indicated) and is the toxicity reported induced by HCA *per se* or, instead, was it caused by the source of HCA/GC tested? These issues are particularly acute inasmuch as the results of Kim *et al*<sup>[1]</sup> are at variance with numerous published studies involving both animals and humans, several of which indicate that HCA actually exerts a protective effect upon the liver.

Differences among various sources of HCA can be quite significant, yet against copious evidence, it too often is assumed that all sources of HCA are the same in terms of physiology. In the study in question, the tested compound is not fully identified. The only information provided is that the tested compound provided “1%, w/w, 60% hydroxyl citric acid” and was provided by Newtree Inc. of the United States (although Newtree appears to be a South Korean company). Whether this material was stabilized with calcium, potassium, sodium, *etc.* or some mixture of these is not revealed. Similarly, no information is provided on the free acid or lactone content, whether the extraction process is novel or established, the amount of residual toxins, such as chloride ion, left in the extract, and so forth and so on. For instance, some HCA calcium salts contain up to approximately six percent halogenated compounds due to improper processing of starting materials that had been dried with the help of sodium chloride—is the material used by Kim *et al*<sup>[1]</sup> one of these?

That the nature of the HCA-containing source is important was made clear years ago in a critical analysis of another study that purported to demonstrate toxicity, in that particular case, testicular toxicity, at high dosages. This was a study by Saito *et al*<sup>[2]</sup>. When examined closely by Burdock *et al*<sup>[3]</sup>, it was determined that the HCA salt tested was very unusual in that it contained a high lactone content and that the weight loss results were not typical of literature on HCA. In this case, the particular animal model also turned out to have been inappropriately chosen. Hence, in an instance of supposed testicular toxicity, there were unacceptable levels of uncertainty about the compound being tested. Moreover, various aspects of the study design and its assumptions proved to be questionable.

Kim *et al*<sup>[1]</sup> remind the reader of the “potential for hepatotoxicity of hydroxycut, a formulation that contains GC among other ingredients<sup>[1]</sup>.” Not mentioned is the fact that after almost two decades of free sale of HCA products, there appear to be no reports of human liver toxicity aside from those involving Hydroxycut and only 8 out of 14 of the Hydroxycut formulas associated with liver toxicity even contained HCA/GC! The safety, including liver safety, of HCA as relates to Hydroxycut and other products was evaluated at length by Stohs *et al*<sup>[4]</sup> and no evidence of toxicity was found. In retrospect, the common denominator in these cases appears to be green tea extracts. Animal models have established the hepatotoxicity of high oral doses of (-)-epigallocatechin-3-gallate<sup>[5]</sup>. Reviews of human usage strongly suggest a causal association, albeit an idiosyncratic one, between green tea consumption and liver damage<sup>[6]</sup>. In contrast, quite a number of reviews

have affirmed that HCA is extremely safe. These include Chuah *et al*<sup>[7]</sup>, Márquez *et al*<sup>[8]</sup> and Stohs *et al*<sup>[9]</sup>.

Published studies involving both animal models and humans indicate that HCA *per se* is either neutral with regard to the liver or actively protective. For instance, GC in a rat model has been tested against toxic challenge to the liver by both ethanol and dexamethasone. Mahendran and Devi<sup>[10]</sup> demonstrated that GC supplementation was sufficient to prevent undesirable changes in the lipid profile on dexamethasone administration and also to protect normal liver phospholipid levels. Likewise, when rats were challenged with ethanol to induce peroxidation damage to the liver, Devi and Mahendran<sup>[11]</sup> found “co-treatment of the rats with *Garcinia cambogia* significantly inhibited the rise in lipid levels and also the peroxidative damage caused by ethanol, which is evident from the improved antioxidant status. The levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase were maintained at near normalcy in *Garcinia cambogia* treated rats”. Similar protection was provided by GC with regard to liver superoxide dismutase, catalase and various glutathione compounds.

The study of Clouatre and Preuss, which lasted eight weeks and involved high-fat/high-sugar diets in rats, found results that are in line with those of Mahendran and Devi<sup>[10-12]</sup>. In healthy and relatively young animals, HCA treatment compared with control led to strong trends towards reduced CRP and tumor necrosis factor- $\alpha$  without exerting significant effects on ALT and AST. These results are similar to those found in a formal safety assessment of a commercial potassium-calcium hydroxycitrate salt (60% HCA) by Soni *et al*<sup>[13]</sup>, which was designed, in part, specifically to look for potential hepatotoxicity. The gavage administration of this salt at doses up to 2500 mg/kg per day for a period of 90 d did not lead to any significant adverse effects, including in the histological examinations of the livers of the test and control arms. Given the high dosage of HCA and the time frame comparable to that of Kim *et al*<sup>[1]</sup>, the results of the study by Soni *et al*<sup>[13]</sup> in the rat rodent model clearly are at variance with the findings in the mouse model and in line with other research reports of HCA's safety. The human equivalent dosage of HCA in the Soni study is approximately 30 g in a 60 kg individual.

There have been at least four published studies of the safety of HCA in humans and these trials reached the same conclusion confirming the safety of the oral consumption of HCA salts. The studies are those of Hayamizu *et al*<sup>[14]</sup>, Hayamizu *et al*<sup>[15]</sup>, Ishii *et al*<sup>[16]</sup> and Hayamizu *et al*<sup>[17]</sup>. None of these studies found any significant adverse effects on the liver. Hayamizu *et al*<sup>[14]</sup> in a study lasting three months, but involving only 1000 mg HCA per day (*i.e.*, 1666 mg of a 60% salt) found no significant change in any liver parameter. Hayamizu *et al*<sup>[16]</sup> found no observed adverse effects 4000 mg HCA per day for ten days and Hayamizu *et al*<sup>[17]</sup> found no adverse effects at 3000 mg HCA for 30 d.

Finally, the issue of GC and inflammation needs to be

addressed more generally. Clouatre *et al*<sup>[18]</sup> were the first researchers to discover that HCA consumption relieves a number of markers of inflammation and this information was confirmed in Clouatre *et al*<sup>[12]</sup>. A number of recent studies now have established these findings regarding HCA and inflammation. A study using rats performed by dos Reis *et al*<sup>[19]</sup> found that the “antiinflammatory effects provided by the *Garcinia cambogia* extract result in an improvement of several parameters analysed (sic) in experimental colitis and could provide a source for the search for new antiinflammatory compounds useful in inflammatory bowel disease treatment.” Similar protective effects have been found by Amin *et al*<sup>[20]</sup> in relation to a high fat diet, metabolic disturbances and brain oxidative dysfunction and by Amin *et al*<sup>[21]</sup> in relation to renal oxidative stress on a high fat and high sucrose diet.

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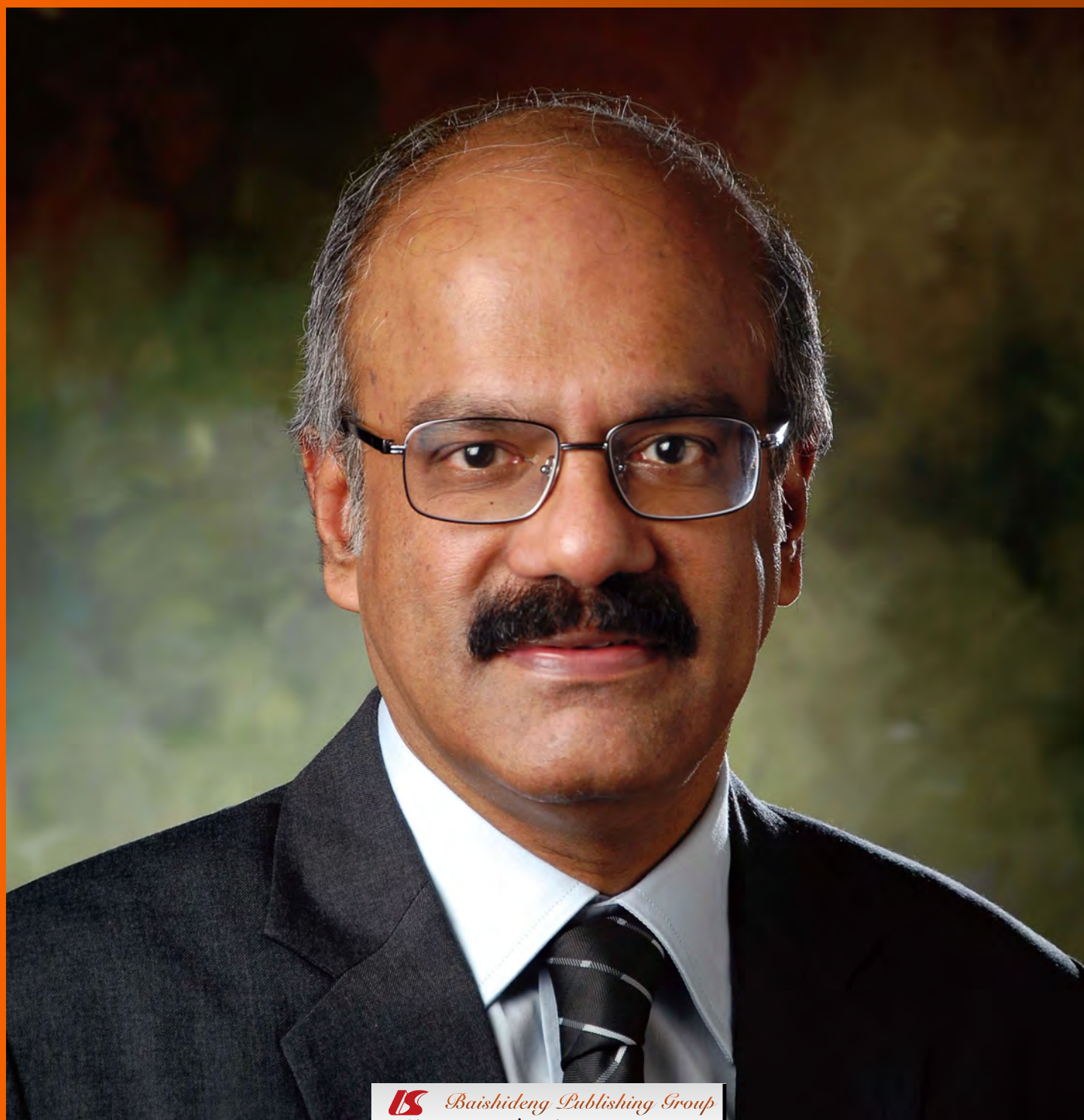
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## Molecular classification of colorectal carcinomas: The genotype-to-phenotype relation

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found in industrialized countries. At present only a minority of CRCs are characterized by a strong genotype-to-phenotype relation. This is due to several additional factors determining phenotype expression. In conclusion, molecular characterization (genotype) is essential to interpret the histological findings (phenotype) and to identify prognostic groups as well as patients for targeted therapy.

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### Abstract

Colorectal carcinomas (CRCs) are frequently found in industrialized countries and lead to a high incidence of malignancy-related mortality. Defined by histomorphological features, CRCs and their pre-invasive lesions are quite heterogeneous. The underlying molecular mechanisms include genomic instability, genomic mutation of tumor suppressor genes or oncogenes, epigenetic changes, and the microRNA network. The molecular mechanisms are guided by repeated clonal selections. The genotype-to-phenotype relation is assumed to be the great challenge of cancer research and the development of effective targeted therapies. At present a strong genotype-to-phenotype relation is characterized only for a minority of CRCs. Consequently, the molecular characterization of CRCs is essential to interpret histological patterns and to identify prognostic groups as well as patients for targeted therapy.

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**Key words:** Carcinogenesis; Colorectal cancer; Molecular pathology

**Core tip:** Colorectal carcinomas (CRCs) are frequently

### INTRODUCTION

Colorectal carcinoma (CRC) is frequently found in industrialized countries and is a leading cause of cancer-related death<sup>[1]</sup>. It is suggested by several studies that the genotype-to-phenotype relation remains the great challenge of basic cancer research<sup>[2,3]</sup>. The important development of DNA sequencing technologies enables clinicians and scientists to perform assessment through to full mutation analysis of CRCs. This approach makes it clear that the number of genetic aberrations in tumor cells is highly variable, including tumors with more than 80 mutations as well as carcinomas harboring fewer than 10 mutations<sup>[4,5]</sup>. However, in addition to cancer-related mutations, several other genetic and epigenetic mechanisms contribute to CRC heterogeneity. This point of view implies that the genotype-to-phenotype relation is more complex than previously assumed and is not only founded by mutations. The basic molecular events determining and guiding development of histologically defined CRC phenotypes (adenocarcinomas and non-glandular variants classified by the World Health Organization) are not elucidated. At present, several histomorphological tu-

mor variants can be distinguished, but specific molecular characteristics reflecting the histotype only exist in small subgroups. For example, microsatellite instability (MSI) is frequently found in mucinous adenocarcinomas and some signet ring cell carcinomas, but not in all tumors of these classes. In addition, microsatellite stability and cytosine-phosphate-guanine (CpG) island hypermethylation are usually, but not always, found in intestinal cribriform comedo-type adenocarcinomas. Because of the molecular heterogeneity of CRCs, identification of basic principles of carcinogenesis has been studied in hereditary CRCs where distinct molecular events were characterized. Familial adenomatous polyposis and hereditary non-polyposis colon cancer (HNPCC) are the most important syndromes that account for the vast majority of hereditary CRCs.

At present, the combination of multiple genetic alterations and clonal selections modified by lifestyle and environmental factors is recognized as the driving force in colorectal carcinogenesis<sup>[6]</sup>. The imbalance between mutation development and cell-cycle control further contributes to tumor development. In the review, major classes of molecular events targeting colorectal carcinogenesis are detailed concerning the genotype-to-phenotype relation.

## GENOMIC INSTABILITY

Genomic instability (GIN) is determined by separate molecular phenomena and describes loss of mutation control by the cell-cycle<sup>[1]</sup>. The important molecular events/pathways of GIN are chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and MSI. In the following, these main pathways are further detailed.

### CIN

The molecular mechanisms underlying CIN, the most common type of GIN, include chromosome rearrangements, sequence changes, chromosomal number alterations, and chromosomal segregation defects. Loss of 18q with the deletion of genes such as *SMAD2*, *SMAD4*, or *DCC*, which is found in up to 70% of primary CRCs, is a common molecular finding in CIN-related tumors<sup>[7]</sup>. The carcinomas almost always have a mutation in the *APC*, while *KRAS* mutations occur in about 50%. CIN-related molecular lesions are found in dysplastic crypt foci. However, it has not been clear up to now whether CIN is a cause or a consequence of malignant cell growth<sup>[1,7]</sup>. It is suggested that CIN acts as a molecular founder and promoter of neoplastic growth.

CIN-related CRCs demonstrate no characteristic histomorphological pattern. They differ in tumor grading, occurrence of necrosis, and accumulation of extracellular mucin. The putative molecular founder event/mutation for the intestinal phenotype of CRCs has not been characterized up to now.

### MSI

MSI is found in up to 15% of so-called sporadic CRCs

and in almost all HNPCC (Lynch syndrome) associated CRCs due to either somatic inactivation of both alleles or an inherited germline mutation to one allele with additional somatic inactivation of the other<sup>[8]</sup>. A mismatch repair function usually corrects deletion/insertion errors during DNA replication. In MSI, sequence corrections resulting in alleles of varying length are not performed. The differences in length are diagnostic in PCR-based strategies using consensus primer panels. In standardized panels for MSI testing, two mononucleotides (BAT25 and BAT26) and three dinucleotide microsatellites (D5S346, D2S123, D17S250) were used<sup>[9]</sup>. MSI CRCs are not usually associated with mutations in *KRAS* or *TP53*. However, genes containing simple repeats such as *EGFR*, *BAX*, and *TGFβRII* are often mutated in these tumors. The *BRAF* status is another variable in MSI CRCs and a prognostic factor. Disease-free survival and overall survival are significantly improved in patients with MSI and non-mutated *BRAF*<sup>[10]</sup>. MSI CRCs do not have chromosomal abnormalities.

On microscopic examination, MSI CRCs are often poorly differentiated, containing mucinous components, have intratumoral lymphocytes, displaying Crohn's like inflammatory response near to the tumor edge, and are plump infiltrative. These morphological features are variably expressed and sometimes absent in MSI CRCs.

### CIMP

The CIMP was originally grouped together with MSI tumors. The islands are CpG rich regions within the genome and especially found in promoter sequences. In carcinogenesis, methylation of CpG islands (so-called type C methylation) leads to transcriptional silencing of genes involved in tumor suppression, apoptosis, DNA repair, and cell-cycle control<sup>[11]</sup>. Genes that are frequently affected by this non-covalent epigenetic modification are *p16*, *MGMT*, and *bMLH1*. The age-related methylation of genes is designated as type A methylation. Based on molecular data, different subgroups of CIMP CRCs are defined. CIMP1 includes carcinomas with frequent MSI and *BRAF* mutation, whereas CIMP2 refers to microsatellite stable carcinomas with a high frequency of *KRAS* mutations. Microsatellite stable carcinomas with frequent *TP53* mutations are commonly CIMP negative<sup>[12]</sup>.

Clinically, CIMP CRCs are commonly found in a proximal location and often have methylation of the *bMLH1* mismatch repair gene. However, over 50% of the CIMP CRCs are microsatellite stable. In general, CIMP CRCs have a poor prognosis and are associated with mutations in *KRAS* and/or *BRAF*. The histological phenotype of CIMP CRCs is not well characterized or defined. In these carcinomas a poor degree of histomorphological differentiation is frequently found reflecting some aspects of MSI. However, despite methylation of the *bMLH1* mismatch repair gene, histomorphological MSI-related histological features are not fully expressed in CIMP CRCs.

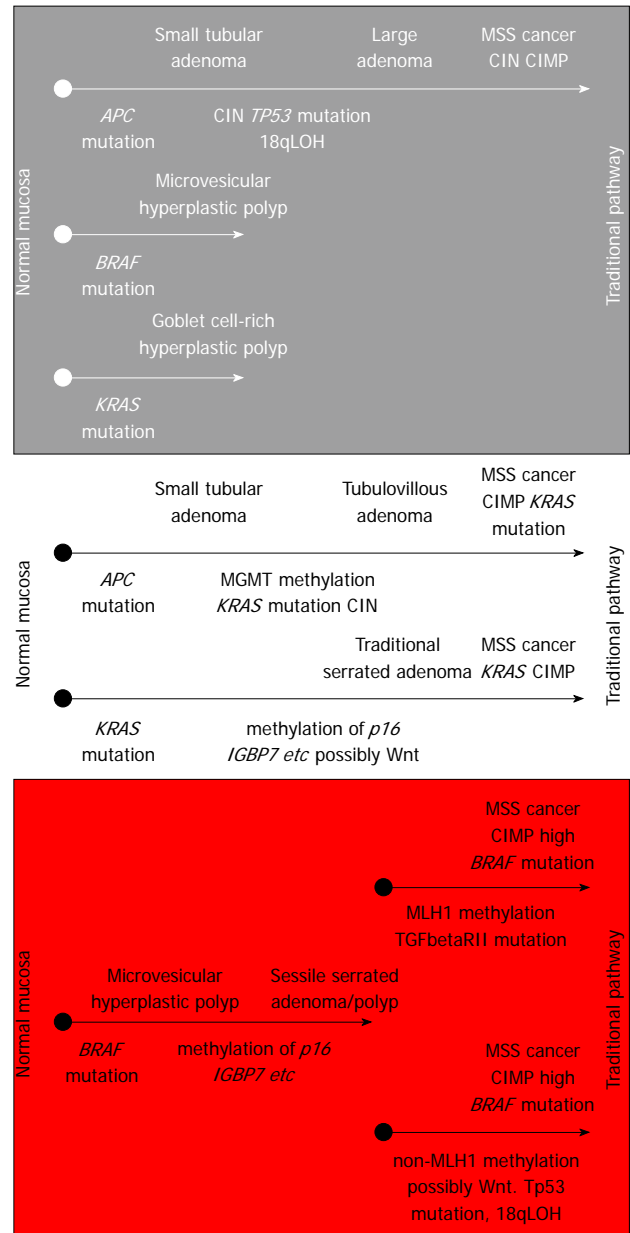
## SERRATED NEOPLASTIC PATHWAY

It has been recognized that about 20% of CRCs arise from a distinct pathway including special molecular and histomorphological features<sup>[1]</sup>. The so-called serrated pathway is associated with a sequence of genetic and epigenetic alterations. Activating mutations of the *BRAF* gene, coding a pro-proliferative, anti-apoptotic serine-threonine kinase, are an early event. The anti-apoptotic *BRAF* function and probably additional failures in the apoptotic pathway of enterocytes are assumed to be crucial in the establishment of serration, where an accumulation of cells is found. The *BRAF* associated proliferative burst is probably followed by up-regulation of *p16INK4a*, acting as a tumor suppressor, and increased secretion of an insulin-like, growth-factor-binding protein 7 (IGFBP7). Silencing of either *p16INK4a* or *IGFBP7* CIMP sensible cells *via* methylation is proposed as essential in the progression to sessile serrated adenoma/polyp (SSA/P)<sup>[13]</sup>.

The phenotypes of serrated polyps vary considerably and the entity mixed polyp reflects the considerable overlap among these lesions<sup>[14]</sup>. SSA/P constitutes about 20% of all serrated polyps and is morphologically defined by the elongation of serrated crypts and distortion of the proliferative zone<sup>[15]</sup>. Several crypts display a dilated, L-shaped, inverted T-shaped or anchor-shaped morphology with excess serration at the crypt base. Progression of SSA/P is associated with the occurrence of cytological dysplasia and development of invasive adenocarcinoma. SSA/P and related adenocarcinomas are preferentially found in the right hemicolon.

Traditional serrated adenomas (TSA) are morphological variants of serrated adenomas and show considerable differences from SSA/P concerning mutation (*KRAS* mutation in about 25%), localization (left-sided), and methylation status (increased methylation, but not methylation of *MLH1*)<sup>[16]</sup>. In terms of histomorphology, TSAs display an overall complex, often filiform configuration with tall columnar cells, eosinophilic cytoplasm, a centrally placed, elongated, hyperchromatic nucleus, and the formation of so-called ectopic crypts (the relationship of crypts with the adjacent muscularis mucosae is not preserved). The filiform serrated adenoma may represent a TSA subset. Conventional adenomas with serrated architecture are defined as an additional subgroup in the alternative serrated neoplastic pathway<sup>[1]</sup>. These morphologically defined entities are reflected by molecular findings and the model of an alternate pathway in colorectal carcinogenesis<sup>[13]</sup>. Important pathways in colorectal carcinogenesis are summarized in Figure 1.

Given the malignant potential of serrated polyps, two important serrated pathways of colorectal carcinogenesis were characterized: (1) sessile serrated pathway; and (2) traditional serrated pathway. The resulting serrated adenocarcinoma has architectural similarity to a SSA/P that may be accompanied by additional morphological features including trabecular and mucinous areas. However, these CRCs can have *MSI-L* or *MSI-H*, *BRAF*- or *KRAS*-



**Figure 1** Three important molecular pathways in colorectal carcinogenesis are characterized at present. In these models only a basal genotype-to-phenotype relation is given. CIN: Chromosomal instability; CIMP: Cytosine-phosphate-guanine island methylator phenotype.

mutations, and CIMP<sup>[13,14]</sup>. Given the molecular heterogeneity of serrated adenocarcinomas, a strong genotype-to-phenotype relation is not well established at present.

## ADDITIVE MOLECULAR PATHWAYS

In addition to the molecular mechanisms and pathways in colorectal carcinogenesis detailed above, several other molecular events have been characterized<sup>[1]</sup>. These molecular lesions include mutational inactivation of tumor suppressor genes such as *APC*, *TP53*, and *TGF-beta*, activation of oncogene pathways driven by the RAS-RAF-MAPK or the PI3K-Akt signaling, injury of the miRNA network (common changes in CRCs are: up-regulation

of miR-31, miR-183, and miR-17-5; down-regulation of miR-143, and miR-145), and epigenetic changes such as histone modification<sup>[1,17-19]</sup>. Experimental data describing and characterizing the molecular network behind colorectal carcinogenesis are continuously growing and should give more insight into the genotype-to-phenotype relation.

## CONCLUSION

CRC is a heterogeneous disease and a leading cause of cancer-related mortality. At present, a strong genotype-to-phenotype relation, which is assumed to be the great challenge of cancer research and the development of effective targeted therapies, is only defined in a small number of CRC variants. Nevertheless, the molecular understanding of key events and modifying pathways in colorectal carcinogenesis has been essentially improved through CRC classification and therapeutic regimes in molecular terms. However, the scientific progress in the molecular understanding of CRCs calls the paradigm of a strong genotype-to-phenotype relation into question. Factors that govern the expression of pathogenic mutations include genomic aberration in a heterozygote background, the network of products from mutant and wild type genes, and environmental factors. In summary, the molecular characterization of CRCs is essential to interpret histological patterns and to identify prognostic groups as well as patients for targeted therapy.

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## WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

# How antibiotic resistances could change *Helicobacter pylori* treatment: A matter of geography?

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some regimens are limited by their use in very small geographic districts. Moreover, not all therapeutic trials have considered bacterial and host factors affecting the therapeutic outcome. The additional use of probiotics may help to reduce adverse events, but their therapeutic impact is doubtful. In conclusion, the "ideal therapy", paradoxically, appears to be a "utopia", despite the unprecedented volume of studies in the field and the real breakthrough in medical practice made by the discovery and treatment of *H. pylori*. The ample discrepancies observed in the different areas do not encourage the development of therapeutic guidelines that could be valid worldwide. On these bases, one of the main challenges for the future might be identifying a successful solution to overcome antibiotic resistances. In this context, geography must be considered a relevant matter.

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**Key words:** *Helicobacter pylori*; Antibiotic resistance; Geography; Therapeutic regimens; Therapeutic outcome

**Core tip:** The present topic outlines the main data regarding antibiotic resistances, paying particular attention to the discrepant results obtained in different geographic areas worldwide, and even in the same districts. Discordances between *in vitro* and *in vivo* studies are detailed and the possible factors explaining this phenomenon are analyzed. Finally, the challenge for the future of devising a successful solution to overcome antibiotic resistances is highlighted, and geography is suggested as a relevant matter.

## Abstract

Therapeutic management of *Helicobacter pylori* (*H. pylori*) remains an unsolved issue. Indeed, no therapeutic regimen is able to cure the infection in all treated patients, and in many the infection persists despite the administration of several consecutive standard therapies. Although antibiotic resistance reports describe alarming results, the outcome of therapeutic regimens does not seem to parallel this scenario in most cases, since a successful performance is often reached in more than 80% of cases. However, the phenomenon of increasing antibiotic resistance is being closely studied, and the results show controversial aspects even in the same geographic area. For the continents of Europe, America, Asia, Africa, and Oceania, minimal and maximal values of resistance to the main antibiotics (clarithromycin, amoxicillin, metronidazole, and levofloxacin) feature wide ranges in different countries. The real enigma is therefore linked to the several different therapeutic regimens, which show results that often do not parallel the *in vitro* findings even in the same areas. A first aspect to be emphasized is that

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) eradication has undoubted benefits. The bacterium's diffusion worldwide, even if it is decreasing, especially in developed countries, still dictates the treatment of all infected subjects, and the possible legal implications that denial of treatment could have must also be considered. In Table 1, epidemiological studies of the last five years from five continents of the world (Europe, America, Asia, Africa, and Oceania) are reported<sup>[1-14]</sup>. Different infection rates depend on the type of study population (pediatric, adult, or geriatric patients), since increasing age, as well as poor hygienic environmental factors, are well known to have a strong influence. Arresting the bacterium's diffusion still has not been achieved.

Treatment of the infection nowadays is both a simple and, at the same time, complex problem. Alongside the conventional first-line regimen (triple therapy), others have been proposed (sequential, concomitant, quadruple, and miscellaneous) to face the growing problem of antibiotic resistance. For no other infection have so large a number of therapeutic proposals been reported by different working groups. The results, however, often appear conflicting and the same regimen may be extremely effective in one geographic area and deliver disappointing results in another. Finally, although many experts believe that there is no such thing as untreatable *H. pylori*, only ill-treated *H. pylori*, no clinical trial has yielded a successful 100% eradication. We must therefore conclude that "the infallible therapy" does not exist at present. Therefore, the aim of this review is to analyze the different results of therapeutic schemes in different geographic regions, as well as their relationship with the diffusion of antibiotic resistances in the same areas.

## ANTIBIOTIC RESISTANCES

*H. pylori* antibiotic resistances are defined as primary (no previous treatment for bacterium eradication) and secondary (a susceptible strain acquires resistance during a treatment)<sup>[15]</sup>. The main reason for this phenomenon is point mutations of *H. pylori* DNA, often associated with inappropriate antibiotic use<sup>[15]</sup>. Heteroresistance is a condition characterized by the coexistence of susceptible and resistant strains in the same patient. Resistances are currently detected by culture-based and molecular methods. Different tests for both techniques have been described. The main culture-based techniques are the agar dilution method, *E*-test, breakpoint susceptibility test, and modified disk diffusion method. Molecular techniques include polymerase chain reaction (PCR), restriction fragment length polymorphism, allele-specific

PCR, sequencing, real-time PCR, and fluorescent *in situ* hybridization<sup>[16-22]</sup>. Although these tests require sophisticated tools, their performance before starting a treatment for *H. pylori* will undoubtedly significantly improve the therapeutic outcome. However, this strategy is hard to apply in clinical practice owing to the long period necessary before obtaining results, as well as the high costs of routine performance of such methods.

## GEOGRAPHIC DIFFERENCES IN ANTIBIOTIC RESISTANCES

A large number of reports from different geographic areas are available in the literature, showing heterogeneous results. Firstly, we will consider the geographic rates of resistance to each antibiotic, thus underlining differences and consequent clinical implications.

### Clarithromycin

Clarithromycin is a drug that belongs to the macrolide family, and its mechanism of action is the inhibition of protein synthesis by binding and slowing down the activity of the bacterial ribosomal unit<sup>[23]</sup>. The mutations that may cause resistances are point mutations in the 23S rRNA component of ribosomes<sup>[24]</sup>: the three most frequent mutations that may occur are A2143G, A2142G, and A2142C, which are responsible for 90% of cases of primary clarithromycin resistance in *H. pylori* strains isolated in Western countries<sup>[25,26]</sup>. In particular, the A2143G mutation has a much stronger impact on conferring resistance than the other two<sup>[27]</sup>. In Eastern countries (*e.g.*, South Korea) additional mutations, such as T2183C and A2223G, have been frequently found to justify the observed clarithromycin resistance, while A2143G accounted only for 23% of resistant strains<sup>[26]</sup>. This finding, if confirmed in other Asian countries, suggests that point mutations inducing clarithromycin resistance might differ in this continent as compared to those in Europe and North America. In conclusion, resistance to clarithromycin is considered as the cause of most eradication regimen failures<sup>[28]</sup>, as its prevalence is continuously increasing. Moreover, new point mutations have also emerged in South America<sup>[29]</sup>. However, isolated reports may not reflect the real scenario; therefore, it would be more useful to consider epidemiological data about resistance region by region.

In Eastern Asian countries, very high clarithromycin resistances have been recorded. The highest rate of resistance was found in Japan (86.4%) in a study on the efficacy of this antibiotic in a third line regimen<sup>[30]</sup>. Interestingly, in the same country, a lower percentage of 15.2% was detected in another study of first line therapy<sup>[31]</sup>. In any case, in every region of the Far East, the rates of resistance were at least 15%, a percentage that often determines the failure of clarithromycin-based therapy. In another two Japanese studies the rates were 32.4%<sup>[32]</sup> and 18.9%<sup>[33]</sup>, respectively. In China, resistances ranged between 21.5% and 23.8%<sup>[34,35]</sup>, while in Viet-

**Table 1** Epidemiological studies of the last five years from five world continents regarding *Helicobacter pylori* infection prevalence

Country	Ref.	<i>Helicobacter pylori</i> positivity	Test	Population
Southern Europe				
Italy	Dore <i>et al</i> <sup>[1]</sup> , 2012	13.3%	Serology	Children
Portugal	Bastos <i>et al</i> <sup>[2]</sup> , 2013	84.2%	Serology	Adults
Northern Europe				
Sweden	Thjodleifsson <i>et al</i> <sup>[3]</sup> , 2007	11%	Serology	Adults
Norway	Bakkevold <sup>[4]</sup> , 2010	51%	Serology	Adults
Eastern Europe				
Czech Republic	Bures <sup>[5]</sup> , 2012	23.5%	Urea breath test	Combined data
Russia	Svarval' <i>et al</i> <sup>[6]</sup> , 2011	40.48%	Serology	Children-adolescents
America				
United States	Patterson <i>et al</i> <sup>[7]</sup> , 2012	17.1%	Serology	Adults
South America (combined data)	Porras <i>et al</i> <sup>[8]</sup> , 2013	79.4%	Urea breath test	Adults
Asia				
Saudi Arabia	Hanafi <i>et al</i> <sup>[9]</sup> , 2013	28.3%	Serology	Children, adolescents
South Korea	Baik <i>et al</i> <sup>[10]</sup> , 2012	55.7%	Serology	Adults
Africa				
Nigeria	Etukudo <i>et al</i> <sup>[11]</sup> , 2012	30.9%	Serology	Children
Morocco	Benajah <i>et al</i> <sup>[12]</sup> , 2013	75.5%	Biopsy	Adults
Oceania				
Australia	Pandeya <i>et al</i> <sup>[13]</sup> , 2011	15.5%	Serology	Combined data
New Zealand	Fawcett <i>et al</i> <sup>[14]</sup> , 2005	6.2%	Serology	Children, adolescents

nam they were considerably higher (33%)<sup>[36]</sup>. A South Korean study revealed, in a pediatric population, tripled resistance rates within 20 years. Surprisingly, a Malaysian study carried out on 90 gastric samples did not show any strain resistant to clarithromycin: this is the only discordant value in this geographical area<sup>[37]</sup>.

A similar pattern is observed in the Near East, where the percentages range between 14.3% (Iran)<sup>[38]</sup> and 37% (Pakistan)<sup>[39]</sup>. Interestingly, it is noticeable that in the same area, another study found a much lower resistance (17.1%)<sup>[40]</sup>, emphasizing that even in the same geographical region, relevant differences may occur.

In Southern America, the most recent studies report a resistance rate that ranges between 13.6% and 19.5%, illustrating a more homogeneous distribution of resistant strains<sup>[41,42]</sup>. In the United States the continuous migration flows from Mexico and other Latin American countries are causing rapid changes in local *H. pylori* strains, as witnessed by the need for new regimens<sup>[43]</sup>. If we consider that in a 2011 randomized study the success rate of a therapy including levofloxacin, amoxicillin, and clarithromycin was only 73.3%<sup>[44]</sup>, whereas in 1995 a clarithromycin-based therapy achieved eradication in more than 90%<sup>[45]</sup>, this is a remarkable difference.

In Europe, there are ample variations between Northern Europe and Mediterranean countries, where the resistances to clarithromycin are considerably more widespread. Finland and Sweden recorded a rate of 2% and 1.5%<sup>[46,47]</sup>, respectively, whilst in Germany and Norway the rates were 7.5% and 5.9%, respectively<sup>[48,49]</sup>.

Resistance is higher in Central/Eastern Europe [9.3% (95%CI: 0-22)], and is at its highest in Southern Europe [18% (95%CI: 2.1-34.8)]<sup>[50]</sup>. In Italy, the trend is continually on the increase; while in the year 2000 the percentages ranged between 1.8% and 14%<sup>[51-53]</sup>, a few years later resistance had increased up to 24.1%, and will likely have

doubled within 15 years<sup>[54,55]</sup>. As in all of Europe, primary clarithromycin resistance in Italy is highly variable in different geographic areas: 0%-6% in the north, 7%-15% in central areas, and 10%-25% in the south<sup>[56]</sup>. Other Mediterranean countries with high rates of clarithromycin resistance are Greece (40%), Spain (15%-20%), France (17.5%), and Portugal (34.7%)<sup>[57-60]</sup>. In Eastern Europe, the situation is similar to that in Southern Europe; several Bulgarian studies have reported a resistance rate of 18.4%-23.4%<sup>[13,61,62]</sup>. An isolated phenomenon has been observed in Poland; in 2013, resistance is around 22%, with a trend toward a perceptible decrease (it was 34% in 2008)<sup>[63]</sup>. Another strong diffusion of resistant strains has been shown in Lithuania (24.7%)<sup>[64]</sup>.

In Oceania, resistance rates range between 8.7%<sup>[65]</sup> and 15.7%<sup>[66]</sup>, suggesting that there is still a fair option for the use of the antibiotic in those areas.

Finally, the main data about the prevalence of resistance to clarithromycin in Africa show very low values in Gambia and Senegal (0% and 1%, respectively)<sup>[67,68]</sup>, but not in South Africa (15.3%)<sup>[69]</sup>.

### Metronidazole

Metronidazole is a nitroimidazole antibiotic used particularly against anaerobic bacteria and protozoa. It works as a pro-drug: it is non-enzymatically reduced by reacting with reduced ferredoxin, which is generated by pyruvate oxidoreductase, and then the reduced molecule is taken up into bacterial DNA and forms unstable molecules that cause the death of the organism<sup>[70]</sup>. The resistance mechanism to metronidazole is not entirely straightforward<sup>[71,72]</sup>. Clearly, alterations of the *rdxA* gene are of primary relevance, but it has not been possible to identify a clear panel of point mutations which could explain the phenomenon. Moreover, other genes such as *frxA* seem to be involved<sup>[73]</sup>.



Eastern Asian countries are the geographical area where it is possible to detect the highest percentages of resistance: 56.6%-95.4% in China<sup>[34,35]</sup>, 57% in Japan<sup>[30]</sup>, 27.3%-52.9% in South Korea<sup>[74,75]</sup>, 69.9% in Vietnam<sup>[36]</sup>, and 75.5% in Malaysia<sup>[37]</sup>. A very similar pattern has been detected in Africa, where the rates vary between 68.8% and 85%<sup>[67,68]</sup>. Indeed, it is well known that the prevalence is much higher in developing countries (50%-80%) like Mexico (76.3%)<sup>[76]</sup>, Colombia (75.5%)<sup>[41]</sup>, and Brazil (40%)<sup>[42]</sup>. In the Near East, a 69.5% resistance was reported in Saudi Arabia<sup>[77]</sup>, 64.5% in Pakistan<sup>[40]</sup>, and 76.8% in Iran<sup>[38]</sup>.

The scenario appears to be slightly different in Europe, where in a multicenter study the global resistance rate to metronidazole was 33.1% (95%CI: 7.5-58.9), with no significant difference between the north [33% (95%CI: 7.1-69.2)] and south [40.8% (95%CI: 27.3-54.3)], but with a significantly lower prevalence in central and eastern areas [29.2% (95%CI: 17.9-41.5)]<sup>[78]</sup>. However, this report was dated 2001, and in the last ten years the situation appears to have changed surprisingly, featuring a decreasing rate of resistance in northern countries (22.5% in Norway<sup>[49]</sup>, 1.1% in Lithuania<sup>[63]</sup>, 19.9% in the Netherlands<sup>[79]</sup>, and 13% in the United Kingdom<sup>[51]</sup>). In Southern and Central-Eastern Europe however, rates of resistance are much higher (34.9% in France<sup>[59]</sup>, 63.6% in Croatia<sup>[80]</sup>, 37.2% in Germany<sup>[48]</sup>, and 23.3% in Bulgaria<sup>[15]</sup>).

Several Italian studies<sup>[81]</sup> have described a resistance rate of 20%-23.9%<sup>[82-84]</sup>, but the resistant strain could be undergoing a dizzying growth, if we consider that only five years later a single group reported a more than doubled rate (59.3%)<sup>[85]</sup>.

In Oceania, three recent studies reported values of 20%<sup>[86]</sup>, 36%<sup>[87]</sup>, and 43.5%<sup>[65]</sup>, suggesting an overall high rate of resistances.

### Amoxicillin

Amoxicillin is a  $\beta$ -lactam antibiotic included in all current therapeutic regimens for *H. pylori* eradication<sup>[88]</sup>. Amoxicillin acts by interfering with peptidoglycan synthesis, in particular by blocking transporters named penicillin binding proteins<sup>[16]</sup>. This drug was the first antibiotic used for *H. pylori* therapy due to a presumed absence of resistance<sup>[89]</sup>. In almost all studies, percentages of resistance are quite low, and these data seem homogeneous worldwide. No resistances have been detected in Croatia, France, Germany, the Netherlands, Portugal, Spain, or Sweden<sup>[90-96]</sup>. In Italy, resistances range from 0% to 0.2%<sup>[97]</sup>, while in the United Kingdom they range from 0% to 0.4%<sup>[98,99]</sup>. Almost negligible resistances have also been reported in America and Oceania. Data in contrast with this trend have been described in Iran and Japan, with a resistance prevalence of 28.6%<sup>[38]</sup> and 8.2%-15.2%<sup>[30,33]</sup>, respectively. Surprisingly, an extremely high resistance rate (85.6%) has been observed in Cameroon<sup>[81]</sup>.

### Levofloxacin

Levofloxacin is a broad spectrum antibiotic of the

fluoroquinolone drug class which is active against both Gram-positive and Gram-negative bacteria<sup>[100,101]</sup>. It acts by inhibiting DNA gyrase, type II topoisomerase, and topoisomerase IV, an enzyme which is necessary to separate replicated DNA and block cell division<sup>[102]</sup>. Resistance of *H. pylori* to fluoroquinolones is due to point mutations in the quinolone resistance determining regions of gyrA<sup>[103]</sup>.

Levofloxacin has recently appeared in therapeutic regimens for *H. pylori* eradication: in the Maastricht-Florence IV consensus for *H. pylori* treatment, a levofloxacin-containing regimen was proposed as second-line treatment when classical first-line therapy containing clarithromycin failed<sup>[88]</sup>. However, in the last three years, resistant strains are increasing, because of plasmid-mediated horizontally transferable genes encoding quinolone resistance<sup>[104]</sup>, so that more and more levofloxacin-based treatments will likely be ineffective in the future.

An example of this unfavorable trend is evident in Asian countries, where the rates of resistance exceed 10%: 18.4% in Vietnam<sup>[36]</sup>, 20.6% in China<sup>[34]</sup>, and are as high as 63.3% in Pakistan<sup>[39]</sup>. Only Malaysia registered 0%<sup>[37]</sup>, although Japan was also low at 8.2%<sup>[30]</sup>.

In Europe, the overall resistance to levofloxacin, detected in a recent multicentric epidemiologic study, is 14.1%<sup>[105]</sup>, with values ranging between 11.7% in Ireland<sup>[106]</sup> and 29.1% in Germany<sup>[107]</sup>: these last percentages must set the clinician on guard, if we consider that only a few years before, in 2003, a resistance rate of 3.3% was detected in France<sup>[108]</sup>. In Italy, a single study found resistance in 10.6% of strains<sup>[81]</sup>, data confirmed by a recent overview that noted a rate of 11.8% in already treated patients<sup>[109]</sup>. In Africa, resistant strains are also not very widespread: 15% in Senegal and 10.2% in South Africa<sup>[68,69]</sup>.

In America, a rate of 19% was found in Alaska<sup>[110]</sup>, while in South America percentages are higher (23% in Brazil, where a clarithromycin resistance of only 8% means that this last drug is still a good therapeutic option)<sup>[111]</sup>. Surprisingly, no report about levofloxacin resistance in Oceania has yet been made, to the best of our knowledge.

### Other antibiotics

Resistance to tetracycline is very low, or even absent, in most countries. Very low rates have been reported in Spain (0.7%<sup>[95]</sup>), the United Kingdom (0.5%<sup>[98]</sup>), and Hong Kong (0.5%<sup>[112]</sup>). Values lower than 5% are recorded in Germany<sup>[48]</sup> and Lithuania<sup>[64]</sup>. The highest prevalence rates are found in Korea (5.3%<sup>[113]</sup>), Iran<sup>[38]</sup> (18.7%), and Vietnam<sup>[36]</sup> (5.8%). The resistance mechanism has been described as a change in three contiguous nucleotides in the 16S rRNA gene (AGA 926-928RTTC)<sup>[114,115]</sup>.

Rifabutin is a bactericidal antibiotic drug primarily used in the treatment of tuberculosis, and its effect on bacteria is based on DNA-dependent RNA polymerase blockage<sup>[116]</sup>. When it was firstly used in the late '90s, the prevalence of *H. pylori* resistance to this group of antibiotics was extremely low, as these drugs were used

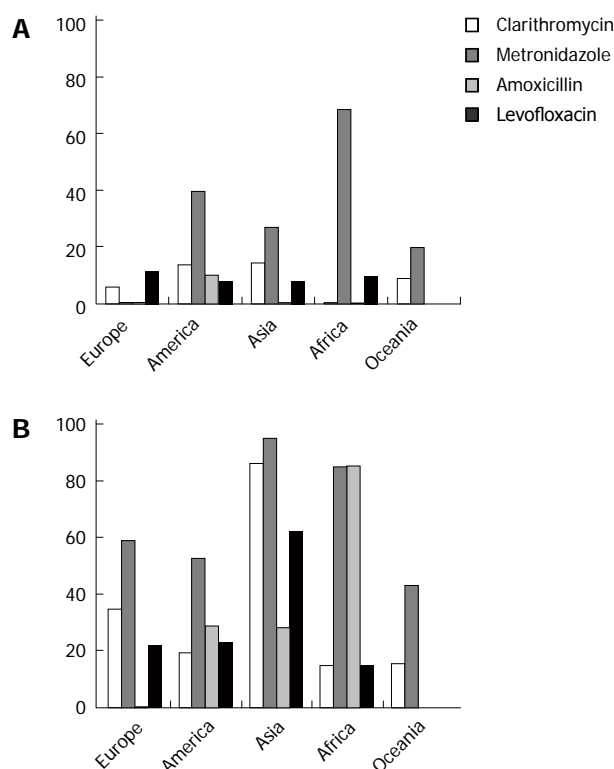


Figure 1 Comparison of the minimal (A) and maximal (B) resistance rates reported in five world continents (Europe, America, Asia, Africa, and Oceania) for the main antibiotics used for *Helicobacter pylori* eradication (clarithromycin, amoxicillin, metronidazole, and levofloxacin).

only in a limited number of patients to treat mycobacterial infections. For example, Heep *et al.*<sup>[117]</sup> did not find a single resistant strain among 81 German patients tested in 1999, nor did Fujimura *et al.*<sup>[118]</sup> among 52 strains in Japan. Even today in some regions such as Brazil, Ireland, and Senegal, no resistant strain has been detected<sup>[42,68,106]</sup>, but data from Malaysia<sup>[37]</sup>, Germany<sup>[48]</sup>, and Iran<sup>[38]</sup> report a resistance rate of 2.2%, less than 5%, and 28.6%, respectively. Resistance is due to point mutations in the *rpoB* gene, as for other bacteria, and occurs in all rifamycin drugs, suggesting a potential risk of cross-resistances between antibiotics of the same family<sup>[119,120]</sup>.

Furazolidone is a nitrofurantoin antibiotic. As a veterinary medicine, it is used to treat salmonids for *Myxobolus cerebralis* infections. In the past, it has been used in humans to treat diarrhea and enteritis caused by bacteria or protozoan infections, but since 1991 it has been recognized by the FDA as a carcinogenic agent and so is no longer used, except in a few developing countries that allow its use for human diseases<sup>[121]</sup>. It was previously used to treat traveler's diarrhea, cholera, and bacteremic salmonellosis. Its use to treat *H. pylori* infections was also proposed<sup>[122]</sup>. Primary furazolidone resistance is rare. In Spain<sup>[123]</sup>, the rates were less than 2%, and in Bulgaria 1.8%<sup>[124]</sup>. Lower values have been described in China<sup>[34]</sup> (0.1%) and Brazil<sup>[42]</sup> (no resistant strains detected). A higher furazolidone resistance rate (9%) was found in Iranian children<sup>[125]</sup>. As the high cost of some drugs,

such as clarithromycin and quinolones, prevents their use in developing countries, where a high prevalence of primary metronidazole resistance is also present, to overcome these limitations, furazolidone-based treatments have been suggested. On the other hand, the low rate of primary *H. pylori* resistance to furazolidone in developed countries may make the use of this drug attractive. In this case, however, it is imperative to consider that furazolidone-based first-line therapy achieves *H. pylori* eradication rates of 75.7% and 79.6%<sup>[126]</sup>, and its use cannot ignore obvious ethical considerations in view of the carcinogenic evidence.

## ANTIBIOTIC RESISTANCES: A COMMENT ON THE DATA IN THE LITERATURE

Figure 1 illustrates the simultaneous minimal and maximal resistance rates in five world continents (Europe, America, Asia, Africa, and Oceania). It is evident that clarithromycin shows a good prospect of success in Africa, Oceania, and few Northern European countries (*e.g.*, Norway), whilst in Southern Europe (*e.g.*, Italy, Spain, Portugal, and Greece) and even South Africa, the risk of failure is high. Metronidazole might be very effective in Lithuania, but its use should be strongly discouraged in Italy and Croatia as well as most American, Asian, and African countries. Amoxicillin appears to be a reliable option in many countries, but it is almost ineffective in Iran and Cameroon. Finally, levofloxacin, which has been proposed as an alternative to clarithromycin in the areas where this last key antibiotic shows a high resistance rate, has been shown to be moderately ineffective worldwide, aside from Oceania, thus confirming the rapid trend toward therapeutic failure. Therefore, proposals for wider use could induce a counterproductive effect.

Among the other antibiotics, tetracycline and rifabutin appear to have low resistance rates, even if the use of the latter drug is limited by the regulations of many countries, where it is not indicated in *H. pylori* infection care, even apart from its high cost. Finally, furazolidone, despite its effectiveness, has been forbidden in many countries.

An obvious point is that, in each country, an ideal specific therapy should be identified. Nevertheless, all the studies on resistances *in vitro* might lack positive feedback *in vivo*. In fact, therapy failure may depend on several factors, of both bacterial and host origin. Not infrequently, different factors act simultaneously in reducing antibiotic therapy efficacy in the same patient. Indeed, a poor compliance to the *H. pylori* eradication regimen is inversely associated with the probability of therapeutic success. Unfortunately, the approved eradication regimens require the combination of 3-4 different drugs in multiple daily doses. Therapy regimen complexity and the onset of side effects are associated with reduced patient compliance. The *in vitro* activity of various antibiotics is greatly reduced or eliminated *in vivo* by the very low pH values encountered in gastric juices. This explains

the need to include a proton pump inhibitor in *H. pylori* eradication regimens. However, a significant variability in gastric acid secretion among different subjects has been reported. A small proportion of subjects show a higher basal acid output in association with normal gastrin values. These hyper-secretor subjects probably have a large parietal cell mass and a low eradication rate<sup>[127]</sup>.

These considerations may partially account for the wide discrepancies demonstrated between studies on resistances *in vitro* and the results of clinical trials *in vivo*. A final factor which might limit the reliability of *in vitro* resistance studies is the different methods used for detection. The methods are complex, expensive, and may often fail even in expert hands, and for this reason, their use is predominantly confined to research purposes.

## MAIN THERAPEUTIC REGIMEN EFFECTIVENESS

Triple therapy is one of the oldest schemes for *H. pylori* eradication. In Europe, it was used successfully in the United Kingdom until 5 years ago<sup>[128]</sup>, achieving an eradication rate of 92%. However, it is characterized by an enormous variability, if we consider the poor rate of 50% calculated in a German study in 2011<sup>[129]</sup>. The possibility of failure was very high in a Turkish study<sup>[130]</sup>, where only 32.7% of eradication occurred. In Asia, a high percentage of eradication was seen in India (82.9%)<sup>[131]</sup>, with a lower percentage in Korea (67.7%)<sup>[132]</sup>. In the American continent, eradication rates range from 78% to 97%<sup>[133,134]</sup>. Data from Africa are more homogeneous, but the weight of resistances affects the possibility of the eradication achieved in 71%-78.2% of cases, in a multicentric and in a Moroccan study, respectively<sup>[135,136]</sup>. The only recent available data from Oceania derive from two trials in which rifampicin was used instead of clarithromycin as second-line regimen, which achieved eradication rates of 95% and 96.6%, respectively<sup>[137,138]</sup>.

Quadruple therapy appears to be more effective than triple therapy, if we consider that in United States, Laine *et al.*<sup>[139]</sup> reported a success rate of 87.7%, and a lower value was seen only in a Canadian study<sup>[140]</sup> (70.8%). However, in Europe, failures are more frequent: quadruple therapy was effective only in 64.8% of cases in a Greek study<sup>[141]</sup>, despite a success rate of 91% in a British trial<sup>[128]</sup>. In Asian countries, the rates seem to be even more discouraging, ranging between 47.1% and 89.5% in Turkey<sup>[130]</sup> and China<sup>[142]</sup>, respectively. No data are available from Africa to the best of our knowledge. Only one Australian study investigated the effectiveness of quadruple therapy, but using a novel combination of a proton pump inhibitor, bismuth subcitrate, rifabutin, and ciprofloxacin as a first-line regimen for patients allergic to penicillin, and achieving an eradication rate of 94.2%<sup>[143]</sup>.

Concomitant therapy is a combination of antibiotics including amoxicillin, metronidazole, clarithromycin, and

a proton pump inhibitor (PPI) for a period of five or seven days. It has proven very effective in Japan, where an eradication rate of 98.1%<sup>[144]</sup> was achieved, but in South Korea the percentage was much lower (63.2%)<sup>[145]</sup>. In two different European studies, the same author reported the minimal and the maximal eradication rates of concomitant therapy as 85.5% and 95.5%, respectively<sup>[146,147]</sup>. In a multicentric Southern American study, this therapy achieved only 78.7% eradication<sup>[148]</sup>. We did not find any results from Africa for this regimen. No further data are available from Oceania.

Sequential therapy is a ten-day therapy that consists of a PPI plus amoxicillin in the first 5 d and a PPI, clarithromycin, and metronidazole in the following 5 d. In Italy it has proven to be very useful compared to other combinations<sup>[149]</sup>; it achieved eradication rates that range between 97.3% in a pediatric population<sup>[150]</sup>, 97% in an elderly population<sup>[151]</sup>, and 89% in a multicentric study involving more than 1000 patients<sup>[83]</sup>. The data from other European countries, however, are very poor. A good performance of this therapy was demonstrated in Africa, with a positive outcome ranging between 89.9% and 94.2%<sup>[136,152]</sup>. However, in South America this scheme appears to be less effective, if we consider the Peruvian percentage of success of 73%<sup>[153]</sup> and overall rate of 81.1%<sup>[148]</sup>. In Asia, a good result was achieved in South Korea<sup>[154]</sup> (92.6% maximum), whilst in China<sup>[155]</sup> only 78.3% eradicated the bacterium. No data are available from Oceania.

Miscellaneous therapy has been recently introduced and includes sequences of different combinations of antibiotics. The main four studies are from the four continents that show promising results: Colombia 94%<sup>[156]</sup>, Italy 85.7%<sup>[157]</sup>, Iran 92.9%<sup>[158]</sup>, and Taiwan 97.4%<sup>[159]</sup>. This regimen requires further confirmation of these excellent results, as well as an accurate evaluation of patient compliance owing to the risk of a large number of side effects and consequent drop-outs.

## THERAPEUTIC REGIMEN EFFECTIVENESS: A COMMENT ON THE DATA IN THE LITERATURE

Although resistance reports describe alarming results, the outcomes of therapeutic schemes do not seem to parallel this scenario in most cases, since a success of more than 80% is often reached. Despite some of the factors that may explain this discrepancy between *in vitro* and *in vivo* results that have been mentioned previously, it is possible that other factors may elucidate this controversial point: (1) most studies are performed in single centers and include populations selected from geographic areas of irrelevant dimensions. This may be an important handicap for the reproducibility of the therapeutic regimen in other areas; (2) the selection of patients is limited to bacterial positivity, often based on non-invasive tests, and does not take into account some bacterial



factors such as the intra-gastric load<sup>[160]</sup>, the possibility of primary resistances (which may even be different in the body and antrum of the same subject)<sup>[161]</sup>, heteroresistance status, and CagA status<sup>[162,163]</sup>. Finally, the presence of coccoid forms<sup>[164]</sup> in the stomach may have clinical relevance, due to the potential reactivation of *H. pylori* in its spiral form following therapy; (3) the results of therapeutic studies may be affected by host factors such as PPI metabolism<sup>[165]</sup>, parietal cell mass<sup>[166]</sup>, and related gastric pH, which is strictly related to antibiotic MIC values, the mucus layer (which affects bacterium/antibiotic contact), the frequent patchy distribution of *H. pylori* in the stomach, and even its persistence in small areas (*e.g.*, cardiac) after apparently successful eradication; and (4) the already outlined technical problems related to resistance detections *in vitro*.

## PROBIOTICS: HAVE THEY A ROLE IN *H. PYLORI* TREATMENT?

The possibility of probiotics interfering with *H. pylori* gastric colonization has been postulated by many authors and, therefore, many studies are available in the literature about the treatment of infected patients with beneficial bacteria supplementation. However, conflicting data have been obtained.

A review of available data showed that clinical trials can be divided into two groups: those using probiotics in association with antibiotic therapy and those using probiotics alone. In the first group, the efficacy of a single strain of probiotics associated with antibiotic triple or quadruple therapy generally resulted in a decrease of side effects such as diarrhea, bloating, nausea, and taste disturbances during treatment<sup>[167,168]</sup>, as well as an improvement of the eradication rate. Some studies have tested the association of a multi-strain probiotic mixture associated with antibiotic therapy. Two of these<sup>[169,170]</sup> showed a reduction in the side effects of antibiotic therapy and a higher eradication rate than that obtained with a single strain, whilst the third study<sup>[171]</sup> did not obtain any significant result.

Among the second group of clinical trials (only probiotics) we found that most studies tested a single probiotic strain, especially *Lactobacillus* species, obtaining a *H. pylori* load reduction as expressed by the urea breath test delta value. Experience by our group confirmed this finding after oral administration of *Lactobacillus reuteri* ATC 55730, not only with a delta value reduction, but also with a semiquantitative fecal antigen decrease<sup>[172]</sup>.

Recently, Szajewska *et al.*<sup>[173]</sup> reported a very interesting meta-analysis of the effects of *Saccharomyces boulardii* supplementation in standard triple therapy, showing a significant effect in both increasing the eradication rate and reducing side effects, in a total of 1307 patients from five randomized controlled trials.

## CONCLUSION

Therapeutic management of *H. pylori* remains an un-

solved issue. Indeed, no therapy regimen is able to cure the infection in all treated patients, and a definite number remain infected despite several consecutive standard therapies. This therapeutic failure is often considered to be the consequence of incorrect treatment rather than treatment limitation, since this appears unacceptable in the antibiotic era. However, no clinical trial has reported an eradication rate of 100% to the best of our knowledge.

Therapeutic failures are attributed to increasing antibiotic resistance. However, this phenomenon has been widely studied and the results show controversial findings even in the same geographic area. For each world continent, minimal and maximal values of resistance to different antibiotics have been reported for different countries, although some regimens appear to be almost unknown in some areas.

Another enigma is the outcomes of several different therapeutic schemes, which often do not parallel *in vitro* findings even in the same areas. Moreover, some schemes are limited by their use in very small geographic districts. Finally, not all therapeutic trials have considered bacterial and host factors affecting the therapeutic outcome.

In conclusion, the “ideal therapy”, paradoxically, appears to be a “utopia”, despite the unprecedented volume of studies in the field and the real breakthrough in medical practice made by the discovery and the treatment of *H. pylori*. A key point could be the possibility, in the near future, to group *in vivo* and *in vitro* studies by geographic areas in order to identify the best therapy, which is certainly related to the local habitat. Indeed, the ample discrepancies observed in the different areas do not encourage the development of therapeutic guidelines that could be valid worldwide. The main challenge for the future might be identifying a successful solution for overcoming antibiotic resistances and, in this context, geography must be considered a relevant matter.

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## WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

# *Helicobacter pylori* and gastric mucosa-associated lymphoid tissue lymphoma: Recent progress in pathogenesis and management

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## Abstract

Recent progress in the research regarding the molecular pathogenesis and management of gastric mucosa-associated lymphoid tissue (MALT) lymphoma is reviewed. In approximately 90% of cases, *Helicobacter pylori* (*H. pylori*) infection plays the causative role in the pathogenesis, and *H. pylori* eradication is nowadays the first-line treatment for this disease, which leads to complete disease remission in 50%-90% of cases. In *H. pylori*-dependent cases, microbe-generated immune responses, including interaction between B and T cells involving CD40 and CD40L co-stimulatory molecules, are considered to induce the development of MALT lymphoma. In *H. pylori*-independent cases, activation of the nuclear factor- $\kappa$ B pathway by oncogenic products of specific chromosomal translocations such as t(11;18)/API2-MALT1, or inactivation of tumor necrosis

factor alpha-induced protein 3 (A20) are considered to contribute to the lymphomagenesis. Recently, a large-scale Japanese multicenter study confirmed that the long-term clinical outcome of gastric MALT lymphoma after *H. pylori* eradication is excellent. Treatment modalities for patients not responding to *H. pylori* eradication include a "watch and wait" strategy, radiotherapy, chemotherapy, rituximab immunotherapy, and a combination of these. Because of the indolent behavior of MALT lymphoma, second-line treatment should be tailored in consideration of the clinical stage and extent of the disease in each patient.

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**Key words:** Gastric lymphoma; Mucosa-associated lymphoid tissue lymphoma; *Helicobacter pylori*; Nuclear factor  $\kappa$ B

**Core tip:** Recent progress in the research regarding the molecular pathogenesis and management of gastric mucosa-associated lymphoid tissue (MALT) lymphoma is reviewed. *Helicobacter pylori* (*H. pylori*) eradication leads to complete disease remission in 50%-90% of cases. In *H. pylori*-independent cases, activation of nuclear factor  $\kappa$ B pathway by chromosomal translocations such as t(11;18)/API2-MALT1, or inactivation of A20 are considered to contribute to the lymphomagenesis. A recent Japanese multicenter study confirmed the excellent long-term outcome of gastric MALT lymphoma after *H. pylori* eradication. Strategies for patients not responding to *H. pylori* eradication should be tailored in consideration of clinical stage and the disease extent in each patient.

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## INTRODUCTION

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT) lymphoma is an indolent non-Hodgkin lymphoma derived from marginal zone B-cells, which occurs in a number of extranodal organs, including the gastrointestinal tract, lung, salivary gland, thyroid, ocular adnexa, liver or skin<sup>[1]</sup>. Among these, the stomach is the most frequent site for MALT lymphoma. Gastric MALT lymphoma comprises 40%-50% of primary gastric lymphomas, 20%-40% of all extranodal lymphomas, 4%-9% of all non-Hodgkin lymphomas, and 1%-6% of all gastric malignancies<sup>[2-5]</sup>. *Helicobacter pylori* (*H. pylori*) plays a causative role in the development of gastric MALT lymphoma, and the eradication of *H. pylori* leads to a complete disease remission (CR) in 50%-90% of cases<sup>[6,7]</sup>.

In the present paper, we review the current knowledge on the etiology, diagnosis and optimal management strategies for patients with gastric MALT lymphoma, with special reference to its association with *H. pylori* infection and efficacy of the eradication therapy.

## PATHOGENESIS OF GASTRIC MALT LYMPHOMA

### *H. pylori*

A link of *H. pylori* with gastric MALT lymphoma was first suggested in 1991 by identification of the bacteria in the vast majority of patients<sup>[8]</sup>. This association was supported by subsequent epidemiological and histopathological studies<sup>[9,10]</sup>. Approximately 90% of patients with gastric MALT lymphoma are infected with *H. pylori*<sup>[7,11,12]</sup>, and about 70% of the cases respond to *H. pylori* eradication<sup>[6,7]</sup>. In such responders, survival of the lymphoma cells depends critically upon the microbe-generated immune responses<sup>[13]</sup>. Laboratory studies demonstrated that the growth of neoplastic B cells is stimulated by tumor-infiltrating *H. pylori*-specific T-cells, which require interaction between B and T cells involving CD40 and CD40L co-stimulatory molecules<sup>[14-19]</sup>. Thus, the genesis of *H. pylori*-dependent gastric MALT lymphoma is now considered as follows: an *H. pylori* infection results in T cell-dependent responses through the classic germinal center reaction, and thus generates reactive B and T cells. The *H. pylori*-specific T cells raised in the reactive component then migrate to the marginal zone/tumor area and provide non-cognate help to autoreactive neoplastic B cells, which may involve stimulation of CD40 and other surface receptors by soluble ligands and cytokines<sup>[13,19]</sup>.

Recently, Munari *et al.*<sup>[20]</sup> reported that high levels of a proliferation-inducing ligand (APRIL) were produced

exclusively by tumor-infiltrating macrophages in *H. pylori*-dependent gastric MALT lymphoma cases, and that macrophages produced APRIL on direct stimulation with both *H. pylori* and *H. pylori*-specific T cells. APRIL is a tumor necrosis factor (TNF) superfamily member known to be important for B-cell development, maturation and survival. It should be noted that APRIL-producing macrophages were dramatically reduced on lymphoma regression induced by *H. pylori* eradication<sup>[20]</sup>. These findings suggest that APRIL may also play some important role in the *H. pylori*-dependent lymphomagenesis.

### Genetic abnormalities

Genetic abnormalities are common in gastric MALT lymphomas. To date, a number of chromosomal translocations have been described in MALT lymphomas. Among these, t(11;18) (q21;q21)/*API2-MALT1*, t(1;14) (p22;q32)/*BCL10-IGH*, t(14;18) (q32;q21)/*IGH-MALT1* and t(3;14) (p13;q32)/*FOXP1-IGH* are replicable<sup>[11,13,21]</sup>. MALT1 and BCL10 proteins are involved in surface immune receptor-mediated activation of the nuclear factor kappa B (NF- $\kappa$ B) transcription factor; the chromosomal translocations involving these genes are believed to exert their oncogenic activities through constitutive activation of the NF- $\kappa$ B pathway, leading to expression of a number of genes important for cell survival and proliferation<sup>[21]</sup>.

In gastric MALT lymphoma, t(11;18)/*API2-MALT1* is the most frequent translocation, which is detected in 15%-24% of cases. The translocation fuses the N-terminal region of *API2* to the C-terminal region of *MALT1* and generates a functional chimeric fusion, which gains the ability to activate the NF- $\kappa$ B pathway<sup>[13,21]</sup>. Clinically, t(11;18) is more frequently associated with absence of *H. pylori* infection, and the majority of the translocation-positive cases do not respond to *H. pylori* eradication therapy<sup>[7,11,21,22]</sup>. Interestingly, t(11;18)-positive cases rarely transform to diffuse large B-cell lymphoma (DLBCL)<sup>[23]</sup>.

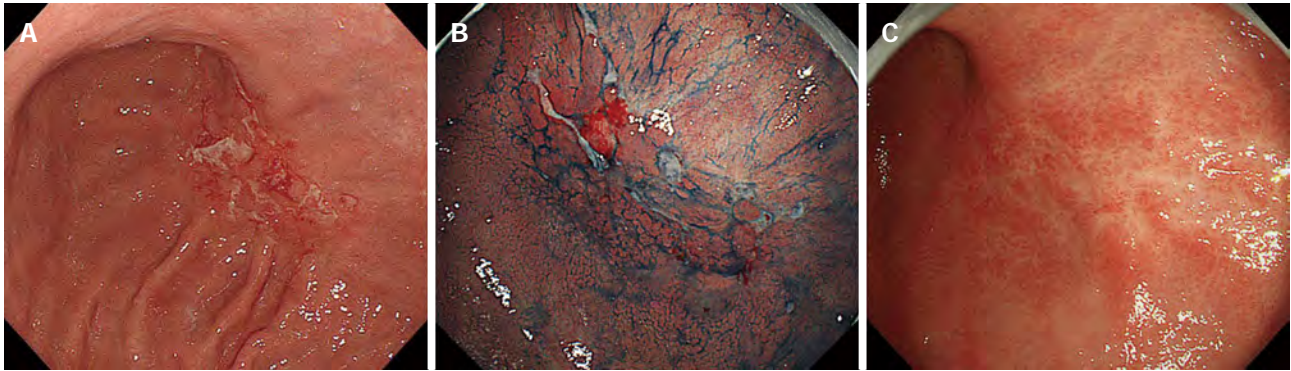
Recently, the TNF- $\alpha$ -induced protein 3 gene (*TNFAIP3*, *A20*), a negative regulator of NF- $\kappa$ B, was identified as the target of 6q23 deletion in many cases of MALT lymphoma<sup>[13,24,25]</sup>. *A20* mutation and deletion, which lead to A20 inactivation, are preferentially found in MALT lymphoma of the ocular adnexa, salivary glands, thyroid and liver. It is considered that A20-mediated oncogenic activities in MALT lymphoma depend on the NF- $\kappa$ B activation triggered by TNF or other unidentified molecules<sup>[13]</sup>. In gastric MALT lymphomas, however, *A20* deletion was detected only in 2 of 29 (7%) cases examined<sup>[25]</sup>. Thus, further investigations are needed to determine to what extent A20 inactivation contributes to the genesis of gastric MALT lymphoma.

## DIAGNOSIS OF GASTRIC MALT LYMPHOMA

### Histopathological diagnosis

The diagnosis of gastric MALT lymphoma should be





**Figure 1** Endoscopic images of gastric mucosa-associated lymphoid tissue lymphoma, superficial type. A, B: Pretreatment images; a superficially depressed lesion with multiple erosions and small ulcers with reddish granular mucosa can be seen on the posterior wall of the angularis; C: Follow-up image 6 mo after *H. pylori* eradication showing regression of the initial lesion.

**Table 1** Lugano staging system for gastrointestinal lymphomas

Stage	Definition	Description
Stage I	Tumor confined to gastrointestinal tract	Single primary site or multiple, non contiguous lesions
Stage II	Tumor extending into abdomen from primary gastrointestinal site	
	Nodal involvement	
	II <sub>1</sub> local	Paragastric (gastric cases) or paraintestinal (intestinal cases) nodal involvement
	II <sub>2</sub> distant	Mesenteric, paraaortic, paracaval, pelvic or inguinal nodal involvement
Stage II E	Penetration of serosa to involve adjacent organs or tissues	Gastrointestinal lesion extending to involve adjacent organs, <i>i.e.</i> , penetration, direct invasion, perforation or peritonitis by lymphoma
Stage IV	Disseminated extranodal involvement, or supra-diaphragmatic nodal involvement	Cases with Ann-Arbor stage III disease should be included

Rohatiner *et al.*<sup>[31]</sup> with modification.

based on the histopathological criteria according to the World Health Organization classification, using tissue specimens appropriately obtained by biopsy or surgery<sup>[1,5,26]</sup>. Histologically, the small to medium-sized neoplastic lymphoid cells (centrocyte-like cells) infiltrate around reactive follicles showing marginal zone growth pattern, which often infiltrate into gastric glands causing destruction of the epithelial cells (lymphoepithelial lesions)<sup>[1,26]</sup>. Immunohistochemically, the neoplastic cells of MALT lymphoma are usually CD20<sup>+</sup>, CD79a<sup>+</sup>, CD5<sup>+</sup>, CD10<sup>+</sup>, CD23<sup>+</sup>, CD43<sup>+</sup>, cyclin D1<sup>+</sup>. Staining for Ki-67 may help in identifying components of DLBCL. Cytogenetic analyses using G-banding, reverse transcription-polymerase chain reaction and/or fluorescence *in situ* hybridization for t(11;18)/*API2-MALT1* or other chromosomal translocations are also useful for confirming the diagnosis<sup>[1,21,26]</sup>.

### Macroscopic diagnosis

The standard macroscopic classifications for gastric lymphomas have not been established. In Western countries, gastric B-cell lymphomas have been endoscopically classified either as ulcerative (34%-69%), mass/polypoid (26%-35%), diffusely infiltrating (15%-40%), or other types<sup>[27-29]</sup>. We previously reported that 197 Japanese cases of primary gastric B-cell lymphoma (MALT lymphomas

and DLBCLs) were macroscopically classified as superficial-spreading (46%), mass-forming (41%), diffuse-infiltrating (6%), or other types (8%)<sup>[30]</sup>. Importantly, the most frequent macroscopic type in gastric MALT lymphomas is superficial type (Figure 1), while that in gastric DLBCLs is mass/polypoid type<sup>[29,30]</sup>.

### Clinical staging

An appropriate clinical staging is mandatory in order to determine the optimal management for malignant lymphomas. For the staging classification in patients with gastric MALT lymphoma, the Ann Arbor staging system with its modifications by Musschoff and Radaszkiewicz (I<sub>1</sub>E, I<sub>2</sub>E, II<sub>1</sub>E, II<sub>2</sub>E, III E, or IV) was recommended in the consensus report of the EGILS (European Gastro-Intestinal Lymphoma Study) group<sup>[26]</sup>. To date, however, the Lugano International Conference (Blackledge) classification (I, II<sub>1</sub>, II<sub>2</sub>, II E, or IV) has been widely applied for the clinical staging in gastrointestinal lymphomas (Table 1)<sup>[31]</sup>. In addition to esophagogastroduodenoscopy, the following are recommended for the initial staging workup: physical examination (including peripheral lymph nodes and Waldeyer's ring), complete hematological biochemical examinations (including LDH and  $\beta$ 2-microglobulin), computerized tomography of abdomen and pelvis, and endoscopic ultrasonography<sup>[26]</sup>.

**Table 2** Groupe d'Etude des Lymphomes de l'Adulte histological grading system for post-treatment evaluation of gastric mucosa-associated lymphoid tissue lymphoma

Score	Lymphoid infiltrate	LEL	Stromal changes	Clinical significance
CR	Absent or scattered plasma cells and small lymphoid cells in the LP	Absent	Normal or empty LP and/or fibrosis	Complete remission
pMRP	Aggregates of lymphoid cells or lymphoid nodules in LP/MM and/or SM	Absent	Empty LP and/or fibrosis	Complete remission
rRD	Dense, diffuse or nodular extending around glands in the LP	Focal LEL or absent	Focal empty LP and/or fibrosis	Partial remission
NC	Dense, diffuse or nodular	Present, "may be absent"	No changes	Stable disease or progressive disease

Copie-Bergman *et al.*<sup>[34]</sup> with modification<sup>[26]</sup>. LEL: Lymphoepithelial lesions; LP: Lamina propria; MM: Muscularis propria; SM: Submucosa; CR: Complete histological response; pMRP: Probable minimal residual disease; rRD: Responding residual disease; NC: No change.

In our opinion, however, ileocolonoscopy, bone marrow aspiration or biopsy, and fluorine-18 fluorodeoxyglucose positron emission tomography should also be included. In addition, endoscopic examinations of the small bowel (balloon-assisted endoscopy or capsule endoscopy) can be considered<sup>[32]</sup>.

## TREATMENT FOR GASTRIC MALT LYMPHOMA

### *H. pylori* eradication

The first-line treatment of all gastric MALT lymphomas is *H. pylori* eradication therapy<sup>[1,26,33]</sup>. In patients with stage I / II<sub>1</sub> disease, CR is achieved in 50%-90% of cases only by *H. pylori* eradication<sup>[6,7]</sup>. Histological evaluation of post-treatment biopsies should be performed according to the Groupe d'Etude des Lymphomes de l'Adulte (GELA) grading system (Table 2)<sup>[26,34]</sup>. Various predictive factors for resistance to *H. pylori* eradication therapy have been described, including absence of *H. pylori* infection, advanced stage, proximal location in the stomach, endoscopic non-superficial type, deep tumor invasion in the gastric wall, and t(11;18)/*API2-MALT1* translocation<sup>[6,7,21,22,26]</sup>.

In a systematic review of the data from 32 published studies that included 1408 patients with gastric MALT lymphoma, the CR rate after *H. pylori* eradication was 78%<sup>[6]</sup>. Recently, we confirmed excellent long-term outcomes of the disease after *H. pylori* eradication by a large-scale multicenter study of 420 Japanese patients with gastric MALT lymphoma<sup>[7]</sup>. In the study, CR was achieved by *H. pylori* eradication in 77% of patients. During the follow-up periods of up to 14.6 years (mean 6.5 years, median 6.04 years), treatment failure was observed in 9% of patients (37 patients; 10 relapse, 27 progression). Probabilities of freedom from treatment failure, overall survival and event-free survival after 10 years were 90%, 95% and 86%, respectively. Table 3 summarizes 28 previously published studies that included more than 20 patients initially treated by *H. pylori* eradication<sup>[7]</sup>. In the 28 studies, CR was achieved in 1361 of 1877 patients (73%), Progressive disease (PD) was observed in 17 of 1576 patients (1.1%), relapse was recorded in 60 of 1203 CR

patients (4.9%), and treatment failure (PD or relapse) was found in 118 of all 1877 patients (6.3%). These data are almost similar to those in our multicenter study<sup>[7]</sup>, except for PD rate (1.1% *vs* 6.4%).

As for the regimen for *H. pylori* eradication therapy, proton pump inhibitor (PPI) + clarithromycin-based triple therapy composed of a double dose of a PPI plus clarithromycin and amoxicillin or metronidazole for 7 or 14 d is recommended<sup>[26,35]</sup>. In the areas where the clarithromycin resistance rate exceeds 15%, use of this drug should be avoided without prior susceptibility testing<sup>[35]</sup>. A pooled data analysis in 1271 patients with gastric MALT lymphoma from 34 studies showed a successful eradication was achieved in 91% of cases after the first-line treatment, and the eradication rate was extended to 98% after the second-line treatment or more attempts<sup>[36]</sup>.

Several studies have demonstrated that *H. pylori* eradication therapy is also effective even in cases with gastric DLBCL<sup>[37,38]</sup>. In those reports, 27%-60% of *H. pylori*-positive patients with DLBCL in stage I / II<sub>1</sub> achieved CR after *H. pylori* eradication. Not only cases with MALT lymphoma component, but also cases without any evidence of MALT lymphoma responded to eradication therapy<sup>[37,38]</sup>. Therefore, *H. pylori* eradication should be tried in *H. pylori*-positive patients with gastric DLBCL.

### Treatments for patients not responding to *H. pylori* eradication

The management strategy for the patient with gastric MALT lymphoma who does not respond to *H. pylori* eradication still remains to be elucidated. While patients with PD or clinically evident relapse should undergo oncological treatment, for patients with persistent histological lymphoma without PD (responding residual disease or no change), a "watch and wait" strategy was recommended up to 24 mo after *H. pylori* eradication in the EGILS consensus report<sup>[26]</sup>.

As for the second-line oncological treatment, radiotherapy is highly effective in localized cases (stage I / II<sub>1</sub>)<sup>[7,26,33]</sup>. While chemotherapy and immunotherapy with rituximab are also effective, these systemic treatments are suitable for cases with an advanced stage<sup>[26,33]</sup>. Recently, the combination of rituximab and chlorambucil<sup>[39]</sup> or fluda-

**Table 3** Review of literature on efficacy of *Helicobacter pylori* eradication for gastric mucosa-associated lymphoid tissue lymphoma *n* (%)

Author, yr	Patients	CR cases	Median FW (yr)	PD	Relapse	Treatment failure <sup>1</sup>
Hancock <i>et al</i> , 2009	199	92 (46)	ND	ND	ND	25 (13)
Wündisch <i>et al</i> , 2006	193	146 (76)	2.3	0	5 (3.1)	5 (2.6)
Wündisch <i>et al</i> , 2005	120	96 (80)	6.3	0	3 (3.1)	3 (2.5)
Stathis <i>et al</i> , 2009	102	66 (65)	6.3	ND	ND	16 (16)
Kim <i>et al</i> , 2007	99	84 (85)	3.4	0	5 (5.9)	5 (5.1)
Nakamura <i>et al</i> , 2005	96	62 (65)	3.2	7 (7.3)	4 (6.4)	11 (11)
Hong <i>et al</i> , 2006	90	85 (94)	3.8	0	8 (9.4)	8 (8.9)
Fischbach <i>et al</i> , 2004	88	73 (83)	3.8	2 (2.3)	4 (5.5)	6 (6.8)
Nakamura <i>et al</i> , 2008	87	57 (66)	3.5	1 (1.1)	1 (1.8)	2 (2.3)
Savio <i>et al</i> , 2000	76	71 (93)	2.3	0	6 (8.5)	6 (7.9)
Terai <i>et al</i> , 2008	74	66 (89)	3.9	0	3 (4.5)	3 (4.1)
Sumida <i>et al</i> , 2009	66	47 (71)	3.3	0	0	0
Weston <i>et al</i> , 1999	58	40 (69)	1.8	0	0	0
Ono <i>et al</i> , 2010	58	48 (83)	6.3	2 (3.4)	1 (2.1)	3 (5.2)
Andriani <i>et al</i> , 2009	53	42 (79)	5.4	0	9 (21)	9 (17)
Akamatsu <i>et al</i> , 2006	47	30 (64)	3.1	1 (2.1)	1 (3.4)	2 (4.3)
Pinotti <i>et al</i> , 1997	44	30 (68)	1.8	0	2 (6.7)	2 (4.6)
Urakami <i>et al</i> , 2000	44	42 (95)	1.7	0	0	0
Ruskoné-Fourmestraux <i>et al</i> , 2001	44	19 (43)	2.9	1 (2.3)	2 (11)	3 (6.8)
Steinbach <i>et al</i> , 1999	34	14 (41)	3.4	2 (5.9)	0	2 (5.9)
Takenaka <i>et al</i> , 2004	33	26 (79)	ND	0	0	0
Chen <i>et al</i> , 2005	32	24 (75)	5.8	0	3 (13)	3 (9.4)
Lee <i>et al</i> , 2004	28	24 (86)	2.0	0	1 (4.2)	1 (3.6)
Montalban <i>et al</i> , 2005	24	22 (92)	4.6	0	1 (4.5)	1 (4.2)
de Jong <i>et al</i> , 2001	23	13 (57)	3.1	1 (4.3)	0	1 (4.4)
Raderer <i>et al</i> , 2001	22	15 (68)	2.1	0	1 (6.7)	1 (4.6)
Dong <i>et al</i> , 2008	22	13 (59)	1.5	0	0	0
Yamashita <i>et al</i> , 2000	21	14 (67)	0.8	0	0	0
Total of above	1877	1361 (73)	3.3	17 (1.1 <sup>2</sup> )	60 (4.9 <sup>3</sup> )	118 (6.3)
Nakamura <i>et al</i> <sup>[7]</sup> , 2012	420	323 (77)	6.04	27 (6.4)	10 (3.1)	37 (8.8)

<sup>1</sup>Progressive disease (PD) or relapse; <sup>2</sup>17/1576 patients; <sup>3</sup>60/1203 complete remission (CR) patients (Nakamura *et al*<sup>[7]</sup> with modification). FW: Follow-up; ND: Not described.

rabine<sup>[40]</sup> provided excellent responses in patients with MALT lymphoma of variable organs, including gastric cases. Surgical resection is nowadays restricted to the management of cases with perforation or bleeding that cannot be controlled endoscopically<sup>[26]</sup>.

## CONCLUSION

While a large amount of clinical evidence has confirmed the validity of *H. pylori* eradication as the first-line treatment for gastric MALT lymphoma, there are many choices for the second-line treatments. Because of the indolent behavior of MALT lymphoma, the strategy for patients not responding to *H. pylori* eradication should be tailored in consideration of the clinical stage and extent of the disease. Despite the recent advances in our understanding of the pathogenesis of gastric MALT lymphoma, there still exist many questions to be answered. Further basic and clinical research is needed to clarify the molecular mechanisms in the development of the disease.

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## WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

# Stool antigen tests for the management of *Helicobacter pylori* infection

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## Abstract

Stool antigen tests (SATs) are noninvasive diagnostic modules for *Helicobacter pylori* (*H. pylori*) infection. Two types of SATs exist for the diagnosis of *H. pylori* infection, one based on enzyme immunoassay (EIA) and another on immunochromatography (ICA). SATs do not require expensive chemical agents or specified equipment; hence, they are less expensive compared with the urea breath test. Both European and Japanese guidelines have shown that EIA-based SATs using monoclonal antibodies are useful for primary diagnosis as well as for the assessment of eradication therapy. ICA-based tests do not require particular equipment and are therefore useful in developing countries. SATs are also useful for the diagnosis of *H. pylori* infection in children and post gastric surgery patients. SATs performed via EIA can assess *H. pylori* infection in a large number of subjects, almost as well as serology. Thus, SATs would be useful or detecting current infection in such a survey to identify and eradicate *H. pylori* infection. The accuracy of SATs is lower when the stool samples are unformed or watery, because *H. pylori*-specific antigens in the stool samples are diluted. Temperature and the interval between stool sample collection and measurement also affect the results of SATs.

The choice of test kit depends on the sensitivity and specificity in each region and the circumstances of each patient.

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**Key words:** *Helicobacter pylori*; Stool antigen test; Diagnosis; Enzyme immunoassay; Immunochromatography

**Core tip:** Stool antigen tests (SATs) are relatively inexpensive noninvasive tests. Several guidelines on *Helicobacter pylori* (*H. pylori*) infection from around the world indicate that SATs using monoclonal antibodies are useful for primary diagnosis as well as for assessing the results of eradication therapy. SATs are also useful for diagnosing *H. pylori* infection in children and post gastric surgery patients. The choice of test kit depends on the accuracy in each population and the circumstances of each patient.

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## INTRODUCTION

Infection by *Helicobacter pylori* (*H. pylori*) has been implicated in the pathogenesis of gastro-duodenal diseases. Several guidelines on *H. pylori* infection from around the world indicate that eradication of *H. pylori* would result in a reduction of the incidence of gastroduodenal diseases, including gastric cancer, and would decrease new infections in future generations<sup>[1,2]</sup>. Following the recommendation of the Japanese guidelines for the management

of *H. pylori* infection (2009 revised edition), in 2013, the Japanese health insurance system approved the coverage of the diagnosis and eradication of *H. pylori* in all infected patients<sup>[3]</sup>. Consequently, an expansion in the role of *H. pylori* diagnostic tests will accompany the increased number of patients undergoing *H. pylori* testing and eradication.

Stool antigen tests (SATs) are noninvasive diagnostic modules for *H. pylori* infection and were introduced after the urea breath test (UBT). Early SATs used an enzyme immunoassay (EIA) based on polyclonal antibodies. While they provided reliable results in the diagnosis of *H. pylori* infection, controversial results were sometimes observed in the post-eradication assessment because of false-positives<sup>[4,5]</sup>. Monoclonal antibody-based techniques generally have higher specificity. SATs based on monoclonal antibodies have been developed, and have been found to be more accurate than those using polyclonal antibodies<sup>[6,7]</sup>. A meta-analysis also showed that the specificity of SATs based on monoclonal antibodies was 0.97 (95%CI: 0.96-0.98)<sup>[8]</sup>. Both European and Japanese guidelines have indicated that SATs using monoclonal antibodies are useful for primary diagnosis as well as for the assessment of eradication therapy<sup>[1,3]</sup>.

Two types of SATs exist for the diagnosis of *H. pylori* infection, one based on EIA and another on immunochromatography (ICA). Although both types of tests are highly sensitivity and specificity, a recent study showed that currently available ICA-based tests provide less reliable results than EIA-based tests<sup>[9]</sup>. However, ICA-based tests are easy to perform and are useful for in-office rapid diagnoses of *H. pylori* infection<sup>[10]</sup>. ICA-based tests do not require specialized equipment; therefore, they would be useful in developing countries.

## DIAGNOSIS

### Comparison with UBT

Among non-invasive diagnostic tests, SAT and UBT have higher accuracy than serological or urinary antibody-based tests<sup>[1,3]</sup>. The American Gastroenterological Association recommends both SAT and UBT for the diagnosis of *H. pylori* infection in patients with dyspepsia<sup>[4]</sup>. While UBT has been considered the most reliable noninvasive test for the diagnosis of *H. pylori* infection, it has several limitations. The cost of UBT is still relatively high because of the price of <sup>13</sup>C-urea (approximately 30.3 USD) and the cost of measuring <sup>13</sup>CO<sub>2</sub>. By contrast, SATs do not require expensive chemical agents and special equipment and hence are less expensive (1400 JPY; approximately 14.2 USD). In addition, patients are required to fast before UBT testing, but not before a SAT. Furthermore, proton pump inhibitor (PPI) administration modulates gastric pH, resulting in lower urease activity of *H. pylori* in the stomach. UBT detects gastric mucosal urease activity; therefore, false-negative results are noted in patients who have been taking PPIs<sup>[11]</sup>. It is therefore generally recommended that PPI administra-

tion be discontinued 2 wk before UBT testing<sup>[11]</sup>. PPIs can similarly influence SAT<sup>[12,13]</sup> results, but some monoclonal antibody-based SATs that are currently available are not affected by PPIs<sup>[14]</sup>. Such SATs, which do not require PPI discontinuation, are useful for the management of *H. pylori* infection in patients with gastroesophageal reflux diseases or those taking nonsteroidal anti-inflammatory drugs.

### Diagnosis in children and post gastric surgery patients

A systematic review and meta-analysis showed that SATs using a monoclonal antibody-based EIA are useful for the diagnosis of *H. pylori* infection in children<sup>[15]</sup>. UBT is also highly accurate in children older than 6 years, while studies from developed countries showed that its specificity was less than 90% in very young children<sup>[16,17]</sup>. By contrast, both monoclonal SAT and UBT were reliable in young children aged 6-30 mo in South American developing countries<sup>[18]</sup>. These results indicate that monoclonal antibody-based SATs are the most effective tests for children in populations with both high and low prevalences of *H. pylori* infection<sup>[18,19]</sup>.

In patients who received distal gastrectomy, the accuracy of UBT was lower than that of a biopsy urease test<sup>[20]</sup>. However, in Japanese patients who underwent distal gastrectomy, the specificity of SAT was 90.5% while that of UBT was only 59.1%<sup>[21]</sup>.

### Mass survey and screening

In mass surveys, with regard to technique and cost, serology has generally been used despite its lower specificity<sup>[3]</sup>. SATs performed *via* EIA can assess *H. pylori* infection in a large number of subjects, almost as well as serology. In 994 healthy Japanese adults who participated in a mass survey, concordance of the results of SAT and serology was over 90%<sup>[22]</sup>. However, in that study, the positivity of SATs was significantly lower than that of serology in 303 subjects with severe atrophic gastritis. In the gastric mucosa of patients with severe atrophic gastritis and intestinal metaplasia, colonization by *H. pylori* is decreased or non-existent. Therefore, in the setting of a mass survey, serology is useful for the detection of both current and past infection. SATs should be used to detect current infection in such a survey to identify and eradicate *H. pylori* infection for the prevention of gastric malignancies.

## ASSESSMENT OF ERADICATION

To date, many studies have demonstrated the usefulness of SATs in the evaluation of the results of eradication therapy. Recent guidelines of the European Helicobacter Study Group (EHSG) recommend both UBT and laboratory-based monoclonal SAT<sup>[1]</sup>. After eradication therapy, the amount of *H. pylori* colonization in the stomach would be reduced, even when eradication therapy was unsuccessful. Therefore, SATs should be performed to detect the reduced number of bacteria. Among laboratory-based monoclonal SATs, the Premier Platinum

HpSA Plus (HpSA ELISA II; Meridian Diagnostics, Inc., Cincinnati, OH, United States), which uses multiple murine monoclonal antibodies, seems to be accurate. We previously demonstrated the significantly higher sensitivity of HpSA ELISA II to that of the Testmate Pylori Antigen EIA (TPAg EIA; Wakamoto Pharmaceutical Co. Ltd., and Kyowa Medex, Tokyo, Japan), which uses a single monoclonal antibody<sup>[23]</sup>. HpSA ELISA II produced a higher positive predictive value, although the TPAg EIA provided efficient results<sup>[24]</sup>.

In the guidelines of the EHSG, laboratory-based tests, but not in-office tests, are recommended for the evaluation of treatment results<sup>[1]</sup>. However, recent observations indicate that some in-office monoclonal antibody-based tests can accurately evaluate the results of eradication treatment<sup>[9,25]</sup>. In-office tests allow physicians to evaluate the results of eradication therapy in a single visit and the next eradication therapy can be started on the same day in non-eradicated patients. In-office tests do not require specialized equipment; therefore, they would be suitable in institutes that cannot measure <sup>13</sup>CO<sub>2</sub>.

PPI administration should be discontinued 2 wk before evaluating treatment results by UBT or SAT<sup>[1]</sup>. However, as described above, the results of certain SATs are not affected by PPIs<sup>[13]</sup>. Actually, in a small series of 22 Japanese patients, we showed that the results of eradication therapy assessed by TPAg EIA during PPI administration were the same those determined by UBT 4 wk after discontinuing PPI in 21 patients<sup>[26]</sup>.

In several guidelines, evaluation of the results of eradication therapy by SATs should be performed at least 4 wk after finishing the treatment<sup>[1,3]</sup>. However, relapse after eradication is considered to be mainly recurrence of the same infection rather than reinfection. Therefore, proposals have been made to extend the timing to 6 or 8 wk after finishing treatment. A monoclonal antibody-based EIA test could determine the treatment results at 6 wk after finishing the treatment as well as 8 wk<sup>[26]</sup>.

## TO BE MENTIONED WHEN PERFORMING SATS

Several factors influence the results of SATs. The accuracy of SATs is lower when the stool samples are unformed or watery, because *H. pylori*-specific antigens in the stool samples are diluted. Therefore, watery stools should not be used, particularly in the determination of the results of eradication therapy. The sensitivity of SATs is also lower in patients with upper gastrointestinal bleeding<sup>[27]</sup>.

Temperature and the interval between stool sample collection and measurement also affect the results of SATs. Such information is available for two kits. Querioz *et al*<sup>[18]</sup> examined the results of HpSA ELISA II and showed a remarkable reduction of the OD value when stool samples were maintained at 37 °C for 48 h. They also retested a stool sample with an OD value of 0.183 after 6 h of incubation at 37 °C and found that the OD value fell below the cutoff (0.120). In samples tested by TPAg EIA

we found that the OD values of initially negative stool samples increased and were almost similar to the cutoff level if the samples were maintained at 40 °C<sup>[9]</sup>. However, OD values were unchanged for up to 7 d at -5 °C-25 °C when stool sample suspensions were stored in their particular collection devices. Therefore, stool samples should be stored at a low temperature and be tested over a short period if the collection devices are not available. To maintain the antigenicity over a longer term, stool samples should be stored at -80 °C.

Differences in the antigenicity of *H. pylori* strains sometimes affect the accuracy of SATs in different populations<sup>[28]</sup>. Therefore, sensitivity and specificity of SATs should be tested in each population before use in the management of *H. pylori* infection.

## CONCLUSION

In summary, SATs are relatively inexpensive noninvasive tests. SATs using monoclonal antibodies are useful for primary diagnosis as well as for the assessment of eradication therapy. SATs are also useful in the management of *H. pylori* infection in children and post gastric surgery patients. In the future, SATs should be used in mass surveys to identify and eradicate *H. pylori* infection for the prevention of gastric malignancies. The choice of test kit depends on the sensitivity and specificity in each region and the circumstances of each patient.

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## WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

### *Helicobacter pylori* and interleukin-8 in gastric cancer

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**Key words:** *Helicobacter pylori*; Interleukin-8; Signals; Gastric cancer; Therapeutics

**Core tip:** There is a close association between gastric cancer and *Helicobacter pylori* (*H. pylori*) infection. *H. pylori* upregulates interleukin-8 (IL-8) gene expression in gastric epithelial cells and the levels of IL-8 may be indicative of poor prognosis. We propose that IL-8 overexpression induced by *H. pylori* plays a major role in gastric cancer development and progression, and that targeting IL-8 may be a promising strategy for gastric cancer treatment.

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## Abstract

*Helicobacter pylori* (*H. pylori*) is a major etiological factor in the development of gastric cancer. Large-scale epidemiological studies have confirmed the strong association between *H. pylori* infection and both cancer development and progression. Interleukin-8 (IL-8) is overexpressed in gastric mucosa exposed to *H. pylori*. The expression of IL-8 directly correlates with a poor prognosis in gastric cancer. IL-8 is multifunctional. In addition to its potent chemotactic activity, it can induce proliferation and migration of cancer cells. In this review, we focus on recent insights into the mechanisms of IL-8 signaling associated with gastric cancer. The relationship between IL-8 and *H. pylori* is discussed. We also summarize the current therapeutics against IL-8 in gastric cancer.

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## INTRODUCTION

Gastric cancer has affected humans for millennia. The risk of gastric cancer appears to evolve over a lifetime as a possible result of changes in diet and lifestyle. In 1984, Marshall and Warren were first to describe the association between peptic ulcer disease and *Helicobacter pylori* (*H. pylori*)<sup>[1]</sup>. *H. pylori* was subsequently causally linked with the development of gastric cancer.

Despite the improved prognosis of gastric cancer resulting from the early diagnosis and development of adjuvant therapy, overall 5-year survival rates for patients with gastric cancer remain disappointing, with a mortality rate of 20% in Western countries and up to 60% in Asian countries<sup>[2]</sup>. Although current combinatory chemotherapeutic regimes result in a median overall survival of up to

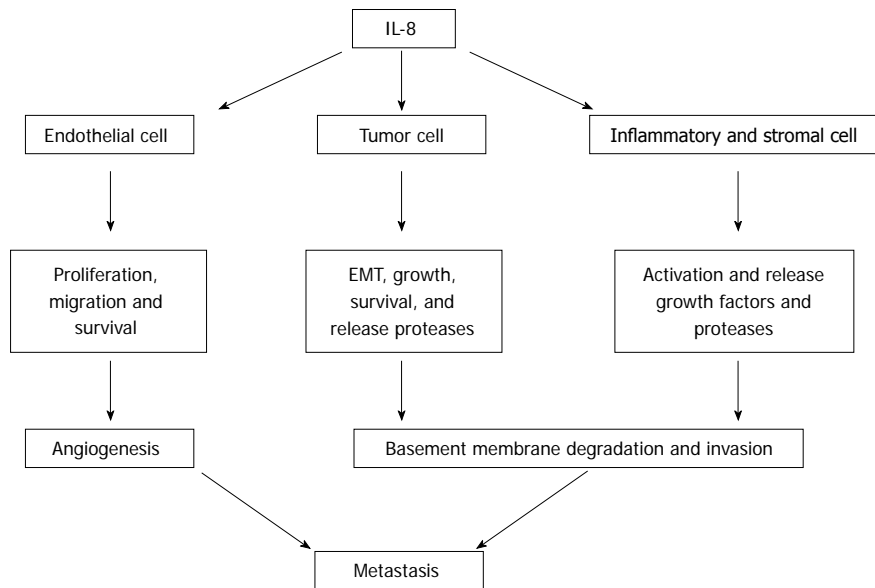


Figure 1 Roles for interleukin-8 in tumor progression and metastasis. EMT: Epithelial-mesenchymal transition; IL-8: Interleukin-8.

11 mo, toxicity is increased<sup>[3,4]</sup>. To overcome the adverse effects, novel chemotherapeutic concepts have focused on the development of targeted therapies for gastric cancer. An understanding of the detailed mechanisms of invasion and metastasis in gastric cancer would be helpful in improving the treatment outcome.

*H. pylori* infection is usually asymptomatic in most hosts, as virtually all carriers develop superficial chronic active gastritis, whereas only about 10% suffer gastric or duodenal ulceration and 0.5%-2.0% develop gastric adenocarcinoma or B cell lymphoma of mucosa-associated lymphoid tissue<sup>[5]</sup>. *H. pylori* colonize the gastric mucosa of 35%-70% of people worldwide and infection with *H. pylori* is the main etiologic factor for development of chronic active gastritis and peptic ulcers<sup>[6,7]</sup>. Epidemiologic data indicate that gastric cancer occurs more frequently in populations with higher rates of *H. pylori* infection, and the World Health Organization has classified this bacterium as a class 1 carcinogen for gastric cancer<sup>[8]</sup>. Animal models have also demonstrated the importance of *H. pylori* in gastric carcinogenesis<sup>[9]</sup>. *H. pylori* infection is important in the process of tissue remodeling, angiogenesis, tumor invasion and metastasis<sup>[10]</sup>, and induces a number of genes in host cells that are potential determinants of inflammation, angiogenesis, and metastasis including interleukin-8 (IL-8), cyclooxygenase-2<sup>[11]</sup>, monocyte chemoattractant protein-1<sup>[12]</sup>, vascular endothelial growth factor<sup>[13]</sup>, and matrix metalloproteinase (MMP)-9<sup>[14]</sup>. However, it remains unclear how *H. pylori* infection activates specific transcription factors and induces gene expression.

IL-8 seems to have significant potential as a prognostic and predictive cancer biomarker. IL-8 was originally identified as a chemoattractant for neutrophils that release angiogenic growth factors, stimulating angiogenesis as a part of cancer progression. As shown in Figure 1, IL-8 increases the proliferation, migration and survival of

endothelial cells, potentiates the epithelial-mesenchymal transition and survival of cancer cells, and activates macrophage and immune responses at the tumor site<sup>[15]</sup>. IL-8 enhances the production and secretion of MMP-2 and MMP-9<sup>[16,17]</sup>, suggesting that it can modulate invasiveness and/or extracellular matrix remodeling in normal physiological conditions and in cancer progression.

An understanding of the basic principles and underlying signals by which *H. pylori* regulates IL-8 may lead to the development of new therapeutic strategies in gastric cancer. With this in mind, we present a brief review.

## A ROLE FOR IL-8 IN GASTRIC CANCER

A significant correlation between high expression levels of IL-8 in gastric mucosa and risk of gastric cancer has been reported<sup>[18]</sup>. Macri *et al.*<sup>[19]</sup> reported that the serum levels of IL-8 act as markers of gastric cancer. Increased expression of IL-8 mRNA in tissue extracts from gastric cancer patients has been associated with some clinicopathological aspects of the disease, including poor prognosis<sup>[20]</sup>. In IL-8 transgenic mice, where expression of human IL-8 is controlled by its own regulatory elements, expression of IL-8 increased tumorigenesis, suggesting that IL-8 might have a crucial role in gastrointestinal cancers<sup>[21]</sup>. These observations indicate that high levels of IL-8 may be associated with poor prognosis as judged by stage and histology, and that IL-8 may be indicative of more aggressive gastric cancers.

The roles for IL-8 in the angiogenesis of gastric cancer have drawn much interest. Since invasion and angiogenesis are all involved in the metastatic process, IL-8 expression in gastric cancer can influence their metastatic capabilities. Upregulation of IL-8 in human gastric carcinomas correlates closely with their angiogenesis<sup>[22]</sup>. Kitadai *et al.*<sup>[23]</sup> reported that the expression of IL-8 directly

correlated with the vascularity of human gastric carcinomas and that IL-8-transfected cells produced rapidly growing, highly vascular neoplasms, compared to control cells. In contrast, inhibition of IL-8 decreases angiogenesis in gastric cancer. Wang *et al.*<sup>[24]</sup> reported that CHIP, a protein that interacts with the carboxy terminus of Hsc70, also interacted with nuclear factor-kappa B (NF- $\kappa$ B), terminating NF- $\kappa$ B activity and inhibiting IL-8-induced angiogenesis. IL-8 stimulates vascular endothelial growth factor (VEGF) expression in endothelial cells *via* CXCR-2 and thereby promotes the activation of VEGF receptors in an autocrine fashion<sup>[25]</sup>. IL-8 has a direct role in angiogenesis by enhancing endothelial cell proliferation and survival in CXCR1- and CXCR2-expressing endothelial cells<sup>[26]</sup>. IL-8 stimulates both endothelial proliferation and capillary tube formation *in vitro*, and both of these effects can be blocked by monoclonal anti-bodies to IL-8. *H. pylori*-derived heat shock protein 60 (HpHSP60) enhances angiogenesis by a CXCR2-mediated signaling pathway<sup>[27]</sup>. Use of an angiogenic array showed that HpHSP60 markedly induced IL-8 and that inhibition of CXCR2, the receptor for IL-8, significantly abolished HpHSP60-induced tube formation. IL-8 has also been linked with cell adhesion and migration in gastric cancer<sup>[23]</sup>. IL-8 activates NF- $\kappa$ B and Akt signals, and induces adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 and CD44 expression in gastric cancer cells<sup>[28]</sup>. Inhibition of IL-8 with small interfering RNA reportedly decreased the adhesion, migration and invasion functions in cancer cells<sup>[23]</sup>.

IL-8 polymorphisms may increase the risk of gastric cancer. Taguchi *et al.*<sup>[29]</sup> reported the association of the *IL-8-251 A/T* polymorphism with higher expression of IL-8 protein, severe neutrophil infiltration and increased risk of atrophic gastritis and gastric cancer. *IL-8-251 T/A* and *IL-8-251 A/A* polymorphisms may be associated with angiogenesis in gastric carcinogenesis in *H. pylori*-infected Koreans<sup>[30]</sup>. In the study, there were significant correlations between MMP-9, angiopoietin-1 concentrations and disease progression in *IL-8-251 A/A* and *IL-8-251 A/T* genotypes. Felipe *et al.*<sup>[31]</sup> reported that patients with the heterozygous *IL-8-251 A/T* genotype, high fat intake and smokers or ex-smokers presented an increased risk of gastric cancer in a Brazilian population. However, the association of IL-8 polymorphisms and gastric cancer is controversial. The IL-8 polymorphism was not consistently associated with gastric cancer risk in a Polish population<sup>[32]</sup>. Furthermore, a meta-analysis of epidemiological studies revealed an overall lack of association between *IL-8-251* gene polymorphisms and risk of gastric cancer; any association is likely to be variable depending on histological type, tumor location, *H. pylori* infection, and ethnicity/country<sup>[33]</sup>.

The downstream signals of IL-8 produced by *H. pylori* have been intensively studied. All biological effects of IL-8 are mediated by two receptors designated CXCR1 and CXCR2. IL-8 binds with high specificity

to CXCR1<sup>[34]</sup> and with less specificity to CXCR2<sup>[35]</sup> expressed on stromal, endothelial and tumor cells. CXCR1, a cell-surface G-protein-coupled receptor, has been associated with tumorigenesis, development and progression of some tumors. Hu *et al.*<sup>[36]</sup> documented that CXCR1 overexpression is associated with late-stage gastric cancer. They reported that knockdown of CXCR1 could inhibit cell proliferation *in vitro* and *in vivo*. Lin *et al.*<sup>[37]</sup> reported that enforced expression of the cysteine-rich 61 (*Cyr61*) gene or treatment with recombinant Cyr61 protein enhanced expression of CXCR1 and CXCR2 in gastric cancer cells. The upregulated functionality of CXCR1 and CXCR2 could facilitate their chemotactic migration toward IL-8 and contribute to transendothelial migration, as well as intravasation. The interaction between IL-8 and epidermal growth factor receptor (EGFR) promotes cell proliferation through transactivation of the receptor by activation of a disintegrin and metalloproteinase<sup>[38]</sup>. IL-8 could induce EGFR phosphorylation, while anti-IL-8 and anti-IL-8 receptor antibodies suppressed EGFR phosphorylation, indicating that *H. pylori*-stimulated IL-8 accelerates the processing of EGFR ligands, and that cleaved EGFR ligands bind and stimulate EGFR in paracrine and autocrine manners to induce cell proliferation.

## SIGNALS INVOLVED IN

### *H. PYLORI*-INDUCED IL-8 IN GASTRIC CANCER

A whole genome analysis of the epithelial response to *H. pylori* exposure revealed *IL-8* as the most markedly up-regulated gene<sup>[39]</sup>. IL-8 appears to play a paramount role in the epithelial cell response to *H. pylori* infection and in the pathological processes leading to gastric disease. IL-8, a CXC chemokine specific for neutrophil granulocyte chemotaxis, has been correlated with the histological severity of gastritis<sup>[40]</sup>. The majority of gastric cancers are end products of an inflammatory process. A chronic *H. pylori* infection is characterized by an inflammation of the gastric mucosa and is accepted as the major cause of chronic gastritis.

IL-8 induction in gastric epithelial cells has been clearly correlated with a functional *cagA* gene<sup>[41]</sup>. In *H. pylori* strains that express *cagA*, cytokine expression has been linked with an elevated inflammatory response *in vivo*<sup>[42]</sup>. *H. pylori* strains are classified as *cagA*-positive or *cagA*-negative according to the presence or absence of *cagA*, respectively<sup>[43]</sup>. *CagA* protein is a major virulence factor of *H. pylori* that has attracted clinical interest as a marker of *H. pylori*-associated disease, having been shown to confer increased gastric cancer risk<sup>[6,44]</sup>. The *cagA* gene is located at one end of the *cag* pathogenicity island (*cagPAI*). The island contains two segments: an upstream *cag* II region and a downstream *cag* I region<sup>[45]</sup>. *PAI* comprises a gene cluster of 40 kbps that encodes a type IV secretion system (T4SS) that functions to translocate *cagA* from epithelium-adherent bacteria into gastric epithelial



cells<sup>[45]</sup>. Once inside the cells, *cagA* is phosphorylated by host cellular kinases, Src<sup>[46,47]</sup> and Abl<sup>[48]</sup>, on a repeating glutamic acid proline-isoleucine-tyrosine-alanine tyrosine phosphorylation motif located at the carboxyl terminus of the protein. *In vitro* examinations of *H. pylori* infection of gastric epithelial cells revealed the requirement of proteins encoded by the *cagPAI*, with the exception of *cagA*, for IL-8 secretion, and the regulation of IL-8 induction by the NF- $\kappa$ B pathway<sup>[44,49]</sup>. However, *H. pylori*-induced pro-inflammatory responses remain controversial<sup>[50-52]</sup>. Ando *et al*<sup>[51]</sup> observed upregulated IL-8 expression in gastric epithelial cells infected with *H. pylori* containing an inactivated *cagA* gene, while Peng *et al*<sup>[52]</sup> reported up-regulation of IL-8 expression in gastric epithelial cells in response to treatment with extracts of *cagA*-positive and *cagA*-negative strains. Bacterial *cagA* expression may not be essential for the upregulation of IL-8 expression in *H. pylori*-infected gastric epithelial cells.

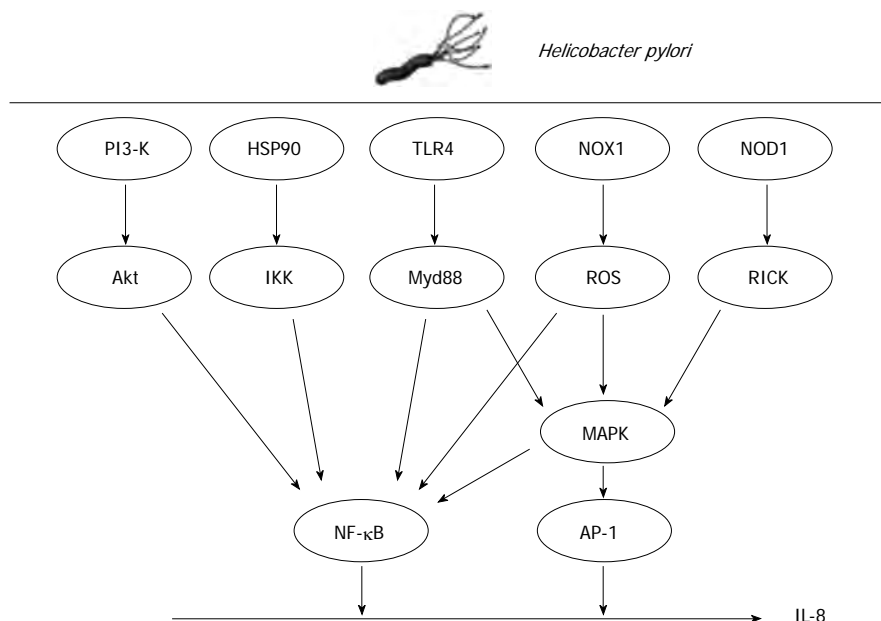
Although it is well known that *H. pylori* upregulates IL-8 expression in gastric cancer cells, the underlying molecular mechanism is not fully understood. Analyses of the genomic structure of IL-8 have revealed many potential targets for regulation at both the transcriptional and post-transcriptional levels. Within its 3'-flanking region, the IL-8 gene contains a repetitive ATTTA motif, which is responsible for destabilization of various cytokine mRNAs<sup>[53]</sup>. Within the 5'-flanking region, the gene contains multiple *cis* elements including a CCAAT box, steroid-responsive element, hepatocyte nuclear factor-1 element, two interferon regulatory factor-1 elements and binding sites for activator protein-1 (AP-1), CCAAT/enhancer binding protein and NF- $\kappa$ B, all of which have been implicated in the induction of IL-8 gene transcription by the aforementioned stimuli<sup>[54]</sup>. As demonstrated by mutation and deletion analyses, these promoter elements are regulated in cell type-specific manners<sup>[55]</sup>. A myriad of intracellular signals have been suggested to mediate the effects of *H. pylori* including production of reactive oxygen species (ROS), and activation of transcription factor NF- $\kappa$ B, AP-1 and mitogen-activated protein kinase (MAPK).

ROS are involved in the pathogenesis of *H. pylori*-associated gastric diseases that include gastric cancer<sup>[56,57]</sup>. Park *et al*<sup>[57]</sup> reported that ROS are produced by NADPH oxidase (NOX1) and induce apoptotic cell death of *H. pylori*-infected gastric epithelial cells. NOX1 induced by *H. pylori* in gastric disease functions in the constitutive production of superoxide anion and hydrogen peroxide<sup>[58]</sup>. Increased expression of NOX1 mRNA moderately increases the generation of superoxide anion, which leads to a reduction in aconitase activity, making NOX1 a good marker of oxidative stress. ROS induced by *H. pylori* stimulate MAPKs, such as extracellular signal-related kinases (ERKs), c-Jun NH<sub>2</sub>-terminal kinases (JNKs) and p38 MAPK, and upregulate transcription of NF- $\kappa$ B<sup>[59]</sup>. Interestingly, IL-8 contributes to the generation of copious quantities of ROS, and can elicit the induction of IL-1 $\beta$ , IL-6, IL-8, IL-12, tumor necrosis factor- $\alpha$ <sup>[60,61]</sup>, and

interferon- $\gamma$ <sup>[60]</sup>. IL-8 activates the CD11b/CD18 dimer, which forms a complex with neutrophils. The complex activates ICAM-1 on the vascular endothelial cell membrane. The resulting tetramer (CD11b/CD18/neutrophil/ICAM-1) infiltrates gastric epithelial cells and facilitates the copious release of ROS through neutrophil NADPH oxidase, resulting in an oxidative burst<sup>[62,63]</sup>. The ROS released from gastric epithelial cells may mediate the chemotactic action of neutrophils and monocytes in *H. pylori*-infected gastric tissues<sup>[56]</sup>.

Co-culture of *H. pylori* with cells can induce IL-8 through the activation of the oxidant-sensitive transcriptional factor NF- $\kappa$ B. ROS are important in this process in *H. pylori*-infected cancer cells<sup>[64]</sup>. NF- $\kappa$ B exists in a latent form in the cytoplasm, bound to the inhibitory protein, I $\kappa$ B. I $\kappa$ B kinase (IKK) directly phosphorylates I $\kappa$ B molecules, leading to the ubiquitin-mediated proteolysis of I $\kappa$ B. The NF- $\kappa$ B dimer that is released from I $\kappa$ B translocates to the nucleus where it activates target genes by binding to the promoter/enhancer region. In addition to ROS, several mechanisms for NF- $\kappa$ B activation by *H. pylori* have been proposed. *H. pylori* induces the phosphorylation of heat shock protein 90 (Hsp90) in gastric epithelial cells<sup>[65,66]</sup>. Hsp90 associates stoichiometrically with the IKK complex, which contributes to the stabilization, activation and shuttling of IKKs to the plasma membrane, because Hsp90 regulates the stability and function of a unique complement of signaling molecules<sup>[67]</sup>. Given that Hsp90 is associated with IKK- $\alpha$  and IKK- $\gamma$  in *H. pylori*-infected gastric epithelial cells<sup>[65]</sup>, the Hsp90-IKK complex may be a target for the pharmacological inhibition of the *H. pylori*-mediated activation of NF- $\kappa$ B signaling. Takeshima *et al*<sup>[68]</sup> reported that NF- $\kappa$ B activation by *H. pylori* requires Akt-mediated phosphorylation of p65. Phosphorylated Akt is detected in epithelial cells of *H. pylori* positive gastric tissues. The application of phosphoinositol-3-kinase inhibitor, dominant-negative Akt and small interfering RNA for Akt suppresses *H. pylori*-induced p65 phosphorylation as well as IL-8 expression, suggesting that Akt signals are involved in *H. pylori*-induced NF- $\kappa$ B activation.

*H. pylori* also activates the transcription factor AP-1 in a *cagPAI*-dependent manner<sup>[69,70]</sup>. The AP-1 complex activated during *H. pylori* infection is composed primarily of c-jun and c-fos heterodimers<sup>[71]</sup>. AP-1 is activated by MAPK and is capable of inducing a strong pro-inflammatory response, often in concert with NF- $\kappa$ B<sup>[71]</sup>. *H. pylori* rapidly activate MAPKs upon contact with gastric epithelial cells<sup>[72]</sup>. MAPK cascades are well characterized pathways that transduce signals from the cell surface to the nucleus. The family includes distinct subgroups: ERKs, JNKs and p38 MAPK. A number of bacterial factors have been implicated in MAPK activation including *vacA*<sup>[73]</sup> and *cagA*<sup>[72]</sup>. The signaling events leading to rapid MAPK phosphorylation during *H. pylori* infection are not well understood, although T4SS is required for ERK phosphorylation of p38 MAPK and JNK<sup>[67]</sup>. *CagA* is capable of activating ERK<sup>[73]</sup>, though ERK can also be



**Figure 2** Scheme of signaling of *Helicobacter pylori*-induced interleukin-8 in gastric cancer cells. PI3-K: Phosphoinositide 3-kinase; HSP: Heat shock protein; TLR: Toll-like receptor; NOX: NADPH oxidase; NOD: Nucleotide binding and oligomerization domain; IKK: IκB kinase; ROS: Reactive oxygen species; RICK: Receptor-interacting protein serine-threonine kinase; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor kappa B; AP-1: Activator protein-1.

activated by *cagA*-independent mechanisms<sup>[74]</sup>, suggesting that *cagA* has an additive role in transcription factor activation. JNK activation during *H. pylori* infection also requires a functional T4SS<sup>[72]</sup>. *H. pylori* peptidoglycan is delivered to the host cell *via* the T4SS, where it is recognized by cytosolic nucleotide binding and oligomerization domain 1 (NOD1)<sup>[75]</sup>. Upon stimulation with purified agonist, NOD1 associates with the receptor-interacting protein serine-threonine kinase 2, triggering a pro-inflammatory response that is characterized by NF-κB activation and IL-8 production<sup>[76]</sup>. In addition to activation of the classical NF-κB pathway, NOD1 is reported to be required for MAPK activation in response to bacterial pathogens. This NOD1-dependent p38 MAPK activation induces IL-8 production<sup>[77]</sup>. Allison *et al*<sup>[77]</sup> observed that NOD1 was necessary for MAPK activation in the early stages of infection and that NOD1 was essential for the activation of both NF-κB and AP-1, as well as the release of IL-8 in response to *H. pylori*. These observations support previous findings that *cagA* induces IL-8 induction *via* the Ras→Raf→Mek→ERK→NF-κB signaling pathway<sup>[78]</sup> and that *cagA* can activate the Ras→ERK pathway<sup>[79]</sup>. Understanding the signals involved in IL-8 expression by *H. pylori* may be beneficial to develop new therapeutics in gastric cancer (Figure 2).

## IL-8 AS A THERAPEUTIC TARGET IN GASTRIC CANCER

Gastric cancer features increased IL-8 expression, suggesting that IL-8 might be a promising therapeutic targeting to prevent cancer progression. Many inhibitors that prevent *H. pylori*-induced IL-8 expression and regulate the

IL-8 downstream signals have been proposed (Table 1).

Polyphenols derived from natural products that include resveratrol, apigenin and anthocyanins inhibit IL-8 induced by *H. pylori*. Resveratrol suppresses the secretion of IL-8 from *H. pylori*-infected gastric epithelial cells. IL-8 secretion is usually regulated by the transcription factor NF-κB and *H. pylori* can induce IL-8 expression by activating a NF-κB pathway in gastric epithelial cells<sup>[80,81]</sup>. Since resveratrol inhibits NF-κB<sup>[82]</sup>, its suppressive effect on IL-8 secretion may correlate with its NF-κB inhibitory activity. Inhibition of IL-8 expression by resveratrol may also be due to modulation of regulatory enzymes like MAPK<sup>[83]</sup>. Anti-oxidant anthocyanins from black soybean may inhibit IL-8 production<sup>[84]</sup>. Cyanidin-3-glucoside, which is abundant in anthocyanins, is reportedly an effective anti-oxidant that inactivates NF-κB by inhibiting phosphorylation of IκB<sup>[85,86]</sup>. Anthocyanins have anti-oxidant effects and the ability to downregulate ROS generation, and decrease the activation of MAPKs induced by *H. pylori*. Apigenin, one of the most common flavonoids, increases IκBα expression, and thus inhibits NF-κB activation and decreases IL-8 expression<sup>[87]</sup>. Apigenin's anti-inflammatory activity has been characterized *in vitro* and *in vivo*<sup>[88,89]</sup>.

Phenyl-thiophenyl propenone RK-I-123 is a small molecule that reportedly reduces the level of ROS and suppresses the activation of NF-κB and AP-1, and the expression of IL-8 in *H. pylori*-infected gastric epithelial cells<sup>[90]</sup>. RK-I-123 was synthesized as a novel propenone compound in an attempt to develop a dual inhibitor of COX-2 and 5-LOX<sup>[91]</sup>. 7-Carboxymethoxy-3',4',5-trimethoxy flavone, abbreviated as DA-6034, is a synthetic derivative of eupatilin that also inhibits IL-8 induced by *H. pylori*<sup>[92]</sup>. DA-6034 promotes the dissociation of the

**Table 1** Inhibitors targeting interleukin-8 in cancer progression

Inhibitors	Mechanisms	Ref.
Resveratrol	Reduces ROS, inhibits MAPK, AP-1 and NF- $\kappa$ B	[82,83]
Anthocyanin	Reduces ROS, inhibits MAPK, AP-1 and NF- $\kappa$ B	[84-86]
Apigenin	Increases the I $\kappa$ B $\alpha$ and thus inhibits NF- $\kappa$ B	[88,89]
RK-I-123	Reduces ROS and inhibits AP-1 and NF- $\kappa$ B	[91]
DA-6034	Dissociates IKK/HSP90 complex and inhibits NF- $\kappa$ B	[92,93]
Rebamipide	Prevents PLD expression <i>via</i> NF- $\kappa$ B	[94,95]
Gefitinib [Iressa <sup>TM</sup> ]	Inhibits EGFR	[98,99]
<i>L. bulgaricus</i>	Inhibits TLR4	[101]
<i>L. acidophilus</i>	Dissociates IKK/Hsp90 complex and inhibits NF- $\kappa$ B	[65,103]
NRF peptide	Disrupts interaction of NRF and NF- $\kappa$ B	[105]
miR-146	Negatively regulates IL-8	[110,111]
miR-155	Inhibits MyD88 <i>via</i> NF- $\kappa$ B	[112,115]
G31P	Synthetic derivative of IL-8	[117]
SCH-527123	CXCR2 inhibitor	[118]

I $\kappa$ B $\alpha$ : Inhibitor of kappa B alpha; RK-I-123: Phenyl-thiophenyl propenone; DA-6034: 7-Carboxymethoxy-3',4',5-trimethoxy flavone; HSP: Heat shock Protein; PLD: Phospholipase D; EGFR: Epidermal growth factor receptor; NRF: NF- $\kappa$ B repressing factor; IL-8: Interleukin-8; miR: MicroRNAs; MAPK: Mitogen-activated protein kinase; IKK: I $\kappa$ B kinase; AP-1: Activator protein-1; ROS: Reactive oxygen species; TLR4: Toll-like receptor 4.

IKK-HspP90 complex and suppresses NF- $\kappa$ B signaling, leading to the inhibition of IL-8 expression in *H. pylori*-infected cells. DA-6034 also inhibits ERK in such cells<sup>[93]</sup>.

Rebamipide [2-[4-chlorobenzoylamino]-3-[2[1H]quinolinon-4-yl] propionic acid; OPC-12759], a mucosal-protective anti-ulcer agent, was reported to inhibit IL-8 in gastric cancer by the regulation of phospholipase D (PLD) expression. Gastric cancer cells infected with *H. pylori* display significant induction of PLD1 expression *via* activation of NF- $\kappa$ B<sup>[94]</sup>. The level of PLD1 protein and I $\kappa$ B $\alpha$  phosphorylation is aberrantly upregulated in *H. pylori*-infected human gastric tissues. Rebamipide is a gastroprotective agent used in the treatment of gastritis and gastric ulcers<sup>[95]</sup>. It protects against gastric mucosa inflammation induced by *H. pylori* by inhibiting neutrophil function<sup>[96]</sup>. Moreover, rebamipide inhibits the growth of gastric cancer cells<sup>[97]</sup>. PLD and IL-8 might be novel targets of rebamipide in *H. pylori*-associated gastric cancer.

Gefitinib (Iressa<sup>TM</sup>, ZD1839) reportedly inhibits epidermal growth factor (EGF) signals and IL-8 production in gastric cancer cells<sup>[98]</sup>. Gefitinib is an orally active, quinazoline-derived agent that inhibits EGF receptor (EGFR)-tyrosine kinase<sup>[99]</sup>. Previous studies have shown that EGFR-mediated signals contribute to the expression of IL-8 and that IL-8 may be involved, at least in part, in EGF/EGFR-induced cancer progression<sup>[100]</sup>. Kishida *et al.*<sup>[98]</sup> employed SN38 (an active metabolite of CPT-11) for activation of EGFR-tyrosine kinase. SN38 activates the EGF/EGFR autocrine loop and induces IL-8 in gastric cancer cells. SN38 induces binding activities in both NF-

$\kappa$ B and AP-1, critical transcription factors for the expression of IL-8, and this reaction is inhibited by gefitinib.

Interestingly, Zhou *et al.*<sup>[101]</sup> suggested that the probiotic application of lactobacilli may inhibit IL-8 production induced by *H. pylori*-activated Toll-like receptor 4 (TLR4). *Lactobacillus bulgaricus* (LBG), a bacterium used in the production of yogurt, is one of the best-studied probiotic microbes. Probiotics are living microorganisms with no or low pathogenicity, which exert beneficial effects on the host. *H. pylori* induces mucosal inflammation including IL-8 production *via* TLR4 signaling<sup>[102]</sup>. Conjugated linoleic acids (CLA) produced by *Lactobacillus acidophilus* (LBA) also decreases the activation of NF- $\kappa$ B and IL-8 expression in *H. pylori*-infected gastric epithelial cells<sup>[103]</sup>. Kim *et al.*<sup>[65]</sup> demonstrated that CLA-containing conditioned medium produced by LBA has anti-inflammatory effects on *H. pylori* infection. In their study, conditioned medium produced by LBA significantly inhibited the activation of the core inflammatory gene signal NF- $\kappa$ B in gastric epithelial cells by dissociation of the complex between Hsp90 and the I $\kappa$ B kinase-subunit. CLA-containing conditioned medium also inhibited the expression of IL-8<sup>[65]</sup>. There is increasing evidence<sup>[104]</sup> that *Lactobacillus* has therapeutic effects on *H. pylori*-related diseases, including enhanced eradication of *H. pylori*, amelioration of resistance to antibiotics, downregulated side effects of antibiotic-based therapy, decreased recurrence of *H. pylori* infection, and inhibition of *H. pylori*-induced apoptosis.

Bartel *et al.*<sup>[105]</sup> suggested that a peptide capable of disrupting the interaction between NF- $\kappa$ B and NF- $\kappa$ B repressing factor (NRF) inhibits *H. pylori*-induced IL-8 expression. In *IL-8* gene expression, exclusively, NRF had two functions. It repressed the basal transcription of *IL-8* gene in unstimulated cells<sup>[106]</sup>, but, following cell stimulation, it was required for the transcriptional activation of the *IL-8* gene. A synthetic peptide corresponding to amino acid 223-238 of NRF interfered with the binding of endogenous NF- $\kappa$ B to NRF interaction, which significantly decreased endogenous *IL-8* gene transcription in response to *H. pylori* infection.

Several microRNAs (miR) are reported to regulate *IL-8* gene expression<sup>[107]</sup>. miRNAs are central regulators of various physiologic processes and their disruption is associated with human diseases<sup>[108]</sup>. Recently, Liu *et al.*<sup>[109]</sup> reported that miR-146a negatively regulated *H. pylori*-induced IL-8 *via* reduced NF- $\kappa$ B activity. miR-146a reportedly suppresses NF- $\kappa$ B activity through the reduction of metastatic potential in cancer cells<sup>[110]</sup>. The authors also reported that miR-146a is the negative regulator of NF- $\kappa$ B activity through the downregulation of IRAK1 and TRAF6 in cancer cells. Perry *et al.*<sup>[111]</sup> found that miR-146a was able to negatively regulate the release of IL-1 $\beta$ -induced IL-8, independent of IRAK1 and TRAF6 signals. miR-155 was also suggested to regulate IL-8 in *H. pylori*-infected gastric epithelial cells. Overexpression of miR-155 reportedly reduced the *H. pylori*-induced IL-8 expression<sup>[112]</sup>. miR-155 has been indicated to play a key role in the regulation of normal immunity or inflammation



response<sup>[113,114]</sup>. Among a number of targets of miR-155, MyD88 is suggested for IL-8 regulation<sup>[115]</sup>. miR-155 may downregulate the protein MyD88 through inhibition of translation. Most TLRs activate MyD88 leading to the nuclear translocation of transcription factors, such as NF- $\kappa$ B and AP-1, and thus transcriptionally regulate IL-8<sup>[116]</sup>. The function of miRNAs during *H. pylori* infection is complex and miR-155 may cooperate with other *H. pylori*-induced miRNAs including miR-146a in response to *H. pylori*. There may be crosstalk between miR-146a and miR-155 in the signal pathways leading to the downregulation of *H. pylori*-induced IL-8 in gastric cancer.

Small molecule inhibitors targeting IL-8 receptors (CXCR1 and CXCR2) have been developed to suppress prostate and colon cancers<sup>[117,118]</sup>. Inhibition of these receptors reduces cell migration and invasion, while increasing apoptosis in cancer cells<sup>[119]</sup>. Liu *et al.*<sup>[117]</sup> synthesized a derivative of the human cytokine IL-8, G31P, with high-affinity for human CXCR1 and CXCR2. G31P treatment significantly reduced prostate cancer cell viability, adhesion and migration capacity. Additionally, G31P inhibited tumor tissue vascularization, which was associated with the decreased expression of vascular endothelial growth factor and NF- $\kappa$ B in orthotopic xenograft tissues. Another small molecule inhibitor targeting CXCR2 is SCH-527123<sup>[118]</sup>. SCH-527123 is able to suppress CXCR2-mediated signal transduction as shown through decreased phosphorylation of the NF- $\kappa$ B, MAPK and Akt pathways in colon cancer cells. The anti-tumor activity of SCH-527123 resulted from inhibition of cancer cell growth, motility, and angiogenesis. In addition to having a direct anti-angiogenic and anti-tumor effect, targeting IL-8 or CXCR2 may also increase chemosensitivity to chemotherapeutics. Wilson *et al.*<sup>[120]</sup> also showed that IL-8/CXCR2 signaling confers resistance to chemotherapeutics (oxaliplatin) through NF- $\kappa$ B activity, which is an important determinant of cancer cell sensitivity to chemotherapeutics.

## CONCLUSION

There is a close association between *H. pylori* infection and gastric cancer. IL-8 is overexpressed in gastric epithelial cells exposed to *H. pylori*. IL-8 is significantly upregulated in both the tumor and its microenvironment, and acts as a key regulator of proliferation, angiogenesis and metastasis. IL-8 expression also contributes to the resistance of gastric cancer to chemotherapeutics. Although anti-IL-8 therapeutic agents are yet to enter preclinical and clinical trials, a large body of published evidence suggests that targeting IL-8 in gastric cancer could have broad-spectrum anti-tumor effects. Many advances have been made since the discovery that IL-8 regulates cell signaling in cancer development and progression independently of chemotaxis during the inflammatory process. Thus, we propose that IL-8 induced by *H. pylori* plays a major role in gastric cancer and that targeting IL-8 may be a promising strategy for the treatment of cancer.

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## WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

# *Helicobacter pylori* $\gamma$ -glutamyl transpeptidase: A formidable virulence factor

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## Abstract

*Helicobacter pylori* (*H. pylori*) produce an enzyme known as  $\gamma$ -glutamyl transpeptidase (HpGGT) that is highly conserved and common to all strains. HpGGT has been gaining increasing attention as an important virulence factor of the bacterium, having been demonstrated to be an important colonization factor in several animal models and has also recently been strongly associated with the development of peptic ulcer disease. From the results of various independent researcher groups, it is clear that HpGGT acts through several pathways to damage gastric epithelial cells including the induction of apoptosis and cell cycle arrest, production of reactive oxygen species leading to DNA damage, promotion of inflammation by increasing cyclooxygenase-2 and interleukin-8 expression, and upregulation of heparin-binding epidermal growth factor-like growth

factor resulting in cell survival and proliferation. In addition, the potential role of HpGGT in promoting gastric carcinogenesis will also be discussed in this review. Apart from affecting the gastric epithelium, HpGGT also has immunomodulatory actions on host immune cells where it displays an antiproliferative effect on T cells by inducing cell cycle arrest and also works with other *H. pylori* virulence factors to skew dendritic cells towards a tolerogenic phenotype, possibly contributing to the persistence of the pathogen in the gastric mucosa.

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**Key words:** *Helicobacter pylori*; Gamma-glutamyl transpeptidase; Pathogenesis; Immunomodulation; Carcinogenesis

**Core tip:** *Helicobacter pylori* produce  $\gamma$ -glutamyl transpeptidase (HpGGT), an important virulence factor associated with the development of peptic ulcer disease. HpGGT acts through several pathways to damage gastric epithelial cells including induction of apoptosis and cell cycle arrest, production of reactive oxygen species, promotion of inflammation and upregulation of heparin-binding epidermal growth factor-like growth factor which may then lead to carcinogenesis. HpGGT also has immunomodulatory actions on immune cells where it displays an antiproliferative effect on T cells and skews dendritic cells towards a tolerogenic phenotype, possibly contributing to the persistence of the pathogen in the gastric mucosa.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a Gram-negative, spiral-shaped bacterium that selectively colonizes the human gastric mucosa. It has been reported to chronically infect at least half of the world's population<sup>[1,2]</sup> and may persist for life in the absence of appropriate treatment. *H. pylori* is a major etiological factor of a range of gastroduodenal diseases including chronic gastritis<sup>[3]</sup> and peptic ulcer disease<sup>[4]</sup>, and has been closely associated with the development of mucosa-associated lymphoid tissue lymphoma<sup>[5]</sup> and even gastric cancer<sup>[6]</sup>.

Since the first isolation of *H. pylori* in 1983<sup>[7]</sup>, numerous virulence factors of the pathogen have been identified including the extensively studied cytotoxin-associated gene A (CagA)<sup>[8]</sup> and vacuolating cytotoxin (VacA)<sup>[9]</sup>. In western countries, strains harbouring CagA and VacA (with s1/mL alleles) have been strongly associated with peptic ulcer disease and gastric cancer<sup>[10,11]</sup>. However, their relevance in East Asia remains unclear as such correlations were not apparent<sup>[12,13]</sup>. From these observations, it can be inferred that CagA and VacA are probably not the only factors contributing to *H. pylori* pathogenesis. There is thus a constant search for other pathogenic factors that could aid in the virulence of the bacterium. One such factor is *H. pylori*  $\gamma$ -glutamyl transpeptidase (HpGGT) which has been gaining increasing attention in recent years and will be the main focus of this review.

## PROPERTIES AND FUNCTIONS OF HpGGT

Similar to mammalian GGTs, HpGGT catalyzes reactions in which a  $\gamma$ -glutamyl moiety is transferred from  $\gamma$ -glutamyl compounds, such as glutathione, to amino acids (transpeptidation) or water (hydrolysis)<sup>[14]</sup>. HpGGT is first translated in a single-chain precursor form which is inactive. The proenzyme then undergoes intramolecular autocatalytic cleavage, resulting in a catalytically active heterodimer comprising a large (40 kDa) and small (20 kDa) subunit. Interestingly, the amino acid sequence of HpGGT is considerably different from the GGTs of other bacterial species, sharing only 52.5%, 47.7% and 38% amino acid sequence identities with *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* GGTs, respectively<sup>[15]</sup>. Among different *H. pylori* strains however, HpGGT is highly conserved with > 97% sequence homology between isolates<sup>[16]</sup>. Notably, HpGGT is also constitutively expressed and is commonly found in all *H. pylori* strains<sup>[15]</sup>, suggesting its importance in the physiology of the bacterium. In further support of this, a subsequent study by Gong and Ho<sup>[17]</sup> demonstrated the importance of HpGGT in the growth of *H. pylori* where strains with higher GGT activity exhibited more profuse growth compared to those having lower GGT activity. Indeed, it was later found that one of the main physiological functions of HpGGT is to metabolize extracellular glutathione and glutamine (substrates that it is unable to uptake directly) as a source of glutamate which is then taken up by the bacterium and subsequently incorporated into the

tricarboxylic acid cycle<sup>[18]</sup>.

## HpGGT and colonization

Although not essential for *in vitro* survival, two pioneer studies on HpGGT had earlier demonstrated the enzyme to be an important virulence factor of the gastric pathogen<sup>[15,19]</sup>. Using the Swiss specific pathogen-free murine model, Chevalier *et al.*<sup>[15]</sup> first described HpGGT to be essential for colonization as *H. pylori* SS1 GGT-deficient mutants could not be recovered from the mice stomachs from 3–60 d post-infection. Interestingly, McGovern *et al.*<sup>[19]</sup> later showed using two different animal models, namely gnotobiotic piglets and C57BL/6 mice, that although the *H. pylori* HpM5 *ggt*-isogenic mutants were still able to colonize the animals, the bacterial load was significantly reduced compared to the parental strain. The differences in animal models and *H. pylori* strains used by both groups could have contributed to the variations observed but nevertheless, both studies had consistently shown that the presence of HpGGT provides an advantage to the bacterium in colonization.

## Association between HpGGT and peptic ulcer disease

The clinical importance of HpGGT was reported by our group in 2010 where *H. pylori* isolates from patients with peptic ulcer disease ( $n = 54$ ) were found to have significantly higher GGT activity ( $P < 0.001$ ) compared to those cultured from patients with non-ulcer dyspepsia ( $n = 44$ )<sup>[16]</sup>. Furthermore, no correlation was observed between HpGGT and other known virulence genes such as *cagA*, *vacA*, *iceA* and *babA*, suggesting a causal link between HpGGT and gastroduodenal diseases. The exact mechanisms detailing how the presence of HpGGT leads to disease development have not been fully elucidated. However, several pathways involving both gastric epithelial cells as well as immune cells have been put forward by various groups and these will be discussed in this review.

## EFFECTS OF HpGGT ON GASTRIC EPITHELIAL CELLS

### HpGGT induces apoptosis

*H. pylori*-induced apoptosis of gastric epithelial cells both *in vitro* and *in vivo* had earlier been described by many researchers<sup>[20–22]</sup>, however the bacterial factor(s) responsible were not clearly defined. By analyzing various *H. pylori* membrane fractions capable of inducing apoptotic cell death in AGS cells, HpGGT was later found to be one of the leading factors involved in the induction of apoptosis by *H. pylori*<sup>[23]</sup>. The pathway by which this occurs is mitochondria-mediated as evident from the accompanying activation of caspases 9 and 3, upregulation of proapoptotic Bax and downregulation of antiapoptotic Bcl-2 and Bcl-xL as well as the release of cytochrome *c* from the mitochondria into the cytosolic space<sup>[24]</sup>. In addition, it has also been shown by Kim *et al.*<sup>[25]</sup> that HpGGT inhibits cell cycle progression at the G<sub>1</sub>-S phase transition and the authors have suggested that this dysregulation

results in the enhancement of apoptosis.

The underlying mechanism as to how HpGGT triggers apoptosis was not addressed in these earlier studies. Interestingly, we had recently reported that exposure of gastric cells to purified native HpGGT resulted in the formation of reactive oxygen species (ROS), in particularly  $H_2O_2$ <sup>[16]</sup> which is a known inducer of apoptosis<sup>[26-28]</sup>. Accordingly, we and others have shown that pro-oxidant products generated by HpGGT through glutathione degradation triggered apoptosis in gastric epithelial cells<sup>[16,29]</sup>, hence providing the link between HpGGT and its ability to induce apoptotic cell death. This model also corroborates with earlier observations whereby *H. pylori* infection was found to be associated with excessive ROS levels<sup>[30,31]</sup> and diminished glutathione levels in the infected gastric mucosa<sup>[32]</sup>.

Intriguingly, apart from gastric cells, HpGGT has also recently been shown to be capable of inducing mitochondria-mediated apoptosis in a human cholangiocarcinoma cell line<sup>[33]</sup>. This suggests that HpGGT-induced apoptosis is not only restricted to gastric epithelial cells and may possibly occur via a common pathway across different cell types. Hence, future studies investigating the effects of HpGGT on other cell lines may be of particular interest.

### **HpGGT is pro-inflammatory**

*H. pylori*-infected subjects develop an inflammatory and immune response towards the pathogen characterized by infiltration of the mucosa by polymorphonuclear and mononuclear leukocytes as well as neutrophils<sup>[34]</sup>. However, this response is ineffective in clearing the bacteria, thereby resulting in chronic gastric inflammation<sup>[35]</sup>. With regard to the role of HpGGT in inflammation, Busiello *et al.*<sup>[36]</sup> showed by using the MKN28 gastric cell line that HpGGT upregulates cyclooxygenase-2 (COX-2) expression and its enzymatic product prostaglandin  $E_2$ , whose role in inflammation has been well established<sup>[37]</sup>. Notably, COX-2 has been found to be overexpressed in various types of cancer including gastric carcinoma<sup>[38-40]</sup> and has roles in promoting cell proliferation, angiogenesis and metastasis<sup>[41-43]</sup>.

In addition, our group had also previously reported that purified native HpGGT stimulated the activation of the transcription factor NF- $\kappa$ B, leading to increased expression and secretion of the pro-inflammatory chemokine interleukin-8 (IL-8) from both AGS and primary gastric epithelial cells<sup>[16]</sup>. HpGGT-induced IL-8 production in gastric cells may thus contribute to the recruitment of immune cells to the sites of infection and the maintenance of chronic inflammation in the gastric mucosa. Importantly, *H. pylori* infection has been associated with elevated levels of gastric IL-8<sup>[44,45]</sup>, a potent neutrophil recruitment factor thought to play a pivotal role in the immunopathogenesis of *H. pylori* infections<sup>[46]</sup>. Collectively, these results strongly support the contributory

role of HpGGT in pro-inflammatory processes.

### **Increase in epidermal growth factor-related peptide expression**

HpGGT upregulates the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF-like growth factor family of proteins and a ligand of epidermal growth factor receptor (EGFR)<sup>[36]</sup>. HB-EGF is first synthesized as a membrane-anchored precursor which is subsequently cleaved at the cell surface, yielding the mature, soluble form<sup>[47]</sup>. Binding of soluble HB-EGF to EGFR activates the Raf/Ras/MEK/Erk and phosphoinositide-3-kinase (PI3K)/Akt pathways which promote cell survival and proliferation<sup>[48,49]</sup>. Importantly, expression of HB-EGF has been reported to be increased in various cancer types including hepatic<sup>[50]</sup>, breast<sup>[51]</sup>, ovarian<sup>[52]</sup> and gastric cancer<sup>[53]</sup>. Furthermore, both expression and protein shedding of HB-EGF have been found to be increased in *H. pylori* infections<sup>[54]</sup> and this has been suggested to contribute to gastric cancer progression by promoting epithelial-mesenchymal transition<sup>[55]</sup>. Till date, the definitive role of HpGGT-induced HB-EGF expression in gastric cells has not been clearly elucidated but its potential role in carcinogenesis would certainly be an area worth investigating in future studies.

### **Disturbing the balance between cell survival and cell death: Link to carcinogenesis?**

It seems contradictory for HpGGT to have both apoptosis- and survival-promoting properties. However, both effects may play different roles during the various events of carcinogenesis. HpGGT-induced apoptosis has been suggested to be important particularly in the early events of carcinogenesis<sup>[23]</sup>. This is because an increase in the rate of apoptosis in a subpopulation of cells could induce a secondary hyperproliferative response where the gastric mucosa attempts to maintain its cell mass<sup>[56]</sup>. Hyperproliferation, coupled with DNA damage induced by HpGGT<sup>[16]</sup>, could then potentially lead to an increase in the mutation rates of important tumor suppressor genes in these cells, resulting in their transformation to a malignant phenotype. In tumor cells that have become apoptosis-resistant, it is then possible that HpGGT-induced COX-2 upregulation in these cells contribute to their continuous survival and proliferation. This postulation is partially supported by the finding that HpGGT-dependent induction of COX-2 mRNA is higher in MKN28 cells compared to AGS cells as observed by Busiello *et al.*<sup>[36]</sup>. Although AGS and MKN28 cells are both carcinoma cells lines, MKN28 cells have a mutation in p53, an important tumor suppressor involved in the control of cell cycle progression and apoptosis<sup>[57]</sup>. Thus, it is plausible that COX-2-induced cell proliferation affects apoptosis-resistant tumor cells to a greater extent, leading to the survival and proliferation of these cancerous cells.

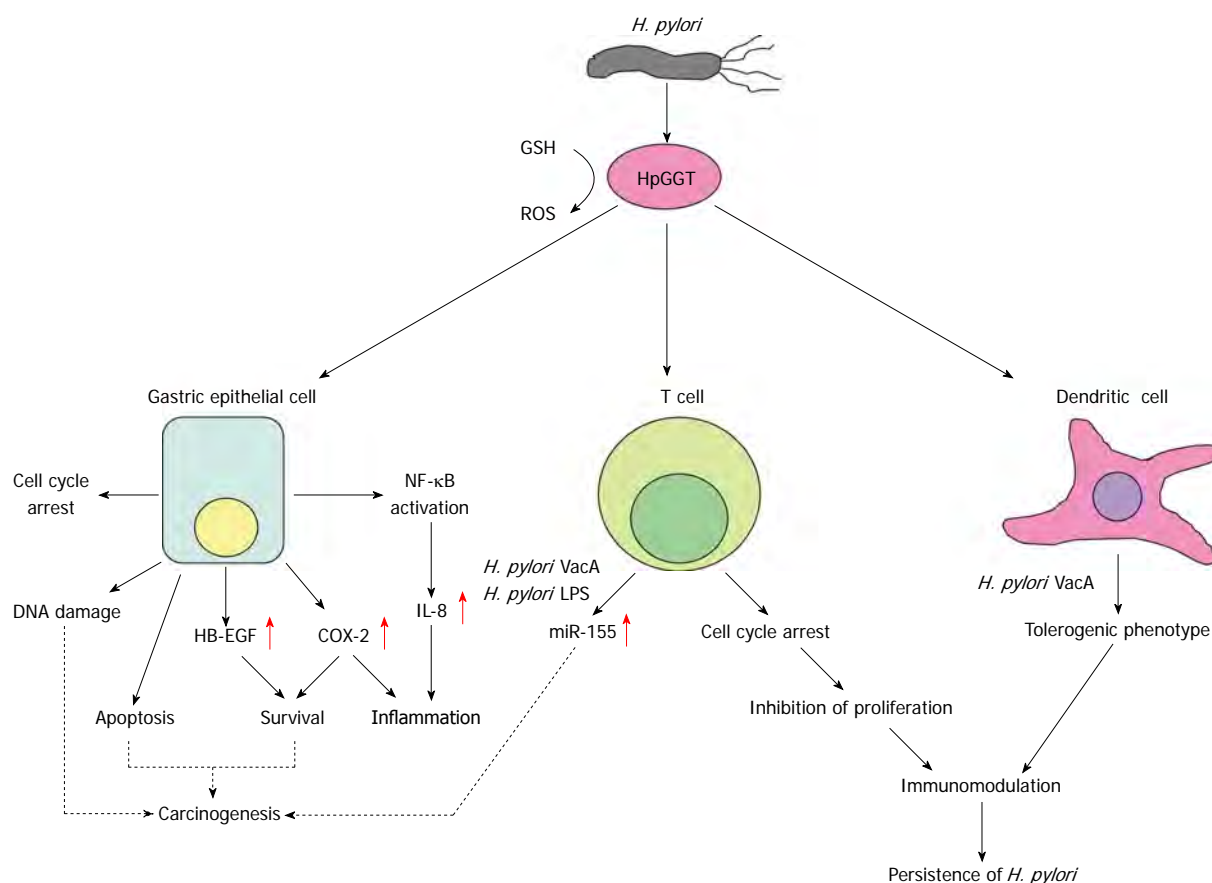


Figure 1 Schematic diagram highlighting important effects of *Helicobacter pylori*  $\gamma$ -glutamyl transpeptidase on both gastric epithelial and immune cells and their implications on carcinogenesis and persistence of *Helicobacter pylori* in the gastric mucosa. COX-2: Cyclooxygenase-2; HpGGT: *Helicobacter pylori* (*H. pylori*)  $\gamma$ -glutamyl transpeptidase; GSH: Glutathione; HB-EGF: Heparin-binding epidermal growth factor-like growth factor; IL-8: Interleukin-8; LPS: Lipopolysaccharide; miR-155: microRNA-155; NF- $\kappa$ B: Nuclear factor-kappa B; ROS: Reactive oxygen species; VacA: Vacuolating cytotoxin. Red arrows indicate upregulation of the respective molecules.

## HpGGT MODULATES THE IMMUNE SYSTEM

Apart from directly influencing gastric epithelial cells, an increasing body of evidence pointing to the role of HpGGT in modulating the immune response is emerging. Being a secreted bacterial protein<sup>[58]</sup>, the possibility of HpGGT interacting with other non-gastric cells is highly possible especially since *H. pylori* is capable of disrupting gastric epithelial barrier function<sup>[59]</sup>. Interestingly, the effects of HpGGT on immune cells have been investigated in various studies and have yielded important results and implications.

### Effects on T cells

In one of the earlier studies investigating the effects of HpGGT on immune effector cells, Schmees *et al.*<sup>[60]</sup> found that HpGGT was capable of abrogating the proliferation of both primary and immortalized human T cells. A corresponding cell cycle arrest at the G<sub>1</sub> phase was observed in these cells which possibly occurred due to disruption of a Ras-dependent signalling pathway. Intriguingly, inhibition of T cell proliferation by HpGGT was found in the same study to be mediated by an apoptosis-indepen-

dent mechanism which is different from that observed in gastric epithelial cells, suggesting that separate mechanisms exist in both cell types. HpGGT-induced inhibition of T cell proliferation has been proposed to have immunosuppressive effects which contribute to the persistence of *H. pylori* infections<sup>[60]</sup>. Interestingly, in a separate study by Beigier-Bompadre *et al.*<sup>[61]</sup>, HpGGT-dependent antiproliferative effect on T cells was found to be modulated by bacterial cholesterol/cholesterol  $\alpha$ -glucoside content, suggesting that HpGGT works with other *H. pylori* factors to shape the immune response during an infection.

Working together with *H. pylori* lipopolysaccharide and vacuolating cytotoxin (VacA), HpGGT was recently reported to upregulate microRNA-155 (miR-155) expression in CCRF-CEM cells, the first study to investigate the regulation of miRNAs by *H. pylori* in T cells<sup>[62]</sup>. Clinically, miR-155 has been shown to be induced upon *H. pylori* infection<sup>[63]</sup> and has also been associated with the development of diffuse large B-cell lymphoma<sup>[64,65]</sup>. In addition, HpGGT-induced miR-155 expression in both CCRF-CEM cells and primary human peripheral blood mononuclear cells was found to be dependent on forkhead box P3 (Foxp3) and requires activation of the cyclic adenosine monophosphate cascade<sup>[62]</sup>. Foxp3 is a tran-



**Table 1** Summary of the effects of *Helicobacter pylori*  $\gamma$ -glutamyl transpeptidase on the host and the possible underlying mechanisms involved

Ref.	Study description	Main findings
Peptic ulcer disease Gong <i>et al</i> <sup>[16]</sup> (2010)	Comparison of GGT activity between <i>H. pylori</i> isolates from PUD ( <i>n</i> = 54) <i>vs</i> NUD ( <i>n</i> = 44) patients.	HpGGT is associated with PUD as strains isolated from PUD patients had significantly higher HpGGT activity compared to those from NUD patients ( <i>P</i> < 0.001).
Gastric epithelium damage by apoptosis Shibayama <i>et al</i> <sup>[23]</sup> (2003)	Identification of apoptosis-inducing factors from <i>H. pylori</i> by testing different purified membrane fractions of the bacteria on AGS cells.	HpGGT is a leading factor in <i>H. pylori</i> -mediated apoptosis induction.
Kim <i>et al</i> <sup>[24]</sup> (2007)	Determination of the pathway involved in HpGGT-induced apoptosis by analyzing levels of caspase-9, -3, Bax, Bcl-2, Bcl-xL and cytochrome c release in AGS cells upon treatment with recombinant HpGGT.	HpGGT induces apoptosis <i>via</i> a mitochondria-mediated pathway.
Kim <i>et al</i> <sup>[25]</sup> (2010)	Examination of the effects of recombinant HpGGT on cell cycle progression in AGS cells.	HpGGT induces cell cycle arrest at the G <sub>1</sub> -S phase transition. (The authors propose this dysregulation enhances apoptosis induction)
Gong <i>et al</i> <sup>[16]</sup> (2010)	Investigation of the effects of HpGGT-induced H <sub>2</sub> O <sub>2</sub> production on apoptosis. AGS cells were incubated with purified native HpGGT and NAC (H <sub>2</sub> O <sub>2</sub> inhibitor) and the activities of caspase-3, -8 and -9 were measured.	HpGGT-mediated oxidative stress is required for HpGGT-associated apoptosis.
Promotion of inflammation Busiello <i>et al</i> <sup>[36]</sup> (2004)	Purification and identification of secreted <i>H. pylori</i> factors involved in the upregulation of COX-2 expression in MKN28 cells.	HpGGT is able to upregulate COX-2 expression and its enzymatic product, prostaglandin E <sub>2</sub> .
Gong <i>et al</i> <sup>[16]</sup> (2010)	Determination of the ability of HpGGT to induce IL-8 production in AGS and primary gastric epithelial cells.	Purified native HpGGT activates NF- $\kappa$ B and upregulates IL-8 production in gastric epithelial cells.
Upregulation of heparin-binding epidermal growth factor-like growth factor Busiello <i>et al</i> <sup>[36]</sup> (2004)	Investigation of the ability of HpGGT to upregulate HB-EGF expression in MKN28 cells and elucidating the underlying host cellular pathways involved using specific pathway inhibitors.	HpGGT upregulates HB-EGF expression <i>via</i> activation of a phosphatidylinositol-3 kinase and p38 kinase-dependent signalling transduction pathway. Increase in HB-EGF promotes cell survival and proliferation.
Modulation of host immune response Schmees <i>et al</i> <sup>[60]</sup> (2007)	Purification and identification of <i>H. pylori</i> factors responsible for inhibition of T cell proliferation.	HpGGT inhibits T cell proliferation by inducing cell cycle arrest in the G <sub>1</sub> phase, possibly through the disruption of a Ras-dependent signalling pathway.
Beigier-Bompadre <i>et al</i> <sup>[61]</sup> (2011)	Characterization of the interdependent effects of VacA, HpGGT and bacterial cholesterol on T cell proliferation using <i>H. pylori</i> and relevant mutants.	HpGGT antiproliferative activity on T cells is modulated by the bacterial cholesterol/cholesterol $\alpha$ -glucoside content.
Fassi Fehri <i>et al</i> <sup>[62]</sup> (2010)	Identification of <i>H. pylori</i> factors involved in the regulation of miRNAs in T cells using miRNA profiling.	HpGGT works with <i>H. pylori</i> VacA and lipopolysaccharide to upregulate miRNA-155 expression in CCRF-CEM cells. This was dependent on Foxp3 transcription factor and requires activation of the cAMP cascade.
Oertli <i>et al</i> <sup>[68]</sup> (2013)	Determination of the role of HpGGT and VacA in dendritic cell reprogramming and development of immune tolerance using <i>in vitro</i> and <i>in vivo</i> models.	Both HpGGT and VacA independently interfere with dendritic cell maturation, possibly contributing to dendritic cell tolerization and hence promoting the persistence of <i>H. pylori</i> infection.

cAMP: Cyclic adenosine monophosphate; COX-2: Cyclooxygenase-2; EGFR: Epidermal growth factor receptor; Foxp3: Forkhead box P3; *H. pylori*: *Helicobacter pylori*; HB-EGF: Heparin-binding epidermal growth factor-like growth factor; HpGGT: *H. pylori*  $\gamma$ -glutamyl transpeptidase; IL-8: Interleukin-8; miRNA: microRNA; NAC: N-acetylcysteine; NF- $\kappa$ B: Nuclear factor-kappa B; NUD: Non-ulcer dyspepsia; PUD: Peptic ulcer disease; VacA: Vacuolating cytotoxin.

scription factor thought to be the master regulator in the development of regulatory T cells (Treg)<sup>[66]</sup>, a subset of T cells with a suppressive activity on immune responses<sup>[67]</sup>. In support of this, mice infected with *ggt*-isogenic mutants were found to have lower Treg counts compared to wild type-infected mice<sup>[68]</sup>. Hence, it was suggested that HpGGT may play an important role in the modulation of the immune system<sup>[62]</sup>.

### HpGGT affects dendritic cells

The ability of *H. pylori* to reprogram dendritic cells (DCs) towards a tolerogenic phenotype has been implicated in

the development of immune tolerance and favors persistence of the bacteria in the gastric mucosa<sup>[69]</sup>. Recently, it has been reported that both VacA and HpGGT play critical roles in DC reprogramming by interfering with their maturation and that this occurred in a manner independent of their suppressive effects on T cells<sup>[68]</sup>. The underlying mechanisms dictating how both factors prevent DC maturation and promote tolerization were not clearly elucidated in the study but it is known that they act via non-redundant pathways since neither of the respective isogenic mutants was capable of rescuing the effect of the other.

## CONCLUSION

*H. pylori* produces a potent virulence factor, HpGGT, which causes injury to host cells through multiple ways (illustrated in Figure 1 and summarized in Table 1), many of which have been implicated in carcinogenesis. To gastric epithelial cells, it induces mitochondrial-dependent apoptosis, cell cycle arrest and production of the pro-inflammatory IL-8. To T cells, it inhibits their proliferation and upregulates miR-155 expression while to DCs, it skews them towards a tolerogenic phenotype. Taken together, it is clear that HpGGT plays an important role in the pathogenesis of *H. pylori* by directly damaging gastric epithelial cells and also in modulating the immune response towards the bacterium, resulting in persistent colonization by the organism. Despite the relatively numerous reports on its effects on the host, much of the underlying mechanisms of how such effects are brought about by HpGGT remain ill-defined. Future studies on the molecular mechanisms responsible for the actions of HpGGT will be required to better understand the role of HpGGT in the pathogenesis of *H. pylori*. This will be particularly important in the consideration of HpGGT as a viable anti-*H. pylori* target. In addition, it could also be worthwhile to evaluate the efficacy of HpGGT as a potential vaccine candidate against *H. pylori* infections especially since the protein is present in all *H. pylori* strains.

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WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

## Nickel trafficking system responsible for urease maturation in *Helicobacter pylori*

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### Abstract

*Helicobacter pylori* (*H. pylori*) is a common human pathogen responsible for various gastric diseases. This bacterium relies on the production of urease and hydrogenase to inhabit the acidic environment of the stomach. Nickel is an essential cofactor for urease and hydrogenase. *H. pylori* has to uptake sufficient nickel ions for the maturation of urease, and on the other way, to prevent the toxic effects of excessive nickel ions. Therefore, *H. pylori* has to strike a delicate balance between the import of nickel ions, its efficient intracellular storage, and delivery to nickel-dependent metalloenzymes when required. The assembly and maturation of the urease enzyme is a complex and timely ordered process, requiring various regulatory, uptake, chaperone and accessory proteins. In this review, we focus on several nickel trafficking proteins involved in urease maturation: NikR, NixA, HypAB, UreEFGH, HspA, Hpn and HpnI. The work will deepen our understanding of how this pathogenic bacterium adapts to severe habitat environments in the host.

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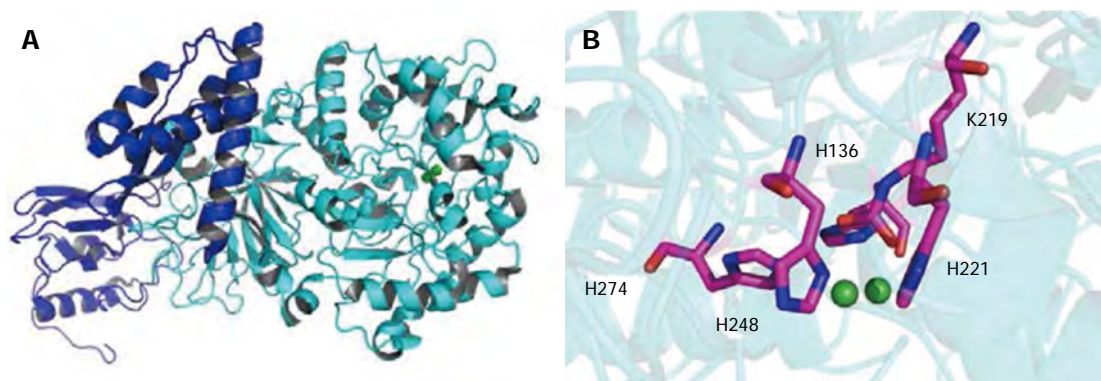
**Key words:** Urease; Histidine-rich protein; NikR; NixA; *Helicobacter pylori*

**Core tip:** *Helicobacter pylori* (*H. pylori*) is responsible for various gastric diseases. The nickel containing urease and hydrogenase are essential for the successful infections of *H. pylori* in the stomach. Nickel is an essential cofactor for urease and hydrogenase. In this review we discussed the various regulatory, uptake, chaperone and accessory proteins involved in the maturation of urease, especially the proteins NikR, NixA, HypAB, UreEFGH, HspA, Hpn and HpnI. The work will deepen our understanding of how this pathogenic bacterium adapts to severe habitat environments in the host.

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### INTRODUCTION

*Helicobacter pylori* (*H. pylori*), a micro-aerophilic Gram-negative spirobacterium, infects around half of the people worldwide and is responsible for gastric diseases



**Figure 1** Structure of *Helicobacter pylori* urease. A: The urease enzyme subunit UreAB (UreA, blue; UreB, cyan; PDB code: 1E9Z); B: The active sites of *H. pylori* urease with the side chains of the enzyme involved in the chelation of the catalytic di-nickel center shown (Ni, green; O, red; N, blue; C, pink). *H. pylori*: *Helicobacter pylori*.

such as chronic gastritis, peptic ulcer and gastric cancer<sup>[1]</sup>. The bacterium is widely present in the mucus layer of the stomach, the mucus glands in the stomach cavity and the surface of gastric epithelial cells as well as within the cells. Due to the wide presence in the differential parts of the stomach, it is difficult to completely eradicate the pathogen during gastric disease therapy<sup>[2]</sup>. The commonly used treatment for *H. pylori* related diseases is the so-called triple therapy, which consists of two antibiotics and either a proton pump inhibitor (PPI) or one kind of bismuth-based colloidal drug<sup>[3,4]</sup>. In some countries, standard triple therapy combining one PPI, amoxicillin and clarithromycin is the best option. However, in countries where clarithromycin resistance rate is over 20%, bismuth-containing quadruple therapy, or non-bismuth sequential or concomitant therapies are the preferred option. The medical and social impact of the discovery of *H. pylori* was acknowledged by the award of the 2005 Noble Prize in Physiology and Medicine to Marshall and Warren.

Around 80% of *H. pylori* cells inhabit the moderately acidic gastric mucus. Once entry into the stomach, the first hurdle for *H. pylori* is to be quickly transmitted through the extremely acidic gastric lumen, exhibiting a median pH of approximately 1.4<sup>[5]</sup>. *H. pylori* multiplies in an environmental pH from 6.0 to 8.0<sup>[6]</sup>, and cannot survive when the pH < 4.0 or > 8.2<sup>[7]</sup>. In order to live in the gastric environment, *H. pylori* has developed various acid-resistant mechanisms. Time-independent acid resistance depends on the high isoelectric points of the inner and outer membrane proteins to reduce proton permeability<sup>[8]</sup>. Acute acid resistance depends on the constitutive synthesis of urease that catalyzes the hydrolysis of urea to ammonia and carbamate, the latter of which is further degraded to ammonia and carbonic acid. The end products are in an equilibrium between their protonated and de-protonated forms, leading to an elevation of the surrounding pH from absolutely acidic to approximately neutral<sup>[9]</sup>. Urease is an oligomeric Ni<sup>2+</sup>-containing heterodimer of UreA and UreB subunits and is essential for *H. pylori* to infect in all animal models so far examined<sup>[10-12]</sup>. The substrate gastric juice urea is able to rapidly

access intrabacterial urease through a pH-gating urea channel, UreI<sup>[13]</sup>, when the periplasmic pH falls < 6.2.

*H. pylori* urease is produced in a high level, accounting for up to 10% of total cellular proteins<sup>[14]</sup>. Expression of urease protein is constitutive<sup>[15]</sup>, primarily due to the housekeeping  $\sigma^{80}$ -dependent promoters for the transcription of both *ureAB* and *ureEFGHI*<sup>[16,17]</sup>. Under *in vitro* growth conditions without additionally added Ni<sup>2+</sup>, only 2% of the active sites were filled with Ni<sup>2+</sup><sup>[18,19]</sup>. Urease produces NH<sub>3</sub> from gastric juice urea with maximal efficiency at millimolar concentrations<sup>[14,20]</sup>, 10<sup>14</sup> times faster than uncatalyzed reactions. The enzymatic hydrolysis of urea causes an abrupt overall pH increase, resulting in negative side effects for human and positive effects in the buffering of the periplasm and maintenance of a proton motive force adequate for ATP synthesis of the bacterium<sup>[21]</sup>. *H. pylori* urease was shown to be a giant 1.1 MDa complex containing 12 subunits of UreA and UreB (Figure 1), with two Ni<sup>2+</sup> needed for enzyme activity<sup>[6,22]</sup>. The assembly of the urease enzyme is a complex, timely ordered process, and the UreEFGH accessory proteins are absolutely necessary<sup>[23,24]</sup>. UreH stabilizes the apoprotein<sup>[23]</sup>; UreF facilitates carbamylation of the Ni<sup>2+</sup>-bridging lysine residue and blocks premature Ni<sup>2+</sup> binding to the active site<sup>[26]</sup>; UreG provides energy during urease assembly<sup>[27]</sup>, and UreE facilitates Ni<sup>2+</sup> incorporation into the active center<sup>[28]</sup>. The hydrogenase accessory proteins HypA and HypB are also necessary to maintain the urease activity, indicating that the bacterium utilizes both maturation systems for the activation of its urease<sup>[18]</sup>. This present review intends to cover the reports and discoveries in the field of nickel trafficking system in urease maturation of *H. pylori*, which may deepen our understanding of how this pathogenic bacterium adapts to severe habitat environments in the host.

## NICKEL REGULATORY PROTEIN NIKR

Bacteria have developed sophisticated mechanisms to regulate levels of intracellular nickel ions, to ensure sufficient nickel for enzyme processes in one way and to

prevent excessive toxic free ions in the other way<sup>[29]</sup>. NikRs, a novel class of ribbon-helix-helix nickel regulatory proteins, are homotetrameric transcription factors that repress and/or activate specific genes in response to nickel availability. *H. pylori* NikR, a tetrameric protein made of two dimeric N-terminal DNA-binding domains (DBD) and C-terminal domains for tetramerization and metal binding (MBD), binds stoichiometric nickel with picomolar affinities<sup>[30,31]</sup>, comparable to NikRs from other species<sup>[32-34]</sup>. The DBD and MBD are connected by a flexible linker, allowing for differential conformations (open, trans and cis) of NikR. In *E. coli* and *Pyrococcus horikoshii*, the apo-NikRs adopt an open conformation, whereas the apo-NikR shows an unusual closed *trans*-conformation and asymmetrical quaternary arrangement, where the DBDs are on the opposite sides of the transmembrane domain<sup>[35]</sup>. Computational and NMR studies suggest that NikR is interconverting among the open, trans and cis forms in solution and nickel binding facilitates the interconversion<sup>[36]</sup>.

At non-physiologically low pH (4.6-5.6), NikR had three types of nickel-binding sites: the final high affinity site (F) with square-planar geometry, the intermediate site (I) involving residues belonging either to the F or external site, and the external sites (X) with an octahedral geometry<sup>[35,37]</sup>. Whereas in physiological conditions (pH 5.6-7.5), NikR binds four low-spin Ni<sup>2+</sup> at the protein tetramerization interface, although differential nickel coordination modes are proposed. Michel's group suggests that two nickels are bound at 4-coordinate square-planar sites with His<sub>3</sub>Cys ligands (*i.e.*, 4-sites) and the other two are coordinated by His<sub>3</sub>(H<sub>2</sub>O)<sub>2-3</sub> in square pyramidal or octahedral geometries (*i.e.*, 5/6 sites)<sup>[37]</sup>. Ciurli's group reports a structure with all four nickel ions bound to 4 sites<sup>[38]</sup>, and the four binding sites are classified into two sets (2/2), with binding affinities differing by one order of magnitude<sup>[39]</sup>. The findings may suggest that an equilibrium exists between the two nickel-bound forms of the protein.

The biological role of NikR is to regulate the transcription of multiple genes as a function of nickel availability<sup>[40,41]</sup>: up-regulated genes in nickel metabolism (*nikABCDE*, *nixA*, *ureA*, *ureB*, *hpn* and *hpn-like*); down-regulated genes in iron uptake and storage (*pfr*, *fur* and *exbB/exbD*), motility (*cheV*, *flaA* and *flaB*), and stress responses to outer membrane proteins (*omp11*, *omp31* and *omp32*)<sup>[40]</sup>. The nickel-responsive binding of NikR to target promoters *pUreA*, *pNikR*, *pexbB* and *pFur* have been characterized by the *in vitro* gel shift and DNase I footprinting studies. Michel's<sup>[42]</sup> group proposed a mechanism for nickel-mediated DNA recognition by NikR. NikR prefers binding Ni at 5/6 sites. Upon addition of two Ni, the ligands are rearranged to two 4-sites. Addition of two more Ni results in mixed coordination geometry (two 4-sites and two 5/6-sites) and makes the protein binding to target DNA. The binding to DNA changes the orientation of the DBD from trans to cis, an orientation that is stabilized at the MBD/DBD interface<sup>[42]</sup>.

Controversial opinions exist for the roles of NikR

in urease activation as a function of pH. One opinion goes that under acidic conditions, the greater availability of Ni<sup>2+</sup> leads to the formation of Ni<sup>2+</sup>-NikR complexes which further increase the expression of urease, Ni<sup>2+</sup> transporter NixA and iron regulator Fur<sup>[43,44]</sup>. Whereas, Pflock *et al.*<sup>[45]</sup> found that a two-component system ArsRS (acid responsive signaling) regulated urease expression in response to low pH, and further proposed that urease expression is mediated by two distinct mechanisms: one in response to increasing Ni<sup>2+</sup> concentration (NikR) and one in response to decreasing pH (ArsR).

## NICKEL UPTAKE

Due to the essential stasis of Ni<sup>2+</sup>-containing urease for the host colonization and infection of *H. pylori*, a constant supply of Ni<sup>2+</sup> into *H. pylori* is required. The concentration of nickel ions in the environment is relatively low: around 30 nM in seawater and 5 nM in freshwater, a condition requiring highly specific importers of Ni<sup>2+</sup> ions for *H. pylori*<sup>[46]</sup>. Thus far, two types of nickel uptake strategies have been identified in *H. pylori*<sup>[46]</sup>: (1) NixA<sup>[47]</sup>, a member of the nickel-cobalt transporter family (Ni-CoT)<sup>[48]</sup>; and (2) the multiple-component ATP-binding protein cassette (ABC)-transporters, which are believed to be a four-gene operon designated as *abcABCD*<sup>[49]</sup>.

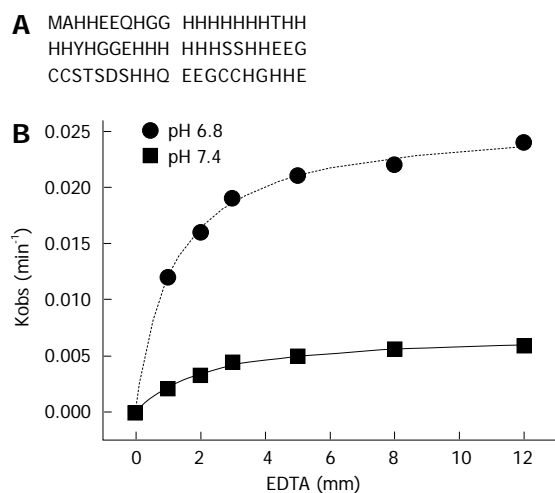
NixA is required for effective *H. pylori* colonization, as disruption of the gene led to reduced colonization<sup>[50]</sup>. NixA is predicted to have eight transmembrane-spanning helices, and transports Ni<sup>2+</sup> with a V<sub>max</sub> of 1750 pmol Ni<sup>2+</sup>/min per 10<sup>8</sup> cells and a K<sub>m</sub> of 11.3 nmol<sup>[51,52]</sup>, thus enabling *H. pylori* to efficiently scavenge nickel ions in the range of 2-11 nmol from the human body<sup>[53]</sup>. NixA transcription was shown to be repressed by NikR in a nickel-dependent manner to prevent excess toxic *in vivo* nickel<sup>[44,54]</sup>.

NixA deletion mutants still retained urease activity in some levels (up to 50% in some strains)<sup>[50,55]</sup>, indicating the existence of an alternative nickel transporter. Further analysis identified the *abcABCD* genes, a component of the ATP-dependent nickel transport system to be potentially involved in NixA-independent nickel uptake, as mutations in *abcCD* decreased urease activity<sup>[49]</sup>. Another work identified FrpB4 to be a potential outer membrane nickel uptake protein as energized by the TonB/ExbB/ExbD machinery<sup>[56]</sup>, indicating that the established iron uptake machinery may be involved in nickel uptake. However, further work is needed to confirm their role and mechanism in nickel transport.

## CHAPERONES

Similar to other bacteria, *H. pylori* has to maintain a delicate balance between the import of nickel ions, its efficient intracellular storage, and delivery to nickel-dependent metalloenzymes when required. Metals, such as nickel, pose problems for the cell because they are required for the growth, whereas they inhibit growth and





**Figure 2** Amino acid sequence of Hpn (A) and the apparent rate of nickel release vs concentrations of ethylenediaminetetraacetic acid at pH 6.8 and 7.4 and the best nonlinear fit of the data (B). EDTA: Ethylenediaminetetraacetic acid.

exhibit toxic effects when present in excess. In this section, we would like to discuss the proteins involved in metallocenter assembly in urease.

### HypA and HypB

HypA and HypB are named to emphasize their roles in the maturation and activation of NiFe hydrogenase (*hyp*, hydrogenase pleiotropic). However HypA and HypB are also found to be accessory proteins for urease<sup>[57]</sup>, as reflected by the reduced urease activity (40-200 folds) upon *hypA* or *hypB* disruption<sup>[18]</sup> and the competition between HypA and UreG for UreE (see below) recognition<sup>[58]</sup>. HypA binds nickel and zinc ions and HypB is a P-loop GTPase to provide energy during nickel insertion in hydrogenase. HypA and HypB exist as homodimers in solution and form heterodimers with each other<sup>[59,60]</sup> with a low affinity ( $K_d$  of  $52.2 \pm 8.8 \mu\text{mol}$ )<sup>[61]</sup>. HypA and HypB also make heterodimers with UreE<sup>[62]</sup> and SlyD<sup>[63]</sup>, respectively in solution. The NMR structure of zinc-bound HypA monomer indicates that the nickel binding site is located at the N-terminus and nickel is bound to four nitrogens in a square planar geometry<sup>[64]</sup>. A thermodynamic study indicates that the zinc binding site has a much higher affinity to zinc than nickel and zinc binding induces a great change in the secondary structure of HypA to exert its structural role in the metalloprotein<sup>[65]</sup>. Further study with XAS showed that HypA dimer has a unique structural flexibility of the zinc site and has roles in sensing nickel binding and pH<sup>[66,67]</sup>: a decrease of pH from 7.2 to 6.3 induces a change of the zinc binding ligands from Cys<sub>4</sub> to Cys<sub>2</sub>His<sub>2</sub> and results in a change of the nickel binding stoichiometry from one Ni per monomer to one Ni per dimer<sup>[66]</sup>. Cys106 and His107 of HypB are required for nickel binding and metal-dependent dimerization<sup>[68]</sup>. Nickel binding of HypB is possibly facilitated by SlyD *via* its IF (insert-in-flap) domain<sup>[63]</sup>. Zinc binding significantly inhibits the GTPase activity of

HypB<sup>[68]</sup>. Nickel binding is reported to either slightly<sup>[68]</sup> or highly<sup>[61]</sup> stimulate the activity of HypB, with reasons for these discrepancies yet unknown. The regulation of HypB activities by metal binding may contribute to the maturation of the hydrogenase and urease.

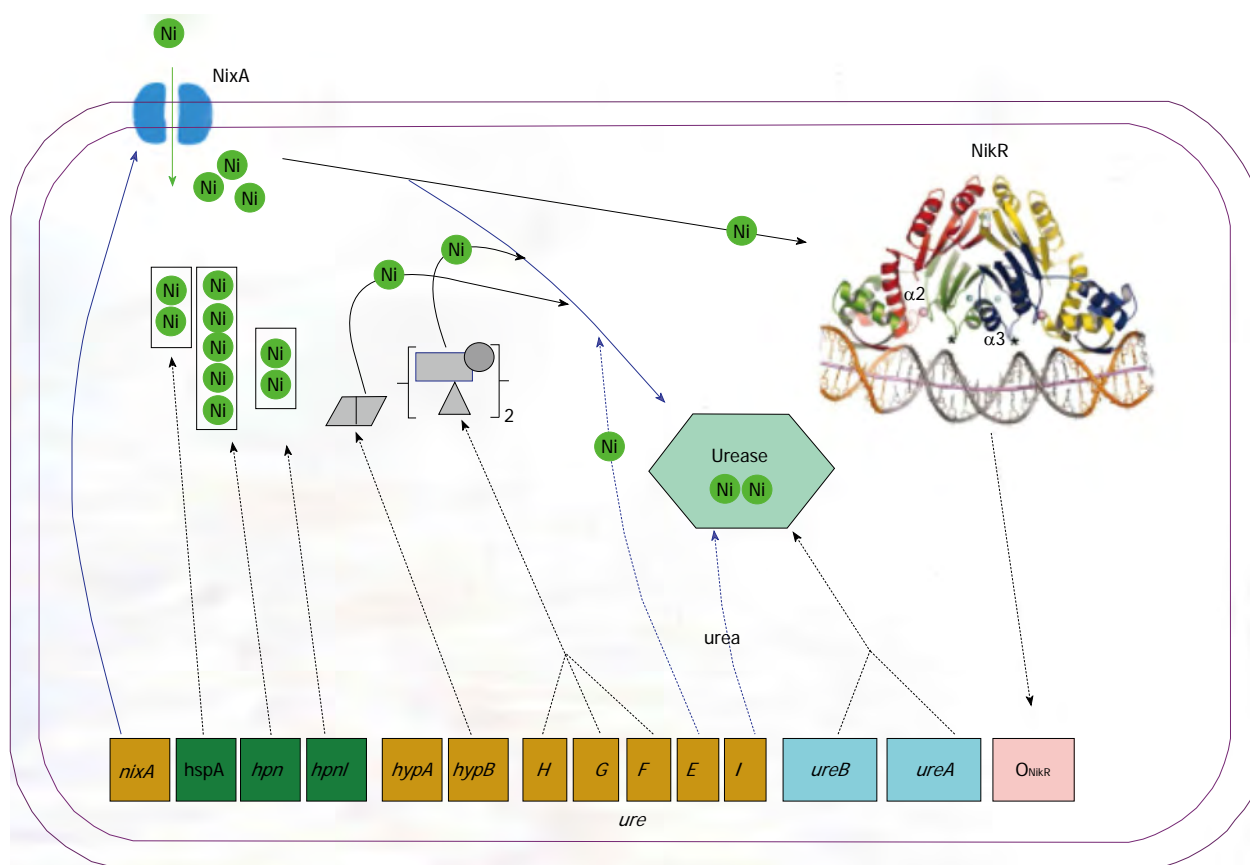
### UreEFGH

UreEFGH is a group of accessory proteins involved in the synthesis of the urease active site<sup>[41]</sup>, which has been excellently covered recently in a review by Farrugia *et al.*<sup>[69]</sup>. This review will only briefly discuss their respective roles. The information about UreH is quite limited primarily due to its insolubility in solution, although it is believed to be the first protein to bind to apo-urease<sup>[70]</sup>. UreE is the chaperone to deliver nickel to urease and UreF activates the GTPase activity of UreG<sup>[29,41]</sup>. UreE is capable of binding Ni and Zn ( $K_d$  of 0.15 and 0.49  $\mu\text{mol}$ , respectively) in a stoichiometry of one per dimer<sup>[71,72]</sup>. Apo-UreE is a dimer and the metal-bound protein is a tetramer (dimer of dimer) formed by the coordination of the metal ion by His104 from each subunit<sup>[73]</sup>. A second UreE crystal structure indicates that Ni is six-coordinate (His102 from one monomer, His102, His152, Glu4 from the other, a water molecule and one unidentified ligand)<sup>[74]</sup>. His152 is disordered in the crystal and could be replaced by UreG residues, thus leading to the transfer of nickel from UreE to UreG. In the calculated structure of UreDEFG through computational modeling, the convex surface of the UreG dimer is in direct contact only with the shallow crevice at the interface of the two UreF monomers through weak van der Waals and polar interactions<sup>[75]</sup>. UreF and UreH can form dimer or heterodimers in solution with concomitant conformational changes in two distinctive regions of UreF<sup>[76]</sup>: (1) the flexible C-terminus becomes ordered to form an extra helix  $\alpha_{10}$  and a loop stabilized by hydrogen bonds involving Arg250; and (2) the first turn of helix  $\alpha_2$  uncoils to expose a conserved residue Tyr48. Both Arg250 and Tyr48 are critical for the heterotrimeric formation of UreG-UreF-UreH and urease maturation<sup>[76]</sup>. One crystal structure of UreGFH indicates that UreFH facilitates UreG dimerization and assembles its metal binding sites by juxtaposing two Cys66-Pro67-His68 motifs at the interface to form the (UreGFH)<sub>2</sub> complex<sup>[77]</sup>.

### HspA, Hpn and Hpn-like

HspA, Hpn and Hpn-like (HpnI) proteins in *H. pylori* are histidine-rich in full or in part. HspA is a bacterial GroES homologue with a unique cysteine- and histidine-rich C-terminal domain<sup>[78]</sup>. HspA binds 2 Ni per monomer with a dissociation constant of 1.1  $\mu\text{mol}$  *in vitro*<sup>[79]</sup>. The *in vivo* work showed that HspA is involved in intracellular nickel sequestration and detoxification, and plays a role as a specific nickel chaperone in the maturation of hydrogenase, while not for urease<sup>[80]</sup>. Hpn (Figure 2A) is a histidine rich protein (accounting for around half of its amino acids) and highly abundant in the cell cytoplasm (approximately 2% of all protein synthesized)<sup>[81]</sup>.





**Figure 3** Complex network controlling urease synthesis and activity in *Helicobacter pylori*. The different levels of control comprise (1) expression of the UreAB structural subunits fine-tuned by acidity and the nickel-dependent transcriptional regulator NikR; (2) nickel uptake into cells via NixA importer; (3) nickel storage in histidine-rich proteins such as Hpn, HpnI and HspA; (4) nickel incorporation into urease as mediated by accessory proteins UreEFGH and HypAB; and (5) urea substrate entry via UreI. *H. pylori*. *Helicobacter pylori*.

The majority of histidines are located within the central part of the protein and include two separated stretches of 6 and 7 consecutive histidine residues. There are two internal short repeats of Glu-Glu-Gly-Cys-Cys, four sets of paired histidine residues and an X-X-His motif at the N-terminus. All these sequence features indicate that this protein would strongly bind metal ions. Mutated strains of *H. pylori* lacking the *hpn* gene are four times more sensitive to ranitidine bismuth citrate, a metal-containing drug widely used to treat *H. pylori* infections, than the wild type<sup>[3,82,83]</sup>. Hpn exists in solution as a range of multimeric forms with the 20-mer to be potentially physiologically relevant<sup>[84]</sup>. The protein can bind nickel in a stoichiometry of five Ni per monomer with a  $K_d$  of 7.1  $\mu\text{mol}$ . Therefore it is possible that nickel may be transferred from Hpn to stronger nickel binding proteins, such as HypA ( $K_d$  of 1.3  $\mu\text{mol}$ ) and HspA ( $K_d$  of 1.8  $\mu\text{mol}$ ). Nickel can be released from Hpn by decreasing pH ( $\text{pH}_{1/2}$  of 6.3) or by adding nickel chelating agent EDTA<sup>[84,85]</sup>, which indicates that Hpn could provide stored nickel ions to the relevant chaperone proteins for the subsequent urease maturation upon intracellular pH decrease. The nickel release from Hpn by EDTA is a two-step process consisting of a rapidly established equilibrium (formation of Hpn-Ni•EDTA,  $K$ ) followed by a rate-determining step (dissociation of Hpn-Ni•EDTA to Ni-EDTA and apo-Hpn,

$k_2$ )<sup>[85]</sup>. The data was fitted in Figure 2B which suggests that lower pH favors both the formation of the Hpn-Ni•EDTA intermediate and its decomposition to the Ni-exchanged products<sup>[85]</sup>. Later work by our group showed that this His-rich protein can form amyloid-like structures and exhibit some cytotoxic effects to gastric epithelia cells<sup>[86]</sup>, indicating that Hpn may be involved in the pathological roles of *H. pylori* other than the nickel storage role in the maturation of nickel specific enzymes<sup>[87]</sup>. HpnI is a histidine- and glutamine-rich protein in *H. pylori*, the N-terminus (46 residues) of which shows 56% identity to Hpn. HpnI binds two nickel ions per monomer in the histidine-rich domain with a dissociation constant of 3.8  $\mu\text{mol}$ <sup>[88]</sup>. Nickel release experiments established that HpnI is similar to Hpn, as nickel can be release from HpnI at acidic pH ( $\text{pH}_{1/2}$  of 4.6) and in the presence of EDTA. One *in vivo* study by Maier's group indicated *H. pylori* can utilize stored nickel ions *via* Hpn and HpnI to aid colonization of the host<sup>[89]</sup>.

## CONCLUSION

*H. pylori* is an established agent causing various gastric diseases. The nickel containing urease and hydrogenase are essential for the successful infections of *H. pylori* in the stomach. Nickel is an essential cofactor for urease

and hydrogenase. Various nickel-binding proteins play key roles in microbial nickel homeostasis by shuttling nickel within the cells. In this review we discussed the regulatory, uptake, chaperone and accessory proteins involved in the maturation of urease, especially the proteins NikR, NixA, HypAB, UreEFGH, HspA, Hpn and HpnI. The proteins function in a coordinated way to mature the urease in an efficient way for the successful inhabitation of the bacterium in the stomach (Figure 3). The work will deepen our understanding of how this pathogenic bacterium adapts to severe habitat environments in the host.

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## WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

# Role of *Helicobacter pylori* virulence factor cytotoxin-associated gene A in gastric mucosa-associated lymphoid tissue lymphoma

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## Abstract

*Helicobacter pylori* (*H. pylori*) infection might initiate and contribute to the progression of lymphoma from gastric mucosa-associated lymphoid tissue (MALT). Increasing evidence shows that eradication of *H. pylori* with antibiotic therapy can lead to regression of gastric MALT lymphoma and can result in a 10-year sustained remission. The eradication of *H. pylori* is the standard care for patients with gastric MALT lymphoma. Cytotoxin-associated gene A (CagA) protein, one of the most extensively studied *H. pylori* virulence factors, is strongly associated with the gastric MALT lymphoma. CagA possesses polymorphisms according to its C-terminal structure and displays different functions among areas and races. After being translocated into B lymphocytes *via* type IV secretion system, CagA deregulates intracellular signaling pathways in both tyrosine

phosphorylation-dependent and -independent manners and/or some other pathways, and thereby promotes lymphomagenesis. A variety of proteins including p53 and protein tyrosine phosphatases-2 are involved in the malignant transformation induced by CagA. Mucosal inflammation is the foundational mechanism underlying the occurrence and development of gastric MALT lymphoma.

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**Key words:** *Helicobacter pylori*; Cytotoxin-associated gene A; Gastric mucosa-associated lymphoid tissue lymphoma; Lymphomagenesis; Molecular mechanism

**Core tip:** Cytotoxin-associated gene A (CagA) protein encoded by *cag* pathogenicity island of *Helicobacter pylori* is a bacterium-derived oncoprotein and is strongly associated with the gastric mucosa-associated lymphoid tissue (MALT) lymphoma. After being translocated into B cells *via* type IV secretion system in ATP-dependent manner, CagA deregulates several pathways in both tyrosine phosphorylation-dependent and -independent manners, and thereby promotes lymphomagenesis. Two important proteins, p53 and protein tyrosine phosphatases-2, are involved in the malignant transformation induced by CagA. In addition, mucosal inflammation is the foundational mechanism underlying the occurrence and development of gastric MALT lymphoma. However, the exact mechanism by which CagA promotes onco-genesis needs further clarification.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*), a spiral-shaped, microaerophilic, Gram-negative bacterium, infects approximately 50% of humans worldwide. *H. pylori* is associated with chronic active gastritis and peptic ulcers, is a risk factor for gastric cancer<sup>[1]</sup> and has been ranked as a class I carcinogen by the International Agency for Research on Cancer since 1994<sup>[2,3]</sup>. *H. pylori* infection might initiate and contribute to the progression of lymphoma from gastric mucosa-associated lymphoid tissue<sup>[4,5]</sup>. Clinical observations have shown that the eradication of *H. pylori* with antibiotic therapy can lead to regression of gastric mucosa-associated lymphoid tissue (MALT) lymphoma in 77.5%-94.0% of patients<sup>[6-9]</sup> and can result in a 10-year sustained remission in up to 64% of cases<sup>[10]</sup>. The eradication of *H. pylori* is the standard care for patients with gastric MALT. The results of a population-based study showed that the incidence of *H. pylori*-positive gastric MALT lymphoma had reduced sharply in the era of anti-*H. pylori* intervention<sup>[11]</sup>. This review summarizes the role of *H. pylori* cytotoxin-associated gene A (CagA) in the development and/or maintenance of gastric MALT lymphoma.

## H. PYLORI CAGA IS CLOSELY RELATED TO THE DEVELOPMENT AND/OR MAINTENANCE OF GASTRIC MALT LYMPHOMA

The CagA protein, encoded by the cytotoxin-associated gene (*cag*) pathogenicity island, is one of the most important *H. pylori* virulence factors<sup>[12,13]</sup> and is causally linked to gastric MALT lymphoma. Fischbach *et al*<sup>[14]</sup> and Eck *et al*<sup>[15]</sup> determined that seropositivity of CagA was present in 89.0%-95.5% of patients with gastric MALT lymphoma, as tested by enzyme-linked immunosorbent assay and Western blot. The seroprevalence rate exceeded the prevalence of chronic active gastritis in the German population. The serological discovery of *cagA*-positive *H. pylori* isolates does not necessarily reflect the current colonization of the gastric mucosa because the immunoglobulin (Ig)A/IgG represents a past immune response. Mucosal-derived antibodies play an important part in the mucosal immune response. CagA-specific mucosal IgA and IgG antibodies occur in almost all patients with *H. pylori*-associated gastric MALT lymphoma<sup>[16,17]</sup>. Sumida *et al*<sup>[18]</sup> showed that in t(11;18)(q21;q21)-negative gastric MALT lymphoma patients, concentrations of anti-CagA IgG were significantly higher in the *H. pylori*-dependent cases than in the *H. pylori*-independent cases, and the *H. pylori*-dependent cases had a better therapeutic effect. The CagA protein can be detected in B lymphocytes in people

infected with *cagA*-positive *H. pylori* strains<sup>[19]</sup>. Kuo further explored that CagA can be detected in the malignant B cells of *H. pylori*-associated gastric MALT lymphoma. The expression of CagA was evaluated using immunohistochemistry and confirmed using immunoblot analyses<sup>[20]</sup>. These findings suggest that gastric MALT lymphoma is associated with *H. pylori* strains expressing the CagA protein. Ohnishi and colleagues transfected C57BL/6 mice with a *cagA*<sup>Hs</sup> (humanized *cagA* gene) expression vector throughout the body or predominantly in the stomach to generate transgenic mice<sup>[21]</sup>. They performed immunoprecipitation, immunoblotting, histological examinations and other analyses of the gastric mucosa from 72-wk-old *cagA*<sup>Hs</sup> mice and determined that CagA induced abnormal proliferation of the gastric epithelial cells and hematopoietic cells, which was followed by the development of gastrointestinal carcinomas and lymphomas of B-cell origin. These results indicate that CagA is involved in the development of gastric MALT lymphoma, which provide the first direct evidence that CagA functions as a bacterium-derived oncoprotein in mammals<sup>[21]</sup>.

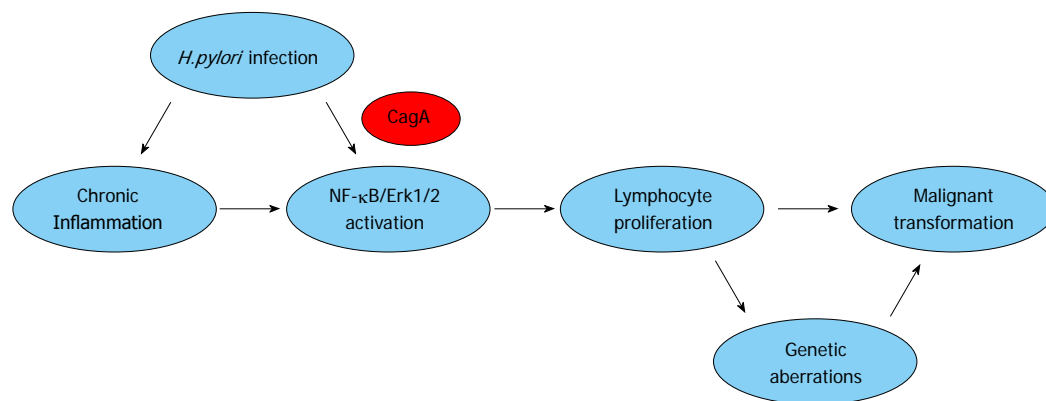
## MOLECULAR MECHANISM OF CAGA INVOLVEMENT IN GASTRIC MALT LYMPHOMA

### The pathogenesis of lymphoma

Lymphomas are malignant tumors that originate in the lymphatic system. Lymphocytes proliferate in response to the stimulation of persistent antigens and repeated infections in patients with immune deficiencies. The deregulation of the cell cycle and apoptosis is important in the pathogenesis of lymphoma. Lymphocytes that lack self-control divide faster than normal cells or survive longer than they should, proliferating in response to antigenic stimulation, which leads to the occurrence of unlimited proliferation and eventual lymphoma. Lymphocytes and lymphoid tissues do not normally exist in the stomach<sup>[22]</sup>. The onset of gastric MALT lymphoma is preceded by the acquisition of MALTs as a result of sustained *H. pylori* infection, which initiates the inflammatory lymphoproliferation<sup>[23,24]</sup>. The persistence of bacterial colonization, acting as immunologic stimuli, results in the recruitment of immune lymphocytes that migrate to and infiltrate the site of *H. pylori* infection in the stomach, which induce and sustain an actively proliferating B-cell population. Eventually, the formation of acquired lymphoid follicles and mucosal associated lymphoid tissues develop<sup>[25-27]</sup> (Figure 1). Much attention has been focused on the role of CagA in malignant transformation of the B cells. CagA may deregulate the host intracellular signaling transduction and lower the threshold for neoplastic transformation<sup>[28]</sup>.

### Structure of the CagA protein

CagA is encoded by the *cagA* gene within the *cag* pathogenicity island, a chromosomal region that simultaneously



**Figure 1** Oncoprotein cytotoxin-associated gene A is involved in the gastric mucosa-associated lymphoid tissue lymphoma development. *H. pylori*: *Helicobacter pylori*; CagA: Cytotoxin-associated gene A; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Erk1/2: Extracellular signal-regulated kinase1/2.

encodes a type IV secretion system (T4SS) specializing in the transfer of CagA from bacteria to the target cells in an ATP-dependent manner<sup>[29,30]</sup>. CagA is the only known effector protein that is translocated by a T4SS<sup>[31-33]</sup>. Analyses of the DNA sequence and molecular phylogenetic trees show that the CagA protein comprises a solid structured N-terminal region<sup>[31,34]</sup> and a variable, intrinsically disordered C-terminal region that is different among strains and exhibits scaffold/hub functions that are responsible for the morphogenetic activity of CagA<sup>[35]</sup>. The C-terminal domain contains repeated tandem five-amino-acid motifs of glutamic acid-proline-isoleucine-tyrosine-alanine (EPIYA). Within the variable region of CagA, there are different intervening sequences between the EPIYA motifs. One copy of EPIYA plus an intervening sequence is identified as an EPIYA segment. The tyrosine residues on the EPIYA (Y) motifs undergo tyrosine phosphorylation. Both the number and type of the EPIYA motifs determine the outcomes of cellular and gastric lesions<sup>[36,37]</sup>. Four unique types of EPIYA motifs (A, B, C and D) have been described based on their flanking amino acid sequences, which contribute to the CagA sequence polymorphism and geographical difference among strains. Almost all CagA contains both EPIYA-A and EPIYA-B motifs. The EPIYA-C motif is usually present in one to three repeats forming the typical Western CagA configuration of ABC, ABCC and ABCCC subtypes. In contrast, the EPIYA-D motif rarely repeats and thus prevalent East Asian CagA strains are ABD combinations<sup>[38]</sup>. The EPIYA-C and EPIYA-D motifs act as phosphorylation sites<sup>[39]</sup>. It is reported that increased number of EPIYA-C could enhance the binding ability to protein-tyrosine phosphatase-2 (SHP-2)<sup>[40]</sup>. Compared with EPIYA-C, EPIYA-D experiences a greater degree of tyrosine phosphorylation and stronger SHP-2-binding affinity, which leads to increased oncogenic potential<sup>[41,42]</sup>. Epidemiological data identified that the incidence rate of gastric MALT lymphoma is higher in East Asia than in Western countries<sup>[11,43-45]</sup>. East Asian might be prone

to gastric MALT lymphoma at least partly, if not all, because most *H. pylori* strains are *cagA*-positive and nearly 90% of CagA carry EPIYA-D motif, 83.6% of which are of EPIYA-ABD genotype<sup>[46]</sup>.

#### ***CagA deregulates intracellular signaling pathways in tyrosine phosphorylation-dependent and -independent manners to initiate pathogenesis.***

**Tyrosine phosphorylation-dependent pathway:** CagA was directly injected from bacteria into attached gastric epithelial cells by a T4SS. Lin *et al.*<sup>[19]</sup> further showed that the translocation of the CagA protein into human B lymphocytes could occur through the T4SS. The delivered CagA activates and stimulates the B lymphocytes, initiating the first step of the B-cell malignant stimulation. In host cells, CagA undergoes tyrosine phosphorylation by c-src/Lyn kinase on specific tyrosine residues of the EPIYA motifs<sup>[19,47]</sup>. The phosphorylated CagA deregulates the intracellular signaling pathways and initiates the malignant transformation of B lymphocytes. CagA specifically binds to intracellular target molecules, including the SHP-2 [Src homology 2 (SH2) domain containing phosphotyrosine phosphatase 2]<sup>[39,41,48,49]</sup>. SHP-2, encoded by *PTPN11*, is a protein tyrosine phosphatase (PTP) and plays a vital role in normal hematopoiesis. SHP2 has two tandem SH2 domains, a PTP domain and a carboxyl-terminal tail which contains multiple tyrosine phosphorylation sites and is rich in proline motifs. In the inactive state, the N-terminal SH2 domain binds the PTP domain and hampers access of potential substrates to the active site. Thus, SHP-2 is auto-inhibited. In contrast, the N-terminal SH2 domain is free from the PTP domain by binding to target phospho-tyrosyl residues, catalytically activating the enzyme by relieving this auto-inhibition<sup>[50]</sup>. Mutation in *PTPN11*, an identified cellular proto-oncogene<sup>[51]</sup>, or aberrant SHP-2 expression/activity positively correlates with the hyperproliferation of leukemic hematopoiesis<sup>[50,52]</sup>. SHP-2 functions as a vital adaptor protein in CagA signaling pathway<sup>[48,49]</sup>. However, *PTPN11*/

SHP-2 has dual roles in different cell types and its oncogenic role is tissue specific<sup>[53]</sup>. Most recent experimental data suggest *PTPN11*/SHP-2 as a tumor suppressor in hepatocarcinogenesis<sup>[54]</sup>. The above pathway depends on tyrosine phosphorylation of CagA.

Zhu *et al.*<sup>[47]</sup> transiently transfected a recombinant retrovirus encoding an inserted *cagA* into conditionally immortalized B lymphocytes. The expressed and phosphorylated CagA was detected in the transfected B cells by Western blot and co-immunoprecipitation analyses, and CagA/SHP-2 complex was detected. The transfection of B lymphocytes with *cagA* significantly increased extracellular signal-regulated kinase1/2 (Erk1/2) phosphorylation, which is negatively regulated by MKP-1 and MKP-6, resulting in the phosphorylation of Bad at Serine 112 of CagA. Erk1/2, activated by CagA, can hamper apoptosis of B lymphocytes by inducing phosphorylation of Bad at Ser-112. *cagA* transfection did not alter the levels of the pro-apoptotic Bcl-2 and Bax. Immunofluorescence staining analysis displayed that CagA-activated Erk1/2 could translocate simultaneously to the cytoplasm and the nucleus, whereas serum-stimulated activated Erk1/2 was located only in the cytoplasm. The evidence indicates that the CagA-activated Erk1/2 can block apoptosis by activating the downstream target molecules, promoting the development of lymphoma. Lin *et al.*<sup>[19]</sup> suggested that CagA translocation, following the phosphorylation of CagA which subsequently binds to and activates endogenous SHP-2, induces the activation of Erk1/2 and mitogen activated protein kinase and the up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> in human B lymphocytes. The step prevents human B lymphocytes from apoptosis, allowing the lymphocytes to acquire survival ability, which contributes to the pathogenesis of lymphoma.

#### Tyrosine phosphorylation-independent pathway:

Umehara *et al.*<sup>[55]</sup> determined that CagA may block the cell cycle progression in the Ba/F3 and gastric epithelial cancer AGS cells, and inhibit the B lymphocyte apoptosis by impairing the p53 and JAK/STAT pathway. The enforced expression of CagA in the interleukin (IL)-3-dependent B-lymphoid cells functions as a G1 inhibitor, suppressing cell proliferation through the inhibition of JAK-STAT pathway and resulting in significant retardation of the G1- to S-phase cell-cycle transition. The IL-3 signal is mainly transmitted by the sequential activations of JAK and STAT. CagA offsets hydroxyurea-induced B-cell apoptosis by disturbing the tumor suppressor p53 accumulation. CagA inhibits the expression of p53 at the level of transcription. Meanwhile, *cagA*-positive *H. pylori* may be involved in the initial stage of gastric MALT lymphoma development, whereas it might not be necessary in the maintenance stage of lymphoma cell proliferation. CagA blocks apoptosis, promoting the accumulation of genetically abnormal cells that should otherwise be removed from the tissue. In IL-3-dependent B cells including BaF3, inhibitors of deoxyribonucleotide

synthesis such as hydroxyurea induce apoptosis in a p53-dependent manner, whereas, DNA-damaging agents such as X-irradiation and cisplatin induce cell death in a p53-independent manner<sup>[56]</sup>. Interestingly, oxidative stress has been reported to be contributed to a variety of gastric disorders such as gastritis and ulcer diseases, especially gastric cancer<sup>[57,58]</sup>. Upon *H. pylori* infection and colonization, CagA might stimulate the response of gastric epithelial cells to oxidative stress and produce, mainly from neutrophils, reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Excessive ROS/RNS causes dysfunction of antioxidant defense mechanism in gastric mucosal, leading to DNA damage, accelerating cell death including apoptosis and subsequent cell proliferation, and resulting in the pathogenesis of gastric disorders as well as carcinogenesis<sup>[58]</sup>. Meanwhile, additional ROS and RNS may decline the expression of Runt domain transcription factor 3 (RUNX3), a marker of oxidative stress, which could restore after *H. pylori* eradication<sup>[58]</sup>. Therefore, RUNX3 acts as a tumor suppressor and is involved in *H. pylori* CagA-dependent gastric carcinogenesis. Moreover, some other molecules have been reported to be correlated with CagA-induced gastric carcinogenesis. Murine double minute 2 (MDM2) might promote pathogenesis of gastric cancer through inactivating the apoptotic and cell cycle arrest function of p53<sup>[59]</sup>. Yet, the role of RUNX3, MDM2 as well as oxidative stress production in CagA-induced gastric MALT lymphoma has been unclear and should be elucidated by further exploration. The malignant transformation from *cagA*<sup>+</sup> *H. pylori* infection into gastric MALT lymphoma should involve multiple steps. CagA has phosphorylation-dependent and -independent activities, and the biological effects of CagA in mammals depend on the cellular context. An imbalance between apoptosis and proliferation is involved in the pathogenesis and development of *H. pylori*-dependent gastric MALT lymphoma<sup>[60]</sup>. CagA inhibits apoptosis and impairs survival in the B cells, resulting in the transformation of MALT lymphoma<sup>[20]</sup> (Figure 2).

## CONCLUSION

Mucosal inflammation is the basic mechanism underlying the occurrence and development of gastric MALT lymphoma. Infection with *H. pylori* induces inflammatory and immune responses in the gastric mucosa. The incapability of the host immune response to clear the bacterial pathogen results in a persistent infection and the subsequent development of chronic gastric inflammation<sup>[61]</sup>. The T-helper 17 (Th17) cells, whose hallmark cytokine is IL-17A, are important for the clearance of extracellular bacteria<sup>[62]</sup>, and they play a role in infection control and precarcinogenesis. IL-17A may contribute to inflammation-associated carcinogenesis<sup>[62,63]</sup>. B7-H2 is among the newer members of the B7 family and is known to have a co-stimulatory function on T cell activity<sup>[64]</sup>. Recent *in vitro* and *in vivo* studies showed that *H. pylori* down-regulates B7-H2 (the positive co-stimulators required for an effi-



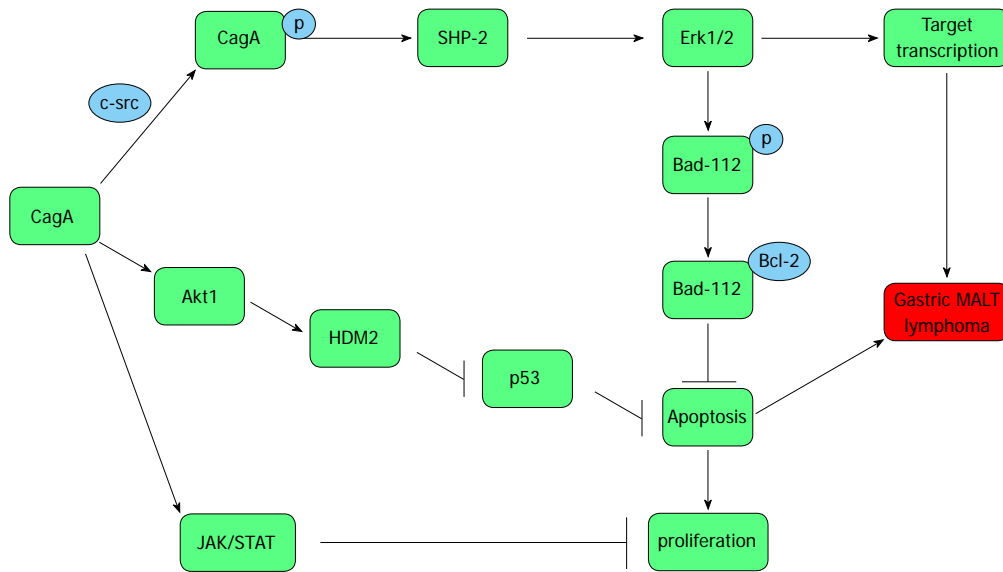


Figure 2 Cytotoxin-associated gene A deregulates intracellular signaling pathways in tyrosine phosphorylation-dependent and -independent manners to initiate lymphomagenesis. CagA: Cytotoxin-associated gene A; Erk1/2: Extracellular signal-regulated kinase1/2; MALT: Mucosa-associated lymphoid tissue; SHP-2: Protein-tyrosine phosphatase-2.

cient effector T cell response) in a CagA-dependent manner in gastric epithelial cells (GECs). CagA-dependent B7-H2 down-regulation in GECs suppresses the Th17-mediated immune response, contributing to outcomes of chronic gastric inflammation and persistent *H. pylori* colonization<sup>[63]</sup>. This process may be involved in gastric carcinogenesis, but the relationship with the development of gastric MALT lymphoma remains unclear. The activation of nuclear factor kappa-light-chain-enhancer of activated B cells and the up-regulation of IL-8 induced by *H. pylori* infections in B lymphocytes lead to the malignant transformation of B cells in a SHP-2-dependent and CagA-independent mechanism<sup>[66-68]</sup>. There is no direct evidence associating CagA, inflammation and gastric MALT lymphoma.

In recent years, microRNAs (miRNAs), a class of small non-coding RNAs that can modulate gene expression at the post-transcriptional level, have been implicated in *H. pylori*-dependent gastric carcinogenesis<sup>[69,70]</sup>. Much data suggest that miRNAs are important in fundamental cellular processes such as proliferation and apoptosis, and miRNAs can function as tumor promoters or suppressors<sup>[71]</sup>; the role of the miRNAs in the association between CagA and gastric MALT lymphoma remains unclear. Gastric MALT lymphoma is considered one of the best models of how infectious pathogens and genetic events lead to oncogenesis<sup>[72,73]</sup>. CagA functions as a typical bacterium-derived oncoprotein in gastric MALT lymphoma pathogenesis, but the molecular mechanism of CagA underlying the development of gastric MALT lymphoma should be further elucidated.

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## Ophthalmologic complications of antiviral therapy in hepatitis C treatment

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### Abstract

Antiviral therapy consisting of interferon-alpha and ribavirin for chronic hepatitis C infection is associated with multi-system side-effects. Ophthalmologic complications are common and can be classified into two groups: interferon-associated retinopathy and atypical adverse events. Interferon-associated retinopathy has been investigated by multiple observational studies that have found widely divergent results. The clinical importance of this complication is, consequently, controversial. This review examines the literature with the specific goal of identifying the most important ophthalmologic issues facing the hepatologist prescribing antiviral therapy. Accordingly, it assesses the incidence of interferon-associated retinopathy, as well as its risk factors, pathogenesis, clinical manifestations and

options for management using data from the observational studies. The likely benefit of a screening program, especially one targeting patients with the highest risk of developing interferon-associated retinopathy, is analysed. Atypical ophthalmologic adverse events occur less frequently than interferon-associated retinopathy during antiviral therapy for chronic hepatitis C infection. They often, however, lead to irreversible vision loss. We examine the reports of these adverse events - in individual case reports or case series and in the observational studies investigating interferon-associated retinopathy - to describe the spectrum of these adverse events, the likely outcome for patients and to highlight the most important areas of future clinical research.

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**Key words:** Interferon; Hepatitis C; Ocular complications; Retinopathy

**Core tip:** Interferon-associated retinopathy is usually a benign, transient phenomenon with no lasting impact on visual function. It occurs in approximately 30% of patients receiving antiviral therapy for chronic hepatitis C infection. The main risk factors for its development appear to be hypertension and diabetes. Unless a clear benefit to patients can be shown, a screening program for the development of interferon-associated retinopathy is not justified. No conclusive evidence exists for a causal link between it and the atypical adverse events of antiviral therapy, which tend to cause irreversible vision loss.

O'Day R, Gillies MC, Ahlenstiel G. Ophthalmologic complications of antiviral therapy in hepatitis C treatment. *World J Gastroenterol* 2013; 19(45): 8227-8237 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8227.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8227>

## INTRODUCTION

With 160 to 170 million infected people worldwide, hepatitis C virus (HCV) presents a major health care problem<sup>[1,2]</sup>. Current standard of care treatment consists of pegylated interferon alpha (PEG-IFN $\alpha$ ) and ribavirin (RBV) for genotypes 2 to 6<sup>[3,4]</sup>. Boceprevir or telaprevir may be added to these for genotype 1 infections<sup>[5]</sup>. Standard of care therapy is associated with side effects in many organs, the majority of which are attributed to interferon. Ophthalmologic complications can be classified into two groups: interferon-associated retinopathy and atypical adverse events.

## BACKGROUND

HCV, first identified in 1989, is a major cause of chronic liver disease<sup>[6,7]</sup>. It is the most common indication for liver transplantation in the Western world<sup>[8]</sup>. The natural course of HCV infection results in chronic disease in approximately 70% of patients, with the remaining 30% clearing the infection spontaneously<sup>[9]</sup>. Patients with chronic hepatitis C (CHC) infection can transmit HCV and are at risk of progression to liver cirrhosis and/or hepatocellular carcinoma<sup>[10]</sup>.

The treatment of chronic hepatitis C infection has evolved over the past 20 years. Interferon alpha (IFN $\alpha$ ) monotherapy was the first drug regimen found to induce viral clearance<sup>[11]</sup>. The combination of IFN $\alpha$  with oral RBV, a synthetic guanosine nucleoside, was subsequently found to increase the rate of viral clearance by 2-3 times<sup>[12,13]</sup>. Pegylation, the process of attaching IFN $\alpha$  to a polyethylene glycol moiety, both increased viral clearance and decreased the frequency of dosing of interferon to once weekly injections<sup>[14,15]</sup>. Most recently, treatment for HCV genotype 1 infection has been amended to include a third drug, either boceprevir or telaprevir, both of which are direct-acting antivirals<sup>[5]</sup>.

## INTERFERON-ASSOCIATED RETINOPATHY

Interferon-associated retinopathy was first described by Ikebe *et al*<sup>[16]</sup> in 1990. It has been widely investigated since then. Our literature review identified 22 English-language reports of observational studies assessing its incidence and clinical features<sup>[17-38]</sup>. These studies all performed ophthalmological examinations during a course of antiviral therapy for chronic hepatitis C monitoring for interferon-associated retinopathy and atypical adverse events. They are summarised in Tables 1 and 2. Table 1 presents studies where more than half of the patients were treated with IFN $\alpha$  based regimens ( $n = 10$ ), whereas Table 2 presents studies with majority PEG-IFN $\alpha$  treated patients ( $n = 12$ ).

### *What are the clinical manifestations of interferon-associated retinopathy?*

Interferon-associated retinopathy can be unilateral or bilateral and typical findings on slit lamp biomicroscopy or fundus photography are cotton wool spots and/or retinal hemorrhages (Figure 1). These lesions usually occur at the posterior pole within 2 disc diameters from the optic disc<sup>[20,39]</sup>. Most commonly, it has a benign course with no impact on vision (Tables 1 and 2). It usually self-resolves during a course of antiviral therapy, or shortly thereafter, without requiring a reduction in dose (Tables 1 and 2).

### *How common is interferon-associated retinopathy?*

The observational studies have found a wide range of incidence of interferon-associated retinopathy during antiviral treatment for chronic hepatitis C infection, from under 4% to over 60% (Tables 1 and 2). Different protocols of ophthalmologic follow up and differences in patient populations are the most obvious causes of these divergent results. Other potential contributors to be considered are RBV combination therapy versus interferon monotherapy and whether different forms and doses of interferon- $\alpha$  are more likely to develop interferon-associated retinopathy.

Observational studies that had infrequent or symptom-initiated ophthalmologic examinations were more likely to find a lower incidence of interferon-associated retinopathy than those with more rigorous ophthalmologic follow up (Tables 1 and 2). Interferon-associated retinopathy most commonly develops between 2 and 12 wk after the initiation of antiviral therapy<sup>[17,22,32,36]</sup>. It is a transient phenomenon lasting from a few weeks to years<sup>[30,32,36]</sup>. Study protocols that did not examine patients multiple times within the first 6 mo of starting treatment were predisposed to underreport rates of interferon-associated retinopathy<sup>[34,38]</sup>. Similarly, most patients who develop interferon-associated retinopathy have no visual symptoms (Tables 1 and 2). Thus, protocols that initiated ophthalmologic review only once a patient became symptomatic would, therefore, also result in underreporting<sup>[33,35]</sup>. Four of the five studies reporting the lowest incidences of interferon-associated retinopathy displayed at least one of these two factors<sup>[33-35,38]</sup>.

Inclusion of patients with retinopathy at baseline skewed studies towards over-reporting of the incidence of interferon-associated retinopathy. No study has specifically assessed the clinical course of patients who have retinopathy from other causes prior to starting antiviral therapy, such as diabetes or hypertension. It is logical, however, that these patients would be at higher risk of having retinopathy during treatment than eyes without retinopathy at baseline. In the 22 observational studies considered in this review, 22 patients were identified as having retinopathy at baseline and 17 (77%) of these had progression of retinopathy<sup>[26,29,31]</sup>. In one trial, half

**Table 1 Incidence of interferon-associated retinopathy in observational studies during which more than half of the patients are treated with interferon- $\alpha$  based regimens for chronic hepatitis C**

Study	IAR incidence	Country	Timing of examinations	Comment
Nagaoka <i>et al</i> <sup>[17]</sup>	22 of 36 (61%)	Japan	Baseline, 2, 4, 8, 16 and 24 wk	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. Age was a risk factor for the development of IAR. HTN and DM were not. Atypical adverse events: nil reported.
d'Alteroche <i>et al</i> <sup>[18]</sup>	36 of 144 (25%) <sup>1</sup>	France	Baseline and then 3 monthly	IAR: No reduced VA in eyes that developed IAR. No dose reduction for management of IAR. HTN (9 of 11), receiving PEG-IFN $\alpha$ and older age were more likely to develop retinopathy. Insufficient numbers with DM ( $n = 1$ ). Atypical adverse events: nil reported.
Okuse <i>et al</i> <sup>[19]</sup>	14 of 73 (19%)	Japan	Baseline, 2, 4, 12 and 24 wk	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. HTN significantly associated with development of IAR (5 of 15), T2DM not (1 of 2). Atypical adverse events: nil reported.
Schulman <i>et al</i> <sup>[20]</sup>	27 of 42 (64%)	United States	Baseline and then 2-3 monthly for 4-20 mo	IAR: therapy discontinued in two patients with multiple CWS, one with mild decrease in VA. All other patients with IAR continued with treatment. High doses of interferon used, up to 5MIU/d. HTN was not predictive of the development of IAR. Insufficient eyes for analysis of DM as risk factor ( $n = 2$ ). Atypical adverse events: permanent peripheral monocular scotoma in 1 patient. Disc edema in 1 patient with a background of rheumatoid arthritis; no long term vision loss.
Jain <i>et al</i> <sup>[21]</sup>	8 of 19 (42%)	Canada	Baseline and then monthly	IAR: no change in VA in any patient with retinopathy. IAR resolved during study period in all but one patient. No dose reduction for management of IAR. Atypical adverse events: nil reported.
Saito <i>et al</i> <sup>[22]</sup>	28 of 81 (35%)	Japan	Baseline and then 2 weekly	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. IAR was more likely in older patients and those with DM and/or HTN. Atypical adverse events: nil reported.
Kadayifcilar <i>et al</i> <sup>[23]</sup>	7 of 20 (35%) <sup>2</sup>	Turkey	Baseline, monthly during treatment and 1 yr after completing treatment.	IAR: one of 7 with CWS at the macular had dose reduction by 1/2 for decreased VA. Full resolution in 4 wk. Otherwise no dose reduction for IAR. 16 of 20 patients had backgrounds of chronic renal failure. Atypical adverse events: unilateral BRVO in 1 patient with a background of CRF resulting in normal visual acuity at 12 mo but residual upper quadrantanopia.
Sugano <i>et al</i> <sup>[24]</sup>	6 of 25 (24%)	Japan	Baseline and then 4 weekly	IAR: not available. Atypical adverse events: not available.
Kawano <i>et al</i> <sup>[25]</sup>	36 of 63 (57%)	Japan	Baseline, 1, 2 and 4 wk and then 4 weekly until 6 mo after completing treatment	IAR: no dose reduction for 35 of 36 patients with IAR. Significantly higher incidence of retinopathy in patients with diabetes (11 of 12) and HTN (4 of 5). Atypical adverse events: severe RH in 1 patient with a background of DM; no long term vision loss.
Hayasaka <i>et al</i> <sup>[26]</sup>	14 of 40 (35%) <sup>3</sup>	Japan	1 mo prior to starting treatment and 2 weekly during treatment.	IAR: no reduced VA in eyes that developed IAR Not clear, but seems that interferon was ceased if developed IAR. Three patients with retinopathy at baseline all showed progression. Atypical adverse events: nil reported.

<sup>1</sup>Twelve patients treated for chronic hepatitis B infection were excluded from the incidence data shown; <sup>2</sup>Sixteen patients treated for chronic hepatitis B infection were excluded from the incidence data shown; <sup>3</sup>Three patients that had baseline diabetic retinopathy were excluded from the incidence data shown. BRVO: Branch retinal vein occlusion; CRF: Chronic renal failure; CWS: Cotton wool spots; DM: Diabetes; HTN: Hypertension; IAR: Interferon-associated retinopathy; MIU: Million international units; PEG: Pegylated; IFN: Interferon; RBV: Ribavirin; RH: Retinal hemorrhage; VA: Visual acuity; VEGF: Vascular endothelial growth factor.

of the patients with retinopathy at baseline had resolution of retinopathy during treatment<sup>[31]</sup>. In the other trials that identified patients with retinopathy, all eyes with baseline retinopathy had progression during the course of treatment.

When patients with baseline retinopathy and studies with suboptimal ophthalmologic follow up are excluded, 313 of 1007 (31%) patients developed interferon-associ-

ated retinopathy with a range of 8%-64% (Tables 1 and 2). The size of this corrected range implies that these factors do not fully explain the wide range of incidence of interferon-associated retinopathy.

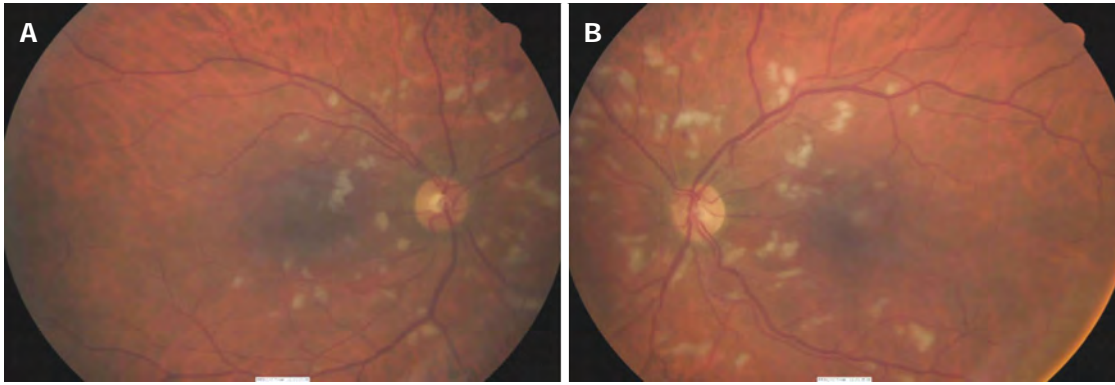
Differences in the dose and type of interferon used in the observational studies have been proposed as key reasons for the wide range of incidence of interferon-associated retinopathy found. Early studies of IFN $\alpha$  for

**Table 2** Incidence of interferon-associated retinopathy in observational studies during which more than half of the patients were treated with pegylated interferon- $\alpha$  based regimens for chronic hepatitis C

Study	IAR incidence	Country	Timing of examinations	Comments
Mousa <i>et al</i> <sup>[27]</sup>	8 of 98 (8%)	Egypt	Baseline, 2, 4, 8, 12 and 24 wk then every 3 mo	IAR: seven of 8 patients with IAR had no reduction in VA. No dose reduction for management of IAR. Combined DM and HTN gave relative risk of 6.5 of developing IAR. Atypical adverse events: vitreous hemorrhage from retinal tears with retinal detachment requiring vitrectomy in 1 patient, final visual outcomes were not described.
Fouad <i>et al</i> <sup>[28]</sup>	22 of 84 (26%)	Egypt	Baseline, 12, 24 and 48 wk and 1 mo after completing treatment	IAR: no reduced VA in eyes that developed IAR. Three patients with IAR developed retinal hemorrhages and treatment was ceased. Logistic regression found HTN (9 of 12) and DM (13 of 16) to be predictors of developing IAR. Atypical adverse events: NAION in 2 patients and optic neuritis in 1 patient, final visual outcomes for these patients were not described.
Vujosevic <i>et al</i> <sup>[29]</sup>	21 of 97 (22%) <sup>1</sup>	Canada	Baseline, 3 and 6 mo and 3 mo after completing treatment	IAR: all patients with pre-existing retinopathy, 9 patients, had worsening of retinopathy during treatment. Factors associated with developing IAR were age, metabolic syndrome, HTN, cryoglobulinemia and pre-existing intraocular lesions. Using multivariate analysis only HTN was a significant predictor of developing IAR. Insufficient number of patients with DM ( $n = 5$ ). Atypical adverse events: bilateral BRVO in one patient with a background of HTN resulting in irreversible vision loss in the left eye only.
Lim <i>et al</i> <sup>[30]</sup>	5 of 10 (50%) <sup>2</sup>	Korea	Baseline and then 3 weekly for 6 mo	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. Atypical adverse events: unilateral CRVO in 1 patient with a background of DM resulting in irreversible vision loss.
Mehta <i>et al</i> <sup>[31]</sup>	18 of 64 (28%) <sup>3</sup>	United States	Baseline, 3 and 6 mo	IAR: no reduced VA in eyes that developed IAR. 1 of 88 ceased treatment for asymptomatic IAR. Male only cohort. HTN and DM not significant predictor of developing IAR. Poor follow up rates - 69% had an eye exam within the first 12 weeks of starting treatment. Atypical adverse events: nil reported.
Kim <i>et al</i> <sup>[32]</sup>	11 of 32 (34%)	Korea	Baseline, 4, 8, 12, 16, 24, 36 wk	IAR: no reduced VA in eyes that developed IAR alone. No dose reduction for management of IAR. All retinal lesions spontaneously resolved. 91% of retinopathy developed within 2 mo, but 1 occurred at 4 mo. HTN significantly associated with development of IAR (6 of 10), T2DM not (1 of 2). Atypical adverse events: unilateral BRVO in 1 patient with background of HTN resulting in irreversible vision loss.
Panetta <i>et al</i> <sup>[33]</sup>	7 of 183 (4%)	United States	Baseline and repeat examination when visually symptomatic	IAR: three patients ceased treatment. Two with visual symptoms associated with IAR. 46% of patients had HTN and 16% had DM - neither predictive of developing IAR. Atypical adverse events: nil reported.
Malik <i>et al</i> <sup>[34]</sup>	3 or 38 (8%)	United Kingdom	Baseline, 3 and 6 mo. Low follow up rates	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. Atypical adverse events: nil reported.
Andrade <i>et al</i> <sup>[35]</sup>	5 of 34 (15%)	Spain	Baseline, at cessation of treatment and when visually symptomatic	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. Higher serum VEGF in patients with retinopathy and/or subconjunctival hemorrhage. Atypical adverse events: cystoid macular edema in 1 patient, final visual outcomes were not described.
Ogata <i>et al</i> <sup>[36]</sup>	25 of 69 (36%)	Japan	Baseline and then regularly for 6 months	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. 46% (13 of 28) treated with IFN $\alpha$ developed IAR compared to 29% (12 of 41) treated with PEG-IFN $\alpha$ . Atypical adverse events: no details.
Chisholm <i>et al</i> <sup>[37]</sup>	5 of 10 (50%)	United Kingdom	Baseline, 2, 4, 8, 12 and 24 wk and 12 wk after completing treatment	IAR: no dose reduction for management of IAR. Atypical adverse events: nil reported.
Cuthbertson <i>et al</i> <sup>[38]</sup>	4 of 25 (16%)	United Kingdom	3 mo after starting treatment or when visually symptomatic	IAR: no reduced VA in eyes that developed IAR. No dose reduction for management of IAR. Atypical adverse events: nil reported.

<sup>1</sup>Nine patients that had baseline retinopathy were excluded from the incidence data shown. All 9 had progression of retinopathy; <sup>2</sup>Thirty-six of the 46 patients treated for chronic hepatitis B infection were excluded from the incidence data shown; <sup>3</sup>Ten patients that had baseline diabetic retinopathy were excluded from the incidence data shown. Five of these had resolution of retinopathy on subsequent eye exams. BRVO: Branch retinal vein occlusion; CRVO: Central retinal vein occlusion; DM: Diabetes; HTN: Hypertension; IAR: Interferon-associated retinopathy; NAION: Non-arteritic anterior ischemic optic neuropathy; PEG: Pegylated; IFN: Interferon; RBV: Ribavirin; VA: Visual acuity; VEGF: Vascular endothelial growth factor.





**Figure 1** Fundus photographs of a 60-year-old male treated with high dose interferon- $\alpha$  for renal cell carcinoma. These images show bilateral, typical interferon-associated retinopathy consisting of cotton wool spots and retinal hemorrhages surround the optic disc.

age-related macular degeneration found that the incidence of interferon-associated retinopathy was dose-dependent<sup>[40]</sup>. Consistent with this, the study with the highest incidence analysed in this review used the highest dose of interferon: 3-10 million units IFN $\alpha$  subcutaneous injection daily<sup>[20]</sup>. It has also been proposed that PEG-IFN $\alpha$ , which has a ten-fold longer serum-half life than conventional IFN $\alpha$ , may cause interferon-associated retinopathy more readily<sup>[36]</sup>. This would contrast with the systemic side effect profile of PEG-IFN $\alpha$ , which appears to be similar to conventional IFN $\alpha$ <sup>[14,15]</sup>. One large study found a significantly higher incidence of interferon-associated retinopathy in patients treated with PEG-IFN $\alpha$  than patients treated with IFN $\alpha$  of 45% *vs* 19%<sup>[18]</sup>. Two other smaller trials have found contradicting non-significant trends<sup>[32,36]</sup>. Ultimately, the significance of this issue is questionable since it is unlikely that small differences in the incidence of interferon-associated retinopathy, which is largely benign, will alter these use of PEG-IFN $\alpha$  over IFN $\alpha$  or the dose used to treat chronic hepatitis C infection.

The effect of ribavirin on the incidence of interferon-associated retinopathy is unclear due to conflicting results found by the observational studies that addressed this issue. It is used for its synergistic effect with interferon therapy, but does not result in HCV eradication as a monotherapy<sup>[12,13]</sup>. Conjunctivitis is the only ophthalmologic adverse event regularly associated with RBV<sup>[41]</sup>. It has, however, been suggested that combination therapy with RBV may increase the risk of interferon-associated retinopathy as compared to interferon monotherapy<sup>[21,30]</sup>. Lim *et al*<sup>[30]</sup> found a significantly higher rate of interferon-associated retinopathy in patients with chronic hepatitis C infection treated with PEG-IFN $\alpha$  and RBV combination therapy than patients with chronic hepatitis B infection treated with PEG-IFN $\alpha$  monotherapy, that is 50% *vs* 14%. These results are difficult to interpret as chronic hepatitis C infection is associated with a hypercoagulable state, which itself may confer an increased risk of developing interferon-associated retinopathy<sup>[42]</sup>. Further studies are required to determine the impact of RBV on the development of interferon-associated retinopathy.

### **Why does interferon-associated retinopathy occur?**

The pathogenesis of interferon-associated retinopathy is yet to be fully elucidated. Its clinical manifestations, cotton wool spots and retinal hemorrhages suggest an ischemic mechanism. These changes are most commonly associated with diabetes or hypertension<sup>[43,44]</sup>. It has been proposed that endothelial dysfunction, as evidenced by the failure of dilatation of retinal arterioles in response to wall shear stress in eyes that subsequently developed interferon-associated retinopathy, is the central process leading to retinal ischemia<sup>[17]</sup>. Endothelial dysfunction, it is proposed, causes platelet aggregation and leukocyte adherence to vascular endothelium<sup>[17]</sup>. These “immune complexes” act as microthrombi and cause focal retinal infarction<sup>[39]</sup>. This hypothesis is supported by data suggesting IFN $\alpha$  may promote pro-thrombotic autoantibody production mediated by T cell activation<sup>[45]</sup>. Further, IFN $\alpha$  may increase production of the highly potent intravascular aggregator of platelets, plasma-activated complement 5<sup>[24]</sup>. Moreover, IFN $\alpha$  increases leukocyte adherence to the vascular endothelium resulting in leukocyte trapping in the retinal microcirculation<sup>[46]</sup>.

### **Does interferon-associated retinopathy causes vision loss?**

Cotton wool spots and retinal hemorrhages are not usually associated with vision loss. They would if they occurred at the central macula, but the *fovea centralis* is avascular. Nevertheless, there are at least two reported cases of irreversible visual disturbance after interferon-associated retinopathy that consisted of cotton wool spots and/or retinal hemorrhages only, i.e. that were not associated with an atypical adverse event<sup>[20,47]</sup>. One patient developed a permanent peripheral monocular scotoma in the same eye due to interferon-associated retinopathy consisting of cotton wool spots and retinal hemorrhages only<sup>[20]</sup>. The other patient developed permanent bilateral reduced visual acuity and visual field defects after isolated interferon-associated retinopathy<sup>[47]</sup>. Such cases, however, are rare; in most patients isolated interferon-associated retinopathy causes no impact on visual func-

tion (Tables 1 and 2). Indeed, in the 1289 patients, only 1 had interferon-associated retinopathy that caused vision impairment<sup>[20]</sup> (Table 1). Importantly, vision loss that occurs whilst taking antiviral therapy is usually due to the development of an atypical adverse event.

### ***Are there any groups that are at greater risk for developing interferon-associated retinopathy?***

Hypertension and diabetes mellitus appear to be risk factors for the development of interferon-associated retinopathy; however, this has not been established unequivocally. Such a finding would be theoretically consistent with the proposed pathogenesis of interferon-associated retinopathy. The same methodological problems that resulted in the diversity in the incidence of interferon-associated retinopathy found by the observational studies described above also apply to this issue. Compounding this, the numbers of patients with diabetes or hypertension that developed interferon-associated retinopathy in most studies were too small to enable meaningful statistical analysis (Tables 1 and 2).

Observational studies of standard of care therapy for chronic hepatitis C infection during which at least 10 patients developed interferon-associated retinopathy identified diabetes and hypertension as its main risk factors<sup>[28,29,31,32]</sup> (Table 2). Fouad *et al*<sup>[28]</sup> performed a comprehensive study of 84 patients treated with standard of care therapy in Egypt with extensive ophthalmologic follow up. Their study, which included a number of patients with hypertension and diabetes, 12 and 16 respectively, found that both predicted the development of interferon-associated retinopathy using logistic regression analysis. By contrast, Mehta *et al*<sup>[31]</sup> found higher rates of interferon-associated retinopathy in patients with hypertension and diabetes, but the differences were not statistically significant. Their study had sufficient numbers of patients with these conditions - 13 patients with diabetes mellitus and 31 with hypertension - however, ophthalmologic follow up was poor with less than 70% of patients receiving an eye exam within 12 weeks of starting standard of care therapy. Both Vujosevic *et al*<sup>[29]</sup> and Kim *et al*<sup>[32]</sup> performed observational studies with good numbers and adequate ophthalmologic follow up. They both found hypertension to be a significant predictor of the development of interferon-associated retinopathy using univariate and multivariate analysis. Diabetes mellitus was not found to be a significant predictor of the development of interferon-associated retinopathy using multivariate analyses in either, but the cohorts only had 5 and 2 patients with diabetes mellitus, respectively. In Vujosevic *et al*<sup>[29]</sup>, a higher percentage of patients with diabetes mellitus developed interferon-associated retinopathy on univariate analysis. There were insufficient numbers of patients with diabetes mellitus in earlier studies involving IFN $\alpha$  to assess its effect<sup>[18-20]</sup>. Studies with adequate numbers of patients with diabetes mellitus tended to find it as a risk factor for the development of interferon-associated retinopathy.

No other patient characteristics that have been assessed have been found to predict the development of interferon-associated retinopathy (Tables 1 and 2). Older age has been suggested to represent a greater risk for its development, but this has not been a consistent finding<sup>[17,19,22,29]</sup>. The larger studies that assessed risk factors identified above did not implicate age, with the exception of Fouad *et al*<sup>[28]</sup>, Vujosevic *et al*<sup>[29]</sup>, Mehta *et al*<sup>[31]</sup> and Kim *et al*<sup>[32]</sup>. An association of age with the development of interferon-associated retinopathy may be because it is also associated with a higher risk of diabetes and hypertension.

### ***If a patient develops interferon-associated retinopathy, what should be done?***

There is a growing body of clinical experience that it is safe to continue standard of care therapy with no dose reduction in patients who develop interferon-associated retinopathy so long as they do not have reduced visual acuity or other visual symptoms which would suggest the development of an atypical adverse event (Tables 1 and 2). Various dose reduction and cessation regimens aiming to minimise the impact of interferon-associated retinopathy have been used. One study described the dose reduction regimens used by two clinicians in their management of 38 patients with interferon-associated retinopathy over 10 years<sup>[18]</sup>. This study did not compare outcomes between the groups. In fact, no formal comparator studies have assessed different strategies of managing standard of care dosing in patients who develop interferon-associated retinopathy. There is, therefore, no good evidence to guide whether interferon therapy should be modified or discontinued when interferon-associated retinopathy has been diagnosed. It is, however, well established that dose reduction of interferon increases the risk of treatment failure. Thus, dose reduction should be considered carefully.

### ***Should we screen for interferon-associated retinopathy?***

No consensus has been reached regarding the need to screen for interferon-associated retinopathy. Cuthbertson *et al*<sup>[38]</sup> argue that due to the low incidence of interferon-associated retinopathy and its generally benign course there is no need for routine screening. By contrast, Vujosevic *et al*<sup>[29]</sup> support a screening program targeting hypertensive patients, who they found to be at greater risk of developing interferon-associated retinopathy. Mousa *et al*<sup>[27]</sup> propose that screening should only be for patients with both diabetes and hypertension, but not those with either in isolation. On the other end of the spectrum, Schulman *et al*<sup>[20]</sup> considered close ophthalmological follow up for all patients as appropriate.

We propose that screening for interferon-associated retinopathy should only be performed if it meets the following criteria: (1) it can be used to predict the patients at risk for developing pathology that causes irreversible visual impairment; and (2) early treatment of these patients will reduce the chance of the development of that pathology. As discussed above, interferon-associated

retinopathy, with a few exceptions, has a generally benign course. Screening for interferon-associated retinopathy may be justified if it can be proved that eyes that develop it are more likely to develop an atypical adverse event, which in turn causes poor visual outcomes. Evidence of such a relationship does not exist to date. In addition, it would need to be established that early detection would enable an intervention that reduces the severity of that atypical adverse event. For example, it would need to be shown that strict risk factor control after the diagnosis of interferon-associated retinopathy prevents the development of an atypical adverse event<sup>[48]</sup>. With the current state of the evidence, a screening program for interferon-associated retinopathy, even one including only those patients at high risk of developing it, does not appear to be justified.

## ATYPICAL ADVERSE EVENTS

Many atypical ophthalmologic adverse events have been encountered during antiviral therapy for chronic hepatitis C infection. The most common of these are retinal vein occlusion (RVO)<sup>[21,29,30,32,49-55]</sup>, and non-arteritic anterior ischemic optic neuropathy (NAION)<sup>[28,49,56-59]</sup>. Other atypical adverse events that have been reported include ocular myasthenia<sup>[60,61]</sup>, optic neuritis<sup>[62]</sup>, Vogt-Koyanagi-Harada disease<sup>[49,63-67]</sup>, ocular sarcoidosis<sup>[68,69]</sup>, ocular toxocariasis<sup>[70]</sup>, neurovascular glaucoma<sup>[71]</sup>, conjunctival hemorrhage<sup>[20,26,35]</sup>, macular edema<sup>[72-74]</sup>, oculomotor nerve palsy<sup>[75]</sup>, trichomegaly<sup>[23]</sup> and retinal detachment<sup>[76]</sup>.

The mechanisms that cause an atypical adverse event may be distinct from the ischemic mechanism thought to be responsible for interferon-associated retinopathy. For example, there is growing evidence that interferon is directly toxic to the optic nerve<sup>[62,77,78]</sup>. Chisholm *et al*<sup>[37]</sup> found high levels of subclinical retinal toxicity, measured as aberration on multifocal electro-retinogram, in patients treated with IFN $\alpha$  and ribavirin. The electro-retinogram changes were not correlated with clinical signs of interferon-associated retinopathy, cotton wool spots and retinal hemorrhages<sup>[37]</sup>.

Atypical complications of antiviral therapy often result in dramatic, irreversible vision loss. In an exhaustive review of NAION that occurred during interferon therapy, half of the 36 documented cases of this complication suffered from permanent visual dysfunction<sup>[56]</sup>. Similarly, in a recent review of RVO during interferon- $\alpha$  therapy, only 4 of 14 cases had full recovery of vision<sup>[53]</sup>. Other atypical complications also lead to long-term visual impairment. In particular, inflammatory complications of antiviral therapy, such as Vogt-Koyanagi-Harada disease, also tend to have poor visual outcomes<sup>[49,67]</sup>. In the 22 observational studies identified by our literature review involving 1287 patients treated with antiviral therapy for chronic hepatitis C infection, 12 (0.93%) patients developed an atypical adverse event. Five (0.39%) of these led to documented irreversible vision loss and 4 (0.31%) did not describe final visual outcomes (Tables 1 and 2).

The relationship between interferon-associated retinopathy and the atypical complications of antiviral therapy is unclear. Indeed, there is limited evidence that atypical adverse events are caused by interferon treatment and not merely due to chance<sup>[56]</sup>. The most common complications, AION and RVO, are both vascular in nature. It has been suggested that there may be common elements between the pathogenesis of these complications and interferon-associated retinopathy<sup>[49,56]</sup>. Certainly, there are many cases in the literature where AION and RVO are concomitant with interferon-associated retinopathy<sup>[51,55,79]</sup>. As the atypical complications are the key causes of vision loss during antiviral therapy and interferon-associated retinopathy is common and well-described, any relationship between them should be explored in depth.

## THE FUTURE

The standard of care regimen is in the process of a major re-evaluation after two major breakthroughs. Firstly, multiple HCV-specific direct-acting antivirals are at various stages of development and two of these have been approved for the treatment of genotype 1 HCV infection<sup>[80-83]</sup>. Secondly, a host genetic polymorphism near the interleukin-28B (IL28B) gene on chromosome 19 that strongly predicts spontaneous and standard of care-induced recovery from infection was identified by four groups in 2009 and 2010<sup>[84-87]</sup>.

Despite the advent of direct-acting antivirals, it is likely that PEG-IFN $\alpha$  will remain an integral part to HCV treatment regimens for the foreseeable future<sup>[5]</sup>. When used as monotherapy, rapid virological resistance develops *in vivo* to the first generation direct-acting antivirals - telaprevir and boceprevir - inhibiting antiviral response<sup>[88]</sup>. Moreover, the antiviral activity of these first generation direct-acting antivirals, the only approved by the FDA, appears genotype specific<sup>[89]</sup>. Accordingly, they are presently recommended for use in genotype 1 chronic HCV only<sup>[5]</sup>. Investigations of second generation direct-acting antivirals are currently under way, so eventually we very likely will have interferon free-regimens<sup>[90,91]</sup>. RBV, however, remains a core component of most of these regimens.

A controversial issue at present is whether patients, particularly those with IL-28B non-responder genotypes, should defer treatment for chronic hepatitis C infection until new, more effective regimens become available<sup>[92]</sup>. Considering that the most common interferon-associated retinopathy seems to be largely benign in most patients, we do not feel that there is enough evidence for the potential risk for ophthalmologic complications to significantly impact this discussion as the most common is largely benign and there is no obvious, established link between interferon and the rarer, more severe adverse events.

## CONCLUSION

In summary, the most common complication of antiviral



therapy for chronic hepatitis C infection is interferon-associated retinopathy. This is usually a benign, self-limiting phenomenon with no lasting impact on visual function. It occurs in approximately 30% of patients undergoing standard of care therapy, however, there is significant variability in its incidence in observational studies. Hypertension and diabetes mellitus appear to be the most important risk factors for its development. The rarer, atypical adverse events of antiviral therapy often cause irreversible vision loss. The most common of these are RVO and NAION. To date, no definitive pathogenic link has been proven between antiviral therapy or interferon-associated retinopathy and any of the various atypical adverse events. If such a relationship can be found, screening for interferon-associated retinopathy may be justified as a means to prevent the development of an atypical adverse event. Newer direct-acting antivirals are likely to outpace further study into this area, making interferon-free antiviral therapy likely in the next 5 years.

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## Cross-talk between the thyroid and liver: A new target for nonalcoholic fatty liver disease treatment

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**Core tip:** The clinical findings that nonalcoholic fatty liver disease (NAFLD) patients have more prevalence of subclinical hypothyroidism and patients with hypothyroidism may develop fatty liver give the evidence that dyslipidemia and fatty liver have some relationship with thyroid dysfunction, and thyroid hormone and its receptor may be a therapeutic target for NAFLD. We review here that thyroid hormone and TR are a potential target for pharmacologic treatments that can benefit NAFLD patients a lot.

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) has been recognized as the most common liver metabolic disease, and it is also a burgeoning health problem that affects one-third of adults and is associated with obesity and insulin resistance now. Thyroid hormone (TH) and its receptors play a fundamental role in lipid metabolism and lipid accumulation in the liver. It is found that thyroid receptor and its isoforms exhibit tissue-specific expression with a variety of functions. TR $\beta$ 1 is predominantly expressed in the brain and adipose tissue and TR $\beta$ 2 is the major isoform in the liver, kidney and fat. They have different functions and play important roles in lipid metabolism. Recently, there are many studies on the treatment of NAFLD with TH and its analogues. We review here that thyroid hormone and TR are a potential target for pharmacologic treatments. Lipid metabolism and lipid accumulation can be regulated and reversed by TH and its analogues.

### INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a burgeoning health problem that affects one-third of adults and is associated with obesity and insulin resistance. Its pathogenesis remains poorly understood, and therapeutic options are limited. Here, we discuss recent treatment insights into NAFLD that focus primarily on its relationship with thyroid function.

### THYROID HORMONE AND ITS RECEPTORS

Thyroid hormone (TH) regulates cellular and tissue me-



tabolism throughout the body. The active form of TH, 3,3',5-triiodo-L-thyronine (T3), controls gene expression in target tissues by binding to its cognate nuclear receptors (TRs), which are ligand-inducible transcription factors. In the presence of T3, TRs activate transcription by binding to T3-response elements (TREs) of the target genes and forming coactivator complexes containing histone acetyltransferase activity<sup>[1]</sup>. In the absence of T3, TRs recruit corepressors, such as nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid receptors, which form a complex with transducin  $\beta$ -like protein 1 and histone deacetylase 3 that has histone deacetylase activity on the promoters of target genes that repress basal transcription<sup>[2]</sup>.

Two TR isoforms, TR $\alpha$  and TR $\beta$ , have been identified. They share high sequence homology in the functional DNA and T3-binding domains, but differ greatly in the lengths and sequences of the amino-terminal A/B domains. Studies of mice deficient in either of these two TR genes or both TR genes indicate that TR isoforms have both redundant roles and specific functions<sup>[3]</sup>. TR $\alpha$ 1, TR $\beta$ 1, and TR $\beta$ 2 isoforms bind T3; however, TR $\alpha$ 2 does not. TR $\alpha$ 2 functions, at least *in vitro*, as a TR $\alpha$ 1 and TR $\beta$ 1 antagonist<sup>[4]</sup>. Activation of TRs affects a multitude of physiological processes ranging from embryonic development to maintenance of energy homeostasis in adults. Excess TH can result in some therapeutically desirable effects, such as increased metabolic rate, increased lipolysis, lowered cholesterol levels, improved heart contractility, and suppressed thyroid-stimulating hormone (TSH) levels. At the same time, systemic thyrotoxicosis can lead to undesirable effects, including tachycardia, arrhythmia, muscle wasting, nervousness, fatigue, and loss of bone mass<sup>[5]</sup>. A series of studies in mice with inactivation or mutation of different TR isoforms<sup>[6-12]</sup>, as well as studies in patients with resistance to TH, suggest that TR isoforms selectively mediate tissue-specific TH responses<sup>[13]</sup>.

There is a tissue-specific expression pattern for TRs. TR $\beta$ 2 is the major isoform in the liver, kidney, and thyroid, and TR $\beta$ 1 is predominantly expressed in the brain and adipose tissue<sup>[14-17]</sup>. There is also a general consensus that TR $\alpha$  mediates the effects of TH on the heart, whereas TR $\beta$  mediates its effects on plasma cholesterol and TSH secretion. Therefore, the development of T3 analogues with preferential binding to TR $\beta$  may induce the beneficial effects of T3 while avoiding undesirable side effects.

## EFFECTS OF TH ON HEPATIC LIPID METABOLISM

TH maintains lipid homeostasis *via* its effects on gene expression in target organs, including the liver and adipose tissues. T3 has profound and diverse effects on lipid metabolism and lipid accumulation in the liver. In the liver, TR $\beta$  is responsible for mediating the majority of the actions of T3, whereas in other tissues, such as the heart

and brown adipose tissue (BAT), TR $\alpha$  is the main mediator of TH effects<sup>[18,19]</sup>.

T3 exerts strong effects on hepatic carbohydrate and lipid metabolism in both anabolic and catabolic states. Elevated levels of T3 in hyperthyroidism are associated with increased lipolysis and lower body weight. In contrast, lower levels of T3 in hypothyroidism are associated with cold intolerance, weight gain, reduced lipolysis, and cholesterol clearance. Mice devoid of all TR isoforms exhibit decreased body temperature and basal metabolic rate, growth retardation, and an increased amount of fat tissue<sup>[20,21]</sup>. T3 increases the expression of several genes involved in hepatic lipogenesis by increasing the expression of lipogenic genes such as fatty acid synthase (FAS), Thrsp (Spot14), acetyl-CoA carboxylase (ACC1)<sup>[22]</sup>, acyl-CoA synthetase 5, fatty acid transporter protein, malic enzyme, and glucose-6-P dehydrogenase. It also induces the expression of genes involved in fatty acid oxidation, such as fatty acid transporter (Fat), fatty acid-binding protein, lipoprotein lipase (LPL)<sup>[23]</sup>, and carnitine palmitoyltransferase-1 alpha (Cpt-1 $\alpha$ )<sup>[24]</sup>. Cpt-1 $\alpha$  is a key rate-limiting enzyme in mitochondrial fatty acid oxidation. Many of these metabolic genes (*e.g.*, malic enzyme, Fas, and Cpt-1 $\alpha$ ) in the liver are directly regulated by the interaction between T3 and TR, as TREs have been identified in promoters of these genes<sup>[25]</sup>. However, the regulation of lipid homeostasis by T3 is complex and tissue dependent, as it involves the coordinated regulation of several target tissues, mainly adipose tissue and the liver. The tissue-dependent manner of lipid regulation *via* TH was uncovered using knockin mice harboring identical mutations in the TR $\alpha$  (TR $\alpha$ 1PV mouse) and TR $\beta$  (TR $\beta$ PV mouse) genes. TR $\alpha$  gene mutations dramatically decrease the mass of both the liver and white adipose tissue (WAT). In contrast, TR $\beta$  gene mutations markedly increase liver mass with an excess deposition of lipids, but no significant abnormality is observed in WAT. Molecular studies showed that the expression of lipogenic genes was decreased in WAT of TR $\alpha$ 1PV mice, but not in TR $\beta$ PV mice. Markedly increased lipogenic enzyme expression and decreased fatty acid  $\beta$ -oxidation activity contribute to adipogenic steatosis and lipid accumulation in the liver of TR $\beta$ PV mice. In contrast, reduced expression of genes critical for lipogenesis mediates decreased liver mass with lipid scarcity in TR $\alpha$ 1PV mice.

TH action is mediated by a complex interaction between TRs and other nuclear receptors, including the PPARs and the liver X receptor (LXR), which respond to circulating metabolite levels<sup>[26,27]</sup>. Cross-talk between TH signaling and these nutrient-responsive factors occurs through a variety of mechanisms, including but not limited to competition for retinoid X receptor (RXR), transcriptional co-factors, DNA-binding sites, or transcriptional cofactors.

Studies in several animal models, including the PPAR $\alpha$  KO mouse, have demonstrated that hepatic steatosis occurs when nuclear receptors involved in metabolic control are inactivated. In both humans and animal

models, obesity is associated with lipid deposition in the liver, which can lead to fibrosis and even cirrhosis<sup>[28,29]</sup>. In both human and murine microarray studies, the greatest change in liver gene expression as a consequence of hepatic lipid accumulation is the downregulation of a set of T3-responsive genes, including genes involved in energy metabolism<sup>[19,30]</sup>.

Autophagy of lipid droplets, termed “lipophagy,” is a major pathway of lipid mobilization in hepatocytes<sup>[31-33]</sup>, and its inhibition has been linked to the development of fatty liver and insulin resistance<sup>[34-36]</sup>. TH is a well-known metabolic regulator of energy expenditure that activates fatty acid  $\beta$ -oxidation in mammals<sup>[37]</sup>. However, the precise mechanism of this effect has not yet been revealed. During periods of starvation, autophagy degrades cytoplasmic materials, producing amino acids and fatty acids that can be used to synthesize new proteins or generate ATP for cell survival<sup>[38]</sup>. Derangement of the autophagic response has been implicated in several pathological hepatic conditions, such as ischemia, reperfusion, viral infections, acute injury,  $\alpha$ 1-antitrypsin deficiency, hepatocellular carcinoma, alcoholic liver disease, and NAFLD<sup>[36,39,40]</sup>.

“Lipophagy”<sup>[31]</sup> leads to the degradation of intracellular lipid droplets, and this process is believed to provide fatty acid substrates for  $\beta$ -oxidation<sup>[41]</sup>. Such lipophagy is coupled to the effects of T3 stimulation in altering the levels of a broad array of hepatic lipid-related metabolites, which is consistent with a key role for T3 as an important regulator of fatty acid delivery to mitochondria and mitochondrial metabolism. Autophagy is a stress-induced catabolic process, conserved in all eukaryotes, involving fusion of autophagosomes with lysosomes and resulting in degradation of cytoplasmic cargo. T3 induces lipophagy in cultured liver cell lines, and it induces hepatic autophagy *in vivo* coupled with ketogenesis, resulting in a lipolytic-metabolomic profile. Moreover, TH stimulation of autophagy and lipid metabolism is TR dependent and modulated by NCoR corepressor activity. These findings suggest that T3 plays an important role in the regulation of hepatic autophagy, which is a critical step for the amelioration of NAFLD.

## THYROID MALFUNCTION IN DYSLIPIDEMIA AND NAFLD PATIENTS

The most frequent metabolic syndrome disorders are dyslipidemia and NAFLD. The pathogenesis of NAFLD is a complex, multifactorial process characterized by insulin resistance and other endocrine disorders. TH can stimulate the expression of uncoupling proteins in the mitochondria of adipocytes and skeletal muscle and modulate adrenergic receptor numbers by enhancing responsiveness to catecholamines<sup>[42]</sup>, thus controlling metabolic and energy homeostasis. TH influences body weight, thermogenesis, lipolysis, and metabolism of cholesterol and bile acids. Thyroid dysfunction is associated with hepatic lipid peroxidation and oxidative stress

in experimental models<sup>[43,44]</sup>, raising the question of the role of hypothyroidism in NAFLD patients. The prevalence of hypothyroidism in patients with NASH is twice as high as in controls<sup>[45]</sup>. NASH is twice as common in postmenopausal compared with premenopausal women, and hormonal replacement therapy decreases the risk of steatosis. This association seems plausible, taking into consideration that thyroid dysfunction can lead to hyperlipidemia, obesity, and insulin resistance<sup>[46]</sup>, all of which are major components of metabolic syndrome<sup>[47,48]</sup> and are implicated in the pathogenesis of NAFLD.

The mechanism of hypothyroidism-induced hyperlipidemia has been shown to be due to a decrease in cholesterol excretion and a marked increase in apoB lipoproteins due to decreased catabolism and turnover secondary to a reduced number of low-density lipoprotein (LDL) receptors on the liver cell surface<sup>[49]</sup>. Thus, common findings in patients with hypothyroid are increased levels of total and LDL cholesterol. In hypothyroidism, a reduced removal rate of triglycerides from plasma and an accumulation of intermediate LDL (IDL) have also been reported. Thus, NAFLD can develop in hypothyroid patients due to increased LDL and deposition of triglycerides in the liver.

In addition to hyperlipidemia and obesity, hypothyroidism has been associated with insulin resistance<sup>[50]</sup>. There is a strong link between insulin resistance and excessive deposition of triglycerides in hepatocytes. A recent study investigated the frequency of metabolic syndrome in hypothyroid patients. These authors studied 100 patients with overt hypothyroidism, 100 patients with subclinical hypothyroid, and 200 healthy controls. The authors found that the HOMA index was higher in the hypothyroid group than in the control ( $P = 0.008$ ) and subclinical hypothyroid groups ( $P = 0.014$ ). Metabolic syndrome prevalence was 44% in the hypothyroid group and 33% in the control group ( $P = 0.016$ )<sup>[51]</sup>.

Thyroid dysfunction commonly occurs in the elderly population, and overt thyroid dysfunction is associated with some liver abnormalities. Xu *et al.*<sup>[52]</sup> performed a cross-sectional study among 878 euthyroid elderly Chinese, in which 227 (25.85%) subjects fulfilled the diagnostic criteria for NAFLD. Patients with NAFLD had significantly lower levels of serum-free thyroxine (FT4) than control patients ( $11.12 \pm 1.43$  pmol/L *vs*  $11.58 \pm 1.47$  pmol/L;  $P < 0.001$ ). The prevalence of NAFLD decreased in proportion to progressively higher serum FT4 levels ( $P < 0.001$ ). Age-, gender-, and smoking status-adjusted correlation analysis showed that serum FT4 levels were negatively correlated with body mass index, waist circumference, and triglyceride and serum uric acid levels (all with  $P < 0.05$ ). Stepwise logistic regression analysis showed that serum FT4 level was significantly associated with the risk for NAFLD. These results suggest that thyroid function, even within the reference range, is associated with NAFLD in elderly people.

TH may interfere with the regulation of lipid and carbohydrate metabolism, and correlate with the severity

of NAFLD; however, these results are still under debate. Mazo *et al.*<sup>[53]</sup> performed a retrospective evaluation of clinical and metabolic correlations between hypothyroidism and NAFLD. Clinical, biochemical, and histological investigations of 103 NAFLD patients exhibiting drug-treated hypothyroidism were conducted. Steatosis was present in 32.0% of the population and nonalcoholic steatohepatitis was present in 68.0%. Females were the majority in both groups. A link was identified between hypothyroidism and markers of glucose and lipid homeostasis, but not with severity of NAFLD.

Hepatic steatosis can progress to hepatocyte injury, inflammation, and fibrosis in the presence of potential synergistic factors such as oxidative stress from  $\beta$ -oxidation, increased expression of inflammatory cytokines by NF- $\kappa$ B-dependent pathways, and adipocytokines<sup>[54-56]</sup>. This is called the “multi-hit hypothesis” and has been used to describe the pathogenesis of NAFLD<sup>[57]</sup>. Lipid peroxidation and oxidative stress are both believed to play important roles in the progression of disease from steatosis to NASH<sup>[56,58]</sup>. Previous experimental data regarding thyroid dysfunction and hepatic lipid peroxidation have shown that, in a state of hyperthyroidism, TH elevation stimulates the metabolic rate, possibly leading to reactive oxygen species generation, lipid peroxidation, and liver cell damage<sup>[43,44]</sup>. On the other hand, reduced levels of oxidative stress accompanying hypothyroidism might be responsible for the experimental results indicating that hypothyroidism protects from hepatic fibrosis<sup>[59]</sup>. This concept correlates with the absence of an association between hypothyroidism and steatosis or NASH. In some studies, mainly with obese NAFLD patients, hypothyroidism appears to contribute to the major components of metabolic syndrome, leading primarily to the accumulation of fat. However during progression to NASH, additional results are needed, with emphases on the role of oxidative stress and lipid peroxidation.

## POTENTIAL PHARMACOLOGIC TREATMENT WITH TH IN BASIC RESEARCH AND CLINICAL PRACTICE

The current pharmacologic treatment for NAFLD is limited, relying mostly on weight loss<sup>[60-62]</sup>. Insulin-sensitizing agents, such as thiazolidinediones, have been shown to decrease hepatic steatosis by promoting fat redistribution to the liver.

### TH

T3 treatment in rats stimulates thermogenesis from fatty acid  $\beta$ -oxidation as a result of lipolysis and increased caloric intake<sup>[63]</sup>. Lipogenesis is also stimulated by T3. However, this effect occurs to a much lesser extent and is mainly seen in the context of restoration of depleted fat stores after a period of energy deficit<sup>[64]</sup>. Previous studies have shown that treatment with T3 itself, or with selec-

tive agonists of TR $\beta$ , may improve the metabolic status of diet-induced obese rodents<sup>[13,65,66]</sup>.

Recently, mice treated with T3 showed a dose-dependent increase in hepatic *FGF21* expression with significant induction at doses as low as 100  $\mu$ g/kg. *FGF21* expression is downstream of the nuclear receptor peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ). PPAR $\alpha$  knockout mice treated with T3 did not have an increase in *FGF21* expression, indicating that hepatic regulation of *FGF21* by T3 in the liver is *via* a PPAR $\alpha$ -dependent mechanism. In contrast, in WAT, *FGF21* expression was suppressed by T3 treatment, with other T3 targets being unaffected. In cell culture studies with an *FGF21* reporter construct, three transcription factors were required for the induction of *FGF21* expression: TR $\beta$ , RXR, and PPAR $\alpha$ . These findings indicate a novel regulatory pathway whereby T3 positively regulates hepatic *FGF21* expression, presenting a novel therapeutic target for diseases such as NAFLD.

In addition, prolonged T3 treatment promotes the catabolism of fatty acids by increasing the expression and activity of Cpt-1 $\alpha$ , a rate-limiting enzyme for transport and  $\beta$ -oxidation of fatty acids in the mitochondria<sup>[25]</sup>. Thus, the catabolism of fatty acids is a cardinal metabolic feature of prolonged hyperthyroidism<sup>[63]</sup>. T3 stimulates the shuttling of free fatty acids (FFAs) for delivery into mitochondria<sup>[67]</sup>. While this process is well described, the T3-regulated cellular pathways that lead to the generation of FFAs from stored lipid droplets in the liver are not very well understood. In that way, T3 treatment is beneficial to patients with high TSH and high FFA levels.

### TR $\alpha$ inhibition

TR $\alpha$  or TR $\beta$  gene knockout mouse models display a range of defects in lipogenesis, lipolysis, cholesterol metabolism, and fatty acid oxidation. Francois<sup>[68]</sup> reported that TR $\alpha$  gene knockout mice are protected from diet-induced hepatic insulin resistance. With the goal of examining whether TR $\alpha$  would be a potential therapeutic target to prevent diet-induced NAFLD and insulin resistance, they assessed insulin action in high-fat diet fed TR $\alpha$  gene knockout (Thra-0/0) and wild-type mice using hyperinsulinemic-euglycemic clamps combined with <sup>3</sup>H/<sup>14</sup>C-labeled glucose to assess basal and insulin-stimulated rates of glucose and fat metabolism. Body composition was assessed by <sup>1</sup>H magnetic resonance spectroscopy, and energy expenditure was measured using indirect calorimetry. Thra-0/0 mice were lighter, leaner, and manifested greater whole-body insulin sensitivity than wild-type mice during the clamp, and these results could be attributed to increased insulin sensitivity both in the liver and peripheral tissues. Increased hepatic insulin sensitivity could be attributed to decreased hepatic diacylglycerol content, resulting in decreased activation of protein kinase C and increased insulin signaling. Therefore, TR $\alpha$  inhibition represents a novel pharmacologic target for the treatment of NAFLD, obesity, and type 2 diabetes.



### TR $\beta$ agonists

The use of TR agonists for the treatment of NAFLD has not been considered viable because TH increases FFA flux from the periphery to the liver, induces hepatic lipogenesis, and therefore could potentially contribute to steatosis. However, specifically targeting TR $\beta$  could provide therapeutic benefit while avoiding the potential of non-selective TR agonists to increase hepatic FFA accumulation. MB07811 is an orally active liver-targeted TR $\beta$  agonist. Cable<sup>[29]</sup> reported a reduction of hepatic steatosis in rats and mice after treatment with MB07811. The purpose of these studies was to assess the effects of MB07811 on whole body and liver lipid metabolism of normal rodents and rodent models of hepatic steatosis. Animal studies showed that MB07811 markedly reduced hepatic steatosis as well as plasma FFA and triglyceride levels. In contrast to MB07811, treatment with T3 induced adipocyte lipolysis *in vitro* and *in vivo*, but had a diminished ability to decrease hepatic steatosis. This finding suggests the influx of FFA from the periphery to the liver may partially counteract the antisteatotic activity of T3. Clearance of liver lipids by MB07811 results from accelerated hepatic fatty acid oxidation, a known consequence of hepatic TR activation, as reflected by increased hepatic mitochondrial respiration rates, changes in hepatic gene expression, and increased plasma acyl-carnitine levels. Transaminase levels remained unchanged or reduced, and no evidence of liver fibrosis or other histological liver damage was observed after treatment with MB07811 for up to 10 wk. Additionally, MB07811, unlike T3, did not increase heart rate or decrease pituitary TSH $\beta$  expression. Therefore, MB07811 represents a novel class of liver-targeted TR agonists with beneficial LDL cholesterol-lowering properties that may provide additional therapeutic benefit to hyperlipidemic patients with concomitant NAFLD.

### LXR activator

TH action is mediated by interactions between TRs and nuclear receptors such as LXR, and Thrsp is known to be regulated by a variety of transcription factors, including TR, PXR, and CAR. Thrsp has been reported to be a lipogenic gene in cultured hepatocytes, suggesting an important role for Thrsp in the pathogenesis of NAFLD. Hepatic overexpression of Thrsp increases triglyceride accumulation with enhanced lipogenesis in the liver of C57Bl/6 mice, whereas hepatic Thrsp gene silencing attenuates the fatty liver phenotype in db/db mice. It has been reported that the LXR activator TO901317 induces Thrsp expression in the liver of wild-type and LXR $\beta$  gene-deficient mice, but not in LXR $\alpha$  or LXR $\alpha/\beta$  double knockout mice. Emerging *in vitro* evidence also points to a critical role for LXR in regulating Thrsp transcription in hepatocytes. New evidence<sup>[69]</sup> also shows that Thrsp is upregulated in the liver of db/db mice and high-fat diet-fed mice, two models of murine NAFLD. The expression of Thrsp depends on LXR $\alpha$  *via* an SREBP1c-dependent mechanism. TO901317 treatment significantly enhances hepatic SREBP1c expression and activity in

wild-type mice but fails to induce Thrsp expression in SREBP-1c gene-deficient mice. TO901317 treatment and LXR $\alpha$  overexpression fail to induce, whereas overexpression of SREBP1c significantly increases, Thrsp promoter activity. Moreover, deletion of the SRE site completely abolishes SREBP1c-induced Thrsp transcription. These findings demonstrate that Thrsp is a lipogenic liver gene that is induced by the LXR agonist through an LXR $\alpha$ -mediated, SREBP1c-dependent mechanism. Thrsp may therefore represent a potential therapeutic target for the treatment of NAFLD.

### TR $\beta$ -specific agonist GC-1

GC-1 is a synthetic TH analogue that is relatively selective for both the binding and activation functions<sup>[13]</sup> of TR $\beta$ 1 over TR $\alpha$ 1. GC-1 has several structural differences with respect to the natural hormone T3, including replacement of the three iodine residues with methyl and isopropyl groups, replacement of the biaryl ether linkage with a methylene linkage, and replacement of the amino acid side chain with an oxyacetic acid side chain<sup>[70]</sup>. GC-1 binds TR $\beta$ 1 with the same affinity as T3 does, but GC-1 binds TR $\alpha$ 1 with an affinity approximately 10 times lower than that of T3, both *in vitro* and *in vivo*<sup>[71]</sup>. The differential effects of GC-1, compared with those of T3, on the thermogenesis by BAT<sup>[72]</sup>, tadpole metamorphosis<sup>[73]</sup>, and the development of bone and central nervous system<sup>[74-76]</sup> may be the result of GC-1 selectivity for TR $\beta$ <sup>[77]</sup>. On the other hand, the selective effects of GC-1 may also be related to the body distribution of the TR isoforms. In agreement with studies in which the TR $\beta$  gene was disrupted<sup>[78]</sup>, GC-1 has almost no effect on the heart, which expresses mainly TR $\alpha$ 1, but does lower serum levels of cholesterol and triglycerides, in agreement with the predominant expression of TR $\beta$ 1 in the liver. Other studies also suggest that the selective actions of GC-1 might be explained by differential tissue uptake, since GC-1 presents a clear tissue-specific accumulation<sup>[79]</sup>. It has been shown, for example, that GC-1 accumulates selectively in the liver as compared in the heart. The tissue/plasma ratio was similar for GC-1 and T3 in the liver but was 30-times lower in the heart<sup>[71]</sup>. It is well known that thyrotoxicosis affects body composition, reducing both fat and lean mass<sup>[80,81]</sup>. In primates, treatment with GC-1 increases oxygen consumption and reduces body weight, but its effects on body composition have not yet been determined. Treatment with GC-1 increases the metabolic rate, has no effect on food intake, and decreases fat mass while sparing lean mass in rats. These data illustrate the potential of GC-1 for the selective activation of TR $\beta$  in rats to induce UCP1 gene expression, while only minimally mediating synergism between TH and the sympathetic nervous system. The use of GC-1 or other TR $\beta$ -selective agonists in rodents and primates has recently been shown to increase energy expenditure and decrease fat mass and plasma levels of cholesterol<sup>[82]</sup>, while sparing the heart<sup>[71]</sup> and skeletal system<sup>[83]</sup>. The TR $\beta$ -specific agonist GC-1 increases energy expenditure and prevents fat



mass accumulation in rats.

The effect of GC-1 on biological processes has not yet been demonstrated. The effects of 6-wk treatment with T3 (daily injections of 3 or 6 µg/100 g body weight) or GC-1 (equimolar doses) on different metabolic parameters in adult female rats were investigated by Villicev<sup>[13]</sup>. Whereas all animals gained weight (17-25 g) equally with T3 or GC-1 treatment, only T3 treatment increased food intake (50%-70%). Oxygen consumption was significantly and equally increased (50%-70%) by T3 and GC-1. Analysis of body composition by dual-energy X-ray absorptiometry (DEXA) revealed that whereas control animals gained about 80% of fat mass, T3- or GC-1-treated animals lost 70%-90% and 20%, respectively. Analysis of the carcasses showed that T3 treatment resulted in a 14%-74% decrease in fat content, whereas GC-1 treatment resulted in only a 15%-23% reduction. The gain in lean mass by DEXA and carcass protein content were unaffected by either T3 or GC-1 treatment. However, the masses of individual skeletal muscles were negatively affected by T3, but only marginally by GC-1. These findings highlight the potential use of GC-1 for the treatment of obesity and metabolic syndrome.

## GC-24

BAT is a tissue specialized in adaptive thermogenesis with the expression of mitochondrial uncoupling protein 1 (UCP1) in response to cold induction. In contrast to WAT, the main function of BAT is to dissipate energy, not to store it. Therefore, the conversion of WAT to BAT is sought as a possible strategy to treat obesity. In rats fed a high-calorie diet, GC-24 confers resistance to diet-induced obesity through the promotion of energy expenditure<sup>[84]</sup>. In addition, a recent case report<sup>[85]</sup> indicates that in a diabetic patient with extreme insulin resistance due to a mutation in the insulin receptor gene, TH induces BAT and ameliorates diabetes.

Overall, TH or TR dysfunction can serve as another mechanism that is related to fatty liver and obesity. Evidence based on animal models and clinical phonemes can lead us to further explore the pathway between thyroid and fatty tissues or the liver. With an understanding of a functional thyroid, we believe that TH analogues and receptor agonists will be potential pharmacologic targets in patients with NAFLD in the near future.

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## Novel therapies for constipation

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prostone, and linaclotide had very different modes of action yet, all three have been shown to be efficacious and safe in the treatment dose for constipation.

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**Key words:** Constipation; Prucalopride; Linaclotide

**Core tip:** When standard laxatives fail in the management of constipation, licensed medication linaclotide and prucalopride show useful efficacy in clinical trials.

### Abstract

Constipation is a common medical problem and when standard laxatives fail it can be difficult to treat. Different aetiologies require tailored therapeutic approaches. Simple constipation may only require dietary manipulation while severe neurological or slow transit constipation may need pharmacologic intervention. Recently new drug therapies have been introduced. PubMed and Ovid were searched for reviews, systematic reviews and meta-analysis published since 2003 using the terms: constipation, Prucalopride, Linaclotide and Lubiprostone. This review summarizes potential novel therapies identified as effective in the management of chronic constipation. Prucalopride is a selective 5-hydroxytryptamine receptor agonist. The prucalopride study was in patients, largely women with idiopathic constipation showed improved spontaneous complete bowel movement (SCBM) at a dose of 2 mg a day with few adverse events reported. Linaclotide is a 14-amino acid peptide guanylate cyclase-C agonist. The linaclotide study was carried out in patients with irritable bowel syndrome, constipation group (IBS-C). There was significant improvement of bowel evacuation and symptom resolution in patients on the active treatment arm. Lubiprostone activates type-2 chloride channels, increasing intestinal fluid secretion. In the trials of this drug, the lubiprostone arms had a greater mean number of SCBM. The novel therapies, prucalopride, lubi-

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### INTRODUCTION

Constipation is a common complaint reported to General practitioners and Gastroenterologists. A systematic review by Peppas *et al*<sup>[1]</sup> found that the mean value of constipation rates in Europe is 17.1% and 15.3% in Oceania. Constipation is a symptom complex. A thorough history and physical examination is paramount in the evaluation of a patient presenting with constipation. The physician should seek to understand the patient's perception of their current bowel habit, compared to the past and should include stool frequency, form and the ease of passage of stool<sup>[2]</sup>. The use of the Bristol Stool Chart may aid the patient in their description of the stool form (Table 1)<sup>[2,3]</sup>.

The presence of alarm features such as unintentional weight loss and rectal bleeding should be excluded during the history taking. The drug history should include the type of laxatives and the dose and duration previously tried. Physical examination should include both an abdominal examination for palpation of any masses or

**Table 1 Bristol stool chart**

Type	Description
1	Separate hard lumps
2	Sausage shaped but lumpy
3	Like a sausage but with cracks on its surface
4	Like a sausage or snake, smooth and soft
5	Soft blobs with clear cut edges
6	Fluffy pieces with ragged edges, a mushy stool
7	Watery, no solid pieces, entirely liquid

palpable stool and a rectal examination, which may reveal evidence of strictures or fissures.

Various definitions of constipation have been used, ranging from a self-reported sense of constipation to the formal criteria used in clinical trials. A practical definition would be a reduced frequency or ease of stool passage that is different to the individual's normal pattern. Constipation can be acute and chronic, with chronic constipation defined as duration of greater than three months<sup>[2]</sup>.

The Rome III criterion for chronic constipation includes the presence of two or more of six symptoms for at least twelve weeks in the preceding six months (Table 2)<sup>[2-4]</sup>. Constipation leads to a reduction in quality of life. A recent systematic review by Belsey *et al.*<sup>[5]</sup> has shown that this reduction is predominant in the mental health domain and is equivalent to chronic conditions such as diabetes.

Constipation can be a primarily functional disorder or secondary to medications, and systemic diseases. Constipation can be classified as normal transit, slow transit, or due to obstructed defaecation<sup>[6]</sup>. A study conducted in Thailand found that 13% of patients had slow transit, 29% had obstructed defaecation, 11% of patients had a mixture of slow transit and obstructed defaecation, with the remaining 47% having normal transit<sup>[7]</sup>.

### Obstructed defaecation

Functional outlet obstruction can occur because of dysfunction of the anal sphincter, pelvic floor muscle dyssynergia or structural abnormalities like obstructing rectoceles<sup>[6]</sup>.

### Slow transit constipation

Patients with slow transit constipation have a reduction in the frequency of high amplitude colonic contractions<sup>[6]</sup>. Scintigraphic measurements indicate that slow transit is more common in the left colon than in the transverse and ascending colon<sup>[8]</sup>. A loss of co-ordination between contractile activity in the rectum and sigmoid colon and a reduced rectal sensory threshold has been implicated in slow transit constipation<sup>[6-10]</sup>.

### Normal transit constipation

In this subgroup the colonic transit time is normal and there is no evidence of functional outlet obstruction on testing. It is the cohort of patients with constipation pre-

**Table 2 Rome III criteria**

- 1 Straining at defaecation on at least 1/4 of occasions.
- 2 Stools that are lumpy/hard on at least 1/4 of occasions.
- 3 Sensation of incomplete evacuation on at least 1/4 of occasions.
- 4 Manual manoeuvres to facilitate at least 25% of defaecations.
- 5 Sensation of anorectal obstruction/blockage at least 25% of defaecations.
- 6 Fewer than 3 bowel movements a week.

dominant irritable bowel syndrome that typically fall into this subgroup<sup>[6]</sup>.

Health professionals have traditionally advised patients presenting with constipation to increase fibre and fluid intake and to exercise. However, the evidence behind this is inconsistent<sup>[11]</sup>. Current guidelines don't make any firm recommendations to support the use of laxatives in chronic constipation<sup>[12-14]</sup>.

In recent years new pharmacological agents have appeared on the market. This review article serves to address the following novel therapies available for the management of primary constipation: Prucalopride, Lubiprostone and Linaclotide.

## PRUCALOPRIDE

Prucalopride is a selective, high affinity 5-hydroxytryptamine receptor agonist, used in patients with severe chronic constipation. There have been 3 pivotal studies of the use of Prucalopride in the management of chronic constipation-Camilleri 2008<sup>[15]</sup>, Tack 2009<sup>[16]</sup> and Quigley 2009<sup>[17]</sup>. They enrolled both men and women, however over 85% of the evaluated patients were female. This has led to the drug being restricted to women only. This is not to say that it is not effective in men, it merely hasn't been adequately tested in them. The recommended dose of Prucalopride is 2 mg as a dose response effect was not obvious between the 2 mg and 4 mg dose tested in the 3 studies. The use of Prucalopride is approved for chronic constipation in women in whom laxatives have failed to provide adequate relief<sup>[18]</sup>.

The study by Camilleri *et al.*<sup>[15]</sup> was a multicentre, randomized, placebo-controlled, trial in 620 patients with severe chronic constipation (< 2 spontaneous, complete bowel movements per week). They found that the proportion of patients with 3 or more spontaneous, complete bowel movements per week was 30.9% of those receiving 2 mg of Prucalopride and 28.4% of those receiving 4 mg of Prucalopride, compared to 12.0% in the placebo group ( $P < 0.001$  for both comparisons). The most frequently reported side effects of the drug have been headache, nausea, and diarrhoea<sup>[15]</sup>.

In the 12 wk study 713 patients recruited patients were given either 2 or 4 mg Prucalopride daily versus placebo. The number of patients achieving > 3 SCBMs/wk was 19.5%;  $P < 0.01$  on 2 mg prucalopride and 23.6;  $P < 0.001$  on 4 mg prucalopride *vs* 9.6% for placebo<sup>[16]</sup>.

Quigley *et al.*<sup>[17]</sup> also demonstrated a similar efficacy

of Prucalopride in 641 patients compared to placebo in chronic constipation. In this 12 wk study 641 patients received either 2 or 4 mg of prucalopride *vs* placebo. In the 2 mg prucalopride group 23.9% had > 3 SCBM per week. In the 4 mg group 23.5% had > 3 SCBM per week ( $P < 0.01$ , in both cases) *vs* 12.1% with placebo<sup>[18]</sup>.

A study from the Asia-Pacific region evaluated the efficacy and safety of the 2 mg dose of prucalopride compared to placebo in patients with chronic constipation. This study found that prucalopride greatly improved bowel function, and patient satisfaction in individuals suffering from chronic constipation over a 12-wk treatment period. It found that prucalopride was safe and was well tolerated by patients<sup>[19]</sup>.

Prucalopride has not been found to have a significant interaction with the hERG potassium channel which was assumed to have been responsible for the development of adverse cardiovascular effects seen with Cisapride<sup>[18,20]</sup>. The three pivotal clinical trials of prucalopride did not demonstrate any relevant electrocardiographic changes<sup>[15-17]</sup>. A recent meta-analysis of seven RCT's of prucalopride found that the number needed to treat (NNT) was 6<sup>[21,22]</sup>.

### Lubiprostone

Chloride channels play a vital role in the transport of fluid and maintaining cell volume and pH in cells and tissues, particularly intestinal epithelial cells. The CIC-2 channel when activated promotes the secretion of intestinal fluid. Lubiprostone activates type-2 chloride channels, increasing intestinal fluid secretion. This may facilitate intestinal transit, thereby increasing the passage of stool<sup>[23]</sup>.

In a multicentre 4 wk trial, Johanson *et al* demonstrated that the use of Lubiprostone in chronic constipation produced a bowel motion within 24-48 h of initial dosing and improved frequency of bowel motions with short term treatment. This double-blinded trial recruited 242 patients with constipation. The patients were randomized to receive either 24 mcg oral Lubiprostone or placebo twice daily for 4 wk. One hundred and twenty patients received Lubiprostone and 122 received placebo. The Lubiprostone arm reported a greater mean number of spontaneous bowel movements at week 1 compared with the placebo arm (5.69 *vs* 3.46,  $P = 0.0001$ ), with an increased frequency of spontaneous bowel movements reported at weeks 2, 3 and 4 ( $P \leq 0.002$ ). Twenty-four hours after the first dose 56.7% of the Lubiprostone group reported a SCBM compared with 36.9% in the placebo group ( $P = 0.0024$ ); within 48 h, 80% and 60.7% of these patients reported a SCBM ( $P = 0.0013$ ), respectively. The two most common treatment-related adverse events were nausea (31.7%) and headache (11.7%)<sup>[24]</sup>.

Barish *et al*<sup>[25]</sup> showed a similar outcome in their multicentre, double-blinded study. A total of 237 patients with chronic constipation were randomized to 4 wk of 24 mcg oral Lubiprostone or placebo twice daily. The Lubipro-

stone arm again had a greater mean number of SCBM at week 1 compared to placebo (5.89 *vs* 3.99,  $P = 0.0001$ ), with a higher proportion having SCBM's in the first 24 h of the initial dose (61.3% *vs* 31.4%,  $P < 0.0001$ )<sup>[25]</sup>.

### Linaclotide

Linaclotide is a 14-amino acid peptide guanylate cyclase-C agonist. It binds to and activates GC-C on the luminal surface of the intestinal epithelium. Activation of GC-C leads to increased cGMP (cyclic guanosine monophosphate) which triggers a signal transduction cascade activating the cystic fibrosis transmembrane conductance regulator. This causes an increase in the secretion of chloride and bicarbonate into the intestinal lumen, resulting in increased luminal fluid secretion and an acceleration of intestinal transit.

A recent 26 wk, randomized, double-blinded trial was done across 102 centres across the United States. The objective of this phase 3 clinical trial was to assess the safety and efficacy of Linaclotide at a daily dosage of 290 mcg *vs* placebo to patients with IBS-C. Based on the recommendations for IBS-C trial design and in the FDA guidance for IBS clinical trials, a responder was defined as a patient who reported: (1) An improvement of > 30% from baseline in average daily worst abdominal pain score; and (2) Increase of > 1 complete spontaneous bowel movement from baseline, both in the same week for > 6/12 wk and 3 other primary end points, based on improvements in abdominal pain and CSBMs for 9/12 wk<sup>[26,27]</sup>.

After the initial screening, 804 patients were recruited. 33.7% of the Linaclotide arm were FDA end point responders *vs* 13.9% of the placebo arm ( $P < 0.0001$ ). The NNT was 5.1, (95%CI: 3.9-7.1). The pain responder criterion of the FDA end point was met by 48.9% of Linaclotide treated patients *vs* 34.5% of placebo-treated patients (NNT = 7.0, 95%CI: 4.7-13.1) and the CSBM responder criterion was met by 47.6% of Linaclotide-treated patients, *vs* 22.6% of placebo patients (NNT = 4.0, 95%CI: 3.2-5.4)<sup>[26]</sup>.

Another 12 wk trial by Rao *et al*<sup>[28]</sup> recruited 800 patients to a double-blinded, parallel group, placebo controlled trial to placebo *vs* 290 mcg linaclotide once daily, followed by a 4 wk randomized withdrawal period. Percent of thirty three point six of the linaclotide-treated patients met the FDA end point compared with 21% of placebo treated patients ( $P < 0.0001$ ) (NNT = 8, 95%CI: 5.4-15.5). Throughout the randomized withdrawal period, patients remaining on linaclotide showed a sustained improvement. The patients that were re-randomized from linaclotide to placebo showed a return of symptoms without any worsening of symptoms relative to baseline<sup>[28]</sup>. The most common adverse effects were GI-related, of which diarrhoea had the highest incidence<sup>[29]</sup>.

## CONCLUSION

Novel therapies such as Prucalopride, Lubiprostone, and

Linaclotide have been shown to be efficacious and safe in the treatment dose for constipation. They have different mechanisms of action influencing and activating colonic motility, secretions and transit time leading to improvement in frequency and consistency of stool and bowel symptoms with greater satisfaction in chronic constipation. Overall, all these new treatment options have been shown to have a good safety profile.

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## Management of portopulmonary hypertension: New perspectives

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### Abstract

Portopulmonary hypertension (PPHTN) is a known complication of cirrhosis. Moderate-to-severe PPHTN implies an extremely poor prognosis. It occurs in 5%-10% of patients referred for liver transplantation (LT), and probably with an higher incidence in patients with large portosystemic shunts. Patients with moderate-to-severe pulmonary hypertension have been previously excluded from LT because of the extremely high surgical risk and since the post-transplant outcome reported was poor. Recently, new perspectives in the management of patients with portopulmonary hypertension are emerging. In fact, some pulmonary vasoactive drugs have become routine in the treatment of patients with idiopathic pulmonary hypertension. These drugs, particularly epoprostenol, have been recently introduced in the treatment of patients with PPHTN, and have been shown to be effective in reducing pulmonary artery pressure as well as pulmonary vascular resistances.

Furthermore, recent studies seem to demonstrate that treatment with pulmonary vasoactive drugs could allow liver transplantation with acceptable surgical risks and excellent survival. Although there are not large series nor prospective studies addressing this topic, the clinical scenario of patients with PPHTN seems to be positively changing.

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**Key words:** Portopulmonary hypertension; Cirrhosis; Liver transplantation; Management; Epoprostenol

**Core tip:** Moderate-to-severe portopulmonary hypertension (PPHTN) implies an extremely poor prognosis and patients are generally excluded from liver transplantation. Recently, some pulmonary vasoactive drugs have become routine in the treatment of patients with idiopathic pulmonary hypertension and have been recently introduced in the treatment of patients with PPHTN. Recent studies seem to demonstrate that treatment with pulmonary vasoactive drugs could allow liver transplantation with acceptable surgical risks and excellent survival. This paper reports a review on management of PPHTN.

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### INTRODUCTION

Portopulmonary hypertension (PPHTN) is a known and uncommon severe complication of cirrhosis, since moderate-to-severe forms have grave prognostic significance with

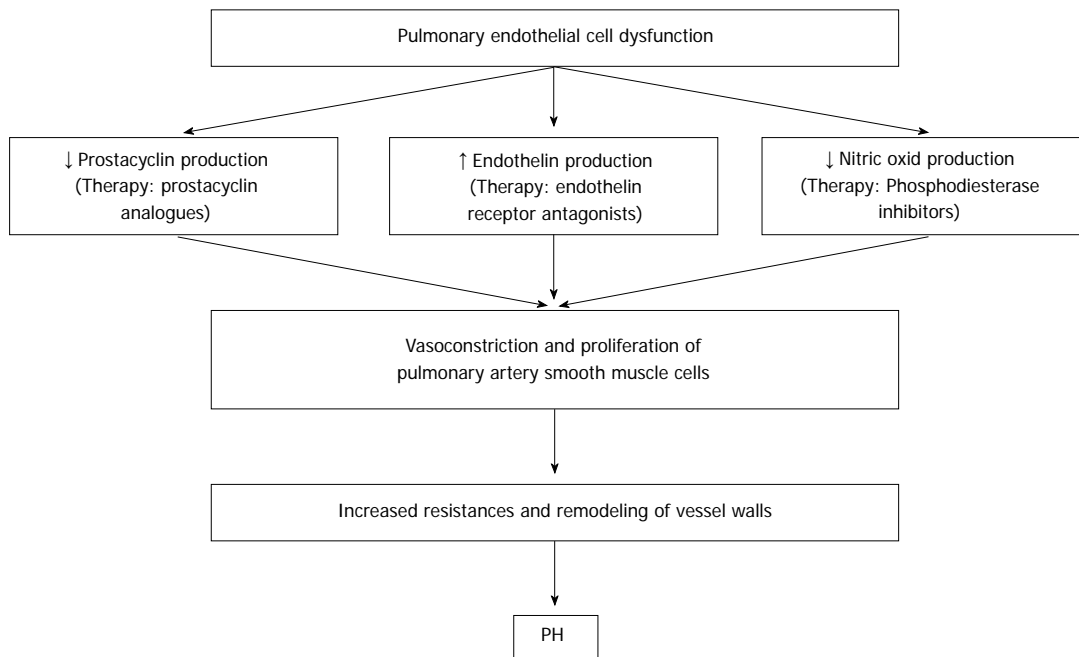


Figure 1 Pathophysiology of pulmonary hypertension and possible drug targets.

the majority of patients succumbing within 2 years<sup>[1-4]</sup>.

PPHTN affects 1%-2% of patients with portal hypertension or cirrhosis and 5%-10% of patients being evaluated for liver transplantation<sup>[5]</sup>.

Diagnosis is suggested by either elevation of the right ventricular systolic pressure or right ventricular dysfunction on echocardiography, but requires confirmation by right heart catheterization<sup>[6-8]</sup>.

## DEFINITION OF PORTOPULMONARY HYPERTENSION

PPHTN definition has evolved over time, specifically with regard to the cutoff for pulmonary vascular resistances (PVR). Initial studies used  $PVR > 120$  dynes  $s\ cm^{-5}$  as abnormal. Subsequent studies and the Consensus Report of the European Respiratory Task Force on pulmonary vascular diseases associated with liver diseases have recommended  $PVR > 240$  dynes  $s\ cm^{-5}$  as the “gold standard”.

Therefore the current consensus diagnostic criteria for PPHTN include mean pulmonary artery pressure (MPAP)  $> 25$  mmHg,  $PVR > 240$  dynes  $s\ cm^{-5}$ , and pulmonary artery occlusion pressure (PAOP)  $< 15$  mmHg<sup>[1,6,7]</sup>.

Since it has been shown that some patients with  $PAOP > 15$  mmHg and  $PVR > 240$  dynes  $s\ cm^{-5}$  may present a trans-pulmonary gradient (TPG) strongly suggestive of obstruction to pulmonary arterial flow, recently new criteria have been proposed by authors from Mayo Clinic<sup>[8]</sup>. These are the following: (1) Portal Hypertension and/or liver disease (clinical diagnosis-ascites/varices/splenomegaly); (2)  $MPAP > 25$  mmHg at rest; and (3)  $PVR > 240$  dynes  $s\ cm^{-5}$ ; and (4)  $PAOP < 15$  mmHg or  $TPG > 12$  mmHg, where  $TPG = MPAP - PAOP$ .

In fact, many patients managed as pulmonary hypertension have elevated left-sided filling pressure, probably due to ventricular interaction. Then, it has been recently proposed that only values of PAOP higher than 18 mmHg exclude the diagnosis of PPHTN<sup>[9-12]</sup>.

## PATHOPHYSIOLOGY AND PATHOGENESIS

PPHTN involves endothelial and smooth muscle proliferation, and have the same features of plexogenic arteriopathy of Idiopathic Pulmonary Hypertension (Figure 1). There is not a clear link between portal hypertension and the development of PPHTN. Because only 5%-10% of patients with portal hypertension develop PPHTN, factors other than portal hypertension must be involved in its development. Moreover, the link between liver dysfunction and PPHTN is not obvious, because PPHTN may develop in cases of portal vein thrombosis or idiopathic hypertension, in absence of any liver dysfunction. Data of a recent retrospective study by Talwalkar *et al*<sup>[13]</sup>, suggest a strong association between large portosystemic shunts, hepatofugal portal blood flow, and PPHTN. These data may support the hypothesis that vasoactive factors from the splanchnic circulation may be pathogenic for PPHTN development. The fact that the most occurring shunt observed in this study was spleno-renal might suggest that the blood flow coming from the spleen, which is involved in the destruction of platelets and prostaglandins delivery, might be of primary importance in the PPHTN pathogenesis. Moreover, presence of large portosystemic shunts, such as those reported in this study, seems to be associated with lack of response to vasoactive treatment.

## PROGNOSIS OF PPHTN AND LIVER TRANSPLANTATION

Moderate-to-severe PPHTN (MPAP  $\geq 35$  mmHg) that is present in up to 5%-10% of patients referred for liver transplantation (LT)<sup>[4,5]</sup>, excludes patients from LT in most of the centers, since post-transplant outcome is poor and because of the high surgical risk<sup>[11-13]</sup>.

A multicenter transplant database has shown that 36% of PPHTN patients died during the immediate post-transparent period due to progressive right ventricular failure, acute respiratory distress syndrome and cardiovascular collapse<sup>[11]</sup>. Moreover, Krowka *et al.*<sup>[14]</sup> reported that only 29% of transplanted patients with untreated PPHTN survived 3 years.

## VASODILATION TREATMENT IN PPHTN

Several vasomodulating and vasodilating drugs have been introduced for the treatment of idiopathic pulmonary hypertension. More recently, vasomodulating and vasodilating drugs, available for the treatment of idiopathic pulmonary hypertension, have also been shown to significantly improve pulmonary hemodynamics in some patients with PPHTN<sup>[5-16]</sup>.

Current medications target three pulmonary hypertension pathways. The first agent, the endothelin receptor antagonists, target the vasoconstrictive endothelin pathway. The second agent targets the prostacyclin pathway, resulting in vasodilation, antiplatelet effect, and vascular remodelling. The third one involves nitric oxide-mediated vasodilation through cyclic guanosine monophosphatase and the inhibition of the phosphodiesterase type 5 enzyme. Therefore, some drugs have a vasodilation effect, while others have remodeling and antiplatelet effects.

Since we do not yet know which is the predominant mechanism which determines pulmonary hypertension in patients with portal hypertension<sup>[4]</sup>, different drugs have been used in the treatment of patients with PPHTN.

The drugs that have been mostly used for the treatment of patients with PPHTN are the prostacyclin analogues, such as Epoprostenol, inhaled Iloprost and Treprostinol. Epoprostenol is a potent pulmonary and systemic vasodilator, that also reduces platelet aggregation<sup>[16-18]</sup>. In PPHTN it has been shown to improve hemodynamics acutely, because of its vasodilator effects<sup>[15]</sup>. Furthermore, in two studies prolonged use of the drug has shown additional improvement<sup>[5,15]</sup>. A recent paper by Awdish *et al.*<sup>[19]</sup> reports the long-term effects of treatment with and without epoprostenol on pulmonary hemodynamics, liver function and survival, in a large retrospective cohort of patients with moderate to severe PPHTN. They showed significant improvements in mean pulmonary artery pressure, pulmonary vascular resistances and cardiac output with epoprostenol after a median of 15.4 mo, and no significant change of liver biochemistry. However, survival seemed not to differ between treatment groups. These studies argue against the hypothesis by Krowka

that epoprostenol might worsen portal hypertension by increasing splenic blood flow and portal system congestion<sup>[15]</sup>. In fact, it is possible to hypothesize that by improving pulmonary hemodynamics and right heart function, epoprostenol could potentially improve liver function<sup>[20]</sup>.

A recent prospective observation study has stressed the utility of early initiation of parenteral prostacyclin therapy in PPHTN patients, so improving 5-year survival, as compared with data of the REVEAL Registry<sup>[20]</sup>.

There are scarce studies concerning the treatment with Treprostinil<sup>[21]</sup>, and inhaled Iloprost<sup>[22]</sup>, sometimes used together with other vasoactive drugs<sup>[23,24]</sup> in patients with PPHTN.

Concerning the endothelin receptor antagonists, such as Bosentan, there are some recent studies that seem to demonstrate a possible hemodynamic improvement and safety of these drugs<sup>[23,25-29]</sup>. Moreover, Bosentan has been administered together with different drugs in some studies: inhaled Iloprost<sup>[23,24,29]</sup>, or with Sildenafil and Iloprost<sup>[24]</sup>.

Phosphodiesterase inhibitor Sildenafil has the advantage of being an oral compound with pulmonary vasoselective action but no hepatotoxicity<sup>[27]</sup>. Few recent retrospective studies, performed in a small number of patients with PPHTN<sup>[30-33]</sup>, seem to show that sildenafil might be effective in monotherapy, and in combination with inhaled prostanoids, leading to hemodynamic improvement. These data have also been confirmed by a recent paper by Krowka<sup>[34]</sup> reporting favourable follow up in seven patients with moderate or severe PPHTN treated with oral combination therapy.

## LIVER TRANSPLANTATION FOLLOWING MEDICAL MANAGEMENT

PPHTN survival in the absence of transplantation has been reported in 38% at 3 years<sup>[1]</sup> and 28% at 5 years<sup>[35]</sup>. Furthermore, it has been demonstrated that PPHTN prognosis is worse than that of idiopathic pulmonary hypertension<sup>[1]</sup>.

PPHTN is a serious problem in the context of liver transplantation. Mild PPHTN (MPAP  $< 35$  mmHg) has scarce perioperative risks<sup>[36,37]</sup>, but moderate disease (MPAP 35-45 mmHg, PVR  $> 250$  dyne scm<sup>-5</sup>) has been associated with a perioperative mortality of 50%-80%<sup>[8,38]</sup>, and a MPAP  $> 50$  mmHg is universally fatal<sup>[14,36,37]</sup>.

Recently numerous studies have demonstrated the possibility of reducing MPAP and PVR, at least in some patients affected by PPHTN, leading to a decrease of perioperative risk due to liver transplantation. Moreover, some studies have shown that PPHTN may resolve following transplantation, presumably by removing the root cause of the problem<sup>[39-41]</sup>.

As a consequence of recent improved knowledge of PPHTN management, a potential therapeutic opportunity arises: PPHTN might initially be controlled with vasodilator therapy and subsequently cured with liver transplantation. Several studies have been performed to examine the feasibility of this hypothesis<sup>[31-42]</sup>. Moreover,



in the last years some retrospective studies have been published reporting patients with PPHTN in whom liver transplantation was successfully performed after treatment with pulmonary vasoactive drugs<sup>[22,43,44]</sup>. In the study by Ashfaq *et al.*<sup>[43]</sup>, 16 of 20 patients with moderate-to-severe pulmonary hypertension (MPAP  $\geq$  35 mmHg) were otherwise considered suitable liver transplant candidates and were treated with vasoactive pulmonary drugs (epoprostenol in 13, bosentan + epoprostenol in 1, sequential; bosentan + diltiazem + epoprostenol, sequential in 1, diltiazem in 1). In these patients MPAP fell to less than 35 mmHg in 12 (75%), and 11 of them underwent liver transplantation. One-year survival was 91%, and 5-year survival was 67%. Nine of 11 patients were off vasodilator therapy after a median of 9.2 mo after transplantation. In patients who failed vasodilator therapy median survival was 8 mo. The Authors conclude that effective pharmacologic control of PPHTN before transplantation is associated with posttransplant survival that is similar to patients transplanted for other indications.

In the study by Sussman *et al.*<sup>[44]</sup>, 8 cirrhotic patients with MPAP  $\geq$  35 mmHg, were treated with continuous intravenous epoprostenol (2-8 ng/kg per minute). In these patients liver transplant was considered if MPAP was lowered to < 35 mmHg. In seven patients the treatment improved hemodynamics within 6.5 mo therapy: mean vascular resistances declined from 410 to 192 dyne s cm<sup>-5</sup>, and cardiac output increased from 6.6 to 10 L/min. Six of the seven responders were listed for transplantation; two died on the waiting list; four were transplanted and remained alive and well after 9 to 18 mo post LT. Epoprostenol was continued throughout surgery and into the post-transplant period. In two of the patients it was possible to stop the vasodilator therapy, whereas in two patients oral medication with bosentan was continued.

In the study by Swanson *et al.*<sup>[41]</sup>, a retrospective screening-right heart catheterization-survival analysis of patients with PPHTN was performed. Patients were categorized in three subgroups: (1) no vasoactive therapy and no transplantation; (2) therapy for pulmonary hypertension alone; and (3) therapy for pulmonary hypertension followed by liver transplantation. Even though it is a relatively small study, it seems to demonstrate that the survival of untreated patients was poor, whereas subgroups of patients selected to medical treatment with or without liver transplantation had better long-term survival. The best survival was in the subgroup of patients in whom liver transplantation was performed following effective medical therapy for pulmonary hypertension. However, it is to stress that this study is retrospective and reports a small number of patients. Moreover, 4 of 5 deaths in the LT group occurred in patients with PAOP  $\leq$  10 mmHg, while only two out of seven LT survivors had PAOP  $\leq$  10 mmHg. The authors hypothesize that lower values of PAOP may be correlated to lower cardiac outputs, and this might contribute to the poor prognosis. In the assessment of patients with PPHTN that are candidate to liver transplantation it is very important to perform an accurate evaluation of right ventricular function, because

the success of undertaking liver transplantation will be determined by the ability of the right ventricle to sustain the increase of cardiac output and of pulmonary vascular resistance that acutely occur at the time of reperfusion that may cause acute right heart failure<sup>[45]</sup>. Therefore, pre-transplant evaluation of right ventricular function by means of right heart catheterization and stress echocardiography is essential. Continuous intraoperative transoesophageal echocardiography has also been recommended for following right ventricular function<sup>[46]</sup>.

## MODEL FOR END-STAGE LIVER DISEASE AND PPHTN

Results of recent studies concerning LT following medical treatment in patients with PPHTN, as discussed above, probably change the clinical scenarios of patients with moderate or even severe pulmonary hypertension and portal hypertension.

Outcome of these patients, traditionally excluded from LT, could benefit from LT if a response to vasoactive therapy is evident. In fact, there is increasing evidence that many patients with PPHTN have important decrease of MPAP and PVR after therapy, so that they have excellent survival following liver transplantation. Furthermore, in some of these patients, vasoactive therapy may be stopped a few months after LT. Moreover, there is not any doubt that many patients who positively respond to medical therapy die on the waiting list for transplantation. Finally, another reason could support early transplant of these patients. In fact, long-term intravenous epoprostenol, probably the most effective drug, is expensive, labor-intensive, requires hospitalization and is difficult to tolerate. These are the reasons why in some studies patients with PPHTN on intravenous therapy are given a MELD exception of 25 points in Region 4<sup>[43]</sup>.

In a recent paper, Krowka *et al.*<sup>[47]</sup> discuss this topic suggesting MELD exception (Meld score = 26 points) when the acceptable candidates satisfy the following criteria: (1) POPH exists with severity characterized by MPAP > 35 mmHg; and (2) a minimum of 12 wk of United States Food and Drug Administration-approved pulmonary arterial hypertension therapy results in the following hemodynamic profile: (1) MPAP < 35 mmHg and PVR < 400 dynes scm<sup>-5</sup>; and (2) satisfactory right ventricular function exists.

## LIVER AND LUNG TRANSPLANTATION

Combined lung and liver transplantation is a therapeutic option for selected patients with coexisting lung and liver diseases such as cystic fibrosis and  $\alpha$ 1-proteinase inhibitor deficiency, and has also been performed in few cases of patients with PPHTN<sup>[48-53]</sup>. Recently, a report concerning 13 consecutive patients who underwent combined lung and liver transplantation has been published. In the whole cohort of patients, 5 with PPHTN and 8 with other severe hepatic and pulmonary diseases (sarcoidosis, cystic

fibrosis, alfa 1-proteinase inhibitor deficiency), patient and graft survival rates, after combined transplantation, were 69% after 1, 62% after 3, and 49% after 5 years<sup>[54]</sup>. However, further studies are needed to confirm the efficacy, as well as the indications of this surgical approach in the management of patients with PPHN.

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## Endoscopic papillary balloon dilation: Revival of the old technique

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### Abstract

Radiologists first described the removal of bile duct stones using balloon dilation in the early 1980s. Recently, there has been renewed interest in endoscopic balloon dilation with a small balloon to avoid the complications of endoscopic sphincterotomy (EST) in young patients undergoing laparoscopic cholecystectomy. However, there is a disparity in using endoscopic balloon papillary dilation (EPBD) between the East and the West, depending on the origin of the studies. In the early 2000s, EST followed by endoscopic balloon dilation with a large balloon was introduced to treat large or difficult biliary stones. Endoscopic balloon dilation with a large balloon has generally been recognized as an effective and safe method, unlike EPBD. However, fatal complications have occurred in patients with endoscopic papillary large balloon dilation (EPLBD). The safety of endoscopic balloon dilation is still a debatable issue. Moreover, guidelines of indications and tech-

niques have not been established in performing endoscopic balloon dilation with a small balloon or a large balloon. In this article, we discuss the issue of conventional and large balloon endoscopic dilation. We also suggest the indications and optimal techniques of EPBD and EPLBD.

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**Key words:** Endoscopic papillary balloon dilation; Endoscopic papillary large balloon dilation; Common bile duct stone; Endoscopic sphincterotomy; Mechanical lithotripsy

**Core tip:** Endoscopic papillary dilation with a dilating balloon is technically simple and effective. However, there is still debate regarding safety, and there is no guideline or consensus of detailed techniques. Because the procedure is performed to treat a common benign condition, it is important to ensure that there are no lethal procedure-related complications. It, however, can lead to potential morbidity and even death. As the foremost priority is patient safety, it should be performed with appropriate techniques in selected patients. Therefore, we suggest the optimal indications and tips for avoiding severe complications of endoscopic papillary balloon dilation with a small balloon or a large balloon.

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### INTRODUCTION

A common bile duct (CBD) stone is one of the most



common indications of endoscopic retrograde cholangiopancreatography (ERCP). In 1974, endoscopic sphincterotomy (EST) was introduced to remove CBD stones<sup>[1]</sup>. It has since become established as the standard treatment for widening the biliary orifice. Although the success rate of ERCP with EST is more than 90%, EST accounts for a major portion of the morbidity and mortality associated with ERCP<sup>[2,3]</sup>.

To avoid complications of EST, endoscopic papillary balloon dilation with a small balloon (EPBD) was introduced as an alternative to EST. Before the development of EPBD, interventional radiologists originally introduced the transpapillary elimination of CBD stones through dilation of the sphincter with a 6 mm balloon in 1981<sup>[4]</sup>. In 1983, Staritz *et al*<sup>[5]</sup> applied this technique to endoscopy during an ERCP procedure. However, EPBD had not been routinely used for the removal of CBD stones in those days because of frequent complications, mainly acute pancreatitis (in patients with sphincter of Oddi dysfunction)<sup>[6]</sup>. Nevertheless, there was renewed interest in EPBD to preserve the function of the biliary sphincter.

As time passed on, various studies reported on the safety, effectiveness, and advantages of EPBD in the East. In contrast, Western studies showed more frequent lethal complications of EPBD compared with EST<sup>[7,8]</sup>. This disparity has led to the different current practices between East and West. Balloon dilation of the intact papilla is rarely used in most Western countries whereas this technique is popularly used in Eastern countries.

Recently, EST followed by endoscopic papillary balloon dilation with a large balloon (EPLBD) was introduced<sup>[9]</sup>. This review discusses conventional EPBD and EPLBD separately, because the concept, potential advantage, indication, and main purpose of EPBD may differ from those of EPLBD, which utilizes a larger balloon. EPBD may be technically simple and easy to use, but there is still debate regarding safety. The aim of this review is to address the concept, outcomes, safety, techniques and advantages of EPBD and EPLBD. In addition, we suggest indications and technical tips for EPBD and EPLBD individually.

## DEFINITIONS AND CONCEPTS

EPBD involves the dilation of the biliary sphincter with a dilating balloon, and is usually performed without EST by using a small-diameter dilating balloon ( $\leq 10$  mm) (Figure 1). The potential advantages of the EPBD over EST are to avoid short-term complications of bleeding and perforation, to preserve the biliary sphincter, and possibly to reduce long-term sequelae of EST<sup>[8,10,11]</sup>. EPLBD is usually defined as the use of a dilating balloon with a diameter of 12 mm or larger in order to remove large stones that require a larger opening of the CBD<sup>[12,13]</sup>. The potential advantages of EPLBD are to reduce the use of mechanical lithotripsy (ML) and to reduce the complications related to full EST in removing large or difficult

CBD stones<sup>[14]</sup>.

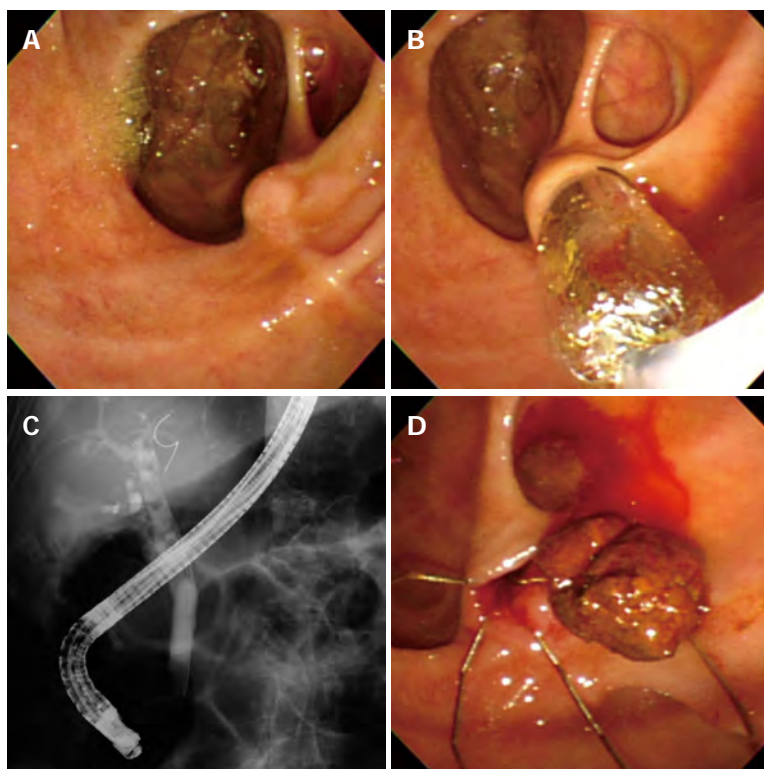
In EPLBD, EST is generally recommended before balloon dilation, because the preceding EST may shift the expansile force toward the CBD rather than the pancreatic orifice. When this combined approach is used, a large endoscopic sphincterotomy is not required. As a result, EPLBD can enlarge the biliary orifice to a greater extent than a standard full EST and create a large biliary orifice (Figure 2). EPLBD may have the advantages of a lower risk of bleeding and perforation over a routine full EST<sup>[14]</sup>. Although EST is generally used at the start of the EPLBD procedure, the safety of large balloon dilation alone without a preceding EST is reported in some studies<sup>[15,16]</sup>. In contrast to EPLBD, the biliary orifice after EPBD is usually less wide than after a full EST. The target stones of EPBD are small- to moderate-sized in minimally dilated CBDs, whereas those of EPLBD are large stones in considerably dilated CBDs (Table 1).

## OUTCOMES

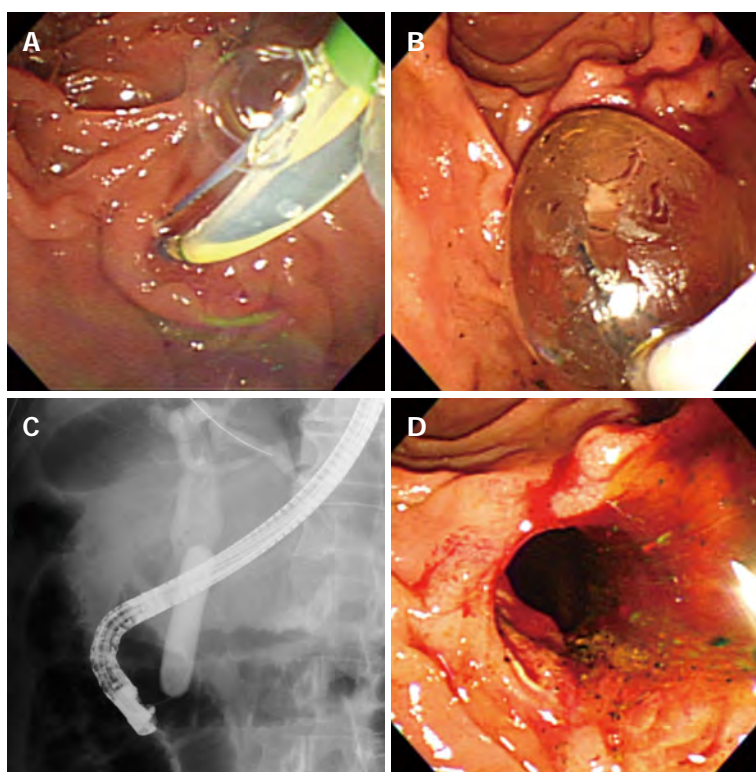
### *Outcome of EPBD compared with that of EST for extraction of bile duct stones*

In a Japanese randomized controlled trial (RCT), EPBD and EST had similar outcomes in the successful removal of bile duct stones (99.3% *vs* 100%) and overall complications (14.5% *vs* 11.8%)<sup>[2]</sup>. In contrast, RCTs from Western countries did not show the same results. In a German RCT, EPBD was inferior to EST in terms of stone removal during the first attempt (77% *vs* 100%)<sup>[7]</sup>. The overall complication rate of EPBD was also higher than that of EST (30.0% *vs* 5.0%). Although the bleeding rate was lower in the EPBD group, cholangitis and pancreatitis developed more frequently than in the EST group. Severe pancreatitis with pancreatic necrosis occurred only in the EPBD group (6.7%). This study was terminated early due to this complication in the EPBD group. Another well-known RCT from the United States reported 2 deaths due to severe pancreatitis developing after EPBD<sup>[8]</sup>. This study was also terminated at the first interim analysis.

Two meta-analyses evaluating the outcome of EPBD compared with EST are available by Baron *et al*<sup>[3]</sup> (8 studies analyzed) and Weinberg *et al*<sup>[17]</sup> (15 studies analyzed). Baron *et al*<sup>[3]</sup> showed that EST and EPBD had comparable overall success rates of stone removal (94.3% *vs* 96.5%). However, in the first attempt without EST, the initial success rate of stone removal was lower in the EPBD group than in the EST group (70.0% *vs* 79.8%). Furthermore, the use of ML was also more prevalent in the EPBD group than in the EST group (20.9% *vs* 14.8%). Overall complication rates were similar in both the EPBD and EST groups (10.5% *vs* 10.3%). However, the rate of pancreatitis was significantly higher in the EPBD group than in the EST group (7.4% *vs* 4.3%) while the rate of bleeding was lower in the EPBD group than in the EST group (0% *vs* 2.0%). Rates of cholangitis and perforation were similar in both groups.



**Figure 1** Endoscopic papillary balloon dilation with a small dilating balloon. A: Huge periampullary diverticulos were noted near the ampulla; B: The 8 mm sized small balloon is gradually inflated with diluted contrast material; inflation is maintained for 30 s; C: Fluoroscopy during balloon dilation shows complete disappearance of the sphincter waist; D: A common bile duct stone was removed by basket through the enlarged biliary orifice.



**Figure 2** Endoscopic papillary large balloon dilation with minor sphincterotomy. A: A minor incision of up to one-third of the papilla was performed over a guidewire; B: The 15 mm sized large balloon is gradually inflated with diluted contrast material; inflation is maintained for 30 s; C: Fluoroscopy during balloon dilation shows complete disappearance of the sphincter waist; D: A large biliary orifice can be seen after balloon dilation.

Weinberg *et al*<sup>[17]</sup> reported that EPBD was statistically less successful for extraction of the stone at the first attempt than EST (73.5% *vs* 80.9%), and the overall success rate of EPBD was slightly lower than that of EST (90.1% *vs* 95.3%). There was no significant difference in short-term complications between the EPBD and EST groups (12.1% *vs* 12.7%). Incidences of bleeding and short-term

infections were significantly lower in the EPBD group than in the EST group. The incidence of perforation was not different between the 2 groups. While many of the complications were similar or lower in the EPBD group than in the EST group, this meta-analysis showed that the incidence of pancreatitis was significantly higher in the EPBD group than in the EST group (8.6% *vs* 4.3%).

**Table 1 Comparison of endoscopic balloon dilation methods according to balloon diameter**

	Small-balloon EPBD	Large-balloon EPBD
Balloon diameter used	≤ 10 mm (6–10 mm)	≥ 12 mm (12–20 mm)
Target stone	Small to moderate sized stones in no or minimally dilated CBD	Large stones in considerably dilated CBD
Endoscopic biliary sphincterotomy	Not performed	Mostly, in conjunction with a small EST <sup>1</sup>

<sup>1</sup>Preceding small-endoscopic biliary sphincterotomy (EST) use may shift the expansile force more toward the common bile duct (CBD) rather than the pancreatic orifice. EPBD: Endoscopic papillary balloon dilation.

Furthermore, in the subgroup analysis, the risk of pancreatitis was higher in younger patients of the EPBD group. These meta-analyses consistently showed that, while EPBD and EST had similar overall success rates for stone removal, acute pancreatitis occurred more frequently in the EPBD group than in the EST group.

### Outcomes of EPLBD

**EPLBD with EST:** In the first introduction of EPLBD by Ersoz *et al.*<sup>[9]</sup>, stone clearance was successful without using ML in patients with large stones (> 15 mm). After this retrospective study, many studies showed that EPLBD could be a useful alternative technique in patients with large CBD stones that were difficult to remove with standard EST.

Recent studies showed that complete stone removal in the first session of EPLBD was accomplished in 89.3% of procedures, and ML was required in 9.5% of patients (including 6 retrospective and 1 prospective trials involving 496 patients)<sup>[14,18–23]</sup>. Overall success of stone removal was 97.6%. Only 8.5% of patients had documented complications, such as bleeding and perforation. Pancreatitis developed in 1.6% of patients. Severe pancreatitis was not reported, contrary to that seen with EPBD.

In a retrospective comparison of EPLBD (with EST) and EST alone (2 studies involving 250 patients)<sup>[24,25]</sup>, EPLBD showed similar outcomes to EST in overall successful stone removal (98.4% *vs* 96.0%) and complications (4% *vs* 6%). However, ML was required significantly more often in the EST group than in the EPLBD group (21.6% *vs* 7.2%). Total procedure time was shorter in the EPLBD group due to less use of ML (13 min *vs* 22 min)<sup>[24]</sup>. Moreover, EPLBD was an effective and safe method for removal of CBD stones in patients with Billroth II gastrectomy, prior biliary sphincterotomy, and periampullary diverticulum<sup>[14,26,27]</sup>.

In prospective randomized comparison studies (2 studies involving 255 patients)<sup>[28,29]</sup>, EPLBD showed similar outcomes to EST for overall success rate of bile duct stones (97.0% *vs* 98.0%) and stone removal at the first attempt (83.5% *vs* 85.9%). Overall use of ML was not different in the EPLBD group and in the EST group (13.4% *vs* 14.1%). The prevalence of overall complications was similar in the EPLBD and EST groups (7.1%

*vs* 7.0%). Moreover, no significant difference was seen in the frequency of pancreatitis, perforation, hemorrhage or cholangitis. In another prospective randomized comparison of EPLBD with EST and ML with EST (involving 90 patients), EPLBD had a similar success rate of stone removal as ML with EST (97.7% *vs* 91.1%), whereas the complication rate was lower in the EPLBD group than in the ML with EST group (4.4% *vs* 20.0%)<sup>[30]</sup>. Cholangitis was less frequent in the EPLBD group than in the ML with EST group (0% *vs* 13.3%). Rates of pancreatitis were similar between the 2 groups.

**EPLBD without a preceding EST:** Although EST is generally used at the start of the EPLBD procedure, only large balloon dilation without a preceding EST is performed for removal of large CBD stones in some studies. In a retrospective study, the overall success rate of EPLBD without a preceding EST was 97.4%, and complete duct clearance with EPLBD alone was performed in 76.3% of patients<sup>[16]</sup>. ML was used in 21.1% of patients. Procedure-related pancreatitis was observed in only one patient (2.6%) and there were no other complications, such as bleeding, perforation, or cholangitis. This study suggested that EPLBD without a preceding EST might be as simple, effective, and safe in patients with large bile duct stones, as EPLBD with a preceding EST. However, the study had a limitation in that there was no comparison of EPLBD without a preceding EST and EPLBD with a preceding EST. Therefore, EPLBD without a preceding EST was not regarded as a routine technique for the removal of large bile duct stones, though it could be an alternative treatment.

## POTENTIAL ADVANTAGES OF EPBD

### Avoidance of bleeding

An important advantage of EPBD over EST is the avoidance of sphincterotomy-induced bleeding. Patients with coagulopathy and those who take anticoagulation medication have a higher risk of EST-induced bleeding<sup>[31,32]</sup>. Several RCTs showed that EPBD might significantly reduce the risk of bleeding compared with EST<sup>[2,8,33]</sup>. In a comparison of bleeding risk in patients with liver cirrhosis and coagulopathy, it was reported that the rate of EST-related hemorrhaging was 30%, whereas the hemorrhagic rate of EPBD was 0%<sup>[34]</sup>. Moreover, a meta-analysis commented that the occurrence of major bleeding was significantly lower in patients treated with EPBD than those treated with EST<sup>[17]</sup>. EPBD is currently regarded as an alternative method to EST in patients with coagulopathy to avoid sphincterotomy-induced bleeding.

### EPLBD

The rate of bleeding after EPLBD was reported as various rates, approximately 0%–8.3%<sup>[23,29,35]</sup>. According to a recent report, severe bleeding occurred less frequently in patients with EPLBD than with EST, though minor bleeding and bleeding in patients with coagulopathy were



excluded<sup>[36]</sup>. However, the rate of bleeding was not significantly different between EPLBD and EST in comparison studies<sup>[28,30]</sup>. Moreover, several reports mentioned that serious massive bleeding had occurred after EPLBD<sup>[37,38]</sup>. Severe bleeding may be caused by the large balloon, and it may lead to surgical intervention or even mortality. These results suggest that EPLBD is not superior to EST with regard to ERCP-related bleeding, unlike EPBD.

### **Preservation of sphincter of Oddi function**

Until now, EST has been widely accepted as an effective and standard technique for the removal of CBD stones; however, EST causes permanent loss of sphincter of Oddi (SO) function. Pneumobilia and duodenal biliary reflux were observed in approximately 50% of patients after EST and almost 100% of patients developed bactericholia and chronic inflammation of the biliary system<sup>[39,40]</sup>. Because laparoscopic cholecystectomy (LC) has been widely performed, preservation of the SO function is needed to avoid complications in young patients undergoing LC<sup>[41]</sup>.

Since EST disrupts the SO function for a long period of time, it is hoped that EPBD reduces damage to SO function compared with EST. Based on an anatomic study in pigs, EPBD showed no rupture of SO smooth muscle, and it was expected to preserve papillary smooth muscle integrity in humans<sup>[42]</sup>. In a manometric study of the SO function<sup>[43]</sup>, EPBD seemed to depress SO function for at least 1 wk. However, 1 mo after EPBD, SO peak pressure and frequency of SO contraction increased significantly, and SO basal and CBD pressure tended to increase compared with the first week's values. These results suggested at least partial recovery of SO function in 1 month after EPBD. In another manometric comparison study of SO function between EPBD with EST, SO basal and peak pressures partially recovered at 1 year, although these values still remained lower than those before EPBD<sup>[44]</sup>. The risk of long-term complications and pneumobilia were also lower in the EPBD group than in the EST group. This study suggested that SO function was preserved to a greater degree than after EST. However, there were studies with different results of preservation of the SO function after EST or EPBD.

In a comparison study, SO function was estimated by measurement of pancreatic enzyme activity in bile aspirated from the CBD<sup>[45]</sup>. According to this study, there were no significant differences in pancreatic enzyme levels from before the procedure *vs* 1 year after the procedure in both EPBD and EST groups. In another prospective study, bacterial cultures of bile were used to evaluate bacterial contamination of the biliary tract after EPBD or EST<sup>[46]</sup>. There was no significant difference in the bacterial cultures at 6 mo or 2 years after the procedures between the EPBD and EST groups. As a result, it is not clear whether the preservation of SO function with EPBD was superior to that of EST, although several studies showed that damaged SO function after EPBD was substantially recovered over time.

Although the preservation of SO function is in-

complete, EPBD is still an attractive method, especially in younger patients, to avoid long-term complications. However, young age is an important risk factor for acute pancreatitis, and acute pancreatitis is more frequent after EPBD. Furthermore, a meta-analysis showed that the pancreatitis risk was higher in younger patients than in older patients in the EPBD group<sup>[17]</sup>. Although EPBD was performed to preserve SO function in younger patients, it is ironic that post-EPBD pancreatitis was more evident in the younger patients.

### **EPLBD**

The preservation of SO function after EPLBD is not clear. Because the acquirement of the large CBD opening after ballooning was the aim of EPLBD, preservation of SO function was not regarded as an important factor in EPLBD. Theoretically, SO function is permanently ablated after EPLBD. From our experience, it is found that SO function does not recover after EPLBD regardless of EST.

## **POTENTIAL ADVANTAGES OF EPLBD**

### **ML**

ML has been commonly used for the management of large CBD stones. EPLBD was developed to reduce the complications related to full EST and to avoid the use of ML for removal of large bile duct stones. In a prospective study of 60 patients, only 3 patients (5%) required adjuvant ML for stone extraction after EPLBD<sup>[23]</sup>. In another RCT, ML was required significantly more often in the EST group than in EPLBD group (25% *vs* 6%)<sup>[24]</sup>. Contrary to previous reports, EPLBD compared with EST alone resulted in similar outcomes in terms of overall successful large CBD stone removal (94.4% *vs* 96.7%) and the use of ML (8.0% *vs* 9.0%) in another RCT<sup>[28]</sup>. Furthermore, there was no difference in the use of ML for large-sized CBD stones in a recent meta-analysis<sup>[47]</sup>, although the overall rate of ML use for various sized stones was less frequent in the EPLBD group than in the EST group. A few discrepancies in the use of ML for removal of large CBD stones have been seen, although many studies report that ML has been used less often in the EPLBD group compared with the EST group. Because the outcomes of the use of ML were not consistent, the choice of EPLBD only to reduce the use of ML in the removal of large CBD stones should be carefully considered.

### **EPBD**

A reduction in the use of ML is not the main purpose of EPBD, unlike EPLBD. Most studies, including 2 meta-analyses, reported that the use of ML was more prevalent in EPBD groups than in EST groups<sup>[3,17,48]</sup>.

## **SAFETY ISSUES**

EPBD and EPLBD are technically simple and effective, but safety is still a debatable issue. As the procedures are performed to treat a common benign condition, it is important to ensure that there are no lethal procedure-



Table 2 Techniques and outcomes of small balloon-endoscopic papillary balloon dilation in randomized controlled trials

Ref.	Patients (n)	Balloon diameter (mm)	Maximum pressure of inflation (atm)	Time of inflation (s)	duration of maximal dilation (s)	Number of ballooning	Overall success rate	Post-EPBD pancreatitis	Bleeding	Perforation	Infection	Death (n)
Arnold <i>et al</i> <sup>[7]</sup>	30	8	10	60		2	77%	20%	0%	0%	10%	0
Bergman <i>et al</i> <sup>[49]</sup>	101	8	Waist	60-120	45-60	1	89%	7%	0%	2%	4%	1 for perforation 2 for pancreatitis
DiSario <i>et al</i> <sup>[6]</sup>	117	8 or less		60		1	97.4%	15.4%	10.5%	0%	1%	
Fujita <i>et al</i> <sup>[2]</sup>	138	4-8	Waist	180	15	1	99%	10.8%	0%	0%	2.9%	0
Lin <i>et al</i> <sup>[53]</sup>	51	8-12	8-12		120/300	1	94.1%	0%	2%	0%	0%	0
Natsui <i>et al</i> <sup>[38]</sup>	41	8	3		120	1	93%	5%	0%	0%	2%	0
Ochi <i>et al</i> <sup>[11]</sup>	51	8	60-80 mmHg	60		3	93%	0%	0%	0%	2%	0
Tanaka <i>et al</i> <sup>[59]</sup>	16	8	8	120		1	100%	19%	0%	0%	0%	0
Ylavianos <i>et al</i> <sup>[60]</sup>	103	10	12	30 or more		Repeated until satisfaction	87.4%	4.8%	0%	0%	1.9%	0
Yasuda <i>et al</i> <sup>[44]</sup>	35	8	6		60	2	100%	5.7%	0%	0%	0%	0

EPBD: Endoscopic papillary balloon dilation

related complications. Although adequate procedural techniques may reduce complications, optimal techniques do not always prevent all complications.

Acute pancreatitis is the most common severe complication of EPBD. In a United States RCT, 2 patients with post-EPBD pancreatitis died<sup>[6]</sup>. Another study reported that one patient died of retroperitoneal perforation after EPBD<sup>[49]</sup>, although perforations are usually rare in EPBD. EPLBD has been regarded as a safe and effective method, regardless of a preceding EST. However, massive bleeding and perforation were occasionally reported in some studies. Life-threatening hemorrhage following EPLBD with a preceding EST was reported, and it was treated with angiographic embolization<sup>[57]</sup>. Four patients died due to EPLBD-related complications in a Korean and Japanese multicenter study<sup>[50]</sup>. Of these 4 patients, 3 died as a result of perforation, and the other died due to delayed massive bleeding. Perforation was a more frequent severe complication of EPLBD, although some patients died due to both bleeding and perforation.

For EPBD, serious pancreatitis has been reported in several studies, although it was showed that discrepancy of complications between East and West. Therefore, the choice of EPBD (in the young patients with CBD stone) only to preserve SO function should be carefully considered. The reason is that the long-term effect from the preservation of SO functions has not proven, until now. For EPLBD, although EPLBD is reported as an effective method in many studies, several reports showed procedure-related deaths due to perforation and delayed bleeding. Therefore, the choice of EPLBD only to reduce the use of ML should be carefully considered. In terms of safety issue, to avoid serious complications, strict selection of patients is of utmost importance in both EPBD and EPLBD.

## TECHNICAL ISSUES

### EPBD

It is not clear why small balloon EPBD has been shown to have a high risk compared with EST in the United States while it is relatively safe in South Korea and Japan. A recent study showed that post-EPBD pancreatitis was more frequent in an EPBD group than in a group with percutaneous transhepatic papillary balloon dilation<sup>[25]</sup>. This suggests that post-EPBD pancreatitis may be associated with detailed procedure protocols, rather than balloon dilation itself. Differences in the detailed methodology might lead to differences in outcome, although there are several possible reasons for the differences in outcome. The EPBD techniques in various RCTs are summarized along with procedure-related complications in Table 2. Technical factors that may be related to the outcomes include balloon diameter, dilation pressure, and dilation time.

Among these technical factors, selection of the optimal balloon diameter is important. Generally, a balloon smaller than the diameter of the CBD is recommended to reduce the damage to the SO and pancreatic orifice<sup>[51]</sup>. If a large CBD stone exceeding the diameter of the papillary orifice remains after EPBD, additional treatment, such as

Table 3 Techniques and outcomes of endoscopic papillary large balloon dilation in various studies

Ref.	Patients (n)	Extent of EST	Balloon diameter (mm)	duration of balloon dilation (s)	Overall success rate	Use of ML	Post-EPBD pancreatitis	Bleeding	Perforation	Infection	Death (n)
Ersoz <i>et al</i> <sup>[6]</sup>	58	Full	12-20	20-45	100%	6.9%	3%	9%	0%	3%	0
Bang <i>et al</i> <sup>[21]</sup>	22	small	10-15	40	100%	9.1%	4.5%	0%	0%	0%	0
Heo <i>et al</i> <sup>[28]</sup>	100	small	12-20	60	97%	8%	4%	0%	0%	1%	0
Garcia-Cano <i>et al</i> <sup>[33]</sup>	30	Variable	10-18	60	94.5%	0%	10%	10%	0%	3.3%	0
Stefanidis <i>et al</i> <sup>[30]</sup>	44	full	15-20	10-12	97.7%	0%	2.2%	2.2%	0%	0%	0
Attasaryya <i>et al</i> <sup>[20]</sup>	103	Full	12-18	60	95%	27.2%	0%	2%	1%	0%	0
Kochhar <i>et al</i> <sup>[18]</sup>	74	small	10-18	60	91.9%	2.7%	2.7%	8.1%	0%	0%	0
Lee <i>et al</i> <sup>[14]</sup>	55	small	15-20	30-60	100%	5.5%	0%	3.6%	0%	0%	0
Misra <i>et al</i> <sup>[22]</sup>	50	Full	15-20	30-45	100%	10%	8%	6%	0%	0%	0
Minami <i>et al</i> <sup>[61]</sup>	88	small	20	30	98.9%	1%	1%	1%	0%	1%	0
Maydeo <i>et al</i> <sup>[23]</sup>	60	Full	12-15	30	100%	5%	0%	8.3%	0%	0%	0
Itoi <i>et al</i> <sup>[24]</sup>	53	Full	15-20	15-30	100%	5.6%	1.9%	0%	0%	1.9%	0
Park <i>et al</i> <sup>[30]</sup>	946	Variable	12-20	30-180	96.9%	10.0%	2.5%	5.9%	0.9%	0.6%	4 (1 for bleeding, 3 for perforation)
Jeong <i>et al</i> <sup>[6]</sup>	38	Without EST	15-18	10-60	97.4%	21.1%	2.6%	0%	0%	0%	0

ML: Mechanical lithotripsy; EPBD: Endoscopic papillary balloon dilation; EST: Endoscopic biliary sphincterotomy.

EST or ML, is often needed.

Other techniques, such as balloon inflation and duration of balloon dilation, were also analyzed in several studies. Techniques of balloon inflation were divided into 2 categories in a recent study<sup>[52]</sup>. In the ungraded inflation method, the balloon was gradually inflated to the target pressure during a fixed time (approximately 30-60 s). In the graded inflation method, the balloon was slowly inflated until the disappearance of the balloon's waist, and then the pressure was maintained for 15 s. In the graded inflation group, the incidence of post-EPBD pancreatitis was significantly lower than in the ungraded inflation group. The result suggested that lower pressure and shorter duration was less traumatic to the papilla, resulting in fewer complications.

Until recently, the optimal duration of balloon dilation had not been established. In most studies and during actual practice, the dilation of EPBD was performed for a short duration of 1 min or less. However, some studies examined a longer duration of balloon dilation and the results showed adequate outcomes with no post-EPBD pancreatitis<sup>[43,53]</sup>. A 5-min EPBD improved the efficacy of stone removal and reduced the risk of post-EPBD pancreatitis, compared with a 1-min EPBD<sup>[54]</sup>. In addition, the duration of EPBD was inversely associated with pancreatitis risk in a meta-analysis, with less than 1-min dilations actually increasing acute pancreatitis<sup>[55]</sup>. Another 2 studies explained that a long duration of balloon dilation served to loosen the SO sufficiently and to resolve compartment syndrome, which involved intramucosal hemorrhaging and edema at the papilla. They suggested that an inadequately loosened SO surrounding the common channel may cause a compartment phenomenon that compresses pancreatic flow and increases the risk of post-EPBD pancreatitis<sup>[3,42]</sup>. Therefore, a long duration (5 min) of EPBD might be preferred over a short duration (less than 1 min) to reduce the risk of post-EPBD pancreatitis.

EPLBD

There are not many analyses of EPLBD technique, although EPLBD has been accepted to be an effective and safe method for large CBD stone removal. The EPLBD techniques of several studies are summarized with procedure-related complications in Table 3. In contrast to EPBD, the important complications of EPLBD are not post-EPBD pancreatitis, but perforation and bleeding. Therefore, the techniques of concern are different from that of EPBD.

Regarding the techniques related to EPLBD, the extent of EST, diameter of the balloon, and the method of balloon inflation are considered the most important. The size

**Table 4** Indications for endoscopic balloon dilation according to balloon diameter

	Small-balloon EPBD	Large-balloon EPBD
Absolute indication	Patients with coagulopathy and need for anticoagulation to avoid sphincterotomy-induced bleeding	No indication
Relative indication	Patients with anatomical abnormalities including gastric bypass surgery (Billroth II gastrectomy) or perampullary diverticulum	Patients with altered anatomy, such as gastric bypass surgery (Billroth II gastrectomy), perampullary diverticulum and prior biliary sphincterotomy
Possible indication	To preserve SO functions	To reduce the use of ML for removal of large CBD stones To avoid full EST-induced bleeding

SO: Sphincter of Oddi; EPBD: Endoscopic papillary balloon dilation; CBD: Common bile duct.

of the CBD stone and diameter of the dilated CBD are significant factors for the selection of balloon size. Among these factors, the diameter of the CBD is regarded more important, because excessive balloon dilation over the CBD diameter might increase the risk of perforation. Therefore, the maximal inflated diameter of balloon should not exceed the diameter of the proximally dilated CBD. Generally, a small EST is recommended to reduce the risk of bleeding, because full EST increases the damage of the large vessel at the papillary roof. A small EST also lowers the risk of perforation, because direct observation of ampullary tearing is possible during balloon dilation.

In a South Korean study, the techniques of larger balloon dilation were recommended to avoid severe complications, such as perforation and massive bleeding<sup>[56]</sup>. If the balloon waist remained at 80% of the maximum inflation capacity, it meant that significant stricture existed in the distal CBD. Excessive inflation for distal CBD stricture could cause a perforation. Therefore, the balloon should be inflated gradually to avoid perforation, with observation of disappearance of the balloon waist at the distal CBD. Unlike EPBD, the duration of ballooning was regarded to be of no importance in the EPLBD, because the small EST might prevent acute pancreatitis<sup>[15]</sup>. In EPLBD, bleeding is not uncommon; however, the bleeding site could be invisible endoscopically. If hemostasis could not be completed by local therapy, the insertion of a fully covered biliary metal stent should be considered for a tamponade effect<sup>[57]</sup>.

## CONCLUSION

Endoscopic papillary dilation with a dilating balloon is an old technique. However, it seems that there is no guideline or consensus on detailed techniques. According to various studies, EPBD and EPLBD for the removal of CBD stones are useful and effective methods. To clini-

**Table 5** Tips for avoiding severe complications of endoscopic papillary balloon dilation

EPBD	EPLBD
1. A balloon smaller than the diameter of the CBD is recommended to reduce damage to the SO and pancreatic orifice.	1. Maximal inflated diameter of balloon should not exceed the CBD diameter.
2. Graded balloon inflation may significantly reduce the incidence of post-EPBD pancreatitis.	2. A small extent of EST followed by large balloon dilation may be recommended, rather than large balloon dilation without EST.
3. If the balloon's waist remains after 2–3 s at maximal balloon inflation, balloon dilation must be stopped immediately.	3. The balloon should be inflated gradually to avoid perforation and bleeding.
	4. If the balloon's waist remains at 80% of the maximum inflation capacity, balloon dilation must be stopped immediately and change to alternative procedures, such as EST and ML.
	5. Close monitoring must be necessary after EPLBD to detect the delayed complications, such as perforation and delayed bleeding.

SO: Sphincter of Oddi; EPBD: Endoscopic papillary balloon dilation; CBD: Common bile duct; EST: Endoscopic biliary sphincterotomy; ML: Mechanical lithotripsy; EPLBD: Endoscopic papillary large balloon dilation.

cians, these methods are very attractive because they are very easy to perform, technically simple, and have a short learning curve. Although EPBD and EPLBD are generally safe, clinicians must remain aware that they can lead to potential morbidity and even death. The foremost priority is the patient's safety, so these methods should not be use indiscriminately, but be performed carefully in selected patients. In addition, doctors should be prepared to use EST or ML if the initial treatment fails. When EPBD and EPLBD are used for the correct indications (Table 4), according to the technical guideline (Table 5), an effective and safe outcome should be expected.

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## Role of the advanced glycation end products receptor in Crohn's disease inflammation

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and helped in drafting the manuscript; Falcone C helped in the conception of the study and in drafting the manuscript; Ardizzone S was clinically responsible for the A series patients, and critically revised the article for important intellectual content; Fociani P helped in collecting the histological samples of the A series patients, evaluated all histological preparations in a blind manner, and critically revised the article for important intellectual content; Danelli P carried out all surgical procedures of the A series patients, and critically revised the article for important intellectual content; Corazza GR critically revised the article for important intellectual content; all the authors reviewed and approved the final version to be published.

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### Abstract

**AIM:** To investigate the level of mucosal expression and the involvement of the receptor for the advanced glycation end products (RAGE) in delayed apoptosis and tumor necrosis factor (TNF)- $\alpha$  production in Crohn's disease (CD).

**METHODS:** Surgical and endoscopic specimens from both inflamed and non-inflamed areas of the ileum and/or colon were collected from 20 and 14 adult CD patients, respectively, and used for the assessment of RAGE expression by means of immunohistochemistry and western blotting analysis. Normal tissues from 21

control subjects were used for comparison. The same polyclonal anti-human RAGE antibody (R and D System) was used in all experimental conditions. RAGE staining was quantized by a score including both the amount of positive cells and intensity of immunoreactivity; cellular pattern was also described. The effects of RAGE blocking on apoptotic rate and TNF- $\alpha$  production were investigated on immune cells freshly isolated from CD mucosa and incubated both with and without the muramyl dipeptide used as antigenic stimulus. Statistical analysis was performed *via* the test for trend, with regression models to account for intra-patient correlations. A 2-sided  $P < 0.05$  was considered significant.

**RESULTS:** In inflamed areas, RAGE expression in both the epithelial and lamina propria compartments was higher than control tissues ( $P = 0.001$  and  $0.021$ , respectively), and a cluster of positive cells were usually found in proximity of ulcerative lesions. Similar results were obtained in the lamina propria compartment of non-inflamed areas ( $P = 0.025$ ). The pattern of staining was membranous and granular cytosolic at the epithelial level, while in the lamina propria it was diffuse cytosolic. When evaluating the amount of protein expression by immunoblotting, a significant increase of both surface area and band intensity ( $P < 0.0001$  for both) was observed in CD inflamed areas compared to control tissue, while in non-inflamed areas a significant increase was found only for band intensity ( $P < 0.005$ ). Moreover, a significantly lower expression in non-inflamed areas in comparison with inflamed areas was found for both surface area and band intensity ( $P < 0.0006$  for both). Finally, RAGE blocking largely affects both the apoptotic rate of mucosal cells (towards an increase in both non-inflamed and inflamed areas of  $P < 0.001$  and  $< 0.0001$ , respectively) and TNF- $\alpha$  secretion (towards a decrease in both non-inflamed and inflamed areas of  $P < 0.05$  and  $< 0.01$ , respectively), mainly in the presence of antigenic stimulation.

**CONCLUSION:** RAGE is up-regulated in CD, especially in inflamed areas, and it appears to play a role in the mechanisms involved in chronic inflammation.

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**Key words:** Apoptosis; Crohn's disease; Chronic inflammation; Immunohistochemistry; Receptor for advanced glycation end products; Tumor necrosis factor- $\alpha$

**Core tip:** Receptor for the advanced glycation end products (RAGE) is a multiligand transmembrane receptor whose activation sustains chronic inflammation. The inhibition of RAGE-ligand interaction has proved successful in an experimental model of Crohn's disease (CD). Our work shows an up-regulation of RAGE expression in both inflamed and non-inflamed mucosa of CD patients in comparison to healthy tissue from control subjects. Moreover, RAGE blocking significantly affects both the apoptotic rate and tumor necrosis factor- $\alpha$  secre-

tion of mucosal immune cells, which are considered to be leading mechanisms in the chronic inflammation of CD. These findings pave the way for a possible use of RAGE blocking agents as a new therapeutic tool in this disabling condition.

Ciccocioppo R, Vanoli A, Klersy C, Imbesi V, Boccaccio V, Manca R, Betti E, Cangemi GC, Strada E, Besio R, Rossi A, Falcone C, Ardizzone S, Fociani P, Danelli P, Corazza GR. Role of the advanced glycation end products receptor in Crohn's disease inflammation. *World J Gastroenterol* 2013; 19(45): 8269-8281 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8269.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8269>

## INTRODUCTION

Dysregulation of immune tolerance towards components of the intestinal microbiota plays a crucial role in the pathogenesis of Crohn's disease (CD)<sup>[1]</sup>. Recently, the receptor for the advanced glycation end products (RAGE) has received a great deal of attention as a key molecule involved in tissue damage occurring in diabetes<sup>[2]</sup>, atherosclerosis<sup>[3]</sup>, neurodegeneration<sup>[4]</sup>, cancer, and inflammation<sup>[5]</sup>. It belongs to the immunoglobulin superfamily and is expressed at low levels by a wide range of differentiated adult cells, including immune cells, but at high levels when activated by its ligands<sup>[6]</sup>. Remarkably, thanks to its ability to recognize a three-dimensional structure rather than a specific amino acid sequence, it binds a broad repertoire of molecules<sup>[6]</sup>; other than the advanced glycation end products, it is also engaged by amyloid- $\beta$  peptides, high-mobility group (HMG) B1 proteins, and S100/calgranulins<sup>[6]</sup>. Since the latter act as damage-associated molecular pattern molecules<sup>[7]</sup>, RAGE is now considered a pattern-recognition receptor<sup>[8]</sup>. It seems very likely that the interaction of RAGE with both S100/calgranulins and HMGB1-proteins plays a proximal role in the inflammatory cascade by triggering the intracellular synthesis of nuclear factor (NF)- $\kappa$ B which, in turn, promotes the transcription of several pro-inflammatory and pro-fibrotic cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and transforming growth factor- $\beta$ , respectively<sup>[9]</sup>. Most importantly, RAGE is also able to bind the  $\beta$ 2-integrin Mac-1, thus determining the recruitment and migration of leucocytes to the site of inflammation<sup>[10,11]</sup>.

As far as CD is concerned, an up-regulation of both RAGE ligands and its mediators has already been found<sup>[12,13]</sup>, with a prominent role played by TNF- $\alpha$ , since blocking with monoclonal antibodies results in both induction and maintenance of remission<sup>[14]</sup>. In addition, the inhibition of the RAGE/HMGB1 pathway in experimental models of colitis led to a significant improvement in both clinical and histological features, with a parallel reduction of the levels of pro-inflammatory cytokines<sup>[15]</sup>. The lack of definitive information about a possible role



of this receptor in human inflammatory bowel disease has prompted us to study RAGE expression in the intestine of CD patients through both immunohistochemistry and western blotting assays, and to investigate its functional role in affecting the apoptotic rate and TNF- $\alpha$  production of mucosal immune cells, which are key mechanisms in causing chronic inflammation and tissue damage<sup>[16]</sup>.

## MATERIALS AND METHODS

### Immunohistochemistry

The immunohistochemical study was performed on archival material from the Pathology Department of the Luigi Sacco Hospital (Milan, Italy), obtained from 20 patients who had undergone surgical procedures of small and/or large bowel resection and/or stricturoplasty for CD (indicated hereafter as A series). Their demographic and clinical features, including body mass index (BMI), disease duration, location, and phenotype according to Montreal classification<sup>[17]</sup>, the CD activity index (CDAI)<sup>[18]</sup>, and the drug therapy followed, all determined at the time of surgery, are shown in Table 1. In all patients, the diagnosis of CD had been established according to widely accepted criteria<sup>[19]</sup> and confirmed at pathology. Specimens from both macroscopically diseased and non-diseased areas were obtained from each patient. Specifically, in order to prevent confounding results due to the proximity to diseased areas, the samples from normal tissue were collected from non-diseased zones at least 50 cm away from the resected area. In addition, surgical specimens from 9 subjects (M/F: 4/5, median age: 65  $\pm$  8.32 years, range: 63-86 years; BMI: 21.8  $\pm$  1.2 kg/m<sup>2</sup>, range: 18.1-24.8 kg/m<sup>2</sup>) who underwent right hemicolectomy for neoplasms, were selected from macroscopically healthy mucosa of both ileum and colon at more than 20 cm from the lesion, and used as controls. Each patient and control signed the informed consent, and all tissues were studied in an anonymous manner in accordance with the recommendations of the local Bio-Ethics Committee.

After tissue sampling during surgery to avoid autolysis artifacts, all specimens were immediately fixed in 40 g/L formaldehyde and embedded in paraffin. Serial 4- $\mu$ m-thick sections were cut from the blocks, mounted on electrostatically charged slides (DIAPATH Super Frost Plus<sup>®</sup>, Menzel-Gläser, Braunschweig, Germany) and dried overnight. After dewaxing and rehydration, the sections were processed for both traditional histology and immunohistochemistry. In addition, samples from both colonic adenomatous polyps<sup>[20]</sup> and normal lung tissue<sup>[21]</sup> were used as positive controls to test both the appropriate pre-treatment and primary antibody dilution. After 0.88 mol/L hydrogen peroxide was applied for 5 min, sections were treated with Tris/EDTA buffer (Target Retrieval Solution, DAKO, Carpinteria, CA, United States) at 1:10 dilution and pH 9.0, and then incubated in a microwave oven in citrate buffer (10 mmol/L, pH 7.0) for three cycles of 5 min each at 650 W, to unmask the antigen. Afterwards,

**Table 1** Demographic and clinical features of Crohn's disease patients *n* (%)

Characteristics	A series ( <i>n</i> = 20)	B series ( <i>n</i> = 14)
Sex		
Male	12	6
Female	8	8
Age (yr)		
Median (range)	41 (25-61)	40 (21-68)
BMI (kg/m <sup>2</sup> )		
Median (range)	25.5 (20-30)	20.7 (17.2-21.9)
Disease duration (mo)		
Median (range)	75 (1-485)	64 (24-360)
Disease location <sup>1</sup>		
L1	14	5
L2	1	2
L3	4	7
L4	None	None
L1-L4	1	None
Disease behavior <sup>1</sup>		
B1	None	6
B2	11	2
B2-B3	8	6
B3	1	None
CDAI		
Median (range)	250 (151-537)	244 (141-296)
Current therapies		
Patients <i>n</i>		
Mesalazine	8 (40)	3 (21.43)
Steroids	2 (10)	3 (21.43)
IS	3 (15)	3 (21.43)
IS + mesalazine	1 (5)	2 (14.29)
Steroids + mesalazine	1 (5)	1 (7.14)
Infliximab	4 (20)	2 (14.29)
IS + infliximab	1 (5)	0

<sup>1</sup>Montreal classification<sup>[17]</sup>. BMI: Body mass index; CDAI: Crohn's disease activity index; IS: Immunosuppressors.

the slides were incubated with CAS-Block solution (Invitrogen Corporation, Carlsbad, CA, United States) for 10 min, as this treatment had been already proved successful in avoiding unspecific ligands<sup>[22]</sup>. The primary antibody we used was the polyclonal anti-human RAGE antibody (R and D System, Minneapolis, MN, United States) at 1:1000 dilution overnight at 4 °C<sup>[22,23]</sup>. Finally, sections were soaked in a stop-wash buffer, rinsed in phosphate-buffered saline, and then incubated with the *Avidin Biotin Complex* (LSAB2 System, HRP, DAKO), followed by the usual reactions to allow color development (Liquid DAB+ Substrate Chromogen System, DAKO) and counterstaining with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO, United States). For the negative control, a goat anti-human IgG1 isotype (R and D System) was used as a primary antibody<sup>[23]</sup>, while in order to determine the origin of lamina propria RAGE<sup>+</sup> cells, seriate sections were processed with the following monoclonal antibodies: anti-CD3 (clone PSI, Novocastra Laboratories, Newcastle, United Kingdom), -CD20 (clone L26, DAKO), -CD138 (Clone B-A38, IQ Products, Houston, TX, United States), -CD68 (clone PG-M1, DAKO), and smooth muscle actin (clone 1A4, DAKO), according to the manufacturers' instructions, whilst neutrophils were identified

through morphology. Finally, in order to quantize RAGE expression, the analysis was split into two compartments, epithelial layer and lamina propria, and all sections were examined by two blinded pathologists under high-power field microscopes (HPFs) (*Nikon ECLIPSE E800*) at constant magnification ( $\times 400$ ). As regards the epithelial layer, the number of positive cells was calculated by using a differential count of at least 500 cells and the results were expressed as a percentage, while the intensity of the staining was estimated by applying an ordinal scale (0-3), where "3" indicated strong staining, "2" moderate staining, "1" weak staining, and "0" no staining. Thereafter, both results were combined together in a score as follows: grade I when less than 10% of epithelial cells were positive and/or had weak immunoreactivity, grade II when 10%-50% of epithelial cells were positive and/or had moderate immunoreactivity, and grade III when more than 50% of epithelial cells were positive and/or had strong immunoreactivity. In the lamina propria compartment, the quantization was performed by applying differential counts of positive cells on 10 HPF per slide, and the results were displayed as the mean number of positive cells. The same ordinal scale and grading score of the epithelial compartment (see above) for quantization of staining intensity and whole expression, respectively, were used. Finally, the pattern of cellular staining was reported as cytosolic (granular or diffuse) and/or membranous.

### Western blot analysis

Two snap-frozen perendoscopic specimens from both macroscopically diseased and non-diseased areas of the colonic mucosa were collected from 14 CD outpatients (indicated hereafter as B series), whose demographic and clinical features are shown in Table 1, and 12 control patients suffering from irritable bowel syndrome (M/F: 3/9; median age: 49 years, range: 21-72 years; BMI: 23.35 kg/m<sup>2</sup>, range: 18.6-25.6 kg/m<sup>2</sup>), all admitted to the Center for the Study and Cure of Inflammatory Bowel Disease at the Fondazione IRCCS Policlinico (Pavia, Italy). The CD patients were taking mesalazine, steroids, azathioprine, or biologics, as single or combined therapy (Table 1), whilst the control patients were drug-free. Each patient gave written informed consent, and approval from the Bio-Ethics Committee was obtained (protocol number: 20110002492).

Mucosal samples were lysed in 200  $\mu$ L ice-cold lysis buffer (53.8  $\mu$ L HEPES pH 7.9 0.93 mol/L, 10  $\mu$ L EDTA pH 8.0 0.5 mol/L, 300  $\mu$ L KCl 1 mol/L, 100  $\mu$ L Nonidet P-40, 4385  $\mu$ L distilled water, 50  $\mu$ L DTT 0.1 mol/L, 50  $\mu$ L PMSF 0.1 mol, 20  $\mu$ L aprotinin, 10  $\mu$ L leupeptin, 50  $\mu$ L NaVO<sub>4</sub> 0.1 mol/L, 10  $\mu$ L NaF 0.5 mol/L), sonicated (Sonifier 150, BRANSON, St. Louis, MO, United States), and incubated for 90 min in the dark. After centrifugation, the supernatants were harvested and the protein concentration was determined using the *Bio-Rad Protein Assay* (BIORAD, Hercules, CA, United States). A total of 50  $\mu$ g of protein from each sample, plus the loading buffer, were boiled and then

loaded onto 0.347 mol/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen-Life Technologies, Carlsbad, CA, United States) and run for 90 min. Proteins were then transferred to nitrocellulose sheets by means of the *PowerEase® 500* electrophoresis instrument (Invitrogen), and non-specific binding was blocked with 50 g/L non-fat milk powder in TBS 100 mL/L solution. Afterwards, the membranes were incubated with the primary antibody (polyclonal anti-RAGE immunoglobulin G 1:2000 dilution, R and D System) overnight on a rocker (Stuart SSL4, Fisher Scientific, Illkirch Cedex, France). After washing, the secondary antibody (polyclonal rabbit anti-goat immunoglobulin G-HRP 1:2000 dilution, DAKO) was applied, and the reaction was developed using a chemiluminescence kit (ECL Plus, GE Healthcare, Buc Cedex, France). Finally, the membranes were aligned with the film (GE Healthcare) in the developing liquid (Kodak, Rochester, New York, NY, United States). Negative control blots were obtained by omitting the primary antibody. Finally, blots were stripped and analyzed for  $\beta$ -actin as an internal control, using a rabbit anti-human polyclonal anti- $\beta$ -actin antibody 1:5000 dilution (Abcam, Cambridge, United Kingdom). Films were acquired using VersaDoc 3000 (BIORAD), and the bands measured in terms of both surface area and intensity by the QuantityOne software (BIORAD) and normalized for  $\beta$ -actin values.

### Functional assay

Six fresh additional perendoscopic mucosal biopsies from both macroscopically diseased and non-diseased colonic areas and peripheral blood samples were collected from seven out of the 14 CD outpatients. After digestion with collagenase A 1 mg/mL (Sigma-Aldrich) in complete medium (X-VIVO-15 plus 50 mL/LHS, penicillin/streptomycin 10000 U/10 mg/mL, gentamicin 2.5 mL/L, amphotericin B 4 mL/L, all by Lonza Group Ltd, Basel, CH) for 90 min, the cellular suspension was passed through a cell filter strainer, 40  $\mu$ m (BD Falcon™, Franklin Lakes, NJ, United States), centrifuged, and washed twice. The lamina propria mononuclear cells (LPMC) thus obtained were plated overnight in 48-well flat bottom tissue culture plates (Sarstedt, Newton, NC, United States) at  $1.0 \times 10^6$  cells/well in complete culture medium plus interleukin-2 40 U/mL (Chiron, Emeryville, CA, United States). After washing,  $0.5 \times 10^6$  LPMC for each well were incubated in the presence of the anti-RAGE antibody or IgG1 isotype antibody (R and D System) as a negative control, at 50 and 100  $\mu$ g/mL for 4 h, and then stimulated overnight in the presence or absence of muramyl dipeptide (MDP) (10  $\mu$ g/mL, Sigma-Aldrich, St. Louis, MO, United States) plus  $1.5 \times 10^6$  autologous irradiated (3000 rads) peripheral blood mononuclear cells used as feeding cells. Finally, the supernatants were collected and stored at -80 °C for evaluation of TNF- $\alpha$  levels by enzyme-linked immunosorbent assay (R and D System), while LPMC were harvested for the detection of the apoptotic rate by flow cytometry (BD FACSCanto, FACS Diva software, BD

**Table 2** Receptor for the advanced glycation end products expression in Crohn's disease diseased areas *vs* control tissues *n* (%)

Score	Patients	Controls	<i>P</i> value
Grading (epithelium)			0.001
I	2 (20)	8 (80)	
II	6 (86)	1 (14)	
III	6 (100)	0 (0)	
Grading (lamina propria)			0.021
I	7 (44)	9 (56)	
II	2 (100)	0 (0)	
III	4 (100)	0 (0)	
Intensity (epithelium)			0.480
0	2 (67)	1 (33)	
1	3 (43)	4 (57)	
2	9 (69)	4 (31)	
Intensity (lamina propria)			0.260
0	4 (50)	4 (50)	
1	4 (50)	4 (50)	
2	5 (83)	1 (17)	
Epithelial positive cells	% (median: 25 <sup>th</sup> -75 <sup>th</sup> )	5 (5-5)	0.008
	55 (20-70)		
Lamina propria positive cells	<i>n</i> /10 hpf (median: 25 <sup>th</sup> -75 <sup>th</sup> )	1 (0-2)	0.030
	5 (1-15)		

Biosciences, San Jose, CA, United States) by using the Annexin V/Propidium Iodide kit (*APOTEST™-FITC*, DAKO).

### Statistical analysis

Continuous variables were described as median and 25<sup>th</sup>-75<sup>th</sup> percentiles, while categorical variables were expressed as counts and percentages. Comparison between data from CD patients and control subjects was performed by using the test for trend and the Mann-Whitney *U* test, as appropriate. Data among CD patients were compared using a general linear or a general ordinal logistic model (according to the type of data), with calculation of the Huber-White robust SE for intra-patient correlation. The Spearman rank correlation test was applied to measure the association between continuous variables. A 2-sided *P* value ≤ 0.05 was considered to be statistically significant. Stata 12.1 (StataCorp LP, College Station, TX, United States) was used for computation.

## RESULTS

### RAGE expression in diseased areas

In both the epithelial and lamina propria compartments, the grading of RAGE expression in CD was significantly higher than in control tissues (test for trend: *P* = 0.001 and 0.021, respectively; Table 2, Figure 1A and B). When splitting the analysis for the two variables considered, there appeared to be no difference in intensity between the groups in either compartment (Table 2, Figure 1C and D), whilst the amount of positive cells, either at epithelial (Figure 2) or lamina propria level (Figure 3), was significantly higher in CD than in control tissues (test for trend: *P* = 0.008 and 0.030, respectively; Table 2, Figure 1E and F). Remarkably, in CD specimens, most of the strongly

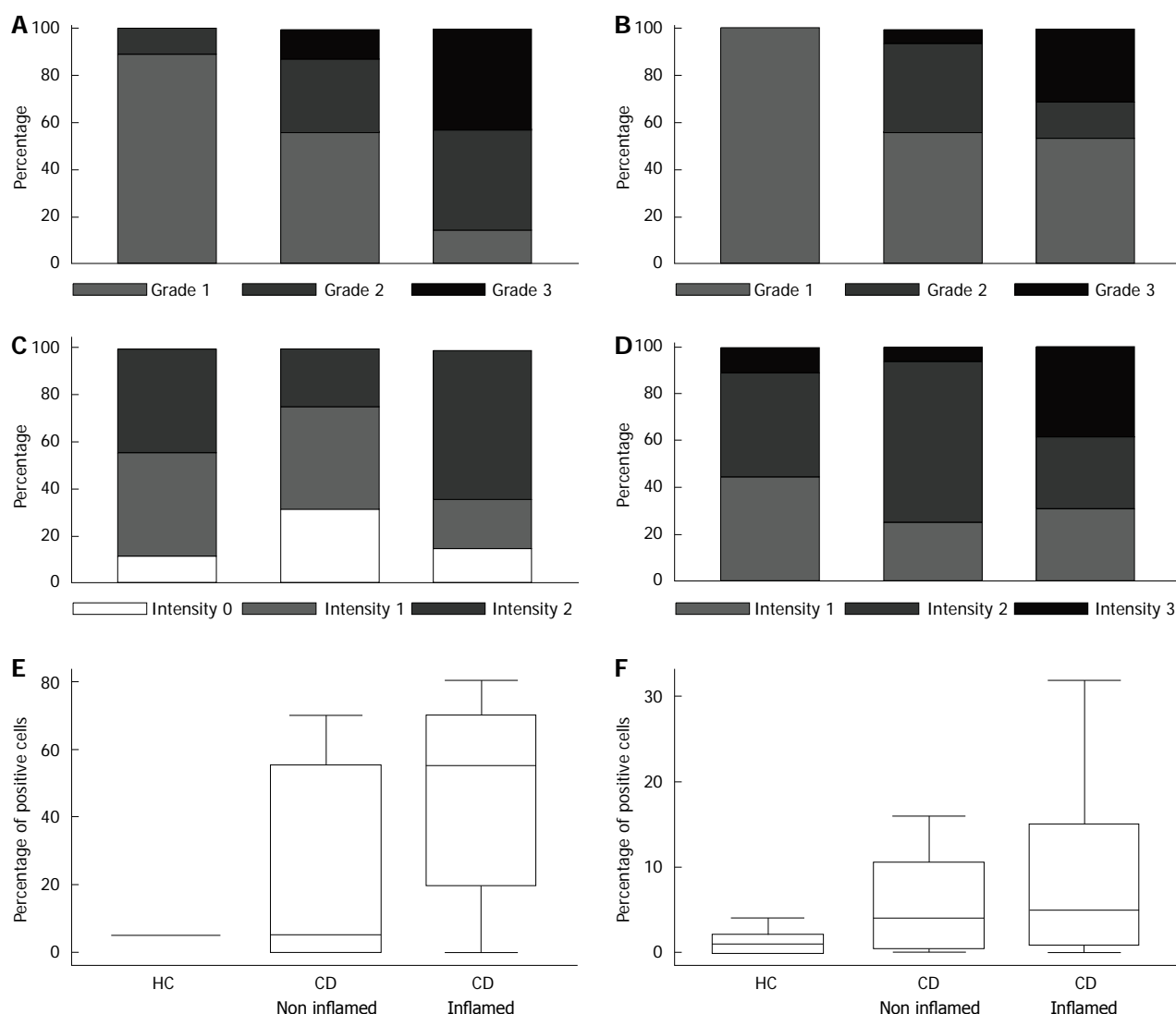
positive cells were found in close proximity to ulcers at epithelial level (Figure 4), while in the lamina propria they were sparsely distributed (Figure 3A and B). Moreover, in all CD samples, the pattern of RAGE expression at epithelial level was both membranous and granular cytosolic (Figure 2B, thin and thick red arrows, respectively), while in the lamina propria it was diffuse cytosolic (Figure 3A and B, black arrows). As regards the type of lamina propria positive cells, the vast majority were plasma cells (median 3, 25<sup>th</sup>-75<sup>th</sup>: 1-10) (Figure 5A) and monocytes/macrophages (median 3, 25<sup>th</sup>-75<sup>th</sup>: 2-12) (Figure 5B), with only a few scattered T lymphocytes and subepithelial myofibroblasts being stained, whilst neutrophils did not display RAGE expression (data not shown). When evaluating the amount of protein expression at mucosal level by immunoblotting, a significant increase of RAGE expression for both surface area and band intensity (*P* < 0.0001 for both) was observed in CD diseased areas with respect to control tissue (Figure 6). Finally, as regards the clinical features, no correlation was found between the grading of RAGE expression with BMI, CDAI score, or the duration, localization, or behavior of the disease, nor with the drug therapy followed (data not shown).

### RAGE expression in non-diseased areas

In both the epithelium and lamina propria compartments, the grading of RAGE expression in CD tissues was generally higher than in control tissues (Figure 1), though statistical significance was achieved only in the lamina propria compartment (test for trend: *P* = 0.025, Table 3). Similarly, no difference between these two groups was found either in intensity (Figure 1) or in the number of positive cells (Figure 1 and Table 3). Within the CD patient group, both grading and amount of RAGE<sup>+</sup> cells tended to be higher in the diseased areas, though significant variations were never reached (Figure 1 and Table 4). As far as the level of RAGE expression was concerned, a significant increase in non-diseased areas of CD patients in comparison with control tissues was observed only for band intensity at Western blot analysis (*P* < 0.005, Figure 6). Moreover, a significantly lower expression in non-diseased areas in comparison with diseased areas was found for both surface area and band intensity (*P* < 0.0006 for both, Figure 6). Finally, a positive correlation between the grading of RAGE expression at epithelial level and BMI was found in the CD group (median 25.5 ± 3.2, range: 20-30, *P* = 0.0116, *r* = 0.55, Figure 7), whilst no correlation was found with the CDAI score, behavior, localization, duration of the disease, nor with current therapies (data not shown).

### Functional assay

In order to investigate whether RAGE blocking has the ability to favor LPMC apoptosis and to reduce the production of the pro-inflammatory cytokine TNF-α, LPMC from both diseased and non-diseased areas of CD patients were incubated with the RAGE blocking antibody and stimulated with MDP, a component of the bacterial cell-wall peptidoglycan, which is present in most

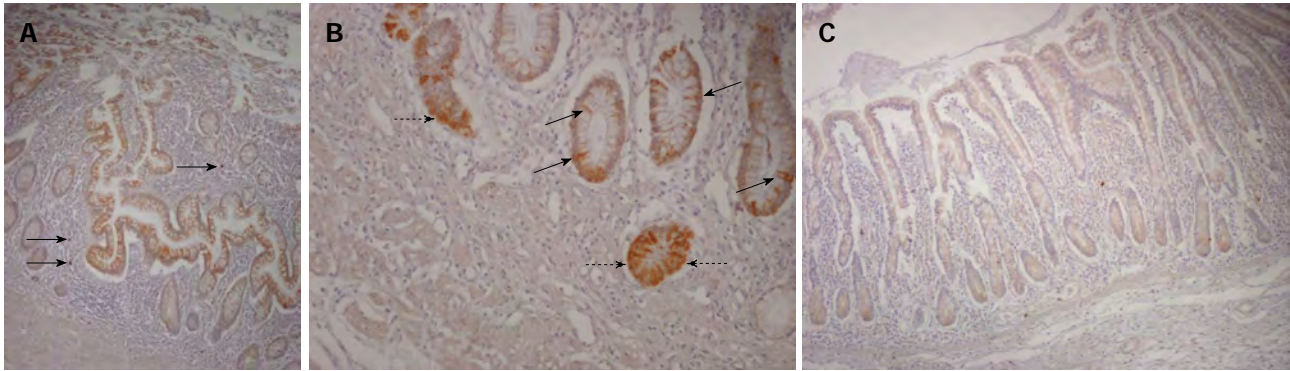


**Figure 1** Receptor for the advanced glycation end products expression. The percentage of samples in each category of both grading (A and B) and intensity (C and D) staining, and the number of positive cells (E and F) are reported. The average values of receptor for the advanced glycation end products (RAGE) immunoreactivity detected at the epithelial (A and C) and the lamina propria (B and D) compartments of patients suffering from Crohn's disease (CD) and the control subjects (HC) are given. In CD patients, the averages of assessments between the areas of diseased mucosa and the areas of non-diseased mucosa were also compared. Moreover, the number of positive cells in the epithelial compartment (E) was calculated by a differential count of at least 500 cells and the results expressed as a percentage, while in the lamina propria (F) compartment, it was performed by differential counts of positive cells on 10 high-power microscopic fields per slide ( $n/10$  HPF), and the results displayed as the mean number of positive cells. A higher, but not statistically significant, number of RAGE+ cells were observed in both diseased and non-diseased areas of CD patients in comparison with normal tissue of HC. The box-plots in panels E and F show median, 25<sup>th</sup>, and 75<sup>th</sup> percentile and extremes.

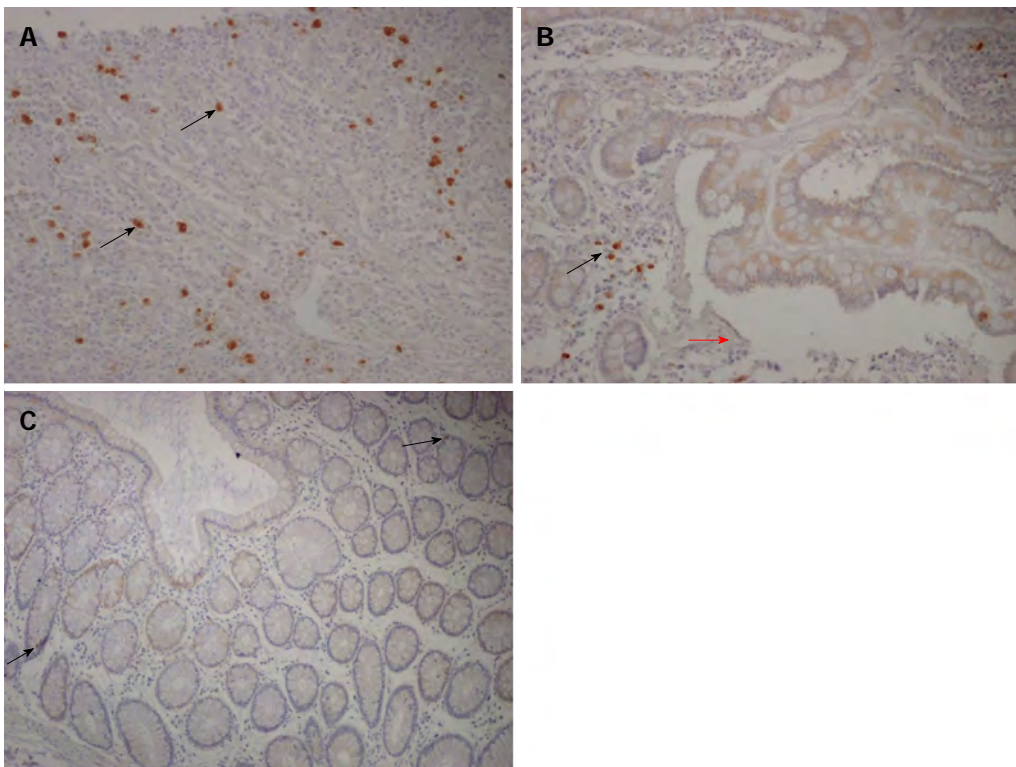
species of the gut microbiota, and whose ligation with the intracellular receptor NOD2 triggers inflammatory cascade in CD<sup>[24]</sup>. As expected, the apoptotic rate of the cell population obtained from non-diseased areas was higher than that found in cells isolated from diseased mucosa, both in the presence and absence of the antigenic stimulus ( $P < 0.001$  for both, Figure 8A and B). When incubating the LPMC from non-diseased areas with the lower concentration of the RAGE-blocking antibody, a significant increase in the apoptotic rate in comparison with the basal values was observed both in the absence ( $P < 0.01$ ) and presence of MDP ( $P < 0.001$ ), while the higher dose only slightly increased the percentages of cell death in both cases (Figure 8A and B). Similar effects

were observed on cells from diseased areas only when applying the higher dose of the RAGE blocking agent ( $P < 0.0001$  for both), since the lower dose was effective only in the presence of MDP stimulus ( $P < 0.005$ , Figure 8A and B). As far as TNF- $\alpha$  secretion is concerned, in the absence of antigenic stimulation, a higher basal level was observed in cultures with cells from diseased mucosa ( $P < 0.0002$ ), and this was consistently reduced by the higher dose of the anti-RAGE agent ( $P < 0.01$ , Figure 8C). Upon antigenic stimulation, both doses of the blocking antibody appeared capable of lowering the level of TNF- $\alpha$  production, both when using cells from diseased ( $P < 0.01$  and  $< 0.005$ , respectively) and non-diseased areas ( $P < 0.05$  and  $< 0.01$ , respectively, Figure 8D).





**Figure 2** Receptor for the advanced glycation end products staining in Crohn's disease and healthy mucosa. A: Almost all cells of the epithelial compartment at both crypt and surface levels showed positive immunostaining (brown cells) in a representative case of Crohn's disease affected areas, with only a few scattered positive cells in the lamina propria (black arrows); B: The pattern of the cellular staining in the crypt zone of a representative case of Crohn's disease affected area is shown, indicating both membranous and cytosolic distribution; C: A representative case of control mucosa is shown, with weak staining of the epithelial cells and only a few scattered positive cells in the lamina propria (receptor for the advanced glycation end products, immunoperoxidase-hematoxylin; original magnification,  $\times 200$ ).

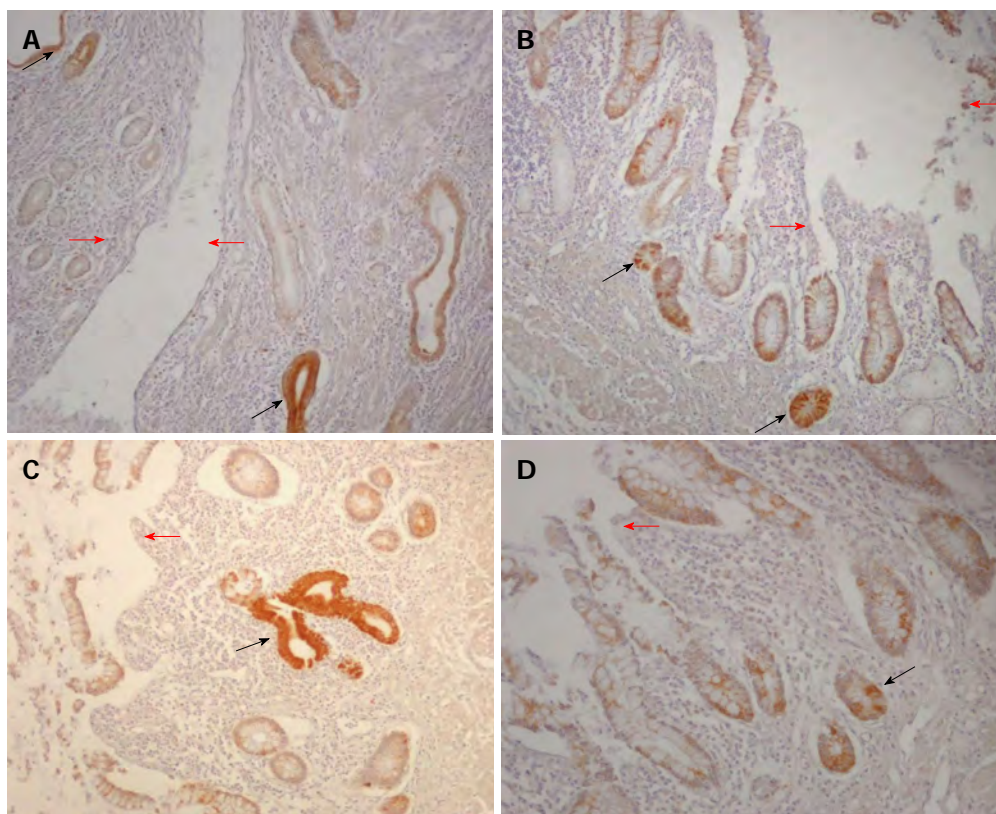


**Figure 3** Receptor for the advanced glycation end products staining in Crohn's disease and healthy mucosa lamina propria. A: Many lamina propria mononuclear cells proved receptor for the advanced glycation end products (RAGE)-positive (the brown cells) in a representative case of Crohn's disease affected areas; B: A cluster of RAGE-positive lamina propria mononuclear cells next to an ulceration (red arrow) in a representative case of Crohn's disease affected area showing a cytosolic distribution; C: A representative case of control mucosa is shown, with weak staining of a few lamina propria mononuclear cells (black arrow) (RAGE immunoperoxidase-hematoxylin; original magnification,  $\times 200$ ).

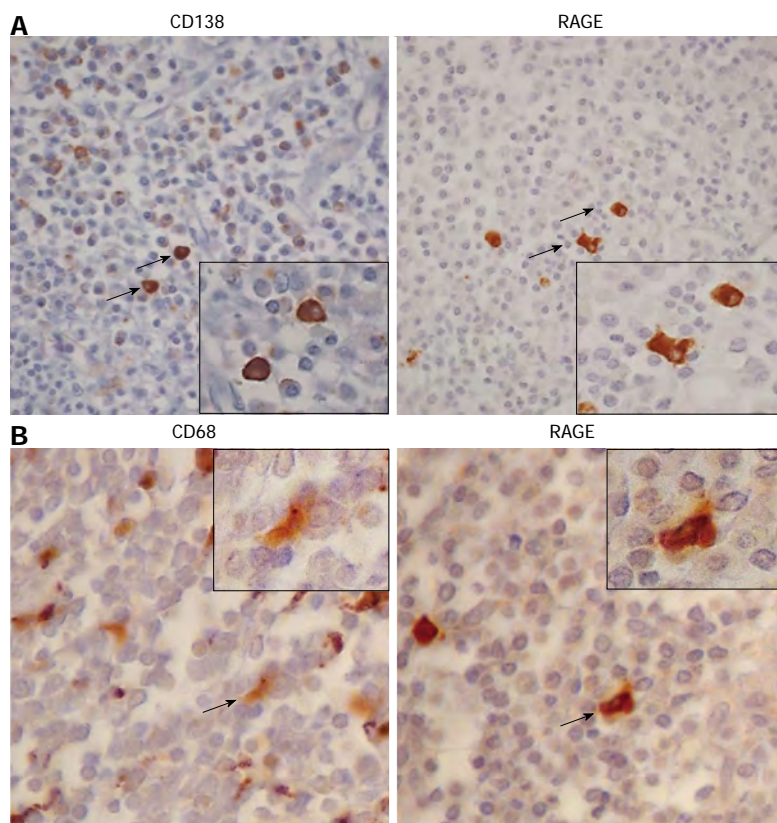
## DISCUSSION

Originally identified in diabetic tissues<sup>[25]</sup>, RAGE was subsequently found to be up-regulated in many pathological conditions<sup>[6]</sup> and, in recent years, it also seems to be involved in the pathogenesis of chronic inflammatory diseases<sup>[26]</sup>, such as rheumatoid arthritis<sup>[27]</sup>, systemic lupus erythematosus<sup>[28]</sup>, and multiple sclerosis<sup>[29]</sup>. As regards the gastrointestinal tract, RAGE has been found to play a role in chronic gastritis due to *Helicobacter pylori* by favor-

ing its adhesiveness to epithelial cells<sup>[30]</sup>, as well as in CD, where an increased expression was found in phagocytes infiltrating inflamed areas<sup>[31]</sup>. Moreover, the administration of HMGB1 blocking agent has proved to be of benefit in experimental models of Crohn's colitis, through a decrease in the expression of RAGE and related pro-inflammatory cytokines<sup>[15]</sup>. On this basis, we first of all investigated RAGE expression by immunohistochemistry on surgical specimens from CD patients where samples of healthy tissue at a certain distance from the workpiece

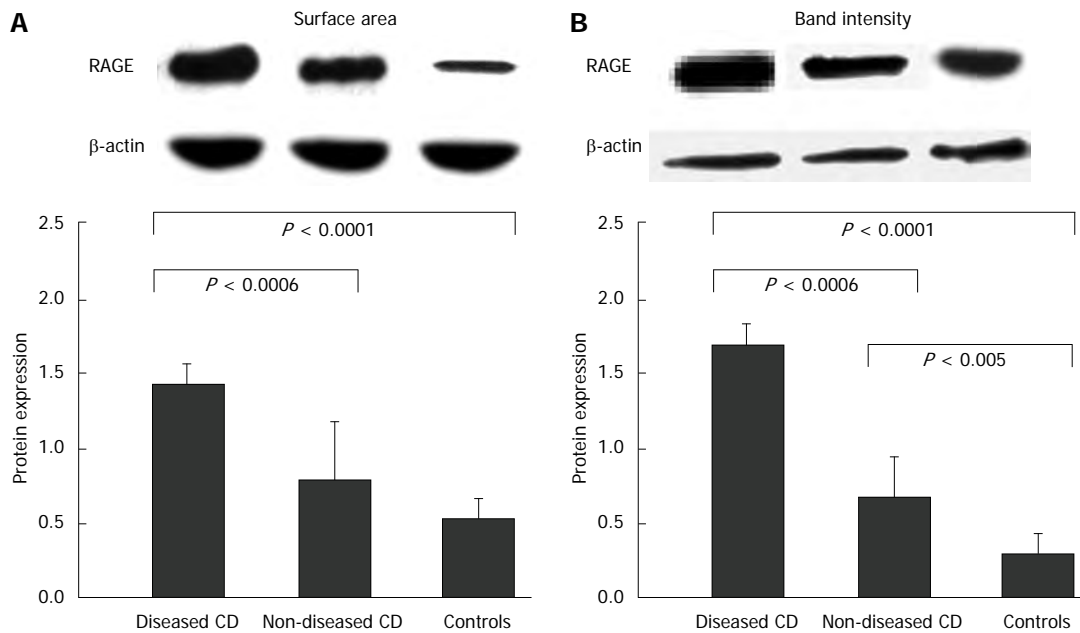


**Figure 4** Receptor for the advanced glycation end products staining in Crohn's disease ulcerative areas. A: Almost all cells of the epithelial compartment in close proximity to an ulceration (red arrows) proved receptor for the advanced glycation end products (RAGE)-positive (the brown cells), with the ulcer-associated cell lineage showing the highest immunoreactivity (black arrows); B and C: A detail of the epithelial cells in the crypt compartment at level of an ulceration (red arrows) showing moderate to strong RAGE-positive staining (black arrows); D: A detail of the epithelial cells of the surface compartment next to an ulceration (red arrow) showing strong RAGE immunoreactivity (black arrows) (RAGE immunoperoxidase-hematoxylin; original magnification,  $\times 200$ ).



**Figure 5** Type of receptor for the advanced glycation end products (+) immune cells. Representative seriate sections of Crohn's disease lamina propria with heavy inflammatory infiltrate, in which the correspondence between the positivity for both CD138 (panel A) indicating the plasma cells, and CD68 (panel B) showing the macrophages, with receptor for the advanced glycation end products (+) cells given (Immunoperoxidase-hematoxylin; original magnification,  $\times 200$ ). The same cellular elements indicated by the black arrows are shown in the boxes at higher magnification (Immunoperoxidase-hematoxylin; original magnification,  $\times 400$ ). RAGE: Receptor for the advanced glycation end products.





**Figure 6** Receptor for the advanced glycation end products expression at mucosal level. Immunoblotting of mucosal samples from both diseased and non-diseased areas of Crohn's disease (CD) patients and from normal areas of control patients with the polyclonal anti-receptor for the advanced glycation end products (RAGE) antibody. The protein levels were measured by scanning densitometry as band area (A) and band intensity (B), expressed as arbitrary units, and normalized towards β-actin levels. In the upper part of both panels, representative cases of band area and intensity with respect to those of β-actin are shown. Specifically, the values for RAGE expression in diseased areas for both band area and band intensity were significantly higher ( $1.48 \pm 0.16$  and  $1.64 \pm 0.14$ , respectively) than those found in non-diseased areas ( $0.71 \pm 0.40$  and  $0.66 \pm 0.23$ , respectively) and healthy mucosa ( $0.44 \pm 0.17$  and  $0.27 \pm 0.15$ , respectively).

**Table 3** Receptor for the advanced glycation end products expression in Crohn's disease non-diseased areas vs control tissues *n* (%)

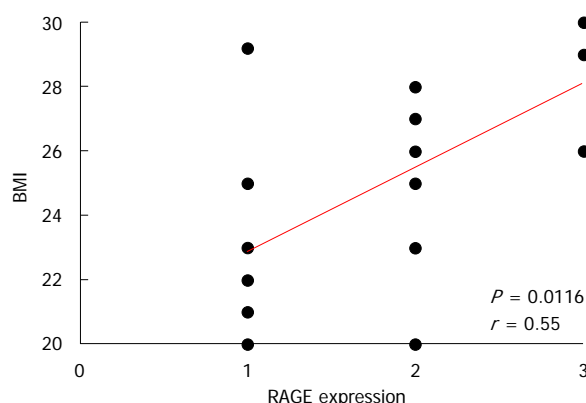
Score	Patients	Controls	P value
Grading (epithelium)			0.091
I	9 (53)	8 (47)	
II	5 (83)	1 (17)	
III	2 (100)	0 (0)	
Grading (lamina propria)			0.025
I	9 (50)	9 (50)	
II	6 (100)	0 (0)	
III	1 (100)	0 (0)	
Intensity (epithelium)			0.210
0	5 (83)	1 (17)	
1	7 (64)	4 (36)	
2	4 (50)	4 (4)	
Intensity (lamina propria)			0.490
0	4 (50)	4 (50)	
1	11 (73)	4 (27)	
2	1 (50)	1 (50)	
Epithelial positive cells	% (median: 25 <sup>th</sup> -75 <sup>th</sup> )	5 (5-5)	0.530
	5 (0-55)		
Lamina propria positive cells	<i>n</i> /10 hpf (median: 25 <sup>th</sup> -75 <sup>th</sup> )	1 (0-2)	0.079
	4 (0-10)		

**Table 4** Receptor for the advanced glycation end products expression in diseased vs non-diseased areas in Crohn's disease patients *n* (%)

Score	Diseased	Non-diseased	P value
Grading (epithelium)			0.17
I	5 (71)	2 (29)	
II	4 (44)	5 (56)	
III	1 (25)	3 (75)	
Grading (lamina propria)			0.53
I	6 (55)	5 (45)	
II	3 (75)	1 (25)	
III	1 (25)	3 (75)	
Intensity (epithelium)			0.37
0	3 (60)	2 (40)	
1	4 (67)	2 (33)	
2	3 (33)	6 (67)	
Intensity (lamina propria)			0.15
0	2 (40)	3 (60)	
1	7 (70)	3 (30)	
2	1 (25)	3 (75)	
Epithelial positive cells	% (median: 25 <sup>th</sup> -75 <sup>th</sup> )	40 (20-60)	0.24
	12.50 (0-50)		
Lamina propria positive cells	<i>n</i> /10 hpf (median: 25 <sup>th</sup> -75 <sup>th</sup> )	5 (1-15)	0.24
	4 (1-13)		

of intestine were also available. This is why we set out to determine whether the activation of the RAGE pathway occurs only within an inflamed *milieu*, or if it represents a predisposing condition which involves the entire length of the intestine in CD. Our results clearly show an increased grade of RAGE expression, in comparison with control tissues, in the entire bowel of CD patients, albeit predominately in macroscopically damaged areas.

Specifically, the vast majority of epithelial cells in the diseased areas, both on the surface and in crypt regions, express RAGE with a whole cellular staining, compared to control tissues where only a few cells were positive. It is worth noting that the highest numbers of positive cells were found close to ulcerative lesions. In the non-diseased tissue, a trend towards up-regulation was evident, but does not reach the statistical significance. Also



**Figure 7** Correlation between receptor for the advanced glycation end products expression and body mass index. A positive correlation between the grading of receptor for the advanced glycation end products (RAGE) expression in the epithelial compartment of non-diseased Crohn's mucosa and body mass index (BMI) was clearly evident.

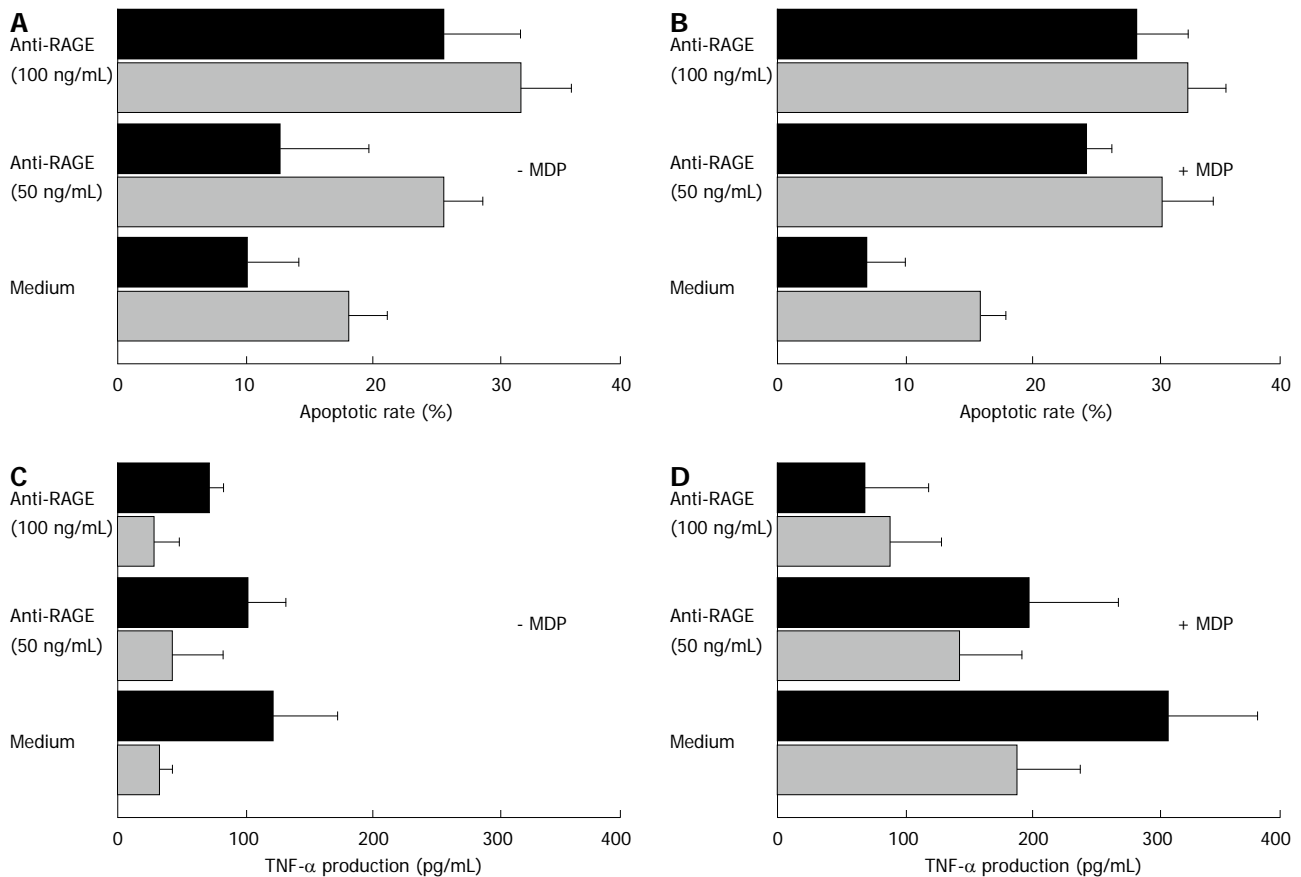
in the lamina propria, the grade of RAGE<sup>+</sup> cells was higher in CD diseased areas compared to healthy tissues, although their distribution did not seem to be associated with ulcerative lesions and the pattern of expression was cytosolic. These differences in both distribution and cellular pattern between epithelial and lamina propria cells seem to indicate a separate role of RAGE, with that in the epithelial cells possibly being a response to external injury, while that in the lamina propria cells mostly being involved in magnification of inflammation. Here, the diffuse cytoplasmic staining may be a consequence of the activated status, whereas the granular cytosolic and membranous staining in the epithelial compartment may represent a secretory pathway<sup>[32]</sup>. Remarkably, the up-regulation of RAGE in epithelial cells under inflammatory stimuli has been shown to mediate the trans-epithelial migration of neutrophils through its binding with the specific  $\beta_2$  integrin CD11b/CD18<sup>[33]</sup>. It is worth noting that we did not find any extracellular positivity in the stromal area, indicating that there is no storage of RAGE in the extracellular matrix. Furthermore, when splitting the results between the two variables included in the grading system, *i.e.*, the intensity of the staining and the number of positive cells, we found that a significant difference was still evident only for the latter. In the non-diseased tissue of CD patients, a significantly higher grade of RAGE expression was found in the lamina propria compartment compared to control tissue. The reason may lie in the small number of samples and the high variability found mainly in the CD group. The results found at immunohistochemistry were confirmed by the analysis of RAGE expression at protein level by immunoblotting on mucosal samples, where an increase in both diseased and non-diseased areas of CD patients in comparison with healthy tissues from irritable bowel syndrome patients was observed. Moreover, the up-regulation of RAGE expression even in non-inflamed areas suggests that in CD, an unbalanced activation of the RAGE pathway may take place along the entire length of the intestine, reaching the

highest levels in inflamed areas where the concentration of its ligands is higher. In this regard, an up-regulation of both S100A12 and HMGB1 in inflammatory bowel disease has been already shown<sup>[12,13]</sup>. In addition, it is recognized that the activation of the RAGE pathway plays a crucial role in regulating apoptosis and autophagy<sup>[34]</sup>, and in favoring the differentiation of T cells towards a T helper-1 phenotype<sup>[35]</sup>, both being leading mechanisms in generating tissue damage in CD<sup>[1,36,37]</sup>. Therefore, we explored RAGE blocking's ability to affect the delayed apoptosis and TNF- $\alpha$  production of LPMC isolated from diseased and non-diseased mucosa of CD patients, and found that pre-treatment with the anti-RAGE antibody induces a dose-dependent increase of the apoptotic rate, with the effect clearly evident also at a lower dose in the presence of MDP. The latter represents the main bacterial wall component which is recognized by NOD2, an intracellular pattern recognition receptor whose variants are associated with CD<sup>[38]</sup>. The binding of MDP to mutated NOD2 is followed by activation of pro-inflammatory pathways mainly regulated by nuclear factor- $\kappa$ B<sup>[39]</sup>. It is interesting to note that the latter molecule is also the transducer of the RAGE pathway upon activation by S100/calgranulins and HMGB1 proteins<sup>[9]</sup>. Our further evidence of a significant and dose-dependent decrease of TNF- $\alpha$  production, mainly in those cells from inflamed mucosa following RAGE blocking, fits perfectly into this context. It seems likely, therefore, that a progressive accumulation of RAGE ligands in those tissues primed by genetic and/or environmental factors leads to an increased expression which, in turn, causes magnification rather than dampening of the inflammation<sup>[26,39]</sup>.

Finally, as far as clinical features are concerned, at variance with the epithelial compartment of the non-diseased areas, where a positive correlation between the grading of RAGE expression and BMI was clearly evident, no correlation was found in diseased areas, probably by virtue of the high concentration of ligands, which *per se* leads to an increased expression that overcomes any systemic regulation<sup>[40]</sup>. Also, no correlation with the duration, localization, behavior, activity of the disease, or current therapies was observed in any condition. Notably, in the diseased areas, we did not find any correspondence between the zones of high RAGE<sup>+</sup> cells density in the epithelial compartment and those in the lamina propria. It is conceivable, therefore, that this might depend on different timing or mechanisms of activation of the RAGE pathway in the two compartments, which deserves further investigation. Also, the limited size of our sample groups, dictated by the need to obtain surgical specimens from macroscopically healthy tissue at a certain distance from the workpiece of intestine, implies that larger studies are needed in order to confirm our evidence.

In conclusion, the evidence we obtained of an increased expression of RAGE on both epithelial and lamina propria cells in CD, mainly in macroscopically injured areas, represents a further step up in the understanding of its pathogenesis, thus paving the way for future thera-





**Figure 8 Functional assays.** The *in vitro* apoptotic rates of lamina propria mononuclear cells (LPMC) incubated in the absence or presence of two different concentrations of the anti-receptor for the advanced glycation end products (RAGE) blocking antibody, and with or without the muramyl dipeptide (MDP) used as antigenic stimulation are given in panels A and B. The analysis was carried out by flow cytometry, and the mean percentage values  $\pm$  SD of at least three experiments for each condition were the following: in the absence of MDP (A), LPMC from non-diseased mucosa (grey bars) showed a spontaneous apoptotic rate of  $18.4 \pm 3.1$ , and a value of  $26.9 \pm 2.8$  and  $32.7 \pm 4.6$  in the presence of 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, respectively; when using LPMC from diseased mucosa (black bars), a value of spontaneous apoptotic rate of  $10.1 \pm 4.2$  was found, while when incubating with 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, values of  $13.0 \pm 6.2$  and  $26.1 \pm 5.5$  were found, respectively. In the presence of MDP (B), LPMC from non-diseased mucosa showed a spontaneous apoptotic rate of  $15.6 \pm 2.1$ , and values of  $31.9 \pm 3.8$  and  $33.3 \pm 2.9$  in the presence of 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, respectively; when using LPMC from diseased mucosa, a value of spontaneous apoptotic rate of  $7.8 \pm 2.6$  was found, while when incubating with 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, values of  $24.1 \pm 2.2$  and  $28.0 \pm 4.1$ , respectively, were found. The tumor necrosis factor (TNF)- $\alpha$  production of LPMC cultured *in vitro* in the absence or presence of two different concentrations of the anti-RAGE blocking antibody, and with or without the MDP as antigenic stimulation are given in the panels C and D. The analysis was carried out by ELISA assay on culture supernatants, and the mean values  $\pm$  SD were as follows: in the absence of MDP (C), the cytokine level was  $32 \pm 14$  pg/mL in the cultures with LPMC from non-diseased areas, and  $124 \pm 48$  pg/mL in those with LPMC from diseased areas; when incubating with 50 and 100 ng/mL of the anti-RAGE blocking antibody, values of  $41 \pm 38$  and  $27 \pm 21$  pg/mL for LPMC from non-diseased areas, and of  $102 \pm 29$  and  $67 \pm 11$  pg/mL for LPMC from diseased areas, respectively, were observed. In the presence of MDP (D), the TNF- $\alpha$  level was  $184 \pm 49$  pg/mL in the cultures with LPMC from non-diseased areas, and  $307 \pm 68$  pg/mL in those with LPMC from diseased areas; when incubating with 50 and 100 ng/mL of the anti-RAGE blocking antibody, values of  $138 \pm 50$  and  $87 \pm 41$  pg/mL for LPMC from non-diseased areas, and of  $198 \pm 68$  and  $71 \pm 47$  pg/mL for LPMC from diseased areas, respectively, were observed.

peutic use of RAGE blocking agents. Although it does not seem to be the primary cause, once set in motion, the RAGE pathway may be considered to contribute significantly to chronic inflammation.

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## COMMENTS

### Background

Crohn's disease (CD) is a disabling, lifelong, inflammatory disease which affects the gastrointestinal tract in a discontinuous manner, and whose lesions depend on a dysregulated immune response towards antigens of the gut microbiota. The receptor for the advanced glycation end products (RAGE) is a multiligand transmembrane receptor, whose activation sustains chronic inflammation.

### Research frontiers

The inhibition of RAGE-ligand interaction was proved successful in an experimental model of CD. However, the information available on the role of RAGE in human inflammatory bowel disease is scanty.

## Innovations and breakthroughs

In this study, the authors show an up-regulation of RAGE expression in the entire bowel of Crohn's patients, although prevalently in macroscopically affected areas, compared to healthy tissue of control subjects, as evaluated by both immunohistochemistry and Western blot analysis. Moreover, RAGE blocking significantly affects both the apoptotic rate and tumor necrosis factor- $\alpha$  secretion of mucosal immune cells, which are the key mechanisms involved in chronic inflammation and tissue damage.

## Applications

This study contributes to their understanding of the pathogenesis of CD and provides a scientific basis for future therapeutic use of RAGE blocking agents.

## Terminology

RAGE is a multiligand receptor which belongs to the immunoglobulin superfamily, and is natively present on the surface of monocyte/macrophage lineage cells and vascular cells. It is constitutively expressed during embryonic development, but progressively down-regulated in adulthood, where it is present at low levels in most normal tissues. The exceptions are the skin and lungs, where expression remains high throughout life. An increased expression of RAGE was found in a number of different pathological conditions, including chronic inflammatory diseases, neurodegenerative disorders, ageing, and cancer.

## Peer review

The authors investigated the involvement of RAGE in CD and found an enhanced expression in both the macroscopically diseased and non-diseased intestine, together with an attenuated inflammatory response of mucosal immune cells following RAGE blocking. The study is well designed and conducted, and the results are clearly presented. This is an excellent study whose only limitation is the relatively small sample size.

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## Overexpressed miRNA-155 dysregulates intestinal epithelial apical junctional complex in severe acute pancreatitis

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### Abstract

**AIM:** To investigate whether miRNA-155 (miR-155) dysregulates apical junctional complex (AJC) protein expression in experimental severe acute pancreatitis (SAP).

**METHODS:** Twenty-four male BALB/c mice were randomly assigned to two groups: the SAP group ( $n = 12$ ) receiving sequential intraperitoneal injection of 50  $\mu$ g/kg caerulein and 10 mg/kg lipopolysaccharide over 6 h, and the control group ( $n = 12$ ) receiving intraperitoneal injection of normal saline. Animals were sacrificed 3 h following the last injection for collection of blood samples and pancreas and distal ileal segment specimens. Routine pancreas and intestine histology was used to assess SAP pathology and intestinal epithelial barrier damage. Levels of serum amylase, diamine oxidase (DAO), and tumor necrosis factor (TNF)- $\alpha$  were determined using commercial kits. Total RNA samples

were isolated from intestinal epithelial specimens and reversely transcribed into cDNA. miR-155 and RhoA mRNA expression profiles were determined using quantitative real-time polymerase chain reaction. Target genes for miR-155 were predicted using the miRTarBase database, RNA22 and PicTar computational methods. Western blotting was performed to quantitate the protein expression levels of the target gene RhoA, as well as zonula occludens (ZO)-1 and E-cadherin, two AJC component proteins.

**RESULTS:** Intraperitoneal injection of caerulein and lipopolysaccharide successfully induced experimental acute pancreatic damage (SAP *vs* control,  $10.0 \pm 2.0$  *vs*  $3.2 \pm 1.2$ ,  $P < 0.01$ ) and intestinal epithelial barrier damage ( $3.2 \pm 0.7$  *vs*  $1.4 \pm 0.7$ ,  $P < 0.01$ ). Levels of serum amylase ( $21.6 \pm 5.1$  U/mL *vs*  $14.3 \pm 4.2$  U/mL,  $P < 0.01$ ), DAO ( $21.4 \pm 4.1$  mg/mL *vs*  $2.6 \pm 0.8$  mg/mL,  $P < 0.01$ ), and TNF- $\alpha$  ( $61.0 \pm 15.1$  ng/mL *vs*  $42.9 \pm 13.9$  ng/mL,  $P < 0.01$ ) increased significantly in SAP mice compared to those in control mice. miR-155 was significantly overexpressed in SAP intestinal epithelia ( $1.94 \pm 0.50$  fold *vs*  $1.03 \pm 0.23$  fold,  $P < 0.01$ ), and RhoA gene containing three miR-155-specific binding sites in the three prime untranslated regions was one of the target genes for miR-155. RhoA ( $22.7 \pm 5.8$  folds *vs*  $59.6 \pm 11.6$  folds,  $P < 0.01$ ), ZO-1 ( $46 \pm 18$  folds *vs*  $68 \pm 19$  folds,  $P < 0.01$ ), and E-cadherin proteins ( $48 \pm 15$  folds *vs*  $77 \pm 18$  folds,  $P < 0.01$ ) were underexpressed in SAP intestinal epithelia although RhoA mRNA expression was not significantly changed in SAP ( $0.97 \pm 0.18$  folds *vs*  $1.01 \pm 0.17$  folds,  $P > 0.05$ ).

**CONCLUSION:** TNF- $\alpha$ -regulated miR-155 overexpression inhibits AJC component protein syntheses of ZO-1, and E-cadherin by downregulating post-transcriptional RhoA expression, and disrupts intestinal epithelial barrier in experimental SAP.

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**Key words:** miRNA-155; Severe acute pancreatitis; Intestinal barrier dysfunction; Apical junctional complex

**Core tip:** Apical junction complex (AJC) damage leads to intestinal barrier dysfunction and disease progression in severe acute pancreatitis (SAP) by an unknown mechanism. We reported for the first time that miRNA (miR)-155, a major mediator regulating early-stage inflammatory process, was overexpressed in experimental SAP intestinal epithelia as induced by circulating tumor necrosis factor (TNF)- $\alpha$ . RhoA gene, a predicted targeted gene for miR-155, was underexpressed at the post-transcriptional level, accompanied by downregulation of ZO-1 and E-cadherin expression; two key component proteins of AJC. Our study demonstrated that the TNF- $\alpha$ /miR-155/RhoA/ZO-1/E-cadherin signaling pathway contributed to intestinal barrier dysfunction in complicated SAP.

Tian R, Wang RL, Xie H, Jin W, Yu KL. Overexpressed miRNA-155 dysregulates intestinal epithelial apical junctional complex in severe acute pancreatitis. *World J Gastroenterol* 2013; 19(45): 8282-8291 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8282.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8282>

## INTRODUCTION

Severe acute pancreatitis (SAP) is a serious medical condition with a high mortality rate. Intestinal barrier dysfunction in SAP patients leads to endotoxemia and multiple organ failure, and contributes to disease progression<sup>[1-3]</sup>. It is helpful to elucidate the pathogenetic mechanisms underlying intestinal barrier damage in SAP for successful treatment of patients who are critically ill in the great majority of cases.

Intestinal mucosal barrier is composed of a layer of crosslinked, highly differentiated epithelial cells located at the apical junction complex (AJC). A tight AJC maintains epithelial cell polarity, reduces the gap between epithelial cells, and decreases epithelial permeability. Therefore, the AJC can prevent intestinal toxins, pathogens and other exogenous substances from entering into the submucosal tissues and the circulation through the epithelial layer. The AJC is believed to be the mainstay structure that maintains the intestinal barrier and prevents enteric endotoxemia, and damage can lead to intestinal barrier dysfunction<sup>[4-8]</sup>.

The mechanisms underlying AJC damage in SAP remain elusive. The structure and function of AJC depend on the regulation of its component proteins. miRNA is a newly established protein regulator<sup>[9-11]</sup> and is critically involved in maintaining epithelial junctions and polarity, as well as cytoskeletal remodeling in tumors<sup>[12-14]</sup>. Tang *et al*<sup>[15]</sup> reported that the expression of zonula occludens (ZO)-1 protein, a major AJC component, was downregulated

by miRNA (miR)-212 in Caco-2 cells and patients with alcoholic liver disease and intestinal barrier dysfunction. Ye *et al*<sup>[16]</sup> reported that downregulated expression of occludin, the component protein of AJC, was regulated by miR-122 in mouse and Caco-2 cells and resulted in intestinal barrier dysfunction. Aside from their critical role in maintaining intestinal epithelial junctions and regulating AJC proteins, miRNAs, especially miR-155, are also implicated in inflammatory processes<sup>[17,18]</sup>, which normally occur at the early stage of SAP. It has been also reported that miR-155 is a major inflammatory mediator<sup>[17-21]</sup> and regulates the inflammatory response and carcinogenesis<sup>[22]</sup>. However, it is yet to be investigated how miR-155 regulates AJC protein expression in intestinal epithelial cells.

The objectives of this study were to investigate how miR-155 regulates AJC protein expression, to predict target genes for miR-155, and to identify the possible miR-155 regulatory pathways in SAP.

## MATERIALS AND METHODS

### Laboratory animals and experimental protocols

The animal experimentation protocol was approved by the Animal Research Committee at First People's Hospital, Shanghai Jiaotong University. Twenty-four male BALB/c mice weighing 25 g were purchased from Shanghai Laboratory Animal Center and housed at room temperature (25 °C) with a 12-h light/dark cycle. All animals were given free access to rodent chow and tap water *ad libitum* and acclimated for  $\geq 3$  d prior to the experiment.

Following 12 h fasting, all experimental mice were randomly divided into two groups, the SAP group ( $n = 12$ ) and the control group ( $n = 12$ ). An experimental SAP mouse model was established as previously reported<sup>[23]</sup>. In the SAP group, intraperitoneal injection of 50  $\mu$ g/kg caerulein dissolved in normal saline (Bachem, Bubendorf, Switzerland) was repeated at hourly intervals for 6 h followed by intraperitoneal injection of 10 mg/kg *Escherichia coli* lipopolysaccharide (LPS) dissolved in normal saline (Sigma-Aldrich, St Louis, MO, United States). In the control group, the same volume of normal saline in place of caerulein and LPS was injected at hourly intervals for 6 h.

All animals were euthanized by injecting 3% intraperitoneal pentobarbital (0.1 mL/100 g; Dongchang Chemical, Shanghai, China) at 3 h after the last injection. Blood samples and pancreas and distal ileal segment specimens were collected. Blood samples were centrifuged at 3000 rpm for 8 min at 4 °C within 2 h after collection, and the supernatants were collected and stored at -80 °C for further experiments. Pancreas and intestine specimens were immediately fixed in 10% buffered formaldehyde (Dongchang Chemical) for further histological examination. Mucosal tissues of the distal ileal segment, 3-5 cm long, were stripped, snap frozen in liquid nitrogen, and stored at -80 °C for further experiments.

### Histological examination

Paraffin-embedded pancreatic and intestinal tissues were cut into 5- $\mu$ m sections. Sections were stained with hematoxylin and eosin (Sigma-Aldrich) and examined using a light microscope (Olympus, Tokyo, Japan). Acute pancreatic damage, including pancreatic edema, acinar cell necrosis, adipose necrosis and hemorrhage, parenchymal inflammation, and extravascular infiltration, was evaluated according to Schmidt's criteria<sup>[24]</sup>, with a maximum score of 16. Intestinal epithelial barrier damage, including changes in mucosal cells, mucosal structure, parenchymal hemorrhage, and inflammatory cell infiltration, was semiquantitatively evaluated using the following scale: (0) no injury; (1) local atrophy and necrosis of mucosal cells; (2) patchy shedding of mucosal cells, hemorrhage, inflammatory cell infiltration, and intact mucosal structure; (3) large areas of shedding and necrosis of mucosal cells, inflammatory cell infiltration, and destroyed mucosal structure; and (4) extensive necrosis of mucosal cells, inflammatory cell infiltration, loss of mucosal structure, and patchy hemorrhage. All experiments were performed in duplicate.

### Serum amylase, diamine oxidase, and tumor necrosis factor- $\alpha$ assays

Serum amylase (a biomarker of acute pancreatic damage) concentration was measured using a commercially available biochemical kit (Kehua, Shanghai, China) as instructed by the manufacturer; serum diamine oxidase (DAO) (a biomarker of intestinal barrier dysfunction) concentration was determined using ultraviolet UV spectrophotometer; and serum tumor necrosis factor (TNF)- $\alpha$  (a biomarker of systemic inflammation and acute phase reaction) level was determined using an ELSIA kit (R and D Systems, Minneapolis, MN, United States). All experiments were performed in duplicate.

### Western blotting assay of ZO-1 and E-cadherin

Intestinal epithelial specimens were homogenized in ice-cold lysis buffer (Sigma-Aldrich). The soluble lysate was mixed with the loading buffer containing 40% glycerol (Sigma-Aldrich), 200 mmol/L dithiothreitol (Sigma-Aldrich) and 0.04% bromophenol blue (Kehua, Shanghai, China), and boiled for 5 min. SDS-PAGE (Sigma-Aldrich) was used to separate the proteins, and the electroblotting technique was used to transfer the proteins to a polyvinylidene fluoride membrane. The blots were blocked with 5% bovine serum albumin (Beyotime, Jiangsu, China) at room temperature for 1 h and incubated with primary antibodies against ZO-1 (1:200, polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, United States) and E-cadherin (1:1000, polyclonal; Cell Signaling Technology, Danvers, MA, United States) at 4 °C overnight. The blots were further incubated with horseradish-peroxidase-conjugated secondary antibodies (Abcam, Cambridge, United Kingdom) for 1 h. The Fujifilm chemiluminescence detection system (Fuji, Tokyo, Japan) was used to detect and visualize the immunoblot bands. The band

density of the target protein was quantified using Image J software (<http://rsbweb.nih.gov/ij/>) and normalized to that of  $\beta$ -actin (internal control). All experiments were performed in duplicate.

### miR-155 real-time reverse transcriptase polymerase chain reaction

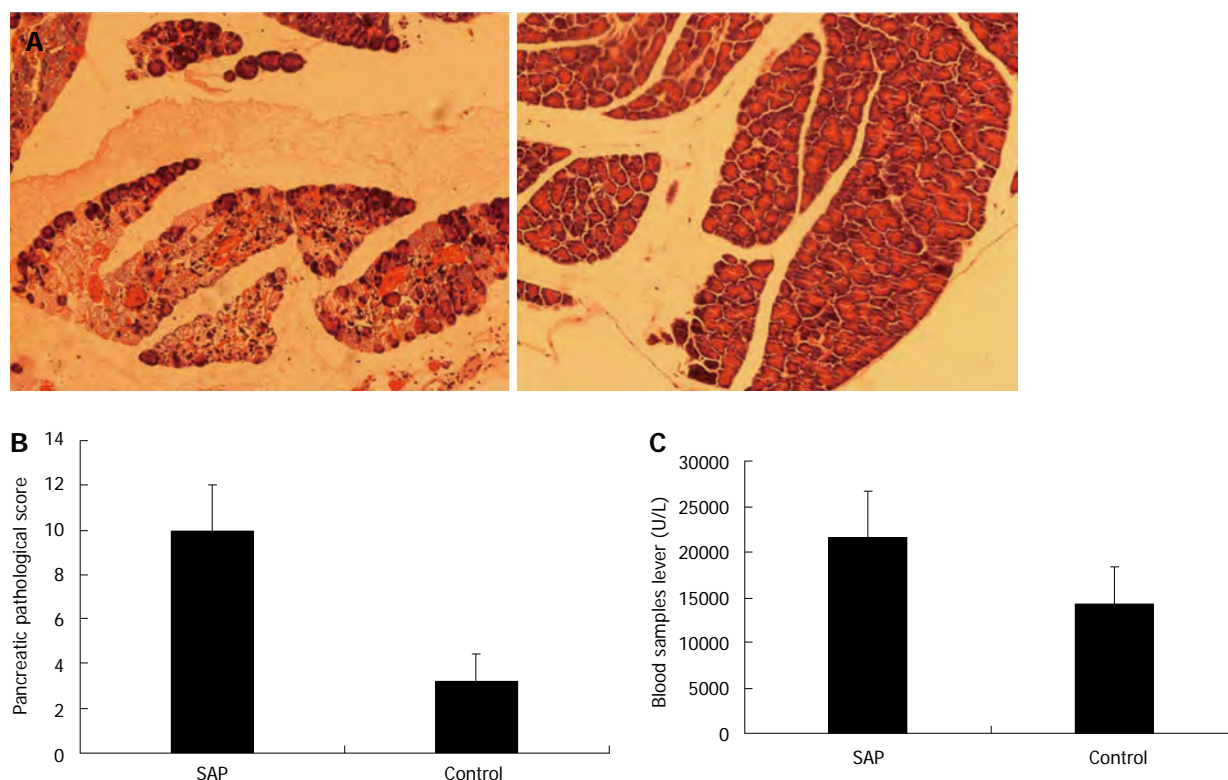
Total RNA samples were isolated from mouse intestinal epithelial specimens using the mirVana miRNA isolation kit (Ambion, Austin, TX, United States). RNA was reversely transcribed into cDNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, United States). miR-155 was detected using stem-loop reverse transcription primers, and U6 served as an internal control. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the TaqMan miRNA assay kit (Applied Biosystems) and quantitated using the 7900HT sequence detection system (Applied Biosystems). The thermal cycling was set as follows: denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 60 °C for 1 min, for a total of 40 cycles. Threshold cycle (Ct) was defined as the fractional cycle number of fluorescence that passed through a given threshold. The  $2^{-\Delta\Delta C_t}$  method was used to determine the relative miR-155 expression level<sup>[25]</sup>. All experiments were performed in duplicate.

### miRNA target gene prediction

Possible target genes for miR-155 were predicted using the miRTarBase database (<http://mirtarbase.mbc.nctu.edu.tw/>), a database curating experimentally validated miRNA-target interactions, and two computational methods, namely, RNA22 (<http://cbcsrv.watson.ibm.com/rna22.html>) and PicTar (<http://pictar.mdc-berlin.de/>). The structural analyses were conducted to confirm the AJC-associated target genes for miR-155. The expression of the possible target gene was validated at the transcriptional and post-transcriptional levels.

### Real-time RT-PCR and Western blotting of Ras homolog gene family, member A

Ras homolog gene family, member A (RhoA) mRNA samples were extracted from intestinal epithelial specimens using the TRIzol reagent (Invitrogen, San Diego, CA, United States) and reverse transcribed into cDNA with the high-capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was determined by qRT-PCR using the SYBR Green PCR Master Mix kit (Applied Biosystems). PCR amplification was performed using the 7900HT sequence detection system (Applied Biosystems), and the thermal cycling was set as follows: denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 75 °C for 1 min, for a total of 40 cycles. All primers were designed using the Primer Premier software (PREMIER Biosoft, Palo Alto, CA, United States) and synthesized by Sangon (Shanghai, China). The sequences of primers were as follows: for RhoA, sense, AGCTTGTGGTAAGACATGCTTG, antisense,



**Figure 1** Pancreas histology and serum amylase level in severe acute pancreatitis and control mice. A: Representative photomicrographs of the pancreas in severe acute pancreatitis (SAP) (left panel) and control (right panel) mice (hematoxylin-eosin,  $\times 100$ ); B: Schmidt's acute pancreatic damage scores in SAP and control mice,  $P < 0.01$  vs control; C: Serum amylase levels in SAP and control mice,  $P < 0.01$  vs control.

GTGTCCCATAAAGCCAACTCTAC; for  $\beta$ -actin (internal control), sense, CCAGGCACCAGGGCGTGATG, antisense, CGGCCAGCCAGGTCCAGACG. The  $2^{-\Delta\Delta Ct}$  method was also used to determine the relative RhoA expression level<sup>[25]</sup>. All experiments were performed in duplicate.

RhoA western blotting assay was performed as mentioned above and using a primary antibody against RhoA (1:2000, polyclonal; Abcam) with  $\beta$ -actin as the internal control.

### Statistical analysis

Statistical analysis was performed using the PASW Statistics version 18.0 software (SPSS Inc., Hong Kong, China). All data were expressed as mean  $\pm$  SD. The means were compared using the two independent samples Student's *t* test, one-way analysis of variance.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Sequential intraperitoneal injection of caerulein and LPS induces experimental SAP

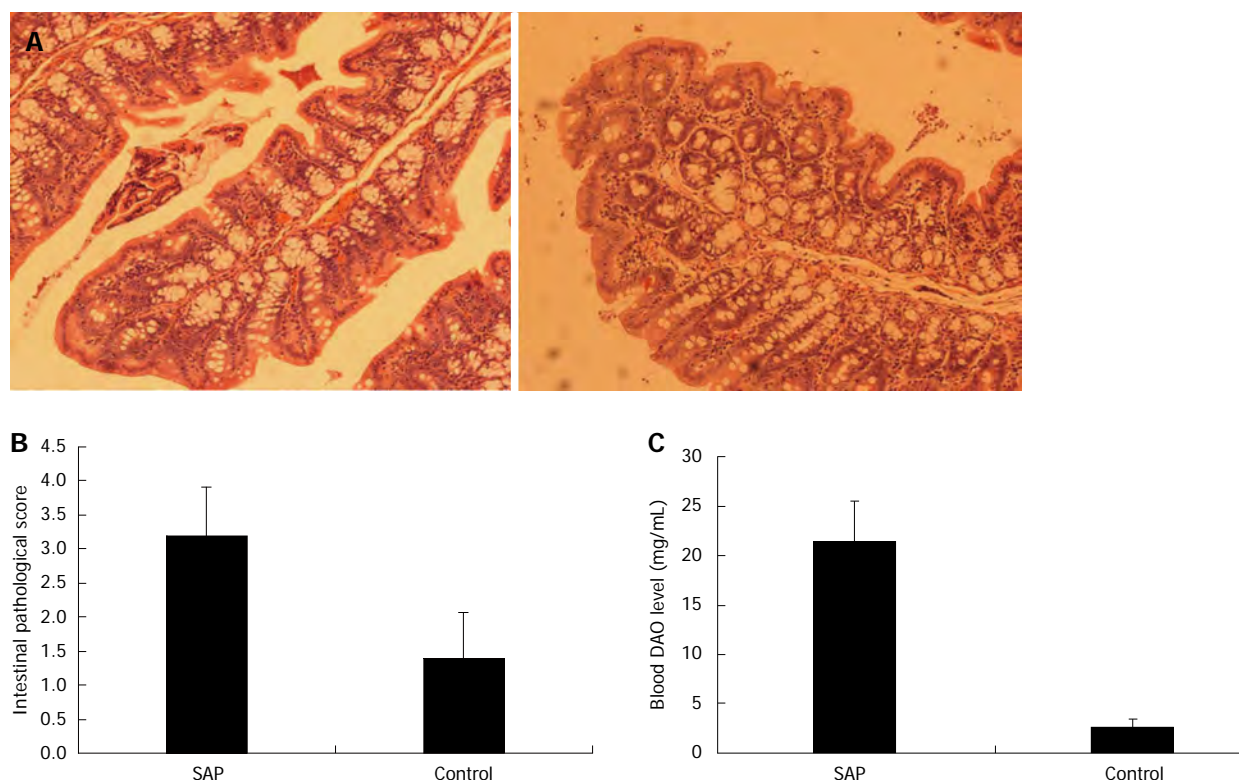
The control group showed no obvious abnormality in the pancreatic tissue on gross and microscopic examination (Figure 1A, right panel). In contrast, the SAP group exhibited patchy hemorrhage and necrosis of the pancreas (Figure 1A, left panel). Moreover, scattered saponification spots on macroscopic examination corresponded to

inflammatory cell infiltration with swollen acinar cells and intercellular space widening on microscopy in the SAP group. The Schmidt's acute pancreatic damage score was significantly higher in the SAP than the control group ( $10.0 \pm 2.0$  vs  $3.2 \pm 1.2$ ,  $P < 0.01$ ; Figure 1B). Serum amylase level was also significantly higher in the SAP than control group ( $21.6 \pm 5.1$  U/mL vs  $14.3 \pm 4.2$  U/mL,  $P < 0.01$ ; Figure 1C). These results showed that sequential intraperitoneal injection of caerulein and LPS successfully induced experimental SAP in mice, manifesting as pancreatic hemorrhage, necrosis, and inflammatory cell infiltration, as well as hyperamylasemia.

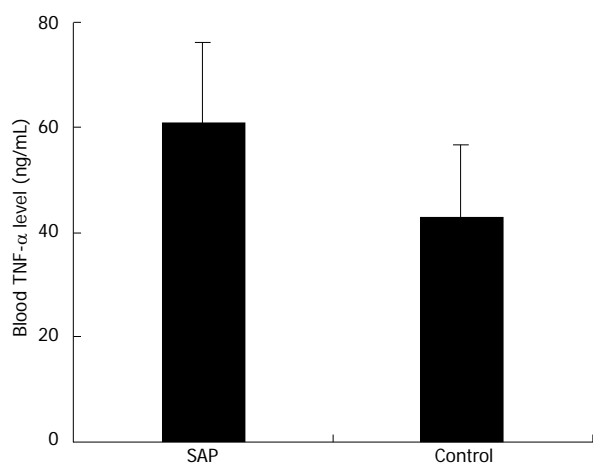
### Caerulein and LPS induces intestinal barrier dysfunction in experimental SAP

Histological examination in the control group showed mild intestinal mucosal congestion and shedding (Figure 2A, right panel), while the SAP group exhibited marked intestinal mucosal congestion and hemorrhage, inflammatory cell infiltration, glandular atrophy, and necrosis and shedding of epithelial cells (Figure 2A). The intestinal epithelial barrier damage score was significantly higher in the SAP than control group ( $3.2 \pm 0.7$  vs  $1.4 \pm 0.7$ ,  $P < 0.01$ ; Figure 2B), and serum DAO level was also significantly higher in the SAP group ( $21.4 \pm 4.1$  mg/mL vs  $2.6 \pm 0.8$  mg/mL,  $P < 0.01$ ; Figure 2C). These results showed that sequential intraperitoneal injection of caerulein and LPS also induced intestinal barrier damage in experimental SAP mice.





**Figure 2** Intestine histology and serum diamine oxidase levels in severe acute pancreatitis and control mice. A: Representative photomicrographs of the intestine in severe acute pancreatitis (SAP) (left panel) and control (right panel) mice (hematoxylin-eosin,  $\times 100$ ); B: Intestinal barrier damage scores in SAP and control mice,  $P < 0.01$  vs control; C: serum diamine oxidase (DAO) levels in SAP and control mice,  $P < 0.01$  vs control.



**Figure 3** Serum tumor necrosis factor- $\alpha$  levels in severe acute pancreatitis and control mice. Serum tumor necrosis factor- $\alpha$  level was measured using ELISA;  $P < 0.01$  vs control. SAP: Severe acute pancreatitis.

### Massive TNF- $\alpha$ release in experimental SAP

Serum TNF- $\alpha$  concentration in the SAP group was significantly higher than in the control group ( $61.0 \pm 15.1$  ng/mL *vs*  $42.9 \pm 13.9$  ng/mL,  $P < 0.01$ ; Figure 3). This showed that a systemic inflammatory response occurred in experimental SAP and resulted in massive release of TNF- $\alpha$ , a major proinflammatory cytokine.

### ZO-1 and E-cadherin are underexpressed in experimental SAP intestinal epithelia

Western blotting analysis showed that the expressions of ZO-1 ( $46 \pm 18$  fold *vs*  $68 \pm 19$  fold,  $P < 0.01$ ; Figure 4A and B) and E-cadherin ( $48 \pm 15$  fold *vs*  $77 \pm 18$  fold,  $P < 0.01$ ; Figure 4C and D) were significantly downregulated in experimental SAP intestinal epithelia as compared to the control group. These results showed that ZO-1 and E-cadherin, two major component proteins of the AJC, were damaged in experimental SAP with intestinal barrier dysfunction.

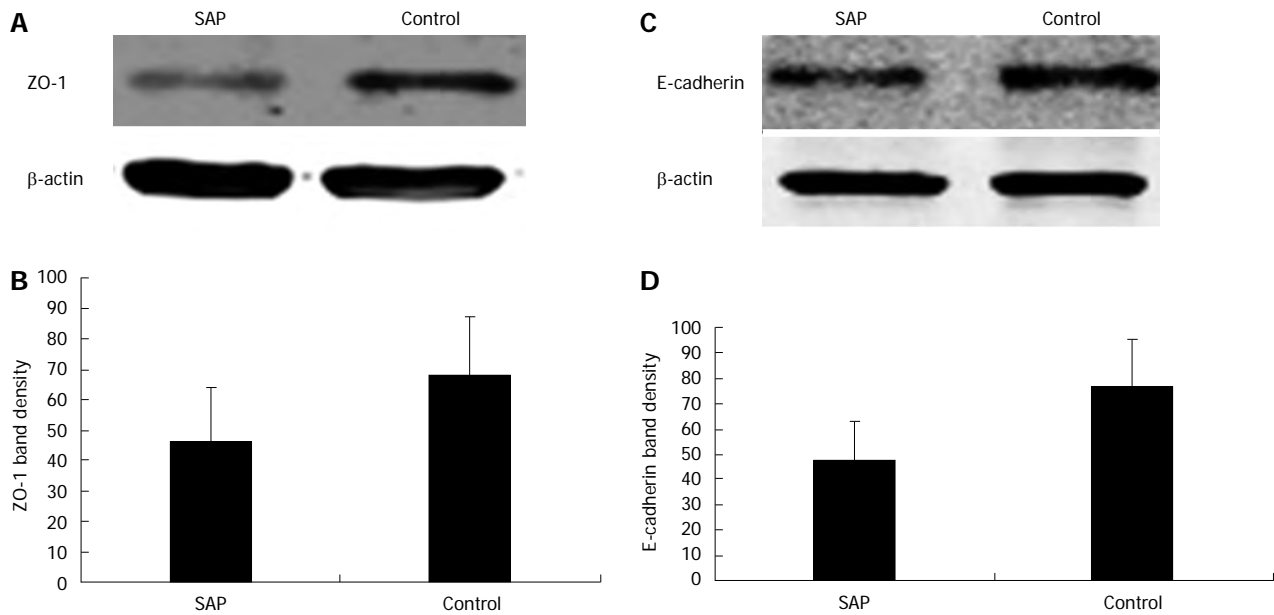
### miR-155 is overexpressed in experimental SAP intestinal epithelia

As shown in Figure 5, miR-155 was significantly overexpressed in intestinal epithelia in the SAP group as compared to the control group ( $1.94 \pm 0.50$  fold *vs*  $1.03 \pm 0.23$  fold,  $P < 0.01$ ). This result showed that miR-155 overexpression might dysregulate AJC protein expression and contribute to intestinal barrier dysfunction in experimental SAP mice.

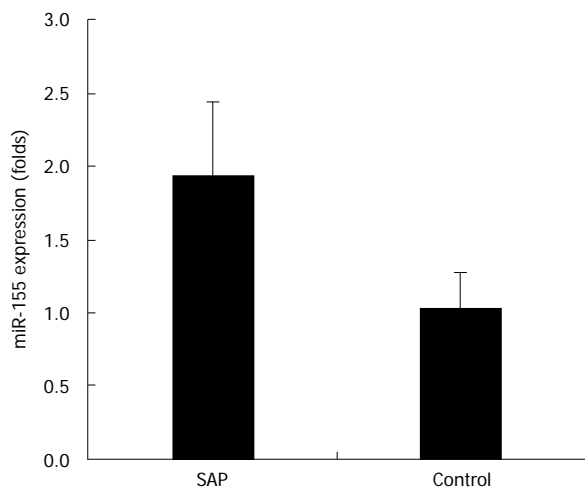
### RhoA is a target gene for miR-155

miRNA regulates its target mRNA by binding to its specific binding site in the 3'-untranslated region (UTR). Prediction with miRTarBase, RNA22, and PicTar ana-



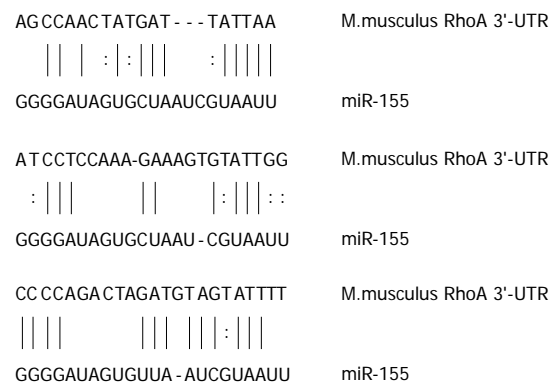


**Figure 4** Expressions of zonula occludens-1 and E-cadherin in the intestinal epithelia of severe acute pancreatitis and control mice. A: Representative western blots showing zonula occludens (ZO)-1 downregulation in SAP mice, with  $\beta$ -actin as an internal control; B: Quantification of ZO-1 expression in SAP and control mice,  $^bP < 0.01$  vs control; C: Representative western blots showing E-cadherin downregulation in SAP mice, with  $\beta$ -actin as an internal control; D: Quantification of E-cadherin expression in SAP and control mice,  $P < 0.01$  vs control. SAP: Severe acute pancreatitis.



**Figure 5** TaqMan miRNA real-time reverse transcriptase polymerase chain reaction for miRNA-155 expression in severe acute pancreatitis and control mice. Quantification of miRNA (miR)-155 expression level was normalized to that of U6 expression;  $P < 0.01$  vs control. SAP: Severe acute pancreatitis.

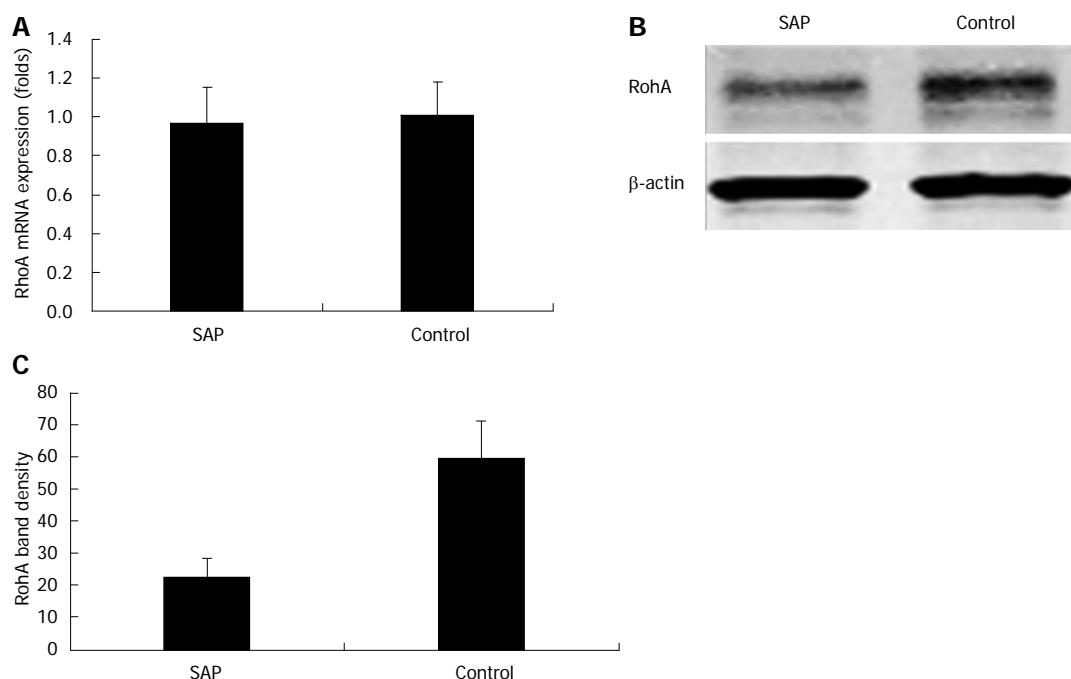
lytical and computational tools showed that RhoA gene encoding a small GTPase protein known to regulate the actin cytoskeleton in the formation of stress fibers and cell division was one of the possible target genes for miR-155. Moreover, structural analysis of miR-155 and RhoA mRNA confirmed that the latter contained three miR-155-specific binding sites in the 3'-UTR (Figure 6). This result showed that miR-155 overexpression might dysregulate AJC protein expression through the RhoA signaling pathway in experimental SAP with intestinal barrier dysfunction.



**Figure 6** Sequence of miRNA-155 and 3'-untranslated region of Ras homolog gene family, member A gene. Ras homolog gene family, member A (RhoA) mRNA contains three miR-155 specific binding sites in the 3'-untranslated region (UTR). Vertical lines and columns indicate the Watson-Crick and wobble base pairing, respectively.

### RhoA is underexpressed in experimental SAP intestinal epithelia

RhoA mRNA expression was not significantly upregulated or downregulated in SAP intestinal epithelia as compared to the control group ( $0.97 \pm 0.18$  fold *vs*  $1.01 \pm 0.17$  fold,  $P > 0.05$ ; Figure 7A). However, western blotting analysis showed that RhoA protein expression was significantly underexpressed in experimental SAP intestinal epithelia ( $22.7 \pm 5.8$  fold *vs*  $59.6 \pm 11.6$  fold,  $P < 0.01$ ; Figure 7B and C). This result suggested that miR-155 overexpression negatively regulated the target gene RhoA expression at the post-transcriptional level, and post-transcriptional RhoA underexpression was implicated in AJC



**Figure 7** Expressions of Ras homolog gene family, member A mRNA and protein in the intestinal epithelia of severe acute pancreatitis and control mice. **A:** Real-time reverse transcriptase polymerase chain reaction showing the relative level of Ras homolog gene family, member A (RhoA) mRNA expression normalized to that of  $\beta$ -actin,  $P > 0.05$  vs control; **B:** Representative western blots showing RhoA protein expression downregulation in severe acute pancreatitis (SAP) mice, with  $\beta$ -actin as an internal control; **C:** Quantification of RhoA protein expression in SAP and control mice,  $P < 0.01$  vs control.

protein dysregulation in experimental SAP with intestinal barrier dysfunction.

## DISCUSSION

Intestinal barrier dysfunction is closely associated with poor prognosis in SAP patients, and is believed to be the primary cause of SAP progression<sup>[26-28]</sup>. It has remained unknown until now how intestinal barrier dysfunction occurs in SAP. To the best of our knowledge, the present work was the first study to report the implication of miR-155 dysregulation in SAP with intestinal barrier dysfunction. Our results showed that a systemic inflammatory response occurred in experimental SAP, as indicated by massive TNF- $\alpha$  release, and miR-155 overexpression was seen in SAP intestinal epithelia with barrier dysfunction. These pathogenetic processes were accompanied with significant underexpression of RhoA, a target gene for miR-155, as well as ZO-1 and E-cadherin, two major component proteins of AJC; all of which contributed to intestinal barrier dysfunction in experimental SAP.

Serum DAO level is regulated by the intestinal mucosal permeability, and is believed to be a reliable biomarker of intestinal barrier function<sup>[29,30]</sup>. Our results showed that serum DAO level increased significantly in experimental SAP. Intestinal barrier dysfunction manifested histologically as necrosis and shedding of epithelial cells in the intestinal mucosal layer as observed in experimental SAP mice. All this biochemical and pathological evidence confirmed that intestinal barrier damage occurred in experimental SAP.

TJs and adherens junctions (AJs) are among the key protein complexes of the AJC<sup>[7,31]</sup>. ZO-1 and E-cadherin

are the major component proteins in TJs and AJs, respectively<sup>[15]</sup>. In TJs, as a cytoskeletal protein, ZO members, including ZO-1, ZO-2, and ZO-3, are linked to the transmembrane protein through its intracellular domains and connected with adjacent epithelial cells *via* its extracellular domains<sup>[32]</sup>. E-cadherin, a transmembrane protein, is connected to adjacent cells *via* its extracellular domains and linked with the cytoskeleton by binding to  $\beta$ -catenin *via* its intracellular domains. These two AJC proteins tightly crosslink epithelial cells in the intestinal mucosa, and maintain the intestinal barrier<sup>[8]</sup>. The AJC is the key structure for maintenance of intestinal barrier function<sup>[4-8]</sup>. The underexpression of ZO-1 and E-cadherin in experimental SAP could result in AJC disruption and consequently compromise intestinal barrier dysfunction.

Our study results showed that miR-155 was significantly overexpressed in experimental SAP intestinal epithelia. miRNA is an endogenous non-coding RNA, at a length of 21-25 nucleotides, which plays a crucial role in the post-transcriptional regulation of gene expression. Moreover, miRNA, especially miR-155, is known to be implicated in regulating the systemic inflammatory response<sup>[33]</sup>, which normally occurs at the early stage of SAP<sup>[17-21]</sup>. Multiple biological functions of miR-155 have been identified, including induction of Toll-like receptor (TLR) activation in monocytes/macrophages, regulation of LPS-induced non-specific immunity, and feedback modulation of the TLR signaling pathway<sup>[22,34]</sup>. More importantly, miR-155 expression can be induced by inflammatory cytokines, such as TNF- $\alpha$  and interferons<sup>[17,22,34-37]</sup>. TNF- $\alpha$ , one of the most important early proinflammatory mediators, is the first factor that is mas-

sively released into the circulation and plays a key role in systemic inflammatory response syndrome<sup>[38,39]</sup>. TNF- $\alpha$  is also destructive for the AJC in other inflammatory diseases. Sasaki *et al*<sup>[40]</sup> reported that the increase in serum TNF- $\alpha$  in inflammatory bowel disease induced by enteric bacteria downregulated expression of ZO-1 protein and E-cadherin and disrupted the AJC. Fries<sup>[41]</sup> found that excessive TNF- $\alpha$  release decreased the protein synthesis of TJs in inflammatory bowel disease, which could be treated with anti-TNF- $\alpha$  therapy by improving TJ protein synthesis. Ma *et al*<sup>[42]</sup> demonstrated that increased serum TNF- $\alpha$  inhibited ZO-1 protein synthesis in Crohn's disease intestinal epithelial cells and consequently resulted in AJC damage. TNF- $\alpha$  release increased in response to systemic inflammation in SAP, which was accompanied by significant overexpression of miR-155 in SAP intestinal epithelia. These results suggested that excessive TNF- $\alpha$  release caused AJC damage by dysregulating miR-155 expression in SAP epithelial cells.

miRNA exerts a regulatory effect by acting on target genes. miRNA binds to argonaute proteins to form the RNA-induced silencing complex, inhibits the transcription of the target genes by acting on its specific binding site in the 3'-UTR<sup>[43]</sup>, and leads to reduced protein synthesis of the target genes<sup>[44]</sup>. The presence of a specific structural pairing between miRNA and its target gene allows for structural analysis to predict the target genes for miRNA. In this study, RhoA was determined to be one of the possible target genes for miR-155. RhoA mRNA contains three miR-155-specific binding sites in the 3'-UTR, which are highly conserved among multiple species, including rats, mice and humans. RhoA, a small GTPase belonging to the Rho family<sup>[45]</sup>, is the major regulator of AJC expression<sup>[46-48]</sup>. RhoA promotes the formation and maintenance of the AJC, and RhoA underexpression leads to disrupted AJC<sup>[49-51]</sup> by blocking protein synthesis of TJs<sup>[52,53]</sup> and AJs<sup>[54]</sup>. Our results showed that RhoA protein rather than mRNA was significantly underexpressed in SAP intestinal epithelia. This indicates that miR-155 overexpression in SAP intestinal epithelia dysregulates RhoA gene expression at the post-transcriptional instead of transcriptional level.

In conclusion, miR-155 plays a crucial role in the structural and functional regulation of the AJC in the intestinal epithelial cells. In SAP, excessive inflammatory response leads to excessive release of inflammatory cytokines, such as TNF- $\alpha$ , and induces miR-155 overexpression in the intestinal epithelia. miR-155 overexpression inhibits RhoA protein synthesis by dysregulating RhoA gene expression at the post-transcriptional level, and consequently downregulates expressions of ZO-1 and E-cadherin; two major component proteins of the AJC. This pathogenetic process eventually results in intestinal barrier dysfunction in SAP.

## COMMENTS

### Background

Intestinal barrier dysfunction is believed to result in endotoxemia and multiple

organ failure, complicating severe acute pancreatitis (SAP). The apical junction complex (AJC) is the mainstay structure that maintains the intestinal barrier and prevents enteric endotoxemia, while AJC damage can lead to intestinal barrier dysfunction. However, the mechanisms underlying AJC damage complicating SAP remain unknown.

### Research frontiers

miRNA (miR) is known to maintain epithelial junctions and polarity, as well as cytoskeletal remodeling, by regulating protein synthesis. miR-dysregulation-induced proinflammatory factors are reported to contribute to AJC disruption in inflammatory bowel disease via an unknown signaling pathway. miR-155 is a major inflammatory mediator emerging at the early stage of the inflammatory process. There is a knowledge gap in the current literature as to whether miR-155 dysregulation is implicated in intestinal barrier dysfunction complicating SAP, and through which signaling pathway dysregulated miR-155 expression interrupts AJC component protein synthesis.

### Innovations and breakthroughs

This successfully created an experimental SAP mouse model with complicating intestinal barrier dysfunction by inducing intraperitoneal inflammatory response, which was validated histologically and biochemically. This study reported for the first time that miR-155 was overexpressed in experimental SAP intestinal epithelia as induced by excess circulating tumor necrosis factor (TNF)- $\alpha$ . RhoA, a small GTPase belonging to the Rho family, is the major regulator of AJC component protein expression. Their target gene prediction test showed that RhoA gene was one of the target genes for miR-155 and was underexpressed in experimental SAP intestinal epithelia at the post-transcriptional level. Downregulated RhoA expression was also accompanied by underexpression of zonula occludens (ZO)-1 and E-cadherin, which are component proteins of tight junctions and adherens junctions - two major constituents of the AJC. Release of proinflammatory factors, such as TNF- $\alpha$ , dysregulates miR-155 expression in intestinal epithelia and consequently disrupts protein synthesis of AJC components, possibly via the TNF- $\alpha$ /miR-155/RhoA/ZO-1/E-cadherin signaling pathway.

### Applications

The results suggest that miR-155 is a key mediator implicated in intestinal barrier dysfunction complicating SAP and other inflammatory gastrointestinal diseases. Inhibitor targeting of the miR-155 signaling pathway may be an effective alternative for treating inflammatory gastrointestinal diseases by repairing intestinal epithelial barrier.

### Terminology

The AJC is a functional unit located at the cell apex and connects the epithelial cells, which is composed of tight junctions and zonula adherens, and desmosomes, and functions to regulate cell polarity, tissue integrity, and intercellular adhesion and permeability. miR-155 is encoded by miR-155 host gene and plays a critical role in regulating inflammatory responses implicated in inflammatory, infectious, and neoplastic disorders. miRTarBase is a computational bioinformatics database accumulating > 3000 miR-target interactions, which are collected by manual survey of pertinent literature through data mining of the text and systematically filtering miR functional studies, and miR-target interactions are normally validated by experimental reporter genes, Western blotting, and microarray and gene sequencing analyses.

### Peer review

The authors demonstrated evidence of SAP. miR-155 expression increased compared to the controls and was associated with a decrease in ZO-1, E-cadherin, and RhoA expression. The authors conclude that these results suggest that SAP decreases intestinal barrier function by decreasing synthesis of key junctional complex proteins via miR-155 signaling.

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## Cooperative inhibitory effect of sinomenine combined with 5-fluorouracil on esophageal carcinoma

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### Abstract

**AIM:** To investigate the inhibitory effects of sinomenine (SIN) combined with 5-fluorouracil (5-FU) on esophageal carcinoma *in vitro* and *in vivo*.

**METHODS:** Esophageal carcinoma (Eca-109) cells were cultured in DMEM. The single or combined growth inhibition effects of SIN and 5-FU on the Eca-109 cells were examined by measuring the absorbance of CCK-8 dye in living cells. Hoechst 33258 staining and an Annexin V/PI apoptosis kit were used to detect the percentage of cells undergoing apoptosis. Western blotting was used to investigate the essential mechanism underlying SIN and 5-FU-induced apoptosis. SIN at 25 mg/kg and 5-FU at 12 mg/kg every 3 d, either combined or alone, was injected into nude mice and tumor growth inhibition and side effects of the drug treatment were observed.

**RESULTS:** SIN and 5-FU, both in combination and individually, significantly inhibited the proliferation of

Eca-109 cells and induced obvious apoptosis. Furthermore, the combined effects were greater than those of the individual agents ( $P < 0.05$ ). Annexin V/PI staining and Hoechst 33258 staining both indicated that the percentage of apoptotic cells induced by SIN and 5-FU combined or alone were significantly different from the control ( $P < 0.05$ ). The up-regulation of Bax and down-regulation of Bcl-2 showed that the essential mechanism of apoptosis induced by SIN and 5-FU occurs *via* the mitochondrial pathway. SIN and 5-FU alone significantly inhibited the growth of tumor xenografts *in vivo*, and the combined inhibition rate was even higher ( $P < 0.05$ ). During the course of chemotherapy, no obvious side effects were observed in the liver or kidneys.

**CONCLUSION:** The combined effects of SIN and 5-FU on esophageal carcinoma were superior to those of the individual compounds, and the drug combination did not increase the side effects of chemotherapy.

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**Key words:** Esophageal carcinoma; Chemotherapy; Sinomenine; 5-Fluorouracil

**Core tip:** The cooperative inhibitory effects of sinomenine (SIN) and 5-fluorouracil (5-FU) on esophageal carcinoma *in vitro* and *in vivo* were investigated. SIN and 5-FU alone or in combination significantly inhibited the proliferation and induced apoptosis of esophageal carcinoma cells in a dose-dependent manner. The essential mechanism underlying SIN and 5-FU-induced apoptosis, as investigated by Western blotting, involved the mitochondrial pathway. No obvious side effects were observed in the liver and kidneys of nude mice. These results indicated that the combined effects of SIN and 5-FU on esophageal carcinoma were superior to the effects of the individual compounds, without an increase in side effects.

Wang J, Yang ZR, Dong WG, Zhang JX, Guo XF, Song J, Qiu S. Cooperative inhibitory effect of sinomenine combined with 5-fluorouracil on esophageal carcinoma. *World J Gastroenterol* 2013; 19(45): 8292-8300 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i45/8292.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8292>

## INTRODUCTION

Esophageal carcinoma is one of the most refractory and common malignant diseases worldwide and is associated with poor disease outcomes<sup>[1,2]</sup>. An estimated 482300 new esophageal cancer cases and 406800 deaths occurred globally in 2008<sup>[2]</sup>. Generally, the primary tumor of most patients can be cured with surgical resection, however, due to early distant metastases, the remainder of patients will eventually succumb to the disease. Despite the advances in surgical methods combined with perioperative treatment that have led to improved prognoses, the overall mortality rate remains low due to early distant metastases<sup>[3-5]</sup>. Systemic chemotherapy is regarded as one of the most effective treatments to improve survival. Although chemotherapy forms an important part of the multidisciplinary treatment approach for metastatic disease, the toxicity of the agents to normal tissues has been the main obstacle to successful treatment. Therefore, to enhance efficacy and reduce toxicity, combined treatments of several chemotherapy regimens are often used.

5-Fluorouracil (5-FU) is universally used as an anti-cancer agent in esophageal carcinoma. Cisplatin and fluorouracil combination therapy has been a standard choice for treating esophageal cancer, for which the median survival time is 9.2 mo for responders and 5.3 mo for non-responders, with a resulting 1-year survival rate of approximately 27.8%-37.6%<sup>[6-8]</sup>. To improve the prognosis of patients with esophageal cancer, more effective novel regimens with high therapeutic effect are urgently needed.

Sinomenine (SIN) is an immunosuppressive compound extracted from the Chinese medicinal plant *Sinomenium acutum* which has been successfully used in Chinese folk medicine to treat various autoimmune diseases for centuries<sup>[9]</sup>. Previous studies have indicated that SIN exhibits a wide range of significant pharmacological activities, including anti-inflammatory, anti-rheumatic, anti-arrhythmic, anti-angiogenesis, analgesic and immunosuppressive effects<sup>[10-12]</sup>. Lu *et al.*<sup>[13]</sup> observed that SIN can promote cell cycle arrest in the G1 phase, which was associated with increased p21/WAP/Cip expression. Additionally, SIN induces caspase-dependent apoptosis, which is involved in the disruption of mitochondrial membrane potential. SIN can also significantly inhibit basic fibroblast growth factor (bFGF)-induced angiogenesis, an effect that may render the compound a prime candidate for possible anti-cancer agents<sup>[12]</sup>.

The aim of this study was to evaluate the effects of SIN combined with 5-FU in terms of their activity

against esophageal carcinoma *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Chemicals and cell culture

SIN (C<sub>19</sub>H<sub>23</sub>NO<sub>4</sub> · 0.3 CHCl<sub>3</sub>) and 5-FU (C<sub>4</sub>H<sub>3</sub>FN<sub>2</sub>O<sub>2</sub>) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States) and dissolved at 100 mmol/L in dimethylsulfoxide (DMSO) for storage at -20 °C. The esophageal carcinoma cell line Eca-109 was obtained from China Center for Type Culture Collection. The growth medium consisted of DMEM (Gibco BRL Gaithersburg, MD, United States) containing 10% fetal bovine serum. Cell cultures were maintained at 37 °C in a 95% humidified atmosphere of 5% CO<sub>2</sub> in air.

### Cell proliferation assay

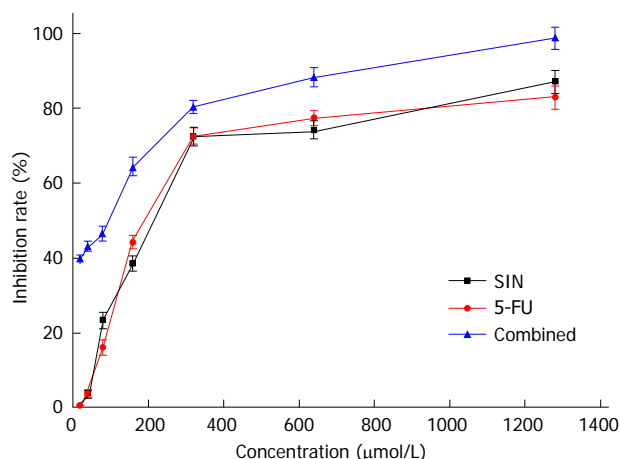
Cell proliferation *in vitro* was determined using the WST-8 Cell Counting Kit-8 (Beyotime Biotechnology, Jiangsu, China) as previously described<sup>[14]</sup>. Generally, cells were seeded in 96-well micro plates at a density of  $5 \times 10^3$ /well in 0.1 mL growth medium. After exposure to different concentrations of SIN (40, 80, 160, 320, 640 μmol/L), 5-FU (40, 80, 160, 320, 640 μmol/L) or SIN + 5-FU for 48 h, 10 μL CCK-8 solution was added to each well. The plates were then incubated for an additional 2 h at 37 °C. The optical density at 450 nm was measured by a microplate reader (BIO-RAD iMark). The procedure was performed in triplicate.

### Annexin V/PI staining for apoptosis

The amount of phosphatidylserine exposed on the extracellular membrane of the apoptotic cells was quantified by the Annexin V-FITC kit. Following incubation with SIN (160 μmol/L) and 5-FU (160 μmol/L) alone or combined for 48 h, adherent cells were harvested by mild trypsinization, washed twice with cold PBS and resuspended in 500 μL binding buffer. After adding 5 μL Annexin V-FITC conjugate and 10 μL Propidium Iodide (PI), the cells were incubated for 15 min at room temperature in the dark. Flow cytometric analysis was performed immediately with FACSCalibur using the CellQuest software.

### Hoechst 33258 assay for apoptosis

The morphological features of apoptotic cells were detected by Hoechst 33258 staining following the manufacturer's protocol (Beyotime Biotechnology, Jiangsu, China). Cells were seeded on sterile cover glasses placed in the 6-well plates. After exposure to SIN (160 μmol/L), 5-FU (160 μmol/L) alone or combined for 48 h, the cells were fixed, washed and stained with 0.5 mL Hoechst 33258 staining solution for 5 min at room temperature in the dark. Then washed twice with PBS and stained nuclei were scored and categorized according to the condensation and staining characteristics of chromatin under a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan). Ten random fields per dish were observed and



**Figure 1** The individual and combined effects of sinomenine and 5-Fluorouracil on the proliferation of esophageal carcinoma cells. The proliferation of Esophageal carcinoma (Eca-109) cells was inhibited by SIN and 5-FU in a dose dependent manner ( $P < 0.05$ ). SIN and 5-FU had significant synergistic effects on inhibiting the proliferation of Eca-109 cells especially at lower concentrations. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

counted.

### Western blot analysis

Whole protein extracts were prepared and analyzed by western blotting using a wet transfer system (Bio-Rad, Hercules, CA, United States). Protein samples (20 μg) were resolved by electrophoresis of SDS-polyacrylamide gels and electro-transferred to nitrocellulose membranes. Transfer efficiency and homogeneous loading was assessed by Ponceau stain. Membranes were blocked for 1 h in 10% non-fat milk, incubated with titrated primary antibody overnight at 4 °C and then labeled with the horseradish peroxidase conjugated secondary antibody for 2 h at ambient temperature. The following antibodies were used: Bax, Bcl-2, GAPDH (Santa Cruz Biotechnology, CA, United States) 1:1000. Antibody reaction was revealed using an enhanced chemiluminescence system (Millipore, Bedford, MA, United States) and then exposed with Kodak X-ray film. Protein band intensity was determined using the CMIASWIN video imaging system (Bio-Rad, Hercules, CA, United States).

### Xenograft tumor model

Animal experiments, which complied with the NIH Guide for the Care and Use of Laboratory Animals, were approved by the committee on Animal Experiment of Wuhan University. Esophageal carcinoma tumors were established in male BALB/c nude mice (4-6 wk, Laboratory Animal Central of Wuhan University) by serial subcutaneous transplantation of Eca-109 cells ( $1 \times 10^7$  cells/mouse). Eighteen mice bearing xenografted tumors were obtained and a further six mice inoculated with saline acted as controls. When the tumors reached approximately 100 mm<sup>3</sup> in size, the mice were divided into four groups ( $P < 0.05$ ): SIN group (25 mg/kg), 5-FU group (12 mg/kg), combination group, and saline control group. Treatment was ad-

ministered *via* intratumoral injection every 3 d. Tumor size was measured 2-3 times per week in two dimensions by calipers, and the volumes were calculated using the following formula: volume (mm<sup>3</sup>) = [length × (width)<sup>2</sup> × π/6].

### Hematoxylin and eosin staining and TUNEL assay

For histologic analysis, tumor tissues were prepared according to a standard protocol, and the paraffin-embedded tumor tissue sections were subsequently stained with hematoxylin and eosin (HE). For TUNEL assay, an in situ apoptosis detection kit (Roche, Branchburg, NJ, United States) was used to detect apoptotic cells in tumor tissue sections. The apoptotic cells were identified, counted (nine random fields per slide), and analyzed under light microscopy (Olympus, Japan).

### Evaluation of side effects

Blood samples were collected by cardiac puncture. The biomarkers of liver and renal injury, such as the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and serum creatinine (Cr), were detected using an OLYMPUS AU5400 analyzer (OLYMPUS, Japan).

### Statistical analysis

All data were expressed as the mean ± SE of the mean, and then subjected to the unpaired Student's *t* test. Statistical significance was defined as a value of  $P < 0.05$ .

## RESULTS

### Inhibition of Eca-109 cell proliferation by SIN and 5-FU

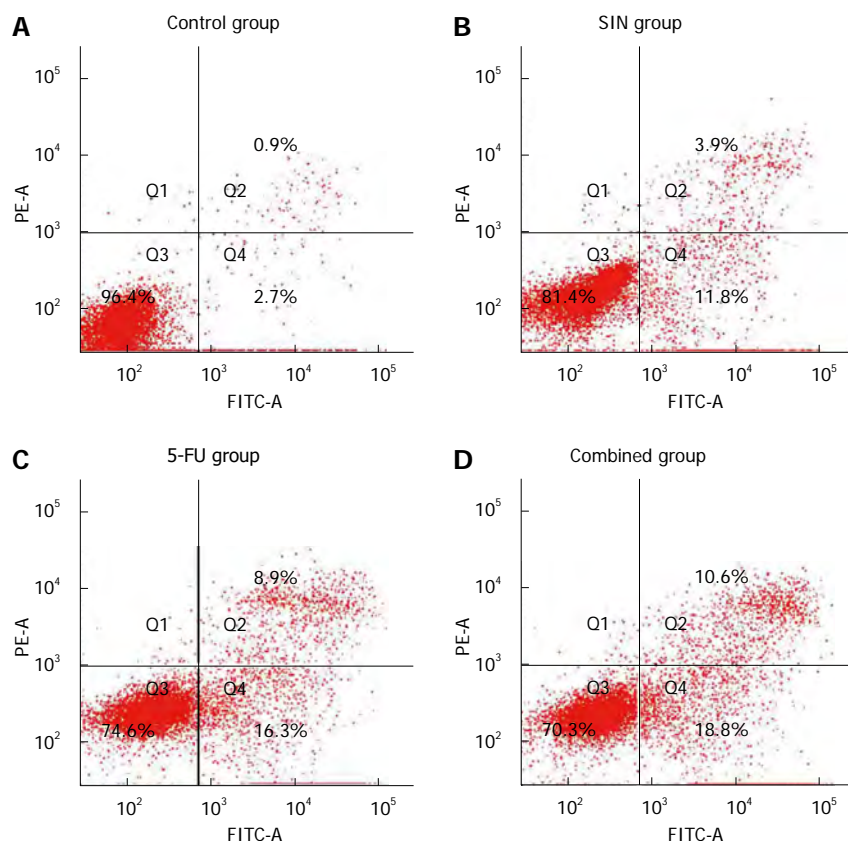
The inhibition of Eca-109 cell proliferation by SIN and 5-FU was assessed by the WSK-8 cell viability assay after 48 h of drug exposure, following 24 h culture in a drug-free medium. As shown in Figure 1, the growth of Eca-109 cells was significantly inhibited in a dose-dependent manner ( $P < 0.05$ ). Significant differences were observed for each concentration of the combined treatment (SIN:5-FU ratio was 1:1) compared to SIN or 5-FU given individually ( $P < 0.05$ ), especially at lower concentrations.

### Apoptosis induced by SIN and 5-FU

Apoptosis induced by SIN and 5-FU was confirmed by Annexin V/PI staining to quantify the amount of phosphatidylserine exposed on the apoptotic cellular membrane. As shown in Figure 2, the percentage of Annexin V-positive/PI-negative cells increased progressively in Eca-109 cells incubated at low concentrations of SIN (160 μmol/L) and 5-FU (160 μmol/L) for 48 h. SIN and 5-FU alone significantly induced apoptosis, as compared with the control group ( $P < 0.05$ ); furthermore, the combined treatment effect was stronger than that of SIN and 5-FU given individually ( $P < 0.05$ , Figure 2).

The morphological features of apoptotic cells were detected by Hoechst 33258 staining. The apoptotic nuclei were assessed by changes revealed by Hoechst staining and were identified by condensed chromatin as well as





**Figure 2** Quantitative flow cytometric measurements of G<sub>2</sub>/M arrest and apoptosis in esophageal carcinoma cells treated with sinomenine and 5-Fluorouracil alone or in combination. The density plots illustrate four cell populations (live, apoptotic, necrotic, and late apoptotic/dead) defined by their fluorescence profiles. SIN and 5-FU alone significantly induced apoptosis compared with the group control ( $P < 0.05$ ), and the combined effects were stronger than those of SIN or 5-FU alone ( $P < 0.05$ ). SIN: Sinomenine; 5-FU: 5-Fluorouracil.

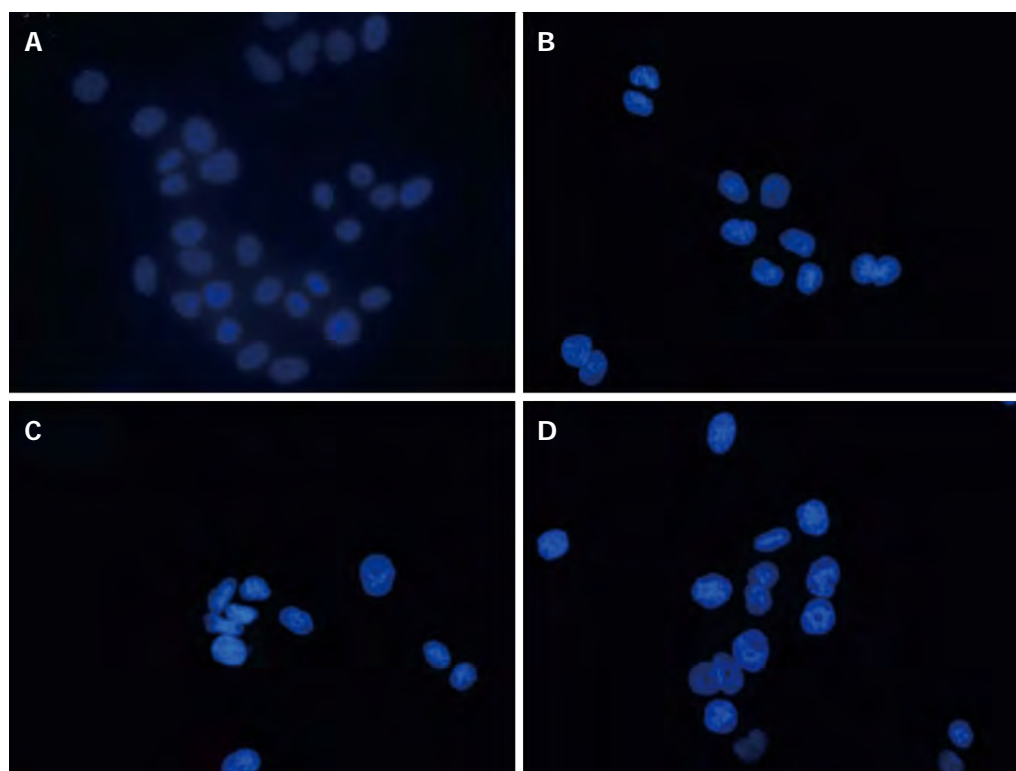
nuclear fragmentation with formation of apoptotic bodies. Ten random fields per dish were observed and counted under a fluorescence microscope. The mean values are expressed as the percentage of apoptotic nuclei per field. Significant changes were detected in the number of apoptotic cells, and the percentage of apoptotic cells induced by SIN and 5-FU combined or alone were significant when compared with the group control ( $P < 0.05$ , Figure 3); furthermore, the apoptotic rate of the combined treatment was greater than those of the individual treatments ( $P < 0.05$ , Figure 3).

#### **SIN and 5-FU induce apoptosis through activation of the mitochondrial pathway**

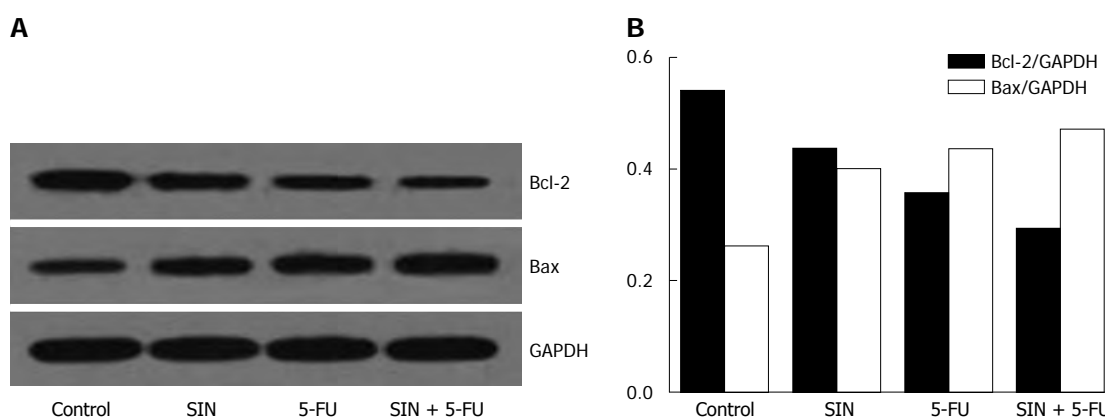
To further investigate the essential mechanism underlying SIN- and 5-FU-induced apoptosis, their effects on the mitochondrial pathway were examined. As shown in Figure 4, SIN (160  $\mu\text{mol/L}$ ) and 5-FU (160  $\mu\text{mol/L}$ ) treatment combined or alone caused an increase in Bax/GAPDH protein levels and a decrease in Bcl-2/GAPDH levels in Eca-109 cells, which led to a decrease in the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio. The apoptotic effect in the combined treatment group was significantly greater than that of the individual treatment groups.

#### **Effect of SIN and 5-FU on tumor development in nude mice**

The effects of SIN and 5-FU on the growth of primary tumor xenografts in nude mice were examined. None of the mice died during the course of the experiment, and all 24 mice successfully grew tumor xenografts. After 14 d growth, the tumor xenografts reached a mean size of 100  $\text{mm}^3$ . The mice were then randomly divided into four treatment groups, and no significant differences were observed in tumor size in the different groups at the start of the study. The results demonstrated that the tumor volumes and weights in the treatment groups were both significantly reduced compared with the saline control group ( $P < 0.05$ , Tables 1 and 2), while the extent of tumor reduction differed for each group. The combination treatment induced greater tumor growth suppression than did SIN or 5-FU alone ( $P < 0.05$ , Table 1), and a difference in tumor weight was also observed between groups ( $P < 0.05$ , Table 2). When compared with the control group, the tumor volume inhibition rate for the combination group was 91.22%, whereas the inhibition rates for the SIN and 5-FU groups were 64.68% and 71.68%, respectively (Table 1). When compared with the control group, the tumor weight inhibition rate for the combination group was 83.38%, whereas the inhibition



**Figure 3** Apoptosis of esophageal carcinoma induced by sinomenine and 5-Fluorouracil alone or in combination. A: Control group; B: SIN group; C: 5-FU group; D: Combined group; apoptotic features were assessed by observing chromatin condensation and fragment staining. SIN: Sinomenine; 5-FU: 5-Fluorouracil.



**Figure 4** Sinomenine and 5-Fluorouracil alone or in combination induced apoptosis in esophageal carcinoma cells through the mitochondrial pathway. Western blotting analysis results of Bcl-2 and Bax protein after SIN (160  $\mu\text{mol/L}$ ) and 5-FU (160  $\mu\text{mol/L}$ ) treatment. GAPDH was used as a loading control. SIN: Sinomenine; 5-FU: 5-Fluorouracil; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

rates for the SIN and 5-FU groups were 63.10% and 61.97%, respectively (Table 2). The results suggest that SIN combined with 5-FU exhibits significant anti-tumor potential *in vivo*.

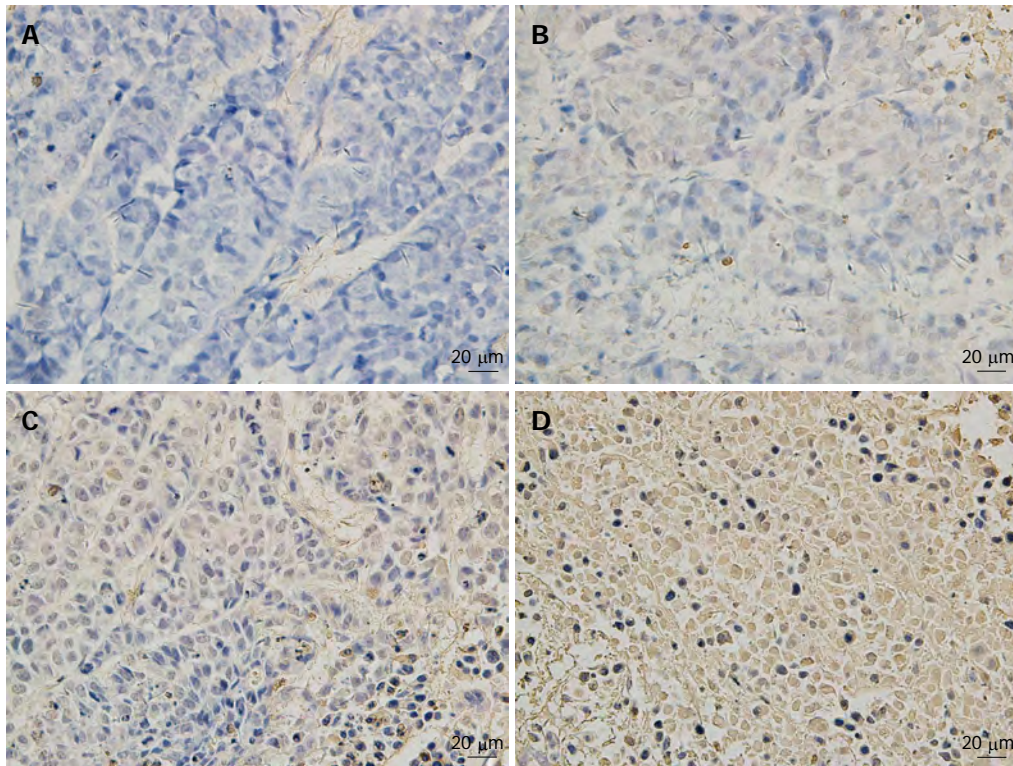
HE staining and TUNEL analysis of the subcutaneous primary tumor sections indicated that SIN and 5-FU individually or in combination induced significant apoptosis of Eca-109 cells *in vivo*, as compared with the saline control group ( $P < 0.05$ , Figure 5), although the degree of apoptosis differed for each group; the apoptotic rate in the combination treatment group was greater than that in the SIN alone and 5-FU alone groups.

The above data indicated that the effects of SIN combined with 5-FU against esophageal carcinoma *in vivo* were superior to that of SIN and 5-FU used individually.

#### Evaluation of side effects

At the end of the experiment, the nude mice were sacrificed and necropsied. No obvious metastasis, hemorrhage, or injury of the liver and kidneys was observed by visual examination.

Blood samples collected by cardiac puncture were used to monitor hepatic and renal toxicity. As biomarkers of liver and renal injury, the activity of ALT and AST,



**Figure 5** Detection of apoptotic cells in xenograft tumor tissue by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. A: Control group; B: SIN group; C: 5-FU group; D: Combined group; SIN and 5-FU induced significant apoptosis of esophageal carcinoma cells *in vivo* compared with the saline control group ( $P < 0.05$ ), and the apoptotic rate of the combination treatment group was more significant than that of the SIN alone and 5-FU alone groups. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

**Table 1** Inhibitory effect of sinomenine and 5-Fluorouracil on esophageal carcinoma tumor volume in nude mice

Group	<i>n</i>	Volume (mm <sup>3</sup> )	Inhibition rate (%)
SIN	6	161.94 (40.96) <sup>bd</sup>	64.68
5-FU	6	129.82 (16.39) <sup>bd</sup>	71.68
SIN + 5-FU	6	40.27 (5.55) <sup>d</sup>	91.22
Control	6	540.20 (66.04)	

Data presented as mean (SD) and are expressed as inhibition rate (%) = (1 - mean of tumor volume of tests/mean of tumor volume of control) × 100%. There were 6 mice per group. <sup>b</sup> $P < 0.05$ , significantly different from SIN + 5-FU group; <sup>d</sup> $P < 0.05$ , significantly different from control group. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

**Table 2** Inhibitory effect of sinomenine and 5-Fluorouracil on esophageal carcinoma tumor weight in nude mice

Group	<i>n</i>	Weight (g)	Inhibition rate (%)
SIN	6	0.218 (0.061) <sup>bd</sup>	63.10
5-FU	6	0.225 (0.088) <sup>bd</sup>	61.97
SIN + 5-FU	6	0.098 (0.066) <sup>d</sup>	83.38
Control	6	0.593 (0.070)	

Data presented as mean (SD) and are expressed as inhibition rate (%) = (1 - mean of tumor weight of tests/mean of tumor weight of control) × 100%. There were 6 mice per group. <sup>b</sup> $P < 0.05$ , significantly different from SIN + 5-FU group; <sup>d</sup> $P < 0.05$ , significantly different from control group. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

as well as the urea and Cr values, were determined to evaluate potential toxicity. The hepatic and renal toxicity induced by SIN and 5-FU alone or in combination is shown in Table 3. Compared to the control group, there were no significant increases in the values of ALT, AST, urea, and Cr ( $P > 0.05$ , Table 3), and no significant differences were observed between the SIN alone, 5-FU alone and combination treatment groups ( $P > 0.05$ , Table 3). At necropsy, the livers and kidneys of the mice from the different treatment groups appeared smooth and normal in color. There was no significant difference in the liver and renal volume or weight between the treated groups and the control group, and the histological pathology examination showed no obvious lesions.

## DISCUSSION

Esophageal carcinoma is one of the most aggressive malignancies and remains a major public health threat globally. In 2008, the number of new esophageal cancer cases was estimated to be 482300 which accounts for 3.8% of all cancers, while the number of deaths was 406800 which accounts for approximately 5.4% of global cancer mortalities<sup>[2]</sup>. More effective treatments, as well as methods for earlier diagnosis, have led to improved survival over recent decades. However, patients with esophageal cancer still exhibit rapid progression and poor prognosis, with a natural disease history of 6-8 mo and a 5-year survival rate of 5%-7%<sup>[15]</sup>, owing to extensive local inva-



**Table 3** Effect of sinomenine combined with 5-Fluorouracil or alone on hepatic and renal function

Group	ALT (U/L)	AST (U/L)	Urea ( $\mu$ mol/L)	Cr ( $\mu$ mol/L)
SIN	45.50 (7.37)	143.67 (28.82)	8.18 (2.04)	16.63 (3.41)
5-FU	45.33 (6.02)	145.33 (18.53)	8.04 (1.20)	15.73 (4.30)
SIN + 5-FU	46.17 (7.17)	146.83 (19.55)	8.28 (1.44)	16.68 (2.68)
Tumor control	45.83 (3.76)	144.67 (17.32)	7.98 (0.70)	16.74 (4.46)
Normal control	46.00 (5.00)	145.17 (11.27)	8.09 (1.03)	16.29 (4.51)

Data presented as mean (SD), with  $n = 6$  mice/group. Groups were treated as follows: SIN (25 mg/kg); 5-FU (12 mg/kg); SIN (25 mg/kg) + 5-FU (12 mg/kg); Tumor control (saline of equal volume); Normal control. No differences were observed in ALT, AST, Urea, and Cr among the groups ( $P > 0.05$ ). SIN: Sinomenine; 5-FU: 5-Fluorouracil; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Cr: Serum creatinine.

sion, lymph node involvement and distant metastases at the time of diagnosis<sup>[16]</sup>. Consequently, more effective chemotherapies have become an important means of extending the survival of esophageal cancer patients.

5-FU is widely used in chemotherapeutic regimens, including those for cancers of the gastrointestinal tract and breast. Although 5-FU-based chemotherapy improves the overall survival of patients with esophageal carcinoma, the response rate is extremely low, and even the combination of 5-FU with newer chemotherapies such as cisplatin and doxorubicin only generates response rates of 25%-35%, with 1-year survival rates of 27%-37%<sup>[6,7]</sup>. To improve the prognosis of patients with esophageal cancer, researchers have undertaken the investigation of novel drugs and combinations of chemotherapeutics.

Over the last few years, interest in exploring the use of traditional medicines for the prevention or treatment of tumors has increased. SIN is an alkaloid derived from the stem of the Chinese medical plant *Sinomenium acutum*, which has been used in traditional Chinese medicine for over 2000 years to treat various rheumatoid diseases with minimal side effects<sup>[17,18]</sup>. The chemical structure of SIN has been clarified, and its potential value in treating rheumatoid arthritis has been recognized by Western medicine. Many studies have indicated that SIN has a wide range of significant pharmacological actions, such as anti-inflammatory, anti-arrhythmic, anti-angiogenesis and immunosuppressive effects. Previous studies have demonstrated that inflammation can affect the angiogenesis and invasion of various types of tumors<sup>[19,20]</sup>. Kok *et al.*<sup>[12]</sup> found out that SIN possesses anti-angiogenic activity in several systems both *in vitro* and *in vivo*. Similarly, in a recent study, SIN was demonstrated to induce apoptosis by modulating NF- $\kappa$ B expression and activity<sup>[21]</sup>, and was able to significantly inhibit NF- $\kappa$ B activity even at  $> 10$  ng/mL<sup>[13]</sup>. Based on these results, SIN was hypothesized to enhance the sensitivity of various cancers to anti-cancer drugs.

In the present study, we investigated the inhibitory effects of SIN combined with 5-FU treatment on esophageal carcinoma *in vitro* and *in vivo*. We found that SIN and 5-FU alone can significantly inhibit the proliferation of

Eca-109 cells in a dose-dependent manner ( $P < 0.05$ ). In addition, the combined effect of SIN and 5-FU on the proliferation of human esophageal carcinoma was superior to that of SIN or 5-FU alone *in vitro* and *in vivo*. We also examined the apoptotic effect induced by SIN combined with 5-FU or administered individually. The results indicated that SIN and 5-FU alone significantly induced apoptosis compared with the control, and that the combined treatment effects were stronger than the individual effects of SIN and 5-FU.

SIN, as a typical anti-arrhythmic drug, has also been reported to inhibit the proliferation and induce apoptosis in various tumors<sup>[22]</sup>. However, the exact mechanisms underlying this apoptotic effect are poorly understood. Apoptosis is a tightly controlled type of cell death, with characteristic effects such as cell shrinkage, membrane blebbing and DNA fragmentation. The signals for apoptosis can be either initiated extrinsically through the death receptor pathway or intrinsically through the mitochondrial pathway<sup>[23,24]</sup>. Mitochondria are considered to play a pivotal role in apoptosis. The mitochondrial pathway has been shown to be an important signaling pathway for apoptosis, and SIN has been proven to utilize this pathway to induce the apoptosis of cancer cells<sup>[25-27]</sup>. 5-FU is well known to inhibit the thymic pyrimidine nucleotidase of tumor cells and affect DNA stability<sup>[28]</sup>. Moreover, 5-FU has been demonstrated to induce the apoptosis of various cancer cells such as breast and colon cancer, with resulting changes in p53<sup>[29]</sup>, caspase-3<sup>[30]</sup> and caspase-8<sup>[31]</sup>. In this study, we confirm that SIN combined with 5-FU treatment can induce apoptosis through the mitochondrial pathway.

A mouse tumor xenograft model was established, and the animals were administered chemotherapy consisting of SIN (25 mg/kg) and 5-FU (12 mg/kg) every 3 d. Similar effects on the proliferation and apoptosis of esophageal cancer cells induced by SIN and 5-FU were also observed *in vivo*. No adverse effects such as gastroenterol disturbance, hemorrhage or kidney dysfunction were observed. The model was also used to evaluate the potential histological pathology and hepatic and renal toxicity resulting from treatment, with no significant differences found between the treatment and control groups. Thus, we conclude that SIN combined with 5-FU demonstrates an anti-cancer effect with no increase in side effects *in vivo*.

In conclusion, SIN combined with 5-FU exhibited a significantly superior anti-cancer effect in comparison to SIN or 5-FU alone. In addition, this study demonstrated that SIN and 5-FU did not increase toxicity *in vivo* when used in combination. Above all, the present study indicates the potential for the combined use of SIN and 5-FU in clinical treatment.

## ACKNOWLEDGMENTS

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for assistance in data collection.

## COMMENTS

### Background

Esophageal carcinoma is one of the most refractory and common malignant diseases and is associated with poor outcome. To enhance the effect of systemic chemotherapy and reduce the toxicity, combined treatments with several regimens are often used. 5-Fluorouracil (5-FU) is universally used as an anti-cancer agent in esophageal carcinoma. Sinomenine (SIN) is an immunosuppressive compound extracted from the Chinese medicinal plant *Sinomenium acutum*; this compound has a wide range of significant pharmacological actions and has been hypothesized to enhance the sensitivity of various cancers to anti-cancer drugs.

### Research frontiers

As an alkaloid derived from a medical plant, SIN has been used in traditional Chinese medicine for over 2000 years. In the area of SIN treatment for cancer, the crucial areas of research are to confirm its effects, investigate the agent's mechanism of action and identify combined systemic chemotherapy regimens to prevent the growth of cancer.

### Innovations and breakthroughs

Few studies have described the anti-inflammatory, anti-rheumatic, anti-cancer, and anti-angiogenesis effects of SIN. The results of this study suggest that the combined effects of SIN and 5-FU on the growth of esophageal carcinoma are superior, with no observed increase in side effects. The essential mechanism underlying SIN- and 5-FU-induced apoptosis, as investigated by Western blotting, involved the mitochondrial pathway.

### Applications

In this study, the combined effects of SIN and 5-FU, as well as their essential mechanism were investigated in esophageal carcinoma. The combined use of these two medicines generated a superior anti-cancer effect without increased side effects. This finding may help to provide novel combined systemic chemotherapy in the treatment of esophageal carcinoma.

### Terminology

SIN: SIN is an alkaloid derived from the stem of the Chinese medical plant *Sinomenium acutum*, which has been used in traditional Chinese medicine for over 2000 years to treat various rheumatoid diseases. The chemical structure of SIN has been clarified, and its potential value in the treatment of immunological and other diseases is recognized by Western medicine.

### Peer review

This is a valuable original study in which the authors examine the inhibitory effects of SIN combined with 5-FU on esophageal carcinoma Eca-109 cells *in vitro* and *in vivo*. The results are exciting and suggest that the combined use of SIN and 5-FU is superior to the effects of the individual agents. This study is important for enhancing the efficacy and reducing the toxicity of chemotherapy regimens for patients with esophageal cancer.

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## Nonalcoholic fatty liver disease is associated with benign gastrointestinal disorders

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### Abstract

**AIM:** To explore associations between nonalcoholic fatty liver disease (NAFLD) and benign gastrointestinal and pancreato-biliary disorders.

**METHODS:** Patient demographics, diagnoses, and hospital outcomes from the 2010 Nationwide Inpatient Sample were analyzed. Chronic liver diseases were identified using International Classification of Diseases, the 9<sup>th</sup> Revision, Clinical Modification codes. Patients with NAFLD were compared to those with other chronic liver diseases for the endpoints of total hospital charges, disease severity, and hospital mortality. Multivariable stepwise logistic regression analyses to assess for the independent association of demographic, comorbidity, and diagnosis variables with the event of NAFLD (vs

other chronic liver diseases) were also performed.

**RESULTS:** Of 7800441 discharge records, 32347 (0.4%) and 271049 (3.5%) included diagnoses of NAFLD and other chronic liver diseases, respectively. NAFLD patients were younger (average 52.3 years vs 55.3 years), more often female (58.8% vs 41.6%), less often black (9.6% vs 18.6%), and were from higher income areas (23.7% vs 17.7%) compared to counterparts with other chronic liver diseases (all  $P < 0.0001$ ). Diabetes mellitus (43.4% vs 28.9%), hypertension (56.9% vs 47.6%), morbid obesity (36.9% vs 8.0%), dyslipidemia (37.9% vs 15.6%), and the metabolic syndrome (28.75% vs 8.8%) were all more common among NAFLD patients (all  $P < 0.0001$ ). The average total hospital charge (\$39607 vs \$51665), disease severity scores, and intra-hospital mortality (0.9% vs 6.0%) were lower among NAFLD patients compared to those with other chronic liver diseases (all  $P < 0.0001$ ). Compared with other chronic liver diseases, NAFLD was significantly associated with diverticular disorders [OR = 4.26 (3.89-4.67)], inflammatory bowel diseases [OR = 3.64 (3.10-4.28)], gallstone related diseases [OR = 3.59 (3.40-3.79)], and benign pancreatitis [OR = 2.95 (2.79-3.12)] on multivariable logistic regression (all  $P < 0.0001$ ) when the latter disorders were the principal diagnoses on hospital discharge. Similar relationships were observed when the latter disorders were associated diagnoses on hospital discharge.

**CONCLUSION:** NAFLD is associated with diverticular, inflammatory bowel, gallstone, and benign pancreatitis disorders. Compared with other liver diseases, patients with NAFLD have lower hospital charges and mortality.

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**Key words:** Nationwide inpatient sample; Nonalcoholic fatty liver disease; Chronic liver disease; Diverticular disease; Pancreatitis; Gallstones; Inflammatory

bowel disease

**Core tip:** This study analyzed the 2010 Nationwide Inpatient Sample to compare outcomes and associations between patients with nonalcoholic fatty liver disease (NAFLD) and other chronic liver diseases. Compared with other liver diseases, NAFLD is associated with diverticular, inflammatory bowel, gallstone, and benign pancreatitis disorders when these latter disorders are considered as either the principal or associated diagnoses on discharge. These associations suggest shared mechanisms of pathology between NAFLD and these benign gastrointestinal disorders. Furthermore, patients with NAFLD have lower hospital mortality and consume fewer healthcare resources compared to patients with other chronic liver diseases.

Reddy SK, Zhan M, Alexander HR, El-Kamary SS. Nonalcoholic fatty liver disease is associated with benign gastrointestinal disorders. *World J Gastroenterol* 2013; 19(45): 8301-8311 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8301.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8301>

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the United States<sup>[1]</sup>. Outpatient primary care and specialist cohort series report prevalence proportions of 25%-46%<sup>[2-4]</sup>. Yet the proportion of hospitalized patients diagnosed with NAFLD is unknown. The prevalence of NAFLD in hospitalized patients maybe similar to outpatient cohorts given the associations between NAFLD and disorders common among hospitalized patients--including diabetes, cardiovascular disease, venous thromboembolism, colorectal cancer, and inflammatory bowel disease<sup>[5-13]</sup>. Conversely, NAFLD may comprise a small percentage of chronic liver disease among hospitalized patients because hepatic complications (such as ascites, variceal bleeding, and hepatocellular carcinoma) are less common with NAFLD compared to hepatitis B, hepatitis C, and alcohol associated liver diseases<sup>[10,14-19]</sup>. Finally, it is unclear if NAFLD is widely recognized by health care providers. This knowledge gap is important given recent small, single institutional studies suggesting relationships between NAFLD and benign digestive and pancreato-biliary disorders including diverticular disease, gallstone disorders, and inflammatory bowel disease<sup>[20-23]</sup>. Recognition of associations between NAFLD and these conditions may reveal insights into the pathologic mechanisms of all disorders.

The objectives of this study were to estimate the prevalence of the diagnosis of NAFLD among hospitalized patients in the United States and to explore associations between NAFLD and benign gastrointestinal and pancreato-biliary disorders.

## MATERIALS AND METHODS

### Database

Data were abstracted from the 2010 Nationwide Inpatient Sample (NIS), Healthcare Cost and Utilization Project, Agency for Healthcare Research and Quality (AHRQ). The 2010 NIS contains discharge information from 1051 non-Federal, short-term, general, and specialty hospitals located in 45 states; approximating a 20% stratified sample of United States community hospitals<sup>[24]</sup>. This study was deemed exempt by the Institutional Review Board at the University of Maryland School of Medicine.

### Sample identification

This study comprised patients with a diagnosis of chronic liver disease and compared patients with NAFLD *vs* any other chronic liver disease. All 25 diagnoses listed in each record were searched in creating each subsample in this study. The International Classification of Diseases, the 9<sup>th</sup> Revision, Clinical Modification (ICD-9-CM) diagnosis code of 571.8 ("other chronic nonalcoholic liver disease") was used to identify the NAFLD subsample. The "other chronic liver disease" subsample was identified using diagnosis codes describing other recognized etiologies of chronic liver disease, chronic liver disease of unknown etiology, and viral infections and errors in mineral metabolism which may lead to chronic liver disease (Table 1). Patient discharge records with diagnoses representing other liver diseases were eliminated from the NAFLD subsample. To eliminate records with possible alcoholic liver disease from the NAFLD subsample, records which included diagnoses pertaining to ethanol abuse, dependence, and/or overdose (Table 1) were removed from the NAFLD subsample. Similarly, records with an ICD-9-CM diagnosis code of 571.8 were eliminated from the "other chronic liver disease" subsample. Nationwide prevalence estimates were calculated using both unweighted and discharge weights, which account for the number of calendar quarters for which each hospital contributed discharges to the NIS<sup>[24,25]</sup>.

### Demographics and diagnoses

The location of patient's residence included central counties of greater than one million population, fringe counties of metropolitan areas of greater than one million population, counties in metropolitan areas of 250000-999999 population, counties in metropolitan areas of 50000-249999 population, and micropolitan counties in areas of less than 50000 population. The reported median income is the median income for the population in the zip code from which the patient pertaining to that particular discharge record resides<sup>[25]</sup>.

All diagnoses were searched to determine the presence of obesity (ICD-9-CM codes 278, 278.0, 278.00, 278.01, 278.02, or 278.03) and dyslipidemia (ICD-9-CM codes 272, 272.0, 272.1, 272.2, 272.3, 272.4, 272.5, 272.8, or 272.9). The presence of associated diagnoses (principal or secondary) were determined using the clinical classification



**Table 1** International Classification of Diseases, 9<sup>th</sup> Revision, Clinical Modification diagnosis codes used to identify disease

ICD-9-CM diagnosis		Diagnosis
The "other liver disease" cohort	571	Chronic liver disease and cirrhosis
	571	Alcoholic fatty liver
	571.1	Acute alcoholic hepatitis
	571.2	Alcoholic cirrhosis of liver
	571.3	Alcoholic liver damage, unspecified
	571.4	Chronic hepatitis
	571.4	Chronic hepatitis unspecified
	571.41	Chronic persistent hepatitis
	571.42	Autoimmune hepatitis
	571.49	Other chronic hepatitis
	571.5	Cirrhosis of liver without mention of alcohol
	571.6	Biliary cirrhosis
	571.9	Other unspecified chronic liver disease without mention of alcohol
	573	Other disorders of liver
	573.1	Hepatitis in viral diseases classified elsewhere
	573.2	Hepatitis in other infectious diseases classified elsewhere
	573.3	Hepatitis, unspecified
	573.8	Other specified disorders of liver
	573.9	Unspecified disorder of liver
	70	Viral hepatitis
	70	Viral hepatitis A with hepatic coma
	70.1	Viral hepatitis A without mention of hepatic coma
	70.2	Viral hepatitis B with hepatic coma
	70.2	Viral hepatitis B with hepatic coma, acute or unspecified without hepatitis delta
	70.21	Viral hepatitis B with hepatic coma, acute or unspecified with hepatitis delta
	70.22	Chronic viral hepatitis B with hepatic coma without hepatitis delta
	70.23	Chronic viral hepatitis B with hepatic coma with hepatitis delta
	70.3	Viral hepatitis b without mention of hepatic coma
	70.3	Viral hepatitis B without mention of hepatic coma, acute or unspecified, without mention of hepatitis delta
	70.31	Viral hepatitis B without mention of hepatic coma, acute or unspecified, with hepatitis delta
	70.32	Chronic viral hepatitis B without mention of hepatic coma without mention of hepatitis delta
	70.33	Chronic viral hepatitis B without mention of hepatic coma with hepatitis delta
	70.4	Other specified viral hepatitis with hepatic coma
	70.41	Acute hepatitis C with hepatic coma
	70.42	Hepatitis delta without mention of active hepatitis B disease with hepatic coma
	70.43	Hepatitis E with hepatic coma
	70.44	Chronic hepatitis C with hepatic coma
	70.49	Other specified viral hepatitis with hepatic coma
	70.5	Other specified viral hepatitis without mention of hepatic coma
	70.51	Acute hepatitis C without mention of hepatic coma
	70.52	Hepatitis delta without mention of active hepatitis B disease or hepatic coma
	70.53	Hepatitis E without mention of hepatic coma
	70.54	Chronic hepatitis C without mention of hepatic coma
	70.59	Other specified viral hepatitis without mention of hepatic coma
	70.6	Unspecified viral hepatitis with hepatic coma
	70.7	Unspecified viral hepatitis c
	70.7	Unspecified viral hepatitis C without hepatic coma
	70.71	Unspecified viral hepatitis C with hepatic coma
	70.9	Unspecified viral hepatitis without mention of hepatic coma
	V02.6	Carrier or suspected carrier of viral hepatitis
	V02.60	Viral hepatitis carrier, unspecified
	V02.61	Hepatitis B carrier
	V02.62	Hepatitis C carrier
	V02.69	Other viral hepatitis carrier
	275	Disorders of iron metabolism
	275.01	Hereditary hemochromatosis
	275.02	Hemochromatosis due to repeated red blood cell transfusions
	275.03	Other hemochromatosis
	275.09	Other disorders of iron metabolism
	275.1	Disorders of copper metabolism
Alcohol abuse, dependence, and/or overdose	291	Alcohol-induced mental disorders
	291	Alcohol withdrawal delirium
	291.1	Alcohol-induced persisting amnesic disorder
	291.2	Alcohol-induced persisting dementia
	291.3	Alcohol-induced psychotic disorder with hallucinations
	291.4	Idiosyncratic alcohol intoxication
	291.5	Alcohol-induced psychotic disorder with delusions

291.8	Other specified alcohol-induced mental disorders
291.81	Alcohol withdrawal
291.82	Alcohol-induced sleep disorder
291.89	Other alcoholic psychosis
291.9	Unspecified alcohol-induced mental disorders
303	Alcohol dependence syndrome
303	Acute alcoholic intoxication
303	Acute alcoholic intoxication in alcoholism unspecified drinking behavior
303.01	Acute alcoholic intoxication in alcoholism continuous drinking behavior
303.02	Acute alcoholic intoxication in alcoholism episodic drinking behavior
303.03	Acute alcoholic intoxication in alcoholism in remission
303.9	Other and unspecified alcohol dependence
303.9	Other and unspecified alcohol dependence unspecified drinking behavior
303.91	Other and unspecified alcohol dependence continuous drinking behavior
303.92	Other and unspecified alcohol dependence episodic drinking behavior
303.93	Other and unspecified alcohol dependence in remission

ICD-9-CM: International classification of diseases, 9<sup>th</sup> revision, clinical modification.

**Table 2 Clinical classification software categories used to identify comorbidities and associated diagnoses**

CCS Category	Diagnosis
98 and 99	Hypertension
49 and 50	Diabetes mellitus
138	Esophageal disorders (excluding ICD-9-CM diagnoses f.or varices)
139	Gastroduodenal ulcer
140	Gastritis and/or duodenitis
141	Other disorders of stomach and duodenum
142	Appendicitis
143	Abdominal and groin hernia
144	Inflammatory bowel diseases
145	Intestinal obstruction
146	Diverticular disease
147	Anal and rectal conditions
148	Peritonitis and intestinal abscess
149	Benign biliary tract disease
152	Benign pancreatic disorders excluding diabetes mellitus
153	Gastrointestinal hemorrhage
154	Noninfectious gastroenteritis

CCS: Clinical classification software categories; ICD-9-CM: International classification of diseases, 9<sup>th</sup> revision, clinical modification.

cation software provided by the AHRQ<sup>[26]</sup> (Table 2). Criteria for the metabolic syndrome include the presence of any three of obesity, diabetes mellitus, hypertension, and dyslipidemia<sup>[27,28]</sup>. Principal discharge diagnoses were also identified by the Disease Staging<sup>®</sup> (Thomson Reuters) classification system<sup>[29]</sup> (Table 3). The all patient refined diagnosis related group (APR-DRG<sup>®</sup> 3M) assesses severity of illness and mortality risk of the DRG pertaining to each patient discharge record<sup>[30]</sup>.

### Statistical analysis

Discrete and continuous variables were compared using  $\chi^2$  and Student's *t* tests with two-sided *P* values. Multivariable stepwise logistic regression analyses to assess for the independent association of each variable with the event of NAFLD were performed. The *P* value for variable entry and stay was 0.05. OR point estimates with 95% Wald confidence limits are reported. Results for those variables that did not stay in each model were not reported. Two

**Table 3 Disease Staging<sup>®</sup> (Thomson Reuters) classification system used to identify principal discharge diagnoses**

Discharge category	Description
GIS03, GIS04, GIS18	Benign anal-rectal disorders
GIS05	Appendicitis
GIS09 or GIS37	Inflammatory bowel disease
GIS10	Diverticular disease
GIS17 or GIS84	Gastroduodenitis
GIS19	Hernia
GIS20	Esophagitis
GIS25-30, HEP11, GIS82-83	Upper gastrointestinal cancers
GIS31	Gastroduodenal ulcer
HEP01 or HEP 84	Gallstone related disorders
HEP12 or HEP85	Non-malignant pancreatitis
HEP81 or HEP82	Hepatobiliary malignancies

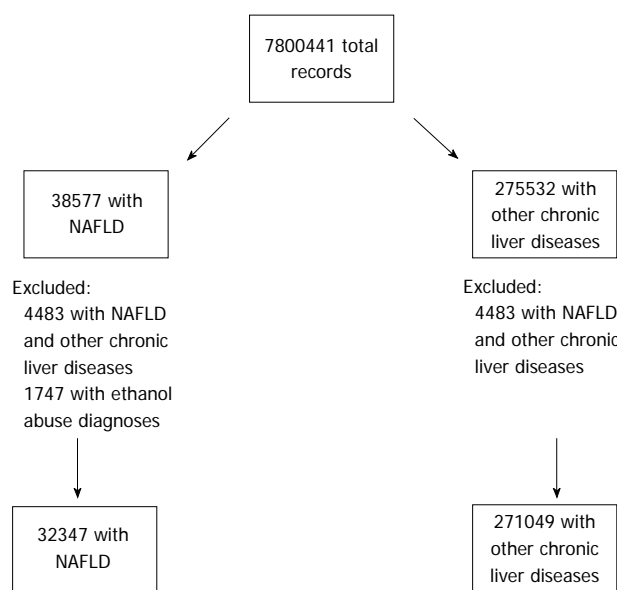
multivariable analyses were performed-one using associated diagnoses and another using principal diagnoses. SAS<sup>®</sup> Version 9.2 (SAS Institute, Inc., Cary, NC, United States) was used to perform all analyses.

## RESULTS

### Patient demographics and comorbidities

Of the 7800441 discharge records in the 2010 NIS, 314109 (4.0%) included a diagnosis describing any chronic liver disease (Figure 1). After excluding patients with diagnoses describing ethanol abuse, dependence, and/or overdose and those with other chronic liver diseases in addition to NAFLD, 32347 (0.4%) records contained the NAFLD diagnosis. Similarly, 271049 (3.5%) records included diagnosis codes describing other chronic liver diseases and did not include NAFLD. When using discharge weighted analyses, 3.9% of all discharge records included a principal or secondary diagnosis describing any chronic liver disease. 0.4% and 3.4% records included diagnoses describing NAFLD and other chronic liver diseases excluding NAFLD, respectively.

NAFLD patients were younger, more often female, and less often black compared to counterparts with other chronic liver diseases (Table 4). Diabetes mellitus, hypertension, obesity, and dyslipidemia were all more common



**Figure 1** Identification of nonalcoholic fatty liver disease and “other chronic liver diseases” subsamples from the Nationwide Inpatient Sample. NAFLD: Nonalcoholic fatty liver disease.

among NAFLD patients. Nearly 29% of the patients in the NAFLD subsample had the metabolic syndrome. NAFLD patients were from higher income areas and more often had private health insurance. The average total hospital charge and length of hospital stay were significantly shorter than among patients with other chronic liver diseases. Rates of NAFLD intra-hospital mortality and advanced APRDRG mortality and disease severity scores were all significantly lower than among patients with other chronic liver diseases.

### Associated gastrointestinal and pancreato-biliary disorders

As a *principal* or *secondary* (e.g., associated) diagnoses, abdominal hernia, appendiceal disorders, benign biliary and pancreatic disorders, diverticular disease, non-variceal esophageal disorders, gastritis/duodenitis, gastroduodenal ulcer, inflammatory bowel disease, and intestinal infection were all significantly more common among NAFLD patients (Table 5). Conversely, gastrointestinal and hepatobiliary malignancies, gastrointestinal hemorrhage, and peritonitis/intra-abdominal abscess were all more common among patients with other chronic liver diseases. NAFLD patients were more likely to have a *principal* diagnosis on discharge of appendicitis, benign pancreatitis, diverticular disease, esophagitis, gallstone disorders, gastroduodenitis, and inflammatory bowel disease. In contrast, benign ano-rectal disorders, gastroduodenal ulcers, and hepatobiliary cancers were more common among patients with other chronic liver diseases.

All variables with statistically significant differences ( $P < 0.05$ ) on univariable analysis (Table 4) were included in multivariable logistic regression models. When considering each gastrointestinal, hepatic, or pancreato-biliary diagnosis as an associated diagnosis, the patterns

of association observed on univariable comparisons were maintained on multivariable logistic regression (Table 5). When considering each disorder as the *principal* diagnosis, appendicitis, benign pancreatitis, diverticular disease, esophagitis, gallstone disorders, Gastroduodenitis, and hernia were all associated with NAFLD on multivariable logistic regression (Table 6). Hepatobiliary cancers were independently associated with other liver diseases. In both analyses, female gender, younger patient age, diabetes mellitus, obesity, dyslipidemia, higher income, and private health insurance were independently associated with NAFLD.

### Subgroup analysis for selected disorders

We performed subgroup analyses of records with a principal discharge diagnosis of diverticular disease, gallstone related disorders or benign pancreatitis. We chose these disorders because of their high prevalence in our sample. When stratified by type of background liver disease, similar differences in ethnicity, gender, comorbidities, health insurance payer, age, hospital charges, and income existed for each subgroup as in the overall sample (Table 7). As in the overall sample, total hospital charges, length of hospital stay, discharge disposition, rates of hospital death and APRDRG mortality and disease severity scores were all greater among patients with other chronic liver diseases in each subgroup.

## DISCUSSION

The diagnosis of NAFLD among hospitalized patients is much less common compared to that noted in outpatient cohort studies<sup>[2-4]</sup>. Our findings that patients with NAFLD were more likely to be female, obese, and non-Black and more likely have diabetes mellitus, hypertension, dyslipidemia, and the metabolic syndrome compared to patients with other chronic liver diseases is in agreement with other studies<sup>[10,14-19]</sup>—suggesting that our methods to identify these patients were accurate. Similarly, patients with other liver diseases were more frequently from low-income regions and less likely to have private insurance as the primary health care payer; reflecting the higher prevalence of hepatitis C viral infections and alcohol abuse in low-income areas. The discrepancy in prevalence of NAFLD in outpatient series compared with hospitalized patients shows that NAFLD is under-recognized in hospital patients and that the impact of NAFLD on clinical outcomes and health care resource utilization is unrecognized.

NAFLD is associated with several benign gastrointestinal and pancreato-biliary disorders. The exceptions were gastrointestinal hemorrhage and peritonitis, which are expected to occur in patients with decompensated liver disease and thus are less likely in NAFLD patients compared to those with other chronic liver diseases<sup>[10,14-19]</sup>. Several studies have established the role of bacterial translocation in nonalcoholic steatohepatitis and severe steatosis<sup>[20,21,31-33]</sup>. This could be a potential mechanism

**Table 4** Demographics and hospital outcomes of discharge records stratified by background liver disease *n* (%)

	NAFLD ( <i>n</i> = 32347)	Other liver diseases ( <i>n</i> = 271049)	<i>P</i> value	Missing
Average age (yr)	52.3 ± 16.5	55.3 ± 15.4	< 0.0001	0
Female gender	19027 (58.8)	112788 (41.6)	< 0.0001	78 (0.0003)
Non-elective admission	25291 (78.4)	231024 (85.4)	< 0.0001	521 (0.2)
Ethnicity			< 0.0001	29349 (9.7)
White	20536 (70.7)	152778 (62.4)		
Black	2798 (9.6)	45634 (18.6)		
Hispanic	4135 (14.2)	32124 (13.1)		
Asian/Pacific Islander	592 (2.0)	5720 (2.3)		
Native American	191 (0.7)	2075 (0.9)		
Other	797 (2.7)	6667 (2.7)		
Diabetes mellitus	14027 (43.4)	78011 (28.9)	< 0.0001	
Hypertension	18413 (56.9)	129031 (47.6)	< 0.0001	
Obesity	11920 (36.9)	21677 (8.0)	< 0.0001	
Dyslipidemia	12262 (37.9)	42299 (15.6)	< 0.0001	
Metabolic syndrome	9286 (28.7)	23888 (8.8)	< 0.0001	
Location			< 0.0001	13512 (4.4)
Central Metropolitan	9115 (28.8)	91426 (35.4)		
Fringe Metropolitan	8540 (27.0)	58794 (22.8)		
Metro below one million	6003 (19.0)	48308 (18.7)		
50000-250000 population	2563 (8.1)	19519 (7.6)		
Micropolitan	3482 (11.0)	24641 (9.5)		
Other	1948 (6.2)	15545 (6.0)		
Median zip code income (\$)			< 0.0001	13104 (4.3)
< 39000	8003 (25.3)	89265 (34.5)		
39000-47999	8140 (25.7)	65082 (25.2)		
48000-62999	8006 (25.3)	58595 (22.7)		
≥ 63000	7488 (23.7)	45713 (17.7)		
Primary payer			< 0.0001	870 (0.3)
Medicare	10429 (32.3)	104892 (38.8)		
Medicaid	4461 (13.8)	65829 (24.4)		
Private	13602 (42.1)	59410 (22.0)		
Self-pay	2454 (7.6)	24729 (9.2)		
No charge	244 (0.8)	2917 (1.1)		
Other	1107 (3.4)	12452 (4.6)		
Major operative procedure	9804 (30.3)	47187 (17.4)	< 0.0001	0
Average total hospital charges (\$)	39607.3 ± 52512	51665 ± 90685	< 0.0001	0
Average length of hospital stay (d)	4.7 ± 6.0	6.6 ± 9.7	< 0.0001	0
Discharge disposition			< 0.0001	420 (0.001)
Routine	25703 (79.5)	167545 (61.9)		
Acute care	660 (2.0)	9190 (3.4)		
Another health facility	2273 (7.0)	39311 (14.5)		
Home Health	3114 (9.6)	30276 (11.2)		
AMA	269 (0.8)	7691 (2.8)		
Other	10 (0.03)	479 (0.2)		
Died in hospital	301 (0.9)	16154 (6.0)	< 0.0001	571 (0.2)
Aprdrg mortality risk			< 0.0001	347 (0.1)
Minor	14347 (44.4)	82849 (30.4)		
Moderate	11678 (36.1)	84343 (31.1)		
Major	4921 (15.2)	63614 (23.5)		
Extreme	1377 (4.3)	40280 (14.9)		
Aprdrg severity			< 0.0001	347 (0.1)
Minor functional loss	814 (2.5)	12242 (4.5)		
Moderate functional loss	18105 (56.0)	88446 (32.6)		
Major functional loss	11156 (35.0)	116365 (43.0)		
Extreme functional loss	2248 (7.0)	53673 (19.8)		
Associated Diagnoses				
Abdominal Hernia	3459 (10.7)	12755 (4.7)	< 0.0001	
Appendiceal disorders	254 (0.8)	728 (0.3)	< 0.0001	
Benign anus-rectum disorders	174 (0.5)	1401 (0.5)	0.62	
Benign biliary disorders	5421 (16.8)	19306 (7.1)	< 0.0001	
Benign pancreatic disorders	3379 (10.5)	15898 (5.9)	< 0.0001	
Diverticular disease	2845 (8.8)	8697 (3.2)	< 0.0001	
Esophageal disorders (non-variceal)	9114 (28.2)	41835 (15.4)	< 0.0001	
Gastritis/duodenitis	2163 (6.7)	11220 (4.1)	< 0.0001	
Gastroduodenal ulcer	1011 (3.1)	7477 (2.8)	0.002	
Gastrointestinal hemorrhage	1119 (3.5)	23651 (8.7)	< 0.0001	
Gastrointestinal malignancies	688 (2.1)	6871 (2.5)	< 0.0001	



Hepatobiliary malignancies	84 (0.3)	7903 (2.9)	< 0.0001
Inflammatory bowel disease	555 (1.7)	2659 (1.0)	< 0.0001
Intestinal infection	886 (2.7)	5904 (2.2)	< 0.0001
Intestinal obstruction	1346 (4.2)	7898 (2.9)	< 0.0001
Peritonitis-abscess	326 (1.0)	5772 (2.1)	< 0.0001
Principal discharge diagnoses			
Appendicitis	217 (0.7)	554 (0.2)	< 0.0001
Benign anus-rectal disorders	163 (0.5)	1694 (0.6)	0.008
Benign pancreatitis	2224 (6.9)	7074 (2.6)	< 0.0001
Diverticular disease	898 (2.8)	1805 (0.7)	< 0.0001
Esophagitis	306 (1.0)	1762 (0.7)	< 0.0001
Gallstone disorders	2622 (8.1)	5978 (2.2)	< 0.0001
Gastroduodenal ulcer	232 (0.7)	2572 (1.0)	< 0.0001
Gastroduodenitis	713 (2.2)	3481 (1.3)	< 0.0001
Gastrointestinal malignancies	338 (1.0)	3017 (1.1)	0.27
Hepatobiliary malignancies	68 (0.2)	2837 (1.1)	< 0.0001
Hernia	204 (0.6)	1497 (0.6)	0.07
Inflammatory bowel disease	242 (0.8)	696 (0.3)	< 0.0001
Intestinal Infection	235 (0.7)	1999 (0.7)	0.83

NAFLD: Nonalcoholic fatty liver disease; AMA: American medical association.

**Table 5** Multivariable logistic regression for factors associated with nonalcoholic fatty liver disease compared to other liver diseases using associated diagnoses

Variable	P value	OR (95%CI)
Age (reference $\leq 70$ yr)	< 0.0001	0.79 (0.76-0.83)
Gender (reference female)	< 0.0001	0.58 (0.57-0.60)
Diabetes	< 0.0001	1.41 (1.37-1.45)
Hypertension	0.05	0.97 (0.94-1.0)
Obesity	< 0.0001	4.47 (4.34-4.61)
Dyslipidemia	< 0.0001	2.35 (2.28-2.42)
Location (reference central metropolitan)	< 0.0001	
50000-250000 population		1.2 (1.1-1.3)
Fringe metropolitan		1.1 (1.1-1.2)
Metro 250000 - one million population		1.2 (1.1-1.2)
Micropolitan		1.4 (1.4-1.5)
Other		1.4 (1.3-1.4)
Income (reference $\geq \$63000$ )	< 0.0001	
\$39000-\$47999		0.80 (0.77-0.83)
\$48000-\$62999		0.86 (0.82-0.89)
< \$39000		0.64 (0.62-0.67)
Payer (reference private insurance)	< 0.0001	
Medicaid		0.42 (0.41-0.43)
Medicare		0.46 (0.44-0.47)
No charge		0.61 (0.53-0.70)
Other		0.55 (0.51-0.59)
Self-pay		0.65 (0.62-0.68)
Associated diagnoses		
Abdominal hernia	< 0.0001	1.70 (1.63-1.79)
Appendiceal disorders	< 0.0001	2.58 (2.19-3.04)
Benign biliary disorders	< 0.0001	2.11 (2.03-2.19)
Benign pancreatic disorders	< 0.0001	1.57 (1.50-1.64)
Diverticular disease	< 0.0001	2.22 (2.11-2.34)
Esophageal disorders (non-variceal)	< 0.0001	1.52 (1.48-1.57)
Gastroduodenal ulcer	< 0.0001	1.41 (1.33-1.49)
Gastrointestinal hemorrhage	< 0.0001	0.41 (0.38-0.44)
Gastrointestinal malignancies	< 0.0001	0.83 (0.76-0.91)
Hepatobiliary malignancies	< 0.0001	0.12 (0.10-0.15)
Inflammatory bowel disease	< 0.0001	1.68 (1.52-1.86)
Intestinal infection	< 0.0001	1.29 (1.19-1.40)
Intestinal obstruction	< 0.0001	1.30 (1.22-1.39)
Peritonitis-abscess	< 0.0001	0.47 (0.42-0.53)

**Table 6** Multivariable logistic regression for factors associated with nonalcoholic fatty liver disease compared to other liver diseases using principal diagnoses

Variable	P value	OR (95%CI)
Age (reference $\leq 70$ yr)	< 0.0001	0.84 (0.81-0.88)
Gender (reference female)	< 0.0001	0.55 (0.53-0.56)
Diabetes	< 0.0001	1.38 (1.35-1.42)
Obesity	< 0.0001	4.75 (4.61-4.89)
Dyslipidemia	< 0.0001	2.51 (2.44-2.58)
Location (reference central metropolitan)	< 0.0001	
50000-250000 population		1.21 (1.14-1.27)
Fringe metropolitan		1.12 (1.09-1.17)
Metro 250000 - one million population		1.15 (1.11-1.20)
Micropolitan		1.44 (1.37-1.51)
Other		1.37 (1.29-1.45)
Income (reference $> \$63000$ )	< 0.0001	
\$39000-\$47999		0.80 (0.77-0.83)
\$48000-\$63000		0.86 (0.83-0.89)
< \$39000		0.64 (0.62-0.67)
Payer (reference private insurance)	< 0.0001	
Medicaid		0.42 (0.40-0.43)
Medicare		0.47 (0.46-0.49)
No charge		0.60 (0.52-0.69)
Other		0.54 (0.50-0.58)
Self-pay		0.62 (0.59-0.65)
Principal discharge diagnosis		
Appendicitis	< 0.0001	3.53 (2.96-4.22)
Benign pancreatitis	< 0.0001	2.95 (2.79-3.12)
Diverticular disease	< 0.0001	4.26 (3.89-4.67)
Esophagitis	< 0.0001	1.69 (1.48-1.93)
Gallstone disorders	< 0.0001	3.59 (3.40-3.79)
Gastroduodenitis	< 0.0001	2.09 (1.91-2.29)
Hepatobiliary malignancies	< 0.0001	0.29 (0.22-0.37)
Hernia	0.01	1.23 (1.04-1.45)
Inflammatory bowel disease	< 0.0001	3.64 (3.10-4.28)

chronic liver diseases<sup>[21]</sup>. Our finding of the association between NAFLD and gallstone disease is in agreement that a recent Italian study which noted a high prevalence of gallstone disease among patients with NAFLD<sup>[22]</sup>. Other series have noted similar findings<sup>[23,34]</sup>.

Patients with NAFLD have better hospital outcomes, less severe disease severity and mortality risk, and utilize

for which diverticular disorders in particular are more commonly associated with NAFLD compared to other

**Table 7** Demographics and hospital outcomes of discharge records of patients with a principal discharge diagnosis of diverticular disease, gallstone disease or benign pancreatitis stratified by background liver disease *n* (%)

	Diverticular disease ( <i>n</i> = 2703)			Gallstone disease ( <i>n</i> = 8600)			Benign pancreatitis ( <i>n</i> = 9298)		
	NAFLD ( <i>n</i> = 898)	OLD ( <i>n</i> = 1805)	<i>P</i> value	NAFLD ( <i>n</i> = 2622)	OLD ( <i>n</i> = 5978)	<i>P</i> value	NAFLD ( <i>n</i> = 2224)	OLD ( <i>n</i> = 7074)	<i>P</i> value
Average age (yr)	55.1 ± 13.5	62.7 ± 14.2	< 0.0001	50.5 ± 16.7	57.7 ± 17.4	< 0.0001	47.8 ± 15.5	51.9 ± 14.0	< 0.0001
Female Gender	501 (55.8)	897 (49.7)	< 0.003	1606 (61.3)	3056 (51.2)	< 0.0001	1058 (47.6)	2796 (39.5)	< 0.0001
Non-elective admission	791 (88.3)	1612 (89.5)	0.36	2305 (88.4)	5137 (86.0)	< 0.0001	2110 (95.1)	6680 (94.5)	< 0.0001
Ethnicity			< 0.0001			< 0.0001			< 0.0001
White	610 (74.5)	1174 (72.1)		1575 (65.3)	3532 (65.5)		1376 (67.8)	3910 (60.8)	
Black	48 (5.9)	236 (14.5)		166 (6.9)	633 (11.7)		196 (9.7)	1355 (21.1)	
Hispanic	128 (15.7)	159 (9.8)		519 (21.5%)	882 (16.4)		339 (16.7)	854 (13.3)	
Other	33 (4.0)	60 (3.7)		152 (6.3)	344 (6.4)		119 (5.9)	313 (4.9)	
Diabetes mellitus	276 (30.7)	440 (24.4)	0.0004	816 (31.1)	1603 (26.8)	< 0.0001	984 (44.2)	1974 (27.9)	< 0.0001
Hypertension	490 (54.6)	1021 (56.6)	0.32	1285 (49.0)	2892 (48.4)	< 0.0001	1222 (55.0)	3524 (49.8)	< 0.0001
Obesity	252 (28.1)	200 (11.1)	< 0.0001	1010 (38.5)	770 (12.9)	< 0.0001	680 (30.6)	561 (7.9)	< 0.0001
Dyslipidemia	319 (35.5)	451 (25.0)	< 0.0001	818 (31.2)	1233 (20.6)	< 0.0001	1093 (49.2)	1359 (19.2)	< 0.0001
Metabolic syndrome	178 (19.8)	195 (10.8)	< 0.0001	567 (21.6)	638 (10.7)	< 0.0001	662 (29.8)	721 (10.2)	< 0.0001
Location			0.02			< 0.0001			< 0.0001
Central Metro	268 (30.6)	537 (30.7)		835 (32.5)	1775 (30.7)		603 (27.8)	2184 (32.6)	
Fringe Metro	247 (28.2)	436 (24.9)		707 (27.5)	1379 (23.9)		600 (27.6)	1554 (23.2)	
Metro below one million	185 (21.1)	322 (18.4)		505 (19.7)	1132 (19.6)		406 (18.7)	1373 (20.5)	
50000-250000	53 (6.1)	147 (8.4)		160 (6.2)	427 (7.4)		185 (8.5)	536 (8.0)	
Micropolitan	81 (9.3)	187 (10.7)		237 (9.2)	651 (11.3)		238 (11.0)	626 (9.3)	
Other	42 (4.8)	119 (6.8)		124 (4.8)	411 (7.1)		140 (6.5)	437 (6.5)	
Median zip code income (\$)			0.0008			< 0.0001			< 0.0001
< 39000	202 (22.8)	502 (28.5)		632 (24.6)	166 (28.9)		556 (25.6)	2334 (34.4)	
39000-47999	209 (23.5)	437 (24.8)		640 (24.9)	1550 (26.9)		534 (24.6)	1734 (25.5)	
48000-63000	233 (26.2)	445 (25.2)		676 (26.3)	1446 (25.1)		564 (25.9)	1527 (22.5)	
> 63000	244 (27.5)	380 (21.5)		618 (24.1)	1107 (19.2)		520 (23.9)	1196 (17.6)	
Primary payer			< 0.0001			< 0.0001			< 0.0001
Medicare	256 (28.5)	866 (48.1)		659 (25.2)	2461 (41.2)		523 (23.6)	2028 (28.8)	
Medicaid	60 (6.7)	181 (10.1)		382 (14.6)	1016 (17.0)		320 (14.4)	1682 (23.9)	
Private	469 (52.2)	562 (31.2)		1229 (46.9)	1687 (28.3)		971 (43.7)	1742 (24.7)	
Self-pay	75 (8.4)	119 (6.6)		215 (8.2)	531 (8.9)		295 (13.3)	1054 (15.0)	
No charge	6 (0.7)	15 (0.8)		27 (1.0)	53 (0.9)		24 (1.1)	128 (1.8)	
Other	32 (3.6)	58 (3.2)		106 (4.1)	219 (3.7)		87 (3.9)	418 (5.9)	
Major operative procedure	109 (12.1)	333 (18.5)	< 0.0001	2002 (76.4)	3327 (55.7)	< 0.0001	294 (13.2)	652 (9.2)	< 0.0001
Average total hospital charges (\$)	26868.7 ± 26364.6	46666.9 ± 80222.8	< 0.0001	40016.5 ± 32898.6	49682.8 ± 65425.9	< 0.0001	32680.5 ± 42691.0	45115.5 ± 83193.4	< 0.0001
Average length of hospital stay (d)	4.2 ± 3.4	6.0 ± 8.3	< 0.0001	4.0 ± 3.4	5.6 ± 6.1	< 0.0001	4.9 ± 4.4	6.2 ± 8.4	< 0.0001
Discharge Disposition			< 0.0001			< 0.0001			< 0.0001
Routine	821 (91.4)	1355 (75.2)		2403 (91.8)	4492 (75.3)		2013 (90.5)	5396 (76.3)	
Acute care	2 (0.2)	33 (1.8)		32 (1.2)	240 (4.0)		45 (2.0)	209 (3.0)	
Another health facility	25 (2.8)	158 (8.8)		66 (2.5)	503 (8.4)		47 (2.1)	480 (6.8)	
Home Health	44 (4.9)	184 (10.2)		101 (3.9)	487 (8.2)		86 (3.9)	361 (5.1)	
AMA	5 (0.6)	17 (0.9)		14 (0.5)	81 (1.4)		22 (1.0)	325 (4.6)	
Other	0	3 (0.2)		0	7 (0.1)		0	4 (0.1)	
Died in hospital	1 (0.1)	53 (2.9)	< 0.0001	3 (0.1)	157 (2.6)	< 0.0001	11 (0.5)	296 (4.2)	< 0.0001
APRDRG Mortality risk			< 0.0001			< 0.0001			< 0.0001
Minor	530 (59.0)	783 (43.4)		1457 (55.6)	2123 (35.5)		1177 (52.9)	2658 (37.6)	
Moderate	278 (31.0)	563 (31.2)		936 (35.7)	2095 (35.1)		746 (33.5)	2308 (32.6)	
Major	82 (9.1)	291 (16.1)		188 (7.2)	1187 (19.9)		238 (10.7)	1318 (18.6)	
Extreme	8 (0.9)	167 (9.3)		37 (1.4)	571 (9.6)		61 (2.7)	785 (11.1)	
APRDRG Severity			< 0.0001			< 0.0001			< 0.0001
Minor functional loss	0	163 (9.0)		1 (0.04%)	377 (6.3)		1 (0.04)	347 (4.9)	
Moderate functional loss	645 (71.8)	747 (41.4)		1828 (69.7)	2371 (39.7)		1318 (59.3)	2721 (38.5)	
Major functional loss	231 (25.7)	697 (38.6)		715 (27.3)	2393 (40.0)		791 (35.6)	2855 (40.4)	
Extreme functional loss	22	197 (10.9)		74	835 (14.0)		112 (5.0)	1146 (16.2)	
	-2.5			-2.8					

NAFLD: Nonalcoholic fatty liver disease; OLD: Other liver diseases; AMA: American medical association.

fewer health care resources compared to patients with other chronic liver diseases (Table 4). These relationships occurred despite the fact that more NAFLD patients underwent major operations, and were maintained in

subgroup analysis of diverticular disease, gallstone disease, and benign pancreatitis (Table 7). Given that hepatic related morbidity more often occurs with other chronic liver diseases (such as hepatitis C and alcoholic liver dis-

ease) compared to NAFLD<sup>[10,14-19]</sup>, these findings suggest that the type and severity of background liver disease plays a vital role in determining overall patient outcomes and health care resource utilization.

There are several limitations to this study. It is unknown how background liver disease diagnoses were derived. Thus, the accuracy of NAFLD in this sample cannot be verified—especially when discharge abstracts used to construct this database were intended for reimbursement and not clinical research purposes. Similar problems regarding the accuracy of entered codes may exist when using ICD-9 diagnosis codes for elements of the metabolic syndrome, gastrointestinal disorders, and pancreato-biliary diseases. Distinctions between simple hepatic steatosis, steatohepatitis, and degrees of fibrosis cannot be made in the NIS. Because medications were not included in the NIS, we were not able to account for drug induced fatty liver disease. This limitation has minimal influence on our conclusions since less than 2% of steatohepatitis is drug induced<sup>[9]</sup>. We attempted to homogenize the NAFLD subsample by using only one ICD-9-DM identifier and eliminating any records listing any other major potential etiology of background liver disease. We focused on patients with any diagnosis of chronic liver disease and postulated that these patients are the most likely to have undergone evaluation for NAFLD. NAFLD may coexist with other chronic liver diseases in a minority of patients<sup>[35-37]</sup>. It is therefore possible that we may have included patients with undiagnosed NAFLD in the “other liver disease” sample. The NIS is a discharge level database where each entry represents a hospital admission and not an individual patient—thus multiple readmissions for a single patient may have biased our results.

In conclusion, NAFLD is widely under diagnosed among hospitalized patients in the United States. NAFLD is associated with diverticular, gallstone, and benign pancreatic disorders. The type of background liver disease is a key factor in hospital outcomes and healthcare resource utilization among hospitalized patients.

## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the developed world. While prevalence proportions among outpatient series are well described, the proportion of hospitalized patients diagnosed with NAFLD is unknown. Moreover, associations of NAFLD to other gastrointestinal disorders are not well established.

### Research frontiers

The important research hotspots related to this article include (1) the prevalence of NAFLD diagnosis among hospitalized patients; (2) outcomes among hospitalized patients with NAFLD; and (3) relationships between NAFLD and other gastrointestinal disorders.

### Innovations and breakthroughs

Most prior reports examining the prevalence of NAFLD are based on outpatient or cohort registry studies—data regarding the prevalence of NAFLD among hospitalized patients are sparse. Few studies have examined hospital outcomes among patients with NAFLD—most focus on long-term survival related to hepatic or cardiovascular complications. Previous studies looking at associations between NAFLD and other gastrointestinal disorders are small, single institution

based, and often biased by patient selection and particular care settings. To overcome these obstacles, the authors used a large database that provides an accurate estimate of the prevalence of NAFLD diagnosis among hospitalized patients across the United States. Analyses of these data show that patients with NAFLD have a lower frequency of hospital mortality and consume fewer healthcare resources compared to those with other chronic liver diseases. Finally, authors' study demonstrates that NAFLD is associated with diverticular, inflammatory bowel, gallstone, and benign pancreatitis disorders independent of demographics or other comorbidities.

### Applications

The study results suggest that suggest that the type of background liver disease plays a vital role in determining overall patient outcomes and health care resource utilization among hospitalized patients. The results also suggest shared mechanisms of disease pathology between NAFLD and diverticular, inflammatory bowel, gallstone, and benign pancreatitis disorders.

### Terminology

A principal diagnosis is the one diagnosis describing the main indication for admission and/or the condition which was the central focus of management during hospitalization. Associated diagnoses include the principal diagnosis, comorbid conditions, and disorders previously managed but not the focus of the particular hospitalization.

### Peer review

The authors mentioned the prevalence of NAFLD and the associations between NAFLD and other common gastrointestinal and pancreato-biliary disorders among hospitalized patients. The authors also discussed the impact of NAFLD on healthcare resource utilization.

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## Biliary tree gastrinomas in multiple endocrine neoplasia type 1 syndrome

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to have an ectopic gastrinoma located in the biliary tree.

**RESULTS:** Seventeen MEN1 patients affected with ZES were analyzed. The mean age was 40 years. Fifteen patients underwent DP and two TP. On histopathological examination, duodeno pancreatic endocrine tumors were found in all 17 patients. Eighty-one gastrinomas were detected in the first three portions of the duodenum. Only one gastrinoma was found in the pancreas. The mean number of gastrinomas per patient was 5 (range 1-16). Malignancy was established in 12 patients (70.5%) after lymph node, liver and omental metastases were found. Three patients exhibited biliary tree gastrinomas as well as duodenal gastrinoma(s). In two cases, the ectopic gastrinoma was removed at the same time as pancreatic surgery, while in the third case, the biliary tree gastrinoma was resected one year after DP because of recurrence of ZES.

**CONCLUSION:** These findings suggest the importance of checking for the presence of ectopic gastrinomas in the biliary tree in MEN1 patients undergoing ZES surgery.

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**Key words:** Gastrinoma; Multiple endocrine neoplasia type 1; Zollinger-Ellison Syndrome; Ectopic gastrinoma; Biliary tree; Duodenopancreatectomy

### Abstract

**AIM:** To describe our patients affected with ectopic biliary tree gastrinoma and review the literature on this topic.

**METHODS:** Between January 1992 and June 2012, 28 patients affected by duodenopancreatic endocrine tumors in multiple endocrine neoplasia type 1 (MEN1) syndrome underwent surgery at our institution. This retrospective review article analyzes our experience regarding seventeen of these patients subjected to duodenopancreatic surgery for Zollinger-Ellison syndrome (ZES). Surgical treatment consisted of duodenopancreatectomy (DP) or total pancreatectomy (TP). Regional lymphadenectomy was always performed. Any hepatic tumoral lesions found were removed during surgery. In MEN1 patients, removal of duodenal lesions can sometimes lead to persistence or recurrence of hypergastrinemia. One possible explanation for this unfavorable outcome could be unrecognized ectopic localization of gastrin-secreting tumors. This study described three cases among the seventeen patients who were found

**Core tip:** Enteropancreatic endocrine neoplasms affect up to 90% of multiple endocrine neoplasia type 1 (MEN1) patients. Gastrinomas are the most common functional enteropancreatic neuroendocrine tumors and were thought to be located almost exclusively in the duodenum in MEN1 patients. This study describes our experience regarding ectopic biliary tree gastrinomas in MEN1 syndrome. Our data doubles the number of cases reported in literature on this topic. Furthermore, the present study brings to light important issues that could

help to establish the best biochemical and oncological cure for such cases, of which clinicians should be aware to improve the management of MEN1 patients.

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## INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) is a familial syndrome characterized by tumors of the parathyroids, the anterior pituitary and the enteropancreatic endocrine system. Enteropancreatic endocrine neoplasms affect up to 90% of MEN1 patients, the incidence increasing with age<sup>[1-3]</sup>. In MEN1, the most common functional enteropancreatic neuroendocrine tumors are gastrinomas, located almost exclusively in the duodenum. According to recent literature, pancreatic localization is extremely rare<sup>[4-6]</sup>. Zollinger-Ellison syndrome (ZES) and/or increased basal or stimulated plasma gastrin levels<sup>[5]</sup> often persist after surgical treatment. This could be caused by either metastatic disease or potentially unrecognized localization of gastrin-secreting tumors.

Interestingly, primary sporadic gastrinomas may arise not only in the pancreas and duodenum, but also in other organs, such as the stomach, ovary, omentum, kidney, jejunum, esophagus, extra-hepatic biliary tree or liver<sup>[7-13]</sup>. All these sites have usually been considered atypical in MEN1 syndrome, or until now, never described. Recently, the extra-hepatic biliary tree and liver were indicated as the sites of primary gastrinomas in three MEN1 patients from two different centers<sup>[14,15]</sup>. Our experience includes a further three MEN1 patients with an ectopic gastrinoma in the biliary tree, thus doubling the number of cases recognized in the international literature. The implications of this phenotype are important in the clinical management of MEN1 ZES.

## MATERIALS AND METHODS

Between January 1992 and June 2012, 28 MEN1 patients affected with duodenopancreatic endocrine tumors were observed and operated on at our institution, following a protocol approved by the Tuscany Region. MEN1 diagnosis was confirmed after genetic testing on germline DNA obtained from peripheral blood leukocytes. Serum gastrin, chromogranin A, pancreatic polypeptide (PP), somatostatin, glucagon and vasoactive intestinal peptide levels were assessed. A preoperative secretin stimulation test (bolus 2 µg/kg, Secrelux®, SANOCHEMIA Pharmazeutika AG) were carried out in all patients and taken as positive where gastrin increased to > 120 pg/dL 2-5 min after secretin infusion. Indications for surgery were

established by the presence of hypergastrinemia, insulinoma or pancreatic nodule(s) over 1 cm in diameter. Abdominal ultrasonography (US), computed tomography (CT) and/or magnetic resonance imaging (MRI), somatostatin receptor scintigraphy (SSRS-Octreoscan), endoscopic ultrasonography (EUS) or selective pancreatic angiography with hepatic venous sampling after selective arterial secretin injection (SASI) test were performed in the majority of patients. Surgical intervention included: (1) complete duodenopancreatic mobilization, and colo-epiploic detachment; (2) inspection and palpation of the pancreas, duodenum, first jejunal loops and gallbladder; (3) abdominal intraoperative US (IOUS); (4) transillumination of the duodenum; and (5) intraoperative serum gastrin measurement by rapid immunoradiometric assay at the induction of anesthesia (basal value), 15, 30 and 45 min after pancreatic surgery. Further postoperative samples were taken after 3, 4 and 24 h. Gastrin values were measured using the rapid radioimmunoassay for serum gastrin, using specific antibodies (100% cross-reactivity) for small gastrin (G-17).

Surgery consisted of duodenopancreatectomy (DP) or total pancreatectomy (TP). Regional lymphadenectomy was performed in all patients and included pancreaticoduodenal, splenic and celiac nodes. Any hepatic tumoral lesions were removed during surgery.

All specimens were available for analysis and were confirmed as being gastrinomas by immunohistochemistry. In accordance with the World Health Organization's criteria for endocrine tumors, we distinguished between microadenomas (< 5 mm) and macromors (> 5 mm). Tumor size, proliferation index, immunohistochemical phenotype, angioinvasion, and evidence of metastatic spread were assessed. The diagnosis of each tumor as a gastrinoma was based on the presence of over 90% of cells displaying positive immunoreaction to gastrin.

During follow-up, fasting serum gastrin, additional entero-hormone levels and secretin provocative tests were performed 3 and 6 mo postoperatively, and yearly thereafter. Cure of hyperestrinism was defined as a normal serum gastrin concentration and a negative secretin test. Abdominal US and/or EUS, CT or MRI were carried out once every two years, or, if necessary, when hormonal levels increased.

## RESULTS

Seventeen of the 28 operated patients (60.7%) were affected with gastrinomas, ten patients with insulinoma and one patient with vipoma. All the patients also had non-functioning tumors in the pancreas. The mean age of patients affected by ZES was 40 years (range 24-63 years): 15 patients underwent DP and two TP. On histological examination, duodenopancreatic endocrine tumors were found in all 17 patients. Immunohistochemical evaluation revealed a total of 81 gastrinomas in the first three portions of the duodenum. In our series, only one gastrinoma was located in the pancreas. The mean number

Table 1 Characteristics, surgical treatment and follow-up of multiple endocrine neoplasia type 1 patients with biliary gastrinomas in our series									
Pt	Sex/age (yr)	MEN1 mutation	Basal gastrinemia (NV < 108 pg/mL)	Previous surgical treatment	Surgical treatment	Postoperative complications	Latest complications and adverse events	Basal serum gastrin postoperative (pg/mL)	Secretin provocative test (at follow-up)
1	F/46	Missense, Leu413Pro exon 9	213.2	Total PTX	TP and removal of a bile duct gastrinoma		Insulin-dependent diabetes mellitus Cholangitis	39.9	NEG
2	F/55	Frameshift, 843del GA, exon 6	87.1	Left nephrectomy for hydronephrosis, total PTX with autotransplantation	WDP	Pancreatojejunal dehiscence for acute pancreatitis→TP with splenectomy	Abdominal abscess	24.8	NEG
3	F/37	Arg415 exon 9stop	204.3	Less than subtotal PTX	(2010) PPDP (2012) Hepatic resection (V-VIII seg) and enucleation of a pancreatic head module	Biliary fistula	Subocclusion	32.4	NEG

PTX: Parathyroidectomy; TP: Total pancreatectomy; WDP: Whipple’s duodenopancreatectomy; PPDP: Pylorus preserving duodenopancreatectomy; MEN1: Multiple endocrine neoplasia type 1.

of gastrinomas per patient was 5 (range 1-16). The largest gastrinoma was 1.8 cm in diameter (mean diameter 0.54 cm), the smallest less than 1 mm in diameter. One apparently primary gastrinoma was detected in a peri-pancreatic lymph node. Overall, malignancy was established in 12 patients (70.5%) after finding lymph node, liver and omental metastases. Two cases, believed to be liver metastases intraoperatively, turned out to be focal nodular hyperplasia on pathological examination.

During follow-up, the secretin stimulation test was positive in only two patients after a mean of 84.8 mo. One (case 3) is discussed below, the other developed nodal recurrence of gastrinoma.

Out of the 17 patients with gastrinoma, three were found to be located in the biliary tree, either at surgery, on histological examination of the surgical specimen or during follow-up. These three cases are detailed below.

Case 1

The first case concerns a 46-year-old woman whose father had suffered from gastric ulcer and died of a myocardial infarction at the age of 61 years. Her mother had colorectal cancer at the age of 78 years. Our patient had had kidney stones since 1992, with recurrent renal colic episodes. She was also affected by type II mellitus diabetes and monoclinal gammopathy. In 1999, she experienced epigastralgia; esophago-gastro-duodenoscopy (EGDS) showed ulceration of the first and second portions of the duodenum. Genetic testing for MEN1 gene mutation was positive (Table 1). Complete biochemical assessment showed elevated levels of calcium, parathyroid hormone (PTH) and gastrin (213.2 pg/mL; VR < 108). Abdominal US revealed multiple nodules over 2 cm in diameter in the head, body and tail of the pancreas. Abdominal CT confirmed the pancreatic lesions, displaying additional smaller nodules. In December 2000, she underwent total parathyroidectomy with eight parathyroid fragments autografted in the non-dominant forearm. After surgery, she was normocalcemic and the PTH serum level was within the normal range.

Serological and radiological examination and a secretin provocative test were performed on follow-up, showing increased gastrin values after stimulation. In 2001, the patient was subjected to TP with gastroduodenal resection and splenectomy (Table 1). During surgery, a nodule was noted in the bile duct and resected. IOUS did not show any hepatic lesions. Gastrin progressively decreased, reaching 42 pg/mL at the end of the surgical procedure. Pathological examination revealed 16 non-functioning neuroendocrine tumors in the pancreas (maximum diameter 3.4 cm in the pancreatic tail) and two neuroendocrine gastrin-positive tumors reaching 1 cm, one in the duodenum and one in the bile duct. The Ki-67 index was less than 1% for all lesions. There was no evidence of lymph node metastasis (Table 2). The patient was discharged after 12 d and is currently on MEN1 follow up.



**Table 2** Pathological findings in multiple endocrine neoplasia type 1 patients with biliary gastrinomas

Pt	Gastrinomas (n)	Size of largest duodenal gastrinoma (cm)	Size of largest biliary gastrinoma (cm)	Other pancreatic endocrine neoplasms (n)	Size of largest NF PEN (cm)	Total tumors (n)	Hepatic findings	Gastrinoma metastases
1	2 (D, BD)	1.0	1.2	16 NF	3.4	18	Negative	Negative
2	4 (3D, 1G)	0.2	0.2	32 NF	1.6	32	Negative	Negative
3	3 (2D, 1BD)	0.3	1.5	37 NF	2.0	40	Negative	Positive (N)

D: Duodenal; BD: Biliary duct; G: Gallbladder; N: Lymph node; PEN: Pancreatic endocrine neoplasms; NF: Non-functioning.

## Case 2

The second case concerns a 55-year-old woman. Her mother had died of chronic renal failure at the age of 86 and her father at 67 of a carcinoid of the small bowel, her sister had died at the age of 46 from neurologic complications of a pituitary prolactinoma.

Our patient's clinical history began at the age of 24 when she developed kidney stones with recurrent renal colic. In 1994, she underwent left nephrectomy for hydronephrosis and hysterectomy for uterine leiomyomatosis. In 1999, she suffered episodes of epigastric pain and EGDS showed the presence of a duodenal ulcer. In 2001, the patient was clinically diagnosed with MEN1 and was referred to our department for a complete serum biochemical assessment, which showed elevated levels of PTH and calcium. A genetic diagnosis of MEN1 was made (Table 1), and in 2002, she underwent total parathyroidectomy, with a parathyroid autograft in the non-dominant forearm. After surgery, she was normocalcemic with normal PTH serum levels.

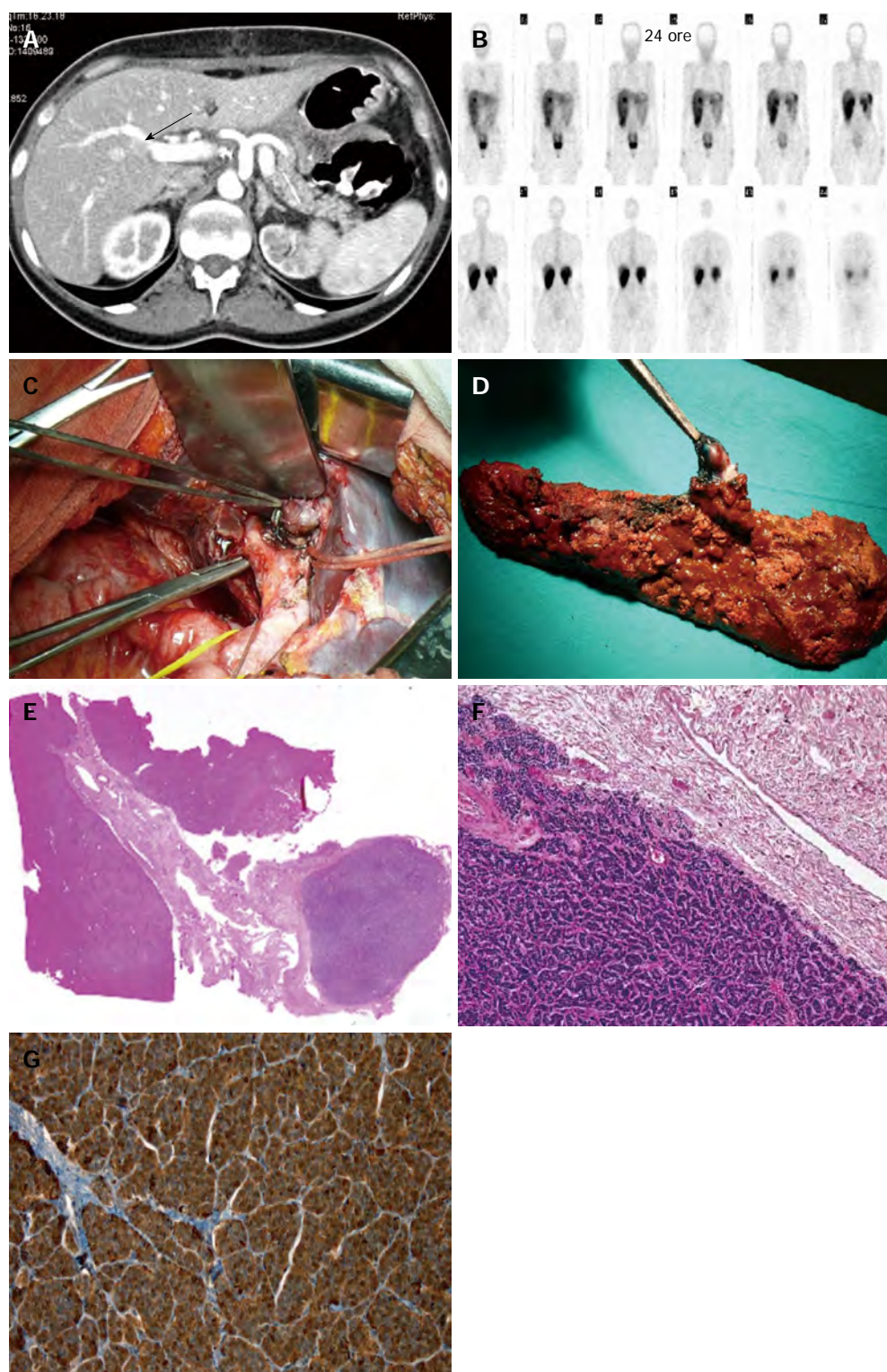
On follow-up, a secretin provocative test showed an increase in gastrin values after stimulation. CT revealed the presence of nodules in the pancreatic head. SSRS confirmed the presence of pathological neuroendocrine tissue in the first and second portions of the duodenum, and in the head of the pancreas. In October 2003, she underwent Whipple's duodenopancreatectomy with regional lymphadenectomy and cholecystectomy (Table 1). Intraoperative US showed no residual pancreatic or hepatic lesions, and gastrin levels decreased to 25 pg/mL at the end of the surgical procedure. Pathological examination revealed 28 non-functioning neuroendocrine tumors of the pancreas (maximum diameter 1.6 cm) and four gastrin-secreting tumors (maximum diameter 0.2 cm), three in the duodenum and one in the gallbladder fundus. The Ki-67 index was less than 1%. There was no evidence of lymph node involvement (Table 2). The postoperative course was complicated by acute pancreatitis and consequent pancreato-jejunal anastomotic dehiscence, thus requiring TP with splenectomy. The patient was discharged after 38 d and is still on follow-up for MEN1.

## Case 3

The third case concerns a 36-year-old woman, whose father had died of myocardial infarction at the age of 52, while her mother and two sisters were in good health. In 2004, she was subjected to left inferior parathyroidectomy for primary hyperparathyroidism, which recurred 4 mo

later. The right superior parathyroid gland was removed, and partial thymectomy was carried out. Intraoperative PTH decreased from 1050 pg/mL at baseline to 150 pg/mL, 20 min after parathyroidectomy. On suspicion of MEN1, the patient was referred to our institution for a genetic test, which revealed mutation of the *MEN1* gene (Table 1). She underwent complete biochemical and radiological examination with CT, but no neuroendocrine lesions of the pancreas were found. MRI was negative for pituitary lesions.

In 2010, she was admitted to our surgical unit for heartburn, abdominal pain and diarrhea. An increase in basal gastrin serum levels (184 pg/mL; NV < 108) and a secretin provocative test confirmed hypergastrinemia (9783 pg/mL). EGDS showed esophagitis and gastroduodenitis with peptic ulcers. The clinical diagnosis of ZES in MEN1 was put forward, and the patient started proton pump inhibitor (PPI) therapy. Abdominal EUS showed a 7-mm nodule in the pancreatic head, together with small submucosal lesions of the first and second parts of the duodenum. In October 2010, she underwent pylorus-preserving duodenopancreatectomy extended to the pancreatic body, with removal of the regional lymph nodes and the gallbladder (Table 1). End-to-side Wirsung-jejunal anastomosis, and common bile duct-jejunal and duodeno-jejunal anastomoses through transmesocolic Roux-en-Y loop were then performed. The IOUS showed no hepatic lesions. Gastrin serum levels progressively decreased, reaching 20 pg/mL at the end of the surgical procedure. Pathology revealed 37 neuroendocrine tumors of the pancreas (maximum diameter 2 cm) and three gastrin positive tumors of the duodenum (maximum diameter 2 mm). Of the 31 lymph nodes examined, two had neuroendocrine tumor tissue positive for gastrin. The postoperative course was uneventful and the patient was discharged 17 d later on PPI therapy (Omeprazole 40 mg × 2/die). One month after surgery, she had abdominal pain and vomiting, and was readmitted to our ward. An upper gastro-intestinal tract X-ray showed slow transit at the level of the anastomotic jejunal loop. Laparotomy was carried out with dissection of adhesions between the small bowel and the great omentum, and the mesocolic defect was closed, suturing it to the stomach. The day after surgery, gastrin levels were measured, revealing a significant increase in serum values (173 pg/mL). Five days later the patient was discharged and given PPI drugs and somatostatin analogs. After two mo, a 12 mm hepatic lesion, posterior to the V-VIII seg-



**Figure 1** Computed tomography scan. A: The tumor (arrow) near the glissonian capsule of the right portal triad sheet; B: The lesion was positive at somatostatin receptor scintigraphy; C: Intraoperative picture: the tumor is close to the hepatic hilum, as indicated by the surgical forceps; D: Surgical resection of the V-VIII hepatic segments: the nodule is shown by the surgical forceps; E: Overview of the neuroendocrine tumor found inside the glissonian capsule of the right portal triad sheet; F: Tumor displaying distinct trabecular pattern; G: Massive gastrin production by the majority of neoplastic cells is shown by immunophenotyping.

ment portal vein branch, was detected by CT and confirmed at US. MRI was subsequently performed to better define the hepatic lesion, which showed a 15 mm nodule in the V segment, immediately below the right portal bifurcation. SSRS confirmed the presence of the hepatic nodule with a high density of SST2 receptor (Figure 1).

Basal gastrin serum levels were within the normal range (45.6 pg/mL), but a secretin stimulation test displayed an increase to 482 pg/mL. In January 2012, the patient was readmitted to our department with epigastric pain, heart-burn and anemia (Hb 7.1) associated with a syncopal episode. An EGDS and a colonoscopy gave negative results.

A SASI test was carried out, producing two gastrin peaks at 1541 and 1217 pg/mL 40 s after stimulation in the hepatic and superior mesenteric arteries, respectively. EUS identified three pancreatic lesions of approximately 0.5 cm in diameter. In February 2012, the patient underwent hepatic resection of the V-VIII segments to remove the lesion, which was found inside the glissonian sheet of the anterior sectional pedicle and not in the liver parenchyma (Figure 1 and Table 1). A 0.7 cm lesion of the residual pancreatic body was also enucleated (Table 2). Intraoperative gastrin levels decreased below the normal range to 32 pg/mL. A gastrin-secreting tumor in the biliary tree and a pancreatic neuroendocrine lesion were found at histology (Figure 1). Both lesions had a Ki-67 index lower than 1%. The patient developed a biliary fistula, which was then treated conservatively. She was discharged after 15 d and is still on follow-up for MEN1.

## DISCUSSION

Primary gastrinomas are classified as duodenal, pancreatic and extraintestinal-extrapancreatic. The pancreas and duodenum are the most frequent sites of sporadic gastrinomas, while the duodenum is almost exclusively the localization of gastrinomas in MEN1 patients<sup>[4-6]</sup>. MEN1-associated gastrinomas occur predominantly in the first three parts of the duodenum, are usually multiple and measure less than 5 mm in diameter<sup>[3-6,16]</sup>. In some cases, duodenal gastrinomas are only found on microscopic examination. From 34% to 85% of duodenal gastrinomas are metastatic at the time of surgery, but tumor spread is usually restricted to the regional lymph nodes<sup>[4-6]</sup>. Pancreatic localization is rare in MEN1 syndrome, sometimes being associated with a more aggressive behavior of the tumor, which may metastasize to the liver<sup>[17]</sup>.

Extraduodenal-extrapancreatic gastrinomas in MEN1 syndrome are rare, while they account for 22% of sporadic gastrinomas according to Norton *et al.*<sup>[18]</sup>. The improved ability to identify gastrinomas in the duodenal wall, either preoperatively or intraoperatively, has resulted in an apparent increase in the incidence of duodenal gastrinomas and decrease in the incidence of occult lesions and primary peripancreatic lymph node gastrinomas. Wu *et al.*<sup>[19]</sup> found extrapancreatic and extraduodenal gastrinomas in only 8 (5%) of 142 patients with ZES, none of whom had MEN1. The sites of these gastrinomas were the ovary, omentum, stomach, jejunum, kidney, biliary tract, and, in 2 cases, the liver<sup>[19]</sup>.

To the best of our knowledge, only twenty cases of hormonally active gastrin-producing or immunostaining gastrin positive neuroendocrine tumors of the extrahepatic biliary tree have been published in literature, 18 in the sporadic setting and two in MEN1<sup>[14]</sup>. The majority of these patients were female and under 50 years of age. The most common symptom was jaundice and, more rarely, peptic ulceration or frank ZES. Generally, basal gastrin serum levels were high and the secretin test was positive. These gastrinomas mainly occurred in the com-

mon bile duct or common hepatic duct, less frequently at the confluence of the hepatic duct. Tumor size varied from a few millimeters to over 2 centimeters. Lymph node or liver metastases were detected in almost 50% of these cases. All these gastrinomas were resectable, and in most cases radical surgery was carried out with no evidence of disease recurrence at either short or long term follow-up.

Primary hepatic gastrinomas are even less frequent than those occurring in the extrahepatic biliary tree, with fewer than 20 cases reported in the literature and only one case described in MEN1<sup>[15,20]</sup>. These gastrinomas are generally single nodules, 1-7 cm in diameter, with high radiotracer uptake at octreoscan. Liver resection resulted in prompt normalization of gastrin serum levels with no evidence of recurrence, suggesting these tumors are primary rather than metastases of occult extrahepatic microgastrinomas. Therefore, only three MEN1 ectopic gastrinomas have been described before the present study: two in the extrahepatic biliary tree and one in the liver<sup>[14,15]</sup>. The clinicopathological characteristics of these primary ectopic gastrinomas are summarized in Table 3. All the patients were young females, with associated hyperparathyroidism, pituitary adenoma, and pancreatic functioning or non-functioning tumors. In all cases, the basal gastrin values were high and the ectopic gastrinoma was single, varying from 6 to 15 mm. The two extrahepatic biliary duct cases were detected intraoperatively, while the hepatic gastrinoma was diagnosed preoperatively at CT and SSRS. Lymph nodes displayed neuroendocrine tumor tissue positivity for gastrin in only one patient. In another, a suspected hepatic metastasis was ablated with radiofrequency and alcoholization. In all but one patient, excision of these ectopic gastrinomas was carried out during DP to remove concomitant duodenal or pancreatic neuroendocrine tumors. These patients were eugastrinemic immediately after surgery and at follow-up.

Our experience regarding MEN1 ectopic gastrinomas is similar to that in the literature: gastrinomas were found incidentally during DP in two patients, and detected in the liver in another after recurrence of ZES. The most likely hypothesis explaining the development of gastrinomas in the biliary tree is the presence of stem cells with the capacity to differentiate into specific endocrine cells. Endocrine cells are widely distributed in the epithelial layer of organs that originate from the primitive gut. Argrophil and somatostatin cells have been identified in the glandular epithelium of both the extrahepatic and intrahepatic bile ducts in adult and infant normal livers<sup>[21,22]</sup>. These cells may increase in number, and many kinds of other endocrine cells appear anew and even proliferate in hepatocytes<sup>[22]</sup>. Endocrine cells also occur in the gallbladder, where metaplastic changes of the mucosa can be induced by lithiasis. Similarly, primary gastrinomas developing in the hepatic parenchyma may originate from endocrine cells within the small peripheral intrahepatic biliary tree. In our third case, the gastrinoma was not localized in the hepatic parenchyma, but along the vascular



**Table 3** Clinicopathological characteristics of multiple endocrine neoplasia type 1 patients with extraduodenal-extrapancreatic gastrinomas reported in the literature

Ref.	Sex/age	Site/size (mm)	Gastrin: basal/ stimulation	Diagnostic imaging positivity	MEN1 stigmata	Surgical treatment	Metastasis	BPC gastrin
Price <i>et al</i> <sup>[14]</sup>	F/55	CD (CBD junction)/6 mm	4500/NR	IOUS, discovered at surgery	pHPT, PA, NF-PETs (n°21/1-6 mm)	Distal splenopancreatectomy; RFA hepatic excision BC	Liver, (solitary)	780 (2 yr)
Price <i>et al</i> <sup>[14]</sup>	F/43	CBD/15 × 12 × 1	580/4300	IOUS, discovered at surgery	pHPT, PA, NF-PET (4), DG (3/1-4 mm); GC, lipoma	PPDP; enucleation; gastric excision	Lymph nodes (CBD HA)	86 (2 yr)
Lee <i>et al</i> <sup>[15]</sup>	F/39	Liver (II-III seg)/16 × 15 × 0.9	447/NR	CCT, SSRS, IOUS	pHPT, PA, insuli- noma	WDP; II-III liver segmentec- tomy	-	34 (po)

pHPT: Primary hyperparathyroidism; PA: Pituitary adenoma; DG: Duodenal gastrinoma; GC: Gastric carcinoid; WDP: Whipple's duodenopancreatectomy; PPDP: Pylorus preserving duodenopancreatectomy; D: Duodenal; CBD: Common bile duct; CD: Cystic duct; G: Gallbladder; N: Lymph node; F: Female.

pedicle, suggesting a possible origin from cells arranged along the bile duct.

Histological findings of gastrinomas almost invariably correspond to well-differentiated neuroendocrine tumors, according to the WHO 2010 classification<sup>[23]</sup>. Immunohistochemically, gastrin can be detected in all tumors: many duodenal gastrinomas are multihormonal, and contain single somatostatin or serotonin expressing cells. Approximately 50% of gastrinomas contain PP, glucagon and/or insulin, as well as gastrin. There is a high risk of gastrinomas behaving malignantly, irrespective of size, with frequent lymph node and liver metastases; pancreatic tumors tend to be worse than duodenal lesions. Malignancy cannot be predicted histologically, except on evidence of angioinvasion or infiltration of surrounding tissues. The proliferation fraction can be taken as an indicator of malignancy and a Ki-67 index greater than 10% is invariably associated with development of metastases<sup>[23]</sup>.

Distinguishing ectopic primary gastrinomas from metastatic gastrinomas is not easy. MEN1 gastrinomas of the duodenum or pancreas can metastasize to the liver and be mistaken for ectopics if the primary tumor goes undetected. Several factors in our MEN1 patients helped to exclude the biliary gastrinomas as being metastatic rather than ectopic. First, segmentary biliary ducts and gallbladder are unusual sites for metastasis. Second, the gastrinomas in all the patients were single lesions and with no disease progression, as commonly occurs in metastatic neuroendocrine tumors. No liver or lymph node metastases were found in two cases, while in the third, diagnosed with duodenal gastrinomas metastatic to the regional lymph nodes, gastrin values normalized rapidly at the end of the first operation, but increased one month later.

Surgical treatment of MEN1 gastrinomas is a matter of debate. Some authors suggest simple enucleation of the duodenal mucosa or limited full-thickness duodenal resection, while others recommend DP<sup>[24,25]</sup>. The majority of patients after duodenal resection, are not cured definitively, and hypergastrinemia, as well as metastatic disease, can occur<sup>[26,27]</sup>. According to Thompson<sup>[28]</sup>, the secretin stimulation test proved negative in only one third

of patients submitted to this procedure, in spite of the fact that most of them (68%) remained eugastrinemic for a long postoperative period. DP has been claimed to promote higher curability rates<sup>[27,33]</sup>. In our experience, surgery is curative after resection of affected pancreatic tissue and enucleation of macroscopic nodules in the residual pancreas. On reviewing the literature, the curability rate of DP with regional lymphadenectomy in MEN1 gastrinomas is more than 85%<sup>[27,31,34-36]</sup>. Indeed, resection of the so-called "gastrinoma triangle", achieved with DP, also allows the removal of lesions located in the common bile duct, the cystic duct or the gallbladder. The frequent relapse in MEN1 gastrinoma patients after duodenal excision, could be caused by residual ectopic gastrinomas that were not removed through conservative surgery, rather than to duodenal recurrence.

The possibility of primary gastrinomas arising in the biliary tree in MEN1 syndrome must always be taken into consideration to ensure that appropriate therapy is used, as this ectopic localization can explain surgical failure in MEN1 ZES. Intraoperative palpation, transillumination and IIOUS of the biliary tree could be useful to locate ectopic gastrinomas. The clinical community should be made aware of these findings to improve the management of MEN1 patients.

## COMMENTS

### Background

Surgical removal of duodenal multiple endocrine neoplasia type1 (MEN1) gastrinomas can lead to persistence or recurrence of hypergastrinemia. A possible explanation for this unfavorable outcome could be unrecognized ectopic localization of gastrin-secreting tumors.

### Research frontiers

Primary gastrinomas arising in the biliary tree can explain surgical failure in the treatment of Zollinger-Ellison syndrome in MEN1 syndrome.

### Innovations and breakthroughs

Only twenty cases of hormonally active gastrin-producing or immunostaining gastrin positive neuroendocrine tumors of the extrahepatic biliary tree have been published in the literature, 18 in the sporadic setting and two cases in MEN1. Primary hepatic gastrinomas are even more infrequent than those of the extrahepatic biliary tree, with fewer than 20 cases reported in the literature to date and only one case described in MEN1.

### Applications

Three out of 17 MEN1 patients were found to be affected by an ectopic gastri-



noma located in the biliary tree. These findings suggest that in MEN1 patients, the presence of ectopic biliary tree gastrinoma is not rare, occurring in 17.6% of cases.

### Terminology

MEN1 is a familial syndrome characterized by tumors of the parathyroids, the anterior pituitary and the enteropancreatic endocrine system. Enteropancreatic endocrine neoplasms affect up to 90% of MEN1 patients. Gastrinomas are the most common functional enteropancreatic neuroendocrine tumor and, according to recent literature, are located almost exclusively in the duodenum in MEN1 patients.

### Peer review

These results lead to doubling of the number of cases described in the literature regarding ectopic biliary tree gastrinomas in MEN1 patients. These findings should be made available to the clinical community to improve the management of MEN1 patients.

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## Gastrojejunostomy in patients with unresectable pancreatic head cancer - the use of Roux loop significantly shortens the hospital length of stay

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### Abstract

**AIM:** To evaluate the use of the Roux loop on the postoperative course in patients submitted for gastroenteroanastomosis (GE).

**METHODS:** Non-jaundiced patients ( $n = 41$ ) operated on in the Department of General and Transplant Surgery in Lodz, between January 2010 and December 2011 were enrolled. The tumor was considered unresectable when liver metastases or major vascular involvement were confirmed. Patients were randomized to receive Roux ( $n = 21$ ) or conventional GE ( $n = 20$ ) on a prophylactic basis.

**RESULTS:** The mean time to nasogastric tube withdrawal in Roux GE group was shorter ( $1.4 \pm 0.75$  vs  $2.8 \pm 1.1$ ,  $P < 0.001$ ). Time to starting oral liquids, soft diet and regular diet were decreased ( $2.3 \pm 0.86$  vs  $3.45 \pm 1.19$ ;  $P < 0.001$ ;  $3.3 \pm 0.73$  vs  $4.4 \pm 1.23$ ,  $P < 0.001$  and  $4.5 \pm 0.76$  vs  $5.6 \pm 1.42$ ,  $P = 0.002$ ; respectively). The Roux GE group had a lower use of prokinetics (10 mg thrice daily for  $2.2 \pm 1.8$  d vs  $3.7 \pm 2.6$  d,  $P = 0.044$ ;

total  $62 \pm 49$  mg vs  $111 \pm 79$  mg,  $P = 0.025$ ). The mean hospitalization time following Roux GE was shorter ( $7.7$  d vs  $9.6$  d,  $P = 0.006$ ). Delayed gastric emptying (DGE) was confirmed in 20% after conventional GE but in none of the patients following Roux GE.

**CONCLUSION:** Roux gastrojejunostomy during open abdomen exploration in patients with unresectable pancreatic cancer is easy to perform, decreases the incidence of DGE and lowers hospitalization time.

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**Key words:** Unresectable pancreatic cancer; Roux and conventional gastroenteroanastomosis; Gastroenteroanastomosis; Delayed gastric emptying; Hospital length of stay

**Core tip:** The lower rate of delayed gastric emptying, which determines lower use of prokinetics after Roux compared to conventional antegastric gastroenterostomy (GE) suggested that prophylactic Roux GE should be performed during surgical exploration of patients with unresectable pancreatic head tumors. The length of hospital stay is shorter following palliative Roux GE, thus the treatment costs of these patients are likely to be smaller. Further research is needed on the cost-effectiveness of prophylactic Roux GE in unresectable pancreatic cancer.

Szymanski D, Durczynski A, Nowicki M, Strzelczyk J. Gastrojejunostomy in patients with unresectable pancreatic head cancer - the use of Roux loop significantly shortens the hospital length of stay. *World J Gastroenterol* 2013; 19(45): 8321-8325 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8321.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8321>

## INTRODUCTION

Adenocarcinoma of the pancreas is a serious public health problem. It accounts only for 2% of new cancer diagnoses in both men and women, but it ranks as the fourth leading cause of cancer-related deaths in the United States<sup>[1]</sup>. Despite recent developments in new imaging techniques and improved staging studies, the incidence rate of early pancreatic cancer has changed little over the last decades. Nonetheless, the surgical treatment of pancreatic cancer is palliative in more than 80%, with median overall survival of 6 mo when diagnosed at the metastatic stage<sup>[2]</sup>.

Nowadays, with increasing incidence of pancreatic cancer, annual costs for therapy have risen rapidly<sup>[3-6]</sup>. The average monthly cost of treatment of a patient with pancreatic cancer is almost \$7000; in patients with terminal disease this can rise to as much as \$6555<sup>[7]</sup>. New anti-cancer agents are the largest expenditure, although the greater part of the costs comes from palliative surgical procedures and postoperative hospitalization care<sup>[8]</sup>. Pancreatic surgeries in high-volume centers are associated with low mortality, but high morbidity<sup>[9-12]</sup>. Postoperative complications increase the duration of the hospital stay and treatment costs.

Limited survival benefit and unfavorable cost-effectiveness make surgery for later stages of pancreatic cancer controversial. From this perspective, the optimal method of palliation is uncertain when tumor unresectability is determined at exploration. Shortening of the length of postoperative hospital stay and associated direct costs is an important part of the development of the new palliative procedures.

The aim of our study was to evaluate the influence of two different surgical techniques for creating gastroenterostomy on the postoperative delayed gastric emptying (DGE) rate and the length of hospital stay in non-jaundiced patients with unresectable pancreatic head cancer.

## MATERIALS AND METHODS

This prospective, randomized study comprised non-jaundiced patients with unresectable pancreatic head tumor ( $n = 41$ ), hospitalized in the Department of General and Transplant Surgery of Medical University in Lodz, who received solitary gastroenterostomy on a prophylactic basis from January 2010 to December 2011. Patients were randomized to receive either antecolic Roux ( $n = 21$ ), or conventional antegastric hand-sewn side-to-side gastroenterostomy ( $n = 20$ ). Before surgery, each patient was allotted a code (Roux or conventional group). Blinded investigators performed all postoperative assessments.

All patients gave signed, written, informed consent for the study. Most of the patients ( $n = 37$ ) originally presented with jaundice in the endoscopic units, where the biliary stents have been inserted. As endoscopic retrograde cholangiopancreatography in Roux-en-Y gastric bypass patients is challenging, anastomoses was performed without the stomach being transected or divided.

Therefore, blockage of the biliary stents causing recurrent jaundice could easily have been managed with stent replacement, without the need of percutaneous drainage. The risk of occlusion of stents increased in our group after 3 mo, thus elective stent exchange at 3-6 mo was performed using the standard technique. Changing a stent was available for the Roux and conventional GE group.

The tumor was considered unresectable when the presence of distant metastases or major vascular involvement was confirmed. Conventional GE was performed in a standard side-to-side antecolic fashion, 20 cm from the ligament of Treitz. Roux GE was constructed as follows. A Roux-en-Y intestinal loop, 60 cm long, was prepared by transecting the jejunum 20 cm from the ligament of Treitz, which was then anastomosed to the stomach in an antecolic fashion to construct a latero-lateral gastro-jejunosomy. The intestinal continuity was restored by a jejuno-jejunal, hand-sewn anastomosis. In all cases, the Tru-cut biopsy of the tumor was obtained. All patients without microscopic diagnosis of the cancer were excluded from the study. Eventually, 21 patients with pancreatic cancer confirmed by pathological report received Roux gastroenterostomy and 20 received the conventional GE. Experienced pancreatic surgeons performed all surgeries. All patients provided written informed consent for the study.

The postoperative course of every patient was documented retrospectively, with special regard to the length of hospital stay as a primary endpoint, as well as prokinetic therapy duration, the number of days of nasogastric tube decompression (NGT), the start of oral fluids, soft diet and solid diet (secondary endpoints). According to recent studies<sup>[13]</sup>, DGE was defined as (1) the nasogastric decompression lasting more than 3 postoperative days (POD) or the need for reinsertion of NGT for persistent nausea and vomiting after POD 3; (2) the inability to tolerate a solid diet by POD 7; or (3) the need for prokinetic agents after POD 10. All data were shown in the text and tables as means  $\pm$  SD.

### Statistical analysis

All statistical calculations were performed using Sigma-Plot version 12.0 (Systat Software Inc., San Jose, CA, United States) with the level of statistical significance set at  $P < 0.05$ . To compare the differences in mean length of hospital stay, time to the postoperative nasogastric tube withdrawal, liquids, liquid diet and full regular diet following Roux and conventional GE, we applied the parametric *t* test and non-parametric Mann-Whitney test. In the *t*-test, equal variance tests were performed to demonstrate differences in the use of prokinetics. All data were shown in the text and tables as means or medians  $\pm$  SD.

## RESULTS

Figure 1 is a flow chart of patient enrollment, randomization and progress through the study. The demographics of the patients from both groups are summarized in Ta-



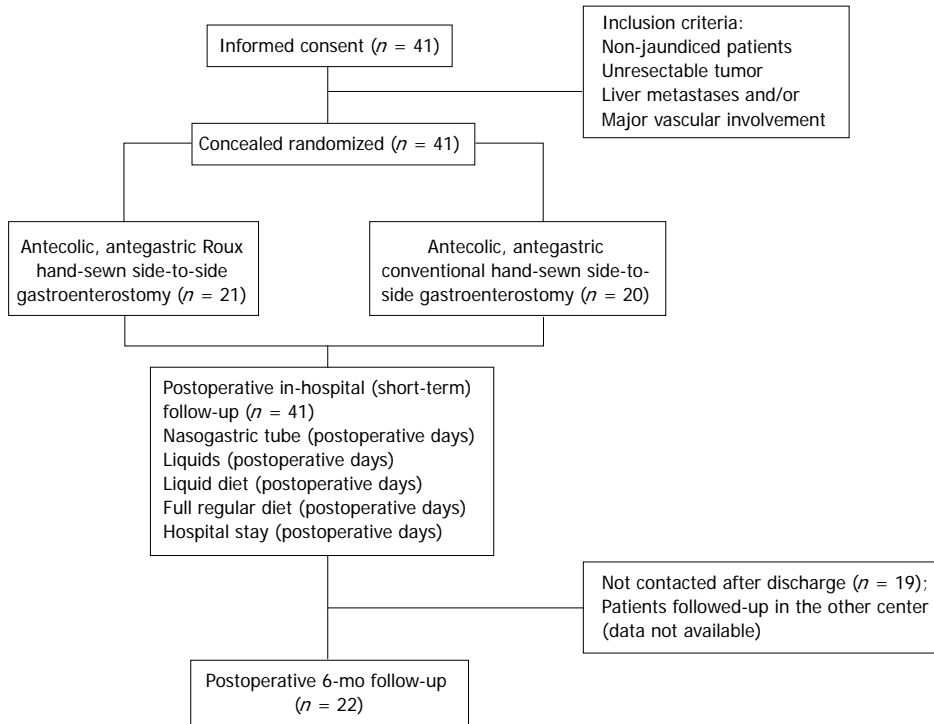


Figure 1 Flow chart of patient enrollment, randomization and progress through the study.

**Table 1 Characteristics and demographics of patients with pancreatic cancer undergoing Roux and conventional gastroenteroanastomosis**

Patients characteristics and demographics	Roux GE	Conventional GE
Age (yr)	60.2 ± 10.6	61.6 ± 8.5
Gender		
Female	10 (50)	8 (40)
Male	10 (50)	12 (60)
Reason for unresectability		
Local	15 (75)	14 (70)
Liver metastases	5 (25)	6 (30)
Indication	Prophylactic	Prophylactic
Reconstruction	Antecolic	Antecolic
Position	Antegastric	Antegastric

Data are expressed as absolute numbers (percentage) or mean ± SD. GE: Gastroenteroanastomosis.

ble 1. The mean operative time of Roux GE was  $55 \pm 25$  min *vs*  $48 \pm 36$  min for conventional GE. Shorter mean time to the postoperative nasogastric tube withdrawal (by 50%,  $P < 0.001$ ), liquids (by 33.3%,  $P < 0.001$ ), liquid diet (by 25%,  $P < 0.001$ ) and full regular diet (by 19.6%,  $P = 0.002$ ) following Roux in contrast to conventional GE was demonstrated (Table 2). No patients required reinsertion of the nasogastric tube. Delayed gastric emptying did not occur after Roux GE, whereas it was confirmed in four cases after conventional GE (20%). The Roux GE group had a lower use of prokinetics compared with conventional GE (10 mg of metoclopramide iv thrice daily for  $2.2 \pm 1.8$  d *vs*  $3.7 \pm 2.6$  d,  $P = 0.044$ ; total  $62 \pm 49$  mg *vs*  $111 \pm 79$  mg,  $P = 0.025$ ; respectively). The mean length of hospital stay was shorter following pallia-

tive Roux GE (7.7 d *vs* 9.6 d;  $P = 0.006$ ). The recurrence of jaundice and cholangitis (23% of patients) and mean survival were comparable in both groups during 6-mo follow-up.

## DISCUSSION

The palliative treatment of patients with unresectable pancreatic cancer is a significant economic burden to public healthcare. As limited survival is expected and the total costs of treatment per incident case are high, there are general concerns about the necessity of palliative procedures, which most frequently surgical in patients with pancreatic cancer. Biliary stents or biliary bypass in patients with jaundice have a definite role because they decrease morbidity; however, performing prophylactic gastroenterostomy is still a matter of debate for several reasons. First, it is difficult to predict the number of patients who will develop duodenal obstruction. Second, delayed gastric emptying is a frequent complication after gastroenterostomy. It is usually not a life-threatening condition and can be treated conservatively, although it compromises quality of life, prolongs the hospital stay and adds to hospital costs in patients with a very limited life expectancy. Recent reports have proved that prophylactic gastroenterostomy should be constructed in patients that are found to have unresectable pancreatic cancer at exploration<sup>[14]</sup>; therefore, it is necessary to decrease the incidence of DGE.

The occurrence of DGE after gastroenterostomy varies from 9% to 26%<sup>[15]</sup>. These differences may reflect considerable variations in DGE definition in previous

**Table 2** Postoperative course following Roux and conventional gastroenteroanastomosis

Postoperative course	Roux GE		Conventional GE		Differences in the Roux and conventional GE ( <i>P</i> value)	Power analysis <sup>1</sup>
	mean $\pm$ SD	Median	mean $\pm$ SD	Median		
Nasogastric tube (postoperative days)	1.4 $\pm$ 0.75	1	2.8 $\pm$ 1.1	3	< 0.001	0.962
Liquids (postoperative days)	2.3 $\pm$ 0.86	2	3.45 $\pm$ 1.19	3	< 0.001	0.86
Liquid diet (postoperative days)	3.3 $\pm$ 0.73	3	4.4 $\pm$ 1.23	4	< 0.001	0.853
Full regular diet (postoperative days)	4.5 $\pm$ 0.76	4	5.6 $\pm$ 1.42	5	0.002	0.784
Hospital stay (postoperative days)	7.7 $\pm$ 3.01	7	9.6 $\pm$ 2.79	10	0.006	0.499

<sup>1</sup>Power *t*-test analysis was performed to justify the sample size. DGE: Delayed gastric emptying; GE: Gastroenteroanastomosis.

studies. However, we used the strict definition of DGE suggested by the International Study Group of Pancreatic Cancer<sup>[13]</sup>.

DGE is a multifactorial problem, which has been linked to tumor involvement of the coeliac axis and interruption of splanchnic innervations, as well as the technique of gastroenterostomy<sup>[15-20]</sup>. Our study confirmed that construction of a gastrojejunal anastomosis with an isolated Roux loop was beneficial (Table 2). The incidence of postoperative DGE was reduced. The procedure was technically simple and convalescence was rapid. Success may depend on already known parameters. Roux anastomosis remains mechanically efficient; however, postoperative gastric motility is temporarily impaired.

The development of laparoscopic surgical methods offers further reduction in costs associated treatment of unresectable pancreatic cancer. Laparoscopic gastric bypass is an effective and safe procedure for patients with gastric outlet obstruction. The hospital stay is shorter than after open surgery and recovery is more rapid<sup>[21]</sup>. It is technically feasible<sup>[22]</sup> for Roux gastrojejunostomy to be utilized for laparoscopic approach<sup>[23-26]</sup>. Thus, it may reveal new perspectives in prophylactic gastrojejunostomy in patients with unresectable pancreatic cancer. If curative resection is not possible, the response to chemotherapy is expected. Minimally invasive laparoscopic Roux GE may enable the patient to receive postoperative chemotherapy earlier. However, until now, the laparoscopic approach in pancreatic cancer has remained limited.

In conclusion, Roux gastroenterostomy should be performed routinely during open abdomen exploration in patients with unresectable pancreatic head carcinoma, because it is easy to perform, is free of specific complications, decreases incidence of DGE, and reduces the length of hospital stay and associated health care costs.

## COMMENTS

### Background

The surgical palliation is an important component of the treatment of pancreatic head cancer. However, the optimal method of palliation is uncertain when tumor unresectability is determined at exploration.

### Research frontiers

Prophylactic gastroenterostomy should be constructed in patients who are found to have unresectable pancreatic cancer at exploration. Nevertheless, delayed gastric emptying is a frequent complication after gastroenterostomy. This research evaluated the use of different surgical techniques for creating gastroenterostomy on the postoperative delayed gastric emptying (DGE) rate and the

hospital length of stay in non-jaundiced patients with unresectable pancreatic head cancer.

### Innovations and breakthroughs

This is the first paper to precisely describe differences in the postoperative course between antecolic Roux and conventional antegastric hand-sewn side-to-side *gastroenterostomy* in patients with unresectable pancreatic head cancer.

### Applications

The study results suggest that Roux instead of conventional antegastric gastroenterostomy should be performed routinely during open abdomen exploration in patients with unresectable pancreatic head carcinoma, because it is easy to perform, is free of specific complications, decreases incidence of DGE, and reduces the length of hospital stay and associated health care costs.

### Peer review

The results evaluate the influence of two different surgical techniques of creating gastroenterostomy on the postoperative delayed gastric emptying rate and the length of stay hospital in non-jaundiced patients with unresectable pancreatic head cancer. This is an interesting randomized study, though it suffers from many vulnerabilities.

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## Gastric endoscopic submucosal dissection: From animal model to patient

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of ESD, as well as complications were registered. In patients, their clinical, endoscopic and histologic evolution was additionally added.

**RESULTS:** Thirty *en bloc* ESDs were carried out in animal models. The mean  $\pm$  SD size of the pieces was of  $28.4 \pm 1.2$  mm, and the time of ESD was  $41.7 \pm 2.4$  min. The time of ESD in the first 15 procedures was  $43.0 \pm 3.0$  min whereas in the next 15 procedures, the time was  $40.3 \pm 3.9$  min,  $P = 0.588$ . The speed in the first 15 ESDs was  $1.25 \pm 0.11$  cm<sup>2</sup>/min vs  $2.12 \pm 0.36$  cm<sup>2</sup>/min in the remaining 15,  $P = 0.028$ . There were no complications. In patients, 5 lesions were resected *en bloc*. The size of the pieces was  $25.2 \pm 5.1$  mm and the time was  $85.0 \pm 25.6$  min. Endoscopic and histological controls did not show evidence of residual neoplastic tissue.

**CONCLUSION:** A sequential ESD training program of a unique endoscopist, based on the practice in porcine models, contributed to learning ESD for its subsequent application in humans, yielding good results in efficacy and safety.

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### Abstract

**AIM:** To assess whether the use of porcine models is useful for learning endoscopic submucosal dissection (ESD), thus contributing to its subsequent application in human patients.

**METHODS:** This study/learning process was carried out in 3 phases: Phase I : *Ex vivo* animal; Phase II : *In vivo* animal; Phase III: Humans. One endoscopist performed 30 gastric ESDs in porcine models, and later 5 gastric ESDs in 5 patients. The ESD was done following the method practiced at the National Cancer Center in Tokyo, Japan. Technical aspects, size, time and speed

**Key words:** Endoscopic submucosal dissection; Porcine models; Animal models; Training

**Core tip:** This study was conducted with the purpose of determining in a prospective manner the results, the efficacy and safety of learning endoscopic submucosal dissection (ESD), in porcine models and assessing whether this practice contributes to subsequent application of this technique in patients. The present study shows interesting findings in this regards. The authors have demonstrated the value of a sequential ESD program and believe that it can contribute to disseminate this technique and encourage its learning mostly in western countries where it is still not a common prac-



tice for different reasons.

González N, Parra-Blanco A, Villa-Gómez M, Gamba A, Taulard A, Silveira A, Sanguinetti A, Olano C, Cohen H. Gastric endoscopic submucosal dissection: From animal model to patient. *World J Gastroenterol* 2013; 19(45): 8326-8334 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8326.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8326>

## INTRODUCTION

Endoscopic submucosal dissection (ESD) is a technique for resection of early neoplastic gastric lesions. It is applied to extirpate large lesions (greater than 15 mm), removing in one bloc more healthy adjacent tissue (horizontal oncologic border) and more submucosal tissue (vertical oncologic border), thus obtaining more complete resections than with the standard mucosectomy and decreasing the probability of relapses<sup>[1-5]</sup>. The ESD was developed in 1999 in Japan, initially for resection of gastric tumors, and later of esophageal, colonic and rectal lesions<sup>[1,2]</sup>. Although it is an effective, safe and widely accepted<sup>[3,4]</sup> technique in experienced hands, its application in Western countries has not yet been extensive due to several reasons. One of them is that the main indication of ESD in Japan is early gastric cancer, which has a high prevalence in that country but is less frequent in the Western world, which makes learning this technique a difficult task<sup>[5]</sup>.

To learn the ESD technique, Japanese authors have suggested a structured and progressive training program, with the recommendation of being initially applied in porcine models. First *ex vivo* and then *in vivo* in order to acquire the necessary technical skill and experience before moving on to practice ESD in patients, mainly when it cannot be performed under the direct supervision of experts<sup>[6]</sup>.

Despite these recommendations, there are not many publications on gastric ESD results in porcine models as part of the training for the practice of this technique<sup>[7-10]</sup>.

### Aims

To prospectively determine the results, efficacy and safety of ESD in porcine models during the training period. To assess whether the practice in porcine models contributes to its subsequent application in patients.

## MATERIALS AND METHODS

One endoscopist with experience in ESD (Parra-Blanco A) trained a younger one (González N), in that technique. This one had previous experience in diagnostic and emergency gastroscopy and therapeutic colonoscopy and in performing endoscopic mucosal resection. During this training period, González N observed 6 ESD procedures, of which 5 were conducted in *ex vivo* models and one in

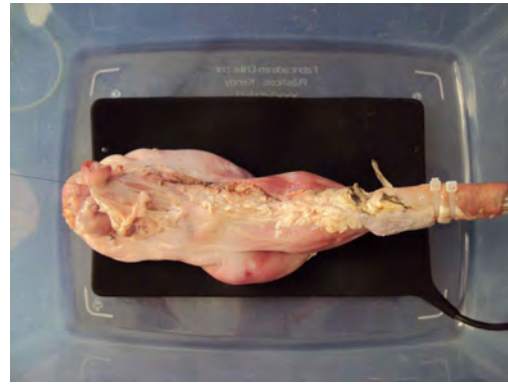


Figure 1 Plastic box with cadaveric model.

the stomach of a live porcine. He additionally performed two gastric dissections in cadaveric models and one dissection of a gastric lesion in an *in vivo* model.

The training was further supplemented with the reading of articles related to ESD, in order to internalize theoretical concepts along with the visualization of videos showing how this technique was applied by Japanese experts.

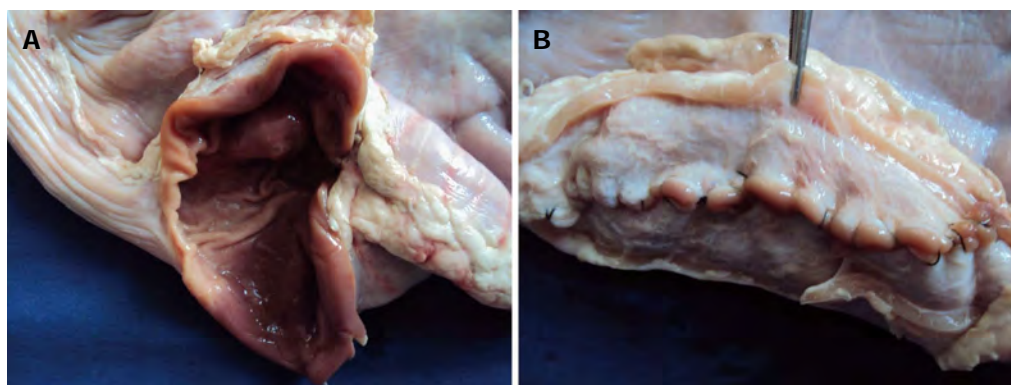
He finally carried out a study/learning process in 3 phases: Phase I : *Ex vivo* animals; Phase II : *In vivo* animals; Phase III: Humans.

### Animal model

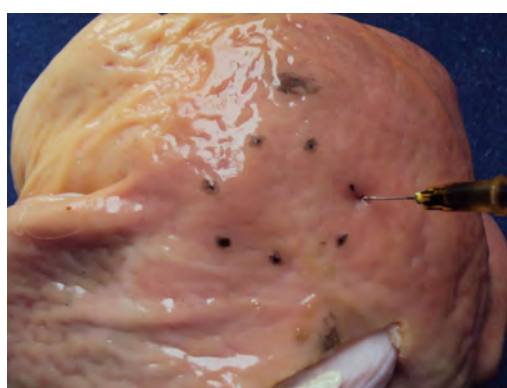
**Ex vivo model:** For the cadaveric stomach models a plastic box (45 cm × 30 cm × 20 cm) that had a plastic tube (overtube) to which the esophagus was connected, was designed (Figure 1). On some occasions, a cut-out 10 mL plastic syringe was used as a connecting device with a 14 mm diameter to connect the esophaguses of small pigs.

Twenty cadaveric stomachs of pigs ranging between 20 and 150 kg were used. In some stomachs 2 dissections were performed. The stomachs, which were stored in the freezer at a temperature of -16 degrees Celsius, were collected 12 h prior to the procedure and were left at room temperature for their subsequent use, in order to preserve the wall elasticity at the moment of performing the procedure. In most cases, the stomachs were opened on the greater curvature so that profuse washing with water and soap could be carried out. This allowed us to perform a thorough cleaning of the mucosa with a brush and later drying it with gauze, thus achieving adequate cleaning without the inconvenience of persistent mucus which is hard to eliminate. Later the stomachs were sutured with coated vicryl suture or prolene suture 3-0 without any insufflation inconvenience whatsoever in any of the cases (Figure 2).

In those cases where the stomachs were closed, a 10 cm long incision was performed with a scalpel on the greater curvature. Another advantage that an open stomach offers is the possibility of carrying out one or more artificial lesions of different diameters and in different topographies through tattooing with Chinese ink or



**Figure 2** The stomachs were sutured with coated vicryl suture or prolene suture 3-0 without any insufflation inconvenience whatsoever in any of the cases. A: Open porcine stomach greater curvature; B: Porcine stomach sutured with vicryl.



**Figure 3** Tattooing artificial lesion with methylene blue.

methylene blue (Figure 3).

To adhere the esophagus to the overtube, plastic clamps were used to prevent the air from coming out and slipping with the movements of the endoscope.

To conduct the study, an Olympus gastroscope (GIF-CV-160: 9.2 mm, diameter/2.8 mm, therapeutic channel) was used and then intended for use in models.

***In vivo* model:** Two domestic female pigs (*Sus scrofa domestica*) of about 30-40 kg underwent ESD. For 48 h they were given a liquid diet and experienced an 8 h-long total fast prior to the procedure. The general anesthesia was administered and controlled by 2 expert veterinarians, who were in charge of monitoring the vital signs and control to prevent complications.

As premedication, 15 mg/kg + 2 ketamine and 1.1 mg/kg intramuscular xylazine were used. For anesthetic induction 5 mg/kg + 0.5 mg/kg (*iv*) midazolam was administered, and for maintenance sedation a dilution of similar amounts of 5% ketamine and 5% midazolam (*iv*), administering approximately 2 mL every 10 min as needed and according to breathing frequency.

Once the procedures were over, a liquid diet was administered for 24 h and then a hypercaloric diet. Omeprazole 20 mg was administered orally every 12 h until the pigs were sacrificed by veterinarians as regulations indicate, following quality standards and required

controls in this type of study. Animal handling was carried out by 2 veterinarians in compliance with the current regulations<sup>[11]</sup>.

### ***Endoscopic submucosal dissection in patients***

After performing 30 ESDs, number recommended by Japanese experts<sup>[11]</sup>, 28 in the cadaveric animal model and 2 ESDs in *in vivo* animal models, 5 gastric dissection procedures were carried out in 5 patients, previous consultation of each case with one of the authors (Parra-Blanco A). The patients were told to discontinue NSAIDs and/or anticoagulants 15 d prior to the procedure, having to present at the moment of the appointment the coagulation test done the day before. The studies were done with an Olympus gastroscope (GIF-CV-160).

The first two cases were carried out in a surgical room and were treated under general anesthesia. A surgical team was present at the operating room in the event that some complications might occur. The other 3 cases were performed in a therapeutic endoscopy room with general anaesthesia. The hospital emergency surgery team was well aware of the procedure.

All the patients were informed of the benefits and of the possible serious complications that might arise with the use of this technique, and signed an informed consent.

An endoscopic control was carried out monthly until the fourth month, with conventional endoscopic vision, chromoendoscopy with FICE using filter number 4 and magnification. A photographic register was made and biopsies were taken of the scar area and of suspicious areas 2 or 3 mo after the procedures.

### ***Technique used for ESD***

The ESD was done in a similar way than the one generally carried out at the National Cancer Center in Japan<sup>[7]</sup>: (1) artificial lesion marking using a coagulation current with needle knife (NK: Needle Knife, Boston, Scientifics, Natick, Massachusetts) or dual knife (Olympus KD-650L); (2) a mixture solution (including 100 mL of normal saline, 1 mL of methylene blue, 1 mL of epinephrine or hyaluronic acid alone), was injected into the submucosa; (3) circumferential incision of 5 mm was made out-

side of the artificial lesion. The incision was performed with NK, dual knife or insulated tip needle knife (IT Knife 2 o, Olympus KD-611L) as single instruments or combined (NK-IT Knife 2 or dual Knife-IT Knife 2). In cases where IT Knife 2 was used, 2 cuts were made with needle knife (hours 6 and 12) to facilitate the incision; on some occasions, it was necessary to perform 1 or 2 more incisions (hours 11 and 2); and (4) the submucosal dissection was performed in all the cases with IT Knife 2, and at times with NK. In the majority of the cases, a transparent cap (Olympus D-201-12704, Tokyo, Japan) was attached to the tip of the gastroscope to provide direct views of the submucosal layer.

For the purpose of facilitating dissection through a better visualization of the plane separation between the submucosa and the muscular layer, in 3 cases ESD was carried out with the clip technique (Resolution Clip. Boston Scientifics R) and an elastic band<sup>[12]</sup>.

The Erbotom ICC 200 ERBE system was used (ERBE, Elektromedizin GmbH, Tübingen, Germany), effect 3, endocut mode with a potency of 120-60 W was used for the circumferential incision; 80-40 W for the submucosal incision, and soft coagulation (40 W) for the haemostasis.

For bleeding control, a coagulation tweezer (Coag-rasper, FD-41OLR) with soft coagulation was used. All the resected pieces were stacked on cork with pins and their measurements were calculated in millimeters and photographically registered. All the procedures were recorded on DVD.

For the prospective analysis of the results obtained, the following variables were registered: (1) lesion topography; (2) size of the resected lesion; (3) total dissection time (circumferential cut and submucosal dissection); (4) surgical instruments and technique used; (5) type of resection (*en bloc* resection or fragmented resection); (6) complications (perforation, bleeding); (7) weight of pig whose stomach was used; and (8) condition of the stomach; fresh (cadaveric but not frozen), thawed (after preservation in freezer) or *in vivo* porcine. In the case of the patients the following variables were registered: whether the ESD was complete (resection with a tumor-free margin in which both the lateral and basal margins were free of tumor cells) or partial (resection in which the tumor extended into the lateral or basal margin, or the margins were indeterminate because of artificial burn effects) and the clinical, endoscopic and histologic evolution.

### Statistical analysis

For the statistical analysis, the description with mean values (standard deviation) for quantitative variables and frequencies for qualitative variables is presented. The Student's *t* test was used (statistical significance threshold of  $\alpha = 0.05$ ) after assumption of normal distribution, to verify differences in terms of qualitative variables. The statistical software used was SPSS 17.0.

## RESULTS

### In animal model

In a period of 5 mo and 13 d, 30 ESDs were performed with an average of 1.4 ESD per week (Table 1). Eighty percent of these ESDs ( $n = 24$ ) were done in frozen stomachs, 13.3% ( $n = 4$ ) in fresh stomachs, and 6.7% (2) in *in vivo* stomachs. Concerning the topography, 90% ( $n = 27$ ) of ESDs were performed in the gastric body, 6.7% (2) in antrum and 3.3% (1), in cardias. In the two *in vivo* models, the regions were antrum and body. In the four fresh stomachs, 3 were in the body and 1 in the antrum. In the 24 thawed stomachs, 23 were in the body and the remaining one in the cardias. The mean  $\pm$  SD size of the pieces was of  $28.4 \pm 1.2$  mm (range: 20-45 mm).

Keeping in mind the condition of the stomach, the mean  $\pm$  SD size for the thawed stomachs was of  $28.8 \pm 1.4$  mm (range: 22.0-45.0 mm). For the fresh stomachs, the mean  $\pm$  SD size was of  $24.5 \pm 0.9$  mm (range: 22.0-26.0 mm). In the cases of *in vivo* stomachs, the mean  $\pm$  SD size was of  $32.5.6 \pm 2.5$  mm (range: 30-35 mm).

According to the topography where the procedure was performed, the following was observed in relation to the size: for the region of the antrum the mean  $\pm$  SD size was of  $28.5 \pm 6.5$  mm (range: 22-35 mm). For the region of the body, the mean  $\pm$  SD size was of  $28.6 \pm 1.2$  mm (range: 20.0-45.0 mm). In the case of the cardias, the size was of 25.0 mm.

The total mean  $\pm$  SD time of the procedure was  $41.7 \pm 2.4$  min (range: 18-70 min). If a cutoff value of 30 mm for the size of the resected piece is established, the dissection time for those lesser than 30 mm was of  $38.0 \pm 2.3$  min, whereas for sizes greater or equal than 30 mm, was  $45.9 \pm 4.0$  min. The difference in dissection time was of 8 min between both size categories with a cutoff value of 30 mm difference which is not significant,  $P = 0.104$ . The mean  $\pm$  SD weight of the pigs where the pieces were obtained from was  $41.7 \pm 6.1$  kg (range: 20-150 kg).

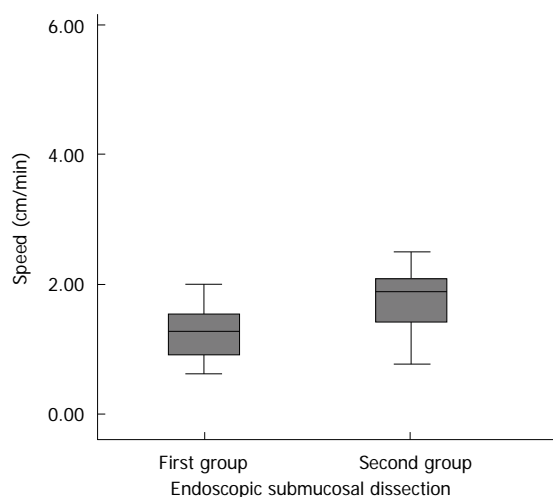
The total mean  $\pm$  SD time of ESD (circumferential cut and submucosal dissection) in the first 15 procedures was  $43.0 \pm 3.0$  min (range: 22-70 min). In the next 15 procedures, the mean  $\pm$  SD time dropped to  $40.3 \pm 3.9$  min (range: 18-70 min), having found no significant differences for the dissection time between the first and the second 15 procedures ( $P = 0.588$ ). The mean  $\pm$  SD size of the pieces in the first 15 procedures was of  $26.1 \pm 1.4$  mm and for the second 15 procedures the mean  $\pm$  SD size increased significantly to  $30 \pm 1.7$  mm ( $P = 0.039$ ). In order to assess if there was any progress in learning, the ESD speed in the second 15 procedures was also calculated and this was compared to the ESD speed in the first 15 procedures. For this purpose, the area of the resected pieces was calculated by using the formula for calculating the area of a circle: ( $\text{Area} = \text{Pi} \times \text{radius}^2$ ) ESD speed was calculated by dividing the area of the resected piece in relation to the total dissection time ( $\text{speed} =$



**Table 1** Technical details 30 endoscopic submucosal dissection in a porcine model

Cases	Topography	Size (mm)	Instrument used	Time dissection (min)	En bloc/pieces	Complications (perforation, bleeding)	Condition of stomach	Weight of the pig (kg)
1	ANTRUM	22	IT,NK	40	BLOC	NO	F	70
2	BODY	24	IT,NK	50	BLOC	NO	F	70
3	BODY	30	IT,NK	43	BLOC	NO	D	150
4	BODY	30	IT (CLIPS)	45	BLOC	NO	D	150
5	BODY	20	IT,NK,	30	BLOC	NO	D	70
6	BODY	20	IT,NK	50	BLOC	NO	D	70
7	BODY	25	IT,NK	45	BLOC	NO	D	50
8	BODY	42	IT,NK	70	BLOC	NO	D	50
9	BODY	21	IT,NK	35	BLOC	NO	D	20
10	BODY	25	IT, NK	35	BLOC	NO	D	20
11	BODY	30	IT,NK	55	BLOC	NO	D	20
12	BODY	25	IT,NK (CLIP)	50	BLOC	NO	D	20
13	BODY	26	IT, DK	40	BLOC	NO	F	20
14	BODY	26	IT,DK	35	BLOC	NO	F	20
15	BODY	25	IT, DK	22	BLOC	NO	D	20
16	BODY	27	IT, DK	27	BLOC	NO	D	20
17	BODY	30	IT, DK	35	BLOC	NO	D	20
18	ANTRUM	35	IT,DK (CLIP)	50	BLOC	NO	IN VIVO	30
19	CARDIAS	25	IT,DK	55	BLOC	NO	D	30
20	BODY	45	IT,DK	70	BLOC	NO	D	30
21	BODY	30	IT,DK	40	BLOC	NO	D	30
22	BODY	35	IT,NK	45	BLOC	NO	D	30
23	BODY	30	IT,DK	45	BLOC	NO	D	30
24	BODY	35	IT,DK	50	BLOC	NO	D	30
25	BODY	30	IT,NK	55	BLOC	NO	IN VIVO	30
26	BODY	20	IT,NK	40	BLOC	NO	D	30
27	BODY	25	IT,NK	36	BLOC	NO	D	30
28	BODY	25	IT,NK	18	BLOC	NO	D	30
29	BODY	30	IT,NK	20	BLOC	NO	D	30
30	BODY	40	IT,NK	19	BLOC	NO	D	30

IT: IT knife2; NK: Needle knife; DK: Dual knife. Condition of stomach: Defrozen (D), Fresh (F).



**Figure 4** Endoscopic dissection speed (cm<sup>2</sup>/min) according endoscopic submucosal dissection group. Shows that the median rate in the first 15 dissections were placed in 1.28 cm<sup>2</sup>/min and in the second group was 1.89 cm<sup>2</sup>/min, showing an increase in the speed of dissection in the second group,  $P = 0.028$ .

area/time).

In the first 15 dissections, the mean  $\pm$  SD speed was  $1.25 \pm 0.11$  cm<sup>2</sup>/min (95%CI: 1.02-1.48). In the case of the second 15 dissections, the mean  $\pm$  SD speed was of  $2.12 \pm 0.36$  cm<sup>2</sup>/min (95%CI: 1.35-2.90) (Figure 4).

Through a Student's *t* test for independent samples, it can be shown that there are statistically significant differences in the speed of both dissection groups ( $P = 0.028$ ).

Due to a possible variation related to the state of the stomach (thawed, fresh, *in vivo*), additionally, only the time of the resections carried out in pre-frozen stomachs in the first 15 ESDs and in the group of the second 15 were analyzed. In the first 15 ESD, 11 resections were performed in thawed stomachs with a mean dissection time of 43.6 min *vs* 13 dissections in thawed stomachs in the second group with a mean time of 38.4 min, these differences were not significant ( $P = 0.386$ ).

In the 30 dissection cases the ESDs were completed achieving the resection of the pieces in one single bloc on all occasions. There were no complications (perforation) in this group, as there was no bleeding in the *in vivo* models.

### In patients

Five surgical procedures in 5 patients with neoplastic lesions (intramucosal carcinoma, 2 adenomatous lesions with low grade dysplasia, and 2 adenomatous lesions with high grade dysplasia) were performed in a period of 6 mo. The endoscopic (Paris classification<sup>[13]</sup>), and histologic characteristics and results are shown in Table 2. The mean  $\pm$  SD size of the lesions was of  $25.2 \pm 5.1$  mm



**Table 2** Endoscopic submucosal dissection results in humans

Topography	Size (mm)	Lesion type <sup>1</sup>	Total time ESD (min)	<i>En bloc</i> resection	Complications	Histology	Complete ESD <sup>2</sup>
Incisura angularis	25	0- II a	65	Yes	No	Adenoma LGD	Yes
Antrum	20	0- II a	60	Yes	No	Intramucosal carcinoma	Yes
Antrum	18	0- II a	30	Yes	No	Adenoma HGD	Yes
Body	18	0- II a	90	Yes	No	Adenoma LGD	Yes
Subcarinal	45	0- II a	180	Yes	Bleeding	Adenoma HGD	Yes

<sup>1</sup>Paris Endoscopic Classification; <sup>2</sup>Anatomopathological study: resection *en bloc*, lateral and vertical margins free of neoplastic tissue. LGD: Low grade dysplasia; HGD: High degree dysplasia; ESD: Endoscopic submucosal dissection.

(range: 18.0–45.0 mm).

In all the cases, the medical instrument used for the dissection was IT-Knife 2. The mean  $\pm$  SD dissection time was  $85.0 \pm 25.6$  min (range: 30–180 min).

In 3 of the procedures in the incisura angularis and in the antrum, the dissection time did not exceed 65 min, reaching 90 min in the high body region and 180 min in the subcarinal area. The piece of lesser size (18.0 mm) was done in 30 min, while the larger lesion (45.0 mm.) lasted 180 min. In one of the procedures some bleeding occurred, which was controlled satisfactorily with a coagulation tweezer.

The five lesions were resected *en bloc*. The pathology study of the pieces reported that the dissections were complete and curative, since no submucosal invasion or vascular or lymphatic involvement was found, nor were detected cell nests of the undifferentiated type in none of the cases (*en bloc* R0 resection).

After the procedures were completed, all the patients went into a surgical room; per oral was discontinued for 24 h; 1000cc *iv* glucophysiological serum was administered plus 2 g of potassium chloride every 12 h, and Omeprazole 40 mg *iv* every 12 h for 72 h. When discharged from hospital, they all received proton pump inhibitors in simple dosage every 12 h until complete scar formation was confirmed by endoscopy.

All of the patients had a good short (immediate post-procedure) and long-term (4 mo post-procedure) clinical evolution without abdominal pain or evidence of digestive bleeding. In the endoscopic controls conducted with conventional vision, intelligent chromoendoscopy with FICE (Filter 4) and magnification, there was no evidence of residual neoplastic tissue. The biopsies conducted in the scar areas, taken 2 or 3 mo after each procedure tested negative for residual neoplastic tissue.

## DISCUSSION

The endoscopic submucosal dissection is a complex and difficult procedure to learn, being conducted mostly in Asian countries, where a large number of superficial gastrointestinal lesions are diagnosed and treated by this technique.

The difficulties inherent to this procedure, the long process of training required to perform it, and the low frequency of superficial gastric lesions diagnosed in Western countries are some of the causes that explain

why very few medical centers outside Japan perform this procedure. Furthermore, access to learning and application of this technique is limited due to lack of Japanese experts in most Western countries. Although outstanding scientific evidence has been published on its applications and results, the publications related to its teaching are scarce, not existing to date a universally accepted standardized learning program on porcine models for subsequent application of this technique in patients.

Gotoda *et al.*<sup>[6]</sup>, have developed training programs on ESD in porcine models. They suggest that these models are a way of rapid rise into the learning curve of this technique in a relatively short period of time and thus may favor its learning in Western countries. They also state that at least 30 submucosal dissections of gastric lesions should be performed to achieve a certain degree of mastery, since at the beginning of the learning curve it is estimated that the perforation rate may reach 20%<sup>[6]</sup>.

In Uruguay, the standardization of ESD teaching does not yet exist and the lack of experts on this technique hampers its dissemination. Due to this inconvenience, as it was formerly pointed out, the practice of ESD in institutions where experts are not available may be developed through the combination of training in animal models before performing endoscopic procedures of this nature in humans. Some authors have described their teaching experience in the acquisition of technical skills using porcine models for the training of ESD for gastric lesions.

Vázquez-Sequeiros *et al.*<sup>[8]</sup>, have described a sequential training program on ESD with the aim of identifying a cheap, safe, efficient and reproducible method for the learning and dissemination of this technique in Spain. According to these authors, such training can be conducted in 4 phases: (1) theoretical phase based on the acquisition of basic knowledge on ESD and in the review of scientific literature; (2) training in an *ex vivo* animal model; (3) training in an *in vivo* animal supervised by an expert; and (4) application of the ESD technique in patients. In this study, 4 endoscopists performed a total of 12 gastric ESDs in porcine models (6 in *ex vivo* model and 6 in *in vivo* model) and later a gastric ESD supervised by an expert in one patient.

Tanimoto *et al.*<sup>[14]</sup> assessed the usefulness of an *in vivo* canine model for ESD practice. They performed 5 esophageal dissections and 5 stomach dissections, completing all the procedures without any complications.

Even though the number and size of the resected pieces were small, the authors recommend the use of this model, which being an *in vivo* model, has the advantage of serving for a more real context and closer to dissections conducted in animals.

Parra-Blanco *et al*<sup>[7]</sup>, have proposed an ESD learning strategy based on an *ex vivo* gastric porcine model and in an *in vivo* porcine model. After an initial ESD learning period conducted in isolated animal stomachs and supervised by an expert, one endoscopist performed a training procedure in esophagus and stomach in an *in vivo* porcine model, dividing the learning period in 2 phases, conducting in each phase 11 ESDs. As the learning process progressed, it was noticed that without existing differences in the size of the resected pieces, only if the gastric resections were considered, the time spent on the dissection phase and the total time were lesser in the second phase as compared to the first one, which suggested the acquisition of a certain skill in ESD performance. However according to the authors, one of the restrictions of this study was the fact that there was no assessment of the impact of previous training on *ex vivo* animal model under supervision, which does not prove if this initial step is necessary to undertake self-learning in animal model. The subsequent impact of animal training in the ESD performance in humans was not evaluated either.

In the present study, the impact of training on the animal model was evaluated early in human patients; therefore, there is a description of the results when the same endoscopist applies the technique in patients after the training program in animal models, thus yielding good results in terms of efficacy and safety.

In relation to the state of the stomachs at the moment of performing the procedures, in most cases, ESDs were performed in pre-frozen porcine stomachs because of its usefulness. However, the authors recommend, if possible, using fresh stomachs due to the fact that the walls are frequently more rigid in pre-frozen organs and, especially, submucosal injection may prove more difficult.

Another important element to be keep in mind is the right size of the organs. Large pigs (greater than 70 kg) have larger stomachs which allow for the performance of multiple resections in different places. In this study, porcine organs ranging from 20 to 150 kg were used. The organs of smaller animals have thinner walls with a greater risk of perforation, and the movements in the stomach are limited due to their reduced size. The use of small stomachs might have the potential benefit of requiring greater skill and therefore the endoscopist will have a greater degree of difficulty. Consequently, the authors recommend the use of small stomachs after an initial practice in large organs in order to acquire greater skill.

Concerning the type of animal model, the *in vivo* porcine model has proven to be more adequate for endoscopic formation in the upper digestive tract<sup>[15-17]</sup>. It is important and recommendable to perform ESD in *in vivo* porcine model as a prior step to the application of

this technique in humans, since this type of model offers several advantages. The principal advantage of the *in vivo* model, as compared to the *ex vivo* model is that the former is more realistic, with presence of peristaltic, intraluminal secretions, and hemorrhage is a possible complication. However, in an initial stage, the basic movements and the ESD strategy may and should be first learned in *ex vivo* models, and probably there is no justifications for using *in vivo* pigs initially, which are more expensive, require more preparation and technical assistance, and it would not be ethically correct<sup>[18]</sup>.

As an objective variable for measuring skill acquisition and learning progress, the following markers of technical skill in ESD have been proposed: decrease in time employed in the procedure, greater speed in the dissection process and/or achieving a greater complete resection rate without need of help from the supervising endoscopist.

Based on the above mentioned, the results of this study suggest that there was progress in the learning curve in animal models. As it can be seen in the results, the time required for the dissection was reduced in the second 15 procedures performed in porcine models. Even though this difference was not significant when compared to the first 15 procedures in animal model, this could be due to the fact that the size of the pieces in the second 15 procedures increased in a significant way. Likewise, it can also be noticed that there was an increase in the dissection speed in the second ESDs, this being the sign of greater acquisition of skill in the technique and therefore progress in its learning.

Distension and elasticity of the stomach walls in different samples (fresh, thawed or *in vivo*) may vary and thus interfere in the dissection time. As it is increased in the frozen models because of lesser distension and elasticity of the tissues, only the times of the dissections performed in frozen stomachs in the first 15 and in the second 15 procedures were compared. In the analysis of the results it was observed that in the second group the procedures were also performed in shorter time, although this difference was not significant. Therefore, the reduction in time cannot be attributed to the state of the stomachs.

One of the restrictions of this study is the fact that very few cases were performed in human patients. Furthermore, it is the experience of just one center and one single endoscopist. The ability and experience of each endoscopist is different, not homogeneous, so this result does not assure its reproducibility.

With respect to the pattern of lesions, usually, the pattern of early gastric cancer is detected as 0-I, II-C or 0-III types in Japan and most of the world. However, in the present study, the most common early gastric cancer was the type II a.

In this article we have discussed the learning curve for ESD, but it is very important to mention that these techniques are useful only after acquiring proficiency in an accurate diagnosis of the early gastrointestinal cancer lesions with the adequate technology and knowledge of

early gastrointestinal cancer classifications (*i.e.*, Vienna and Paris Classifications; Chromoendoscopy and Magnification).

Therefore, before starting the practice of this technique, it is necessary and more important that the endoscopist knows how to thoroughly examine the neoplastic lesion to achieve the identification of endoscopic characteristics that predict the existence of deep invasion, an increased risk of lymph node metastasis and to demarcate the line between the area with cancer and without cancer. This step, prior to the ESD, is very important, because it may avoid unnecessary surgical resection or endoscopy. Furthermore, it is important that before starting to develop this technique, the endoscopist acquires ample experience in therapeutic endoscopy, since he must be able to deal with and solve possible complications during the ESD process, such as bleeding or perforation.

It is worth mentioning that even though the training in ESD in porcine models is safe and accessible, it is recommendable to carry out all of the steps suggested before performing this technique in humans. Besides, it is important to highlight that to define the learning curve only in terms of time, dissection speed or number of procedures conducted is not appropriate since this may vary according to the conditions of each center, and mostly according to the skills of each endoscopist. This is the reason why it is very important to be self-critical, know each one's limitations and to receive permanent feedback, so that the ESD practice can be carried out in a safe and efficient way. When the time comes to move on to performing ESD in humans, it is recommendable that this practice be progressive, starting with resection of lesions in areas with lesser technical difficulty (antrum, incisura angularis) and later ESD in lesions with greater difficulty (lesser curvature, subcardinal region).

Due to the scarce number of patients that can be benefited of the application of ESD in Western countries (mostly due to the low prevalence of early gastric neoplasia), the interval between dissection procedures in humans can be too long. This can be the reason why in this period the technical skills and acquired confidence can decrease. To overcome this situation, the authors recommend that before the performance of this technique in humans, it is necessary to previously perform it in animal model.

In Latin America, the standardization of ESD teaching does not exist and there is shortage of experts in this technique, thus hampering its dissemination. For this reason, it is important to train endoscopy teams to perform ESD so that they can share the knowledge developed on the basis of the experience acquired during the learning curve and to form multicenter endoscopy teams who can do research work, develop this technique in-depth and design structured training programs suited to the needs and resources available.

In conclusion, according to the analysis of the results obtained, the authors conclude that in Uruguay an ESD sequential training program of a unique endoscopist,

initially based on practice in porcine model (performing the number of dissections recommended by Japanese experts), conducted in the absence of experts to supervise the procedures, contributed to the learning of the ESD technique, and yielded promising results in efficacy and safety for its subsequent application in humans, even though the sample is small.

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## COMMENTS

### Background

Endoscopic submucosal dissection (ESD) is a technique for resection of early neoplastic lesions of the gastrointestinal tract, thus obtaining more complete resections than with the standard mucosectomy and decreasing the probability of relapses. However, it is associated with a high rate of complications, but it is an effective and safe technique in experienced hands, and widely accepted. In the absence of experts in western countries, practicing with animal models may be helpful to overcome the learning curve.

### Research frontiers

ESD is an effective and safe technique in experienced hands, and widely accepted. In the present study, the authors demonstrated the feasibility of using porcine models as a learning resource to teach the ESD technique. The results of multicenter studies of ESD in humans after previous training with animal models have to be explored further.

### Innovations and breakthroughs

In Latin American countries there are few training centers at which an endoscopy fellow can be trained in the ESD technique. More ESD training centers in Latin America are needed to offer our patients this technique with good results.

### Applications

ESD has been considered technically difficult. The potential implications of starting such a complex technique in an animal model rather than in human cases are evident. A formal training program is still necessary to teach this technique in western countries and Latin America. For this reason, animal models are an invaluable learning resource. It would be justified to start training with animal models instead of with real patients, if experts are not available to supervise the procedures.

### Terminology

ESD is an endoscopic method for complete *en bloc* resection of early gastrointestinal neoplasms with minimal risk of deeper wall-layer involvement or lymph node metastases. In selected cases, ESD may replace surgery and provide clean margins for accurate histological diagnosis of the lesion borders and complete curative treatment.

### Peer review

This is an interesting investigation that presents the feasibility of using porcine models for ESD training. It provides an alternative way to learn ESD in Western

countries.

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## Clinical significance of serum procalcitonin in patients with ulcerative colitis

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### Abstract

**AIM:** To investigate the association of procalcitonin (PCT) with ulcerative colitis (UC) activity.

**METHODS:** Serum PCT levels, C-reactive protein (CRP) levels, the erythrocyte sedimentation rate, and the white blood cell count were analyzed in 18 patients with UC and 11 healthy volunteers. Serum PCT levels were analyzed by an electrochemiluminescence immunoassay. Severity assessments were based on Truelove and Witts' severity index. Correlation of serum PCT and CRP levels with UC activity was examined. Moreover, we assessed serum PCT and CRP levels in patients with a Mayo endoscopic subscore.

**RESULTS:** Serum PCT levels in severe UC patients ( $n = 7$ ) ( $0.096 \pm 0.034$  ng/mL) were significantly higher

than in mild-to-moderate UC patients ( $n = 11$ ) ( $0.033 \pm 0.012$  ng/mL) and healthy volunteers ( $n = 11$ ) ( $0.035 \pm 0.005$  ng/mL) ( $P = 0.0005$  and  $P < 0.0001$ , respectively). In addition, there was no difference in serum PCT levels between mild-to-moderate UC patients and healthy volunteers. Interestingly, patients with a Mayo endoscopic subscore of 3 points displayed significantly increased levels of serum PCT ( $0.075 \pm 0.043$  ng/mL) compared with patients with a subscore of 2 points ( $0.03 \pm 0.011$  ng/mL) ( $P = 0.0302$ ). Moreover, CRP levels in patients with severe UC or a Mayo endoscopic subscore of 3 points were not significantly higher than in patients with mild-to-moderate UC or a Mayo endoscopic subscore of 3 points.

**CONCLUSION:** Serum PCT levels were significantly correlated with UC activity.

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**Key words:** C-reactive protein; Disease activity; Procalcitonin; Severity; Ulcerative colitis

**Core tip:** To investigate the association of procalcitonin (PCT) with ulcerative colitis (UC) activity, we analyzed 18 UC patients (7 with severe and 11 with mild-to-moderate UC) and 11 healthy volunteers. Serum PCT levels in severe UC patients were significantly higher than in mild-to-moderate UC patients and healthy volunteers. Moreover, patients with a Mayo endoscopic subscore of 3 points displayed significantly increased serum PCT levels compared with patients with a subscore of 2 points. However, serum C-reactive protein was not associated with disease activity or the Mayo endoscopic score. Thus, serum PCT may be a good biomarker for assessing UC activity.

Koido S, Ohkusa T, Takakura K, Odahara S, Tsukinaga S, Yu-

kawa T, Mitobe J, Kajihara M, Uchiyama K, Arakawa H, Tajiri H. Clinical significance of serum procalcitonin in patients with ulcerative colitis. *World J Gastroenterol* 2013; 19(45): 8335-8341 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8335.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8335>

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are idiopathic inflammatory bowel diseases that are generally complicated by systemic or local infection. Clinical, endoscopic, histological, and radiological investigations are typically necessary to make an accurate diagnosis and assessment of disease activity<sup>[1]</sup>; however, disease activity in UC is difficult to assess objectively because clinical disease activity indices depend on several subjective components. Biological markers have also been investigated in the United States for diagnostic purposes, for the assessment of disease activity and the prediction of relapse, and for monitoring the effect of therapy<sup>[2]</sup>. However, traditional markers, such as C-reactive protein (CRP), the erythrocyte sedimentation rate (ESR), and the white blood cell (WBC) count, are still the most common markers used in clinical practice<sup>[3]</sup>. Among these biological markers, CRP is a protein produced by the liver in response to various acute and chronic inflammatory conditions and has been shown to be a good marker for predicting the course and outcome of several diseases<sup>[4]</sup>. Importantly, heterogeneity exists among individuals' immune responses, and elevations in CRP levels are more common in CD than in UC due to differences in acute-phase responses in CD and UC<sup>[5]</sup>. Indeed, only 51% of UC patients with active disease based on colonoscopy have elevated CRP levels<sup>[6]</sup>. This finding suggests that CRP levels add to clinical assessment's ability to predict active mucosal inflammation in UC patients. Thus, the UC biomarkers currently used in clinical practice are of low sensitivity and often do not reliably assess clinical activity<sup>[2]</sup>.

Procalcitonin (PCT), a prohormone composed of 116 amino acids, is the precursor of the calcium homeostasis hormone calcitonin, which is found in thyroid C cells and pulmonary endocrine cells<sup>[7]</sup>. Clinically relevant levels of PCT influence the immunologic responses that contribute to systemic inflammatory responses and septic shock<sup>[8]</sup>. Many studies have indicated that PCT is an excellent marker of bacterial infection in patients with sepsis and its related conditions<sup>[9,10]</sup>. As a marker of disease activity, PCT has also been evaluated in chronic inflammatory and autoimmune conditions, such as pulmonary Wegener's granulomatosis<sup>[11]</sup> and systemic lupus erythematosus/systemic antineutrophil cytoplasmic antibody-associated vasculitis<sup>[12]</sup>; therefore, serum PCT levels may be helpful for predicting the disease activity of UC.

This prospective study aimed to assess the correlation of serum PCT levels with UC activity. Moreover, we

compared PCT levels, CRP levels, the ESR, and the WBC count and evaluated the additional diagnostic benefit of measuring serum PCT levels along with CRP levels in the assessment of disease activity in UC.

## MATERIALS AND METHODS

### Study subjects

This study was conducted prospectively from March 2011 to May 2013 in a single-center cohort. The study protocol was reviewed and approved by the ethics committee of the Jikei Institutional Review Board, Jikei University School of Medicine, and the clinical study committee of Jikei University Kashiwa Hospital. All of the subjects provided written informed consent. The diagnosis of UC was confirmed by a typical history combined with appropriate endoscopic, histopathological, and radiologic findings. The eligibility criteria for study participation were mild-to-severe UC<sup>[13]</sup> with an endoscopy score of at least 1 (erythema, a decreased vascular pattern, and mild friability) on a scale of 0 (normal or inactive) to 3 (spontaneous bleeding and ulceration)<sup>[14]</sup> and/or watery diarrhea at least 5 times/d with visible blood in the stools. Severity assessments were based on Truelove and Witts' severity index<sup>[13]</sup>. After total colonoscopy, the endoscopic findings were evaluated according to the Mayo system, with scores of 0-3<sup>[14]</sup>. Patients with toxic megacolon or concomitant diseases, including diabetes, hematological disorders, any malignancies, obvious infection or sepsis, chronic liver disorder, or any liver or renal diseases were excluded. Subjects who were recruited had venous blood samples drawn to assess PCT levels, CRP levels, the WBC count, and the ESR. Where clinically indicated, stool cultures were performed to exclude infective etiology for diarrhea symptoms.

### Study design

When patients had an established diagnosis of UC, serum PCT levels, CRP levels, the WBC count, and the ESR were examined. Serum PCT levels were analyzed by an electrochemiluminescence immunoassay (ECLIA) at the SRL (Tokyo, Japan), with a lower detection limit of 0.02 ng/mL. The normal value for PCT levels was defined as < 0.05 ng/mL, with a cut-off of  $\geq 0.5$  ng/mL being indicative of bacterial sepsis at the SRL. Based on our laboratory reference, the normal limits for the other inflammatory variables were a CRP level of 0.0-0.3 mg/dL, a WBC count of 3300-8600 count/ $\mu$ L, and an ESR of 2-19 mm/h.

### Statistical analysis

Values are expressed as the mean  $\pm$  SD. The statistical analysis was performed using the Mann-Whitney test and the Kruskal-Wallis one-way analysis of variance on ranks. Proportions were compared using Fisher's exact test and Cramer's  $V$  post-test. The statistical analysis was performed with STAT VIEW software, version J 5.1 (SAS Institute, Inc., Cary, NC, United States), which was used

**Table 1** Baseline characteristics of patients with ulcerative colitis

	Mild-to-moderate <sup>1</sup>	Severe	P value
Age upon entry, yr (mean ± SD)	39.3 ± 12.5	40.0 ± 19.7	> 0.2
Gender, male/female	6/5	7/0	> 0.2
Extent of disease			
Number of patients			> 0.2
Extensive	7	7	
Left-side	4	0	
Proctitis	0	0	
Concomitant medication use			
Number of patients			> 0.2
Sulfasalazine	3	0	
5-ASA	9	7	
Glucocorticoid	6	2	
Immunomodulator	1	0	

<sup>1</sup>Severity assessments were based on Truelove and Witts' severity index. Eighteen ulcerative colitis (UC) patients were included in the study. The distribution of age and sex was not significantly different between mild-to-moderate and severe UC. There was no difference in the extent of disease and concomitant medication use between mild-to-moderate and severe UC. ASA: 5-aminosalicylic acid.

for all analyses.  $P < 0.05$  was considered significant.

## RESULTS

### Baseline characteristics

Eighteen UC patients (mean age:  $39.6 \pm 15.2$  years; 13 male, 5 female) were included in the study. The distribution of age and sex was not significantly different between mild-to-moderate UC (mean age:  $39.3 \pm 12.5$  years; 6 male, 5 female), severe UC (mean age:  $40.0 \pm 19.7$  years; 7 male, 0 female), and the control healthy volunteers (mean age  $40.2 \pm 9.92$  years, 10 male, 1 female) (Table 1). In patients with UC, mild-to-moderate activity was observed in 11 patients (61.1%), and severe activity was observed in 7 (38.9%). Moreover, there was no difference in the extent of disease and concomitant medication use between mild-to-moderate and severe UC (Table 1).

### Biomarkers in severe UC

In this study, serum PCT levels were analyzed by ECLIA at the SRL, with a lower detection limit of 0.02 ng/mL. The normal value for PCT levels has been defined as  $< 0.05$  ng/mL, with a cut-off of  $\geq 0.5$  ng/mL being indicative of bacterial sepsis at the SRL. There was no significant difference in serum PCT levels between patients with mild-to-moderate UC ( $0.033 \pm 0.012$  ng/mL) and healthy volunteers ( $0.035 \pm 0.005$  ng/mL) ( $P = 0.3108$ ) (Figure 1A). Interestingly, patients with severe UC displayed significantly increased levels of serum PCT ( $0.096 \pm 0.034$  ng/mL) compared with patients with mild-to-moderate UC and healthy volunteers ( $P = 0.0005$  and  $P < 0.0001$ , respectively, Figure 1A). Moreover, CRP levels, the ESR, and the WBC count in severe UC were not significantly higher than in mild-to-moderate UC ( $P =$

**Table 2** Sensitivity and specificity of procalcitonin and C-reactive protein for severe ulcerative colitis detection

Marker	Sensitivity	95%CI	Specificity	95%CI
Procalcitonin (ng/mL)				
> 0.0250	100%	59.04-100.0	27.27%	6.022-60.97
> 0.0400	100%	59.04-100.0	72.73%	39.03-93.98
> 0.0550	100%	59.04-100.0	100%	71.51-100.0
> 0.0650	71.43%	29.04-96.33	100%	71.51-100.0
> 0.0850	57.14%	18.41-90.10	100%	71.51-100.0
> 0.1050	42.86%	9.899-81.59	100%	71.51-100.0
> 0.1150	28.57%	3.669-70.96	100%	71.51-100.0
> 0.1350	14.29%	0.3610-57.87	100%	71.51-100.0
C-reactive protein (mg/dL)				
> 0.2500	100%	59.04-100.0	27.27%	6.022-60.97
> 0.5000	85.71%	42.13-99.64	36.36%	10.93-69.21
> 0.7000	85.71%	42.13-99.64	45.45%	16.75-76.62
> 0.9000	85.71%	42.13-99.64	63.64%	30.79-89.07
> 1.400	85.71%	42.13-99.64	72.73%	39.03-93.98
> 2.600	85.71%	42.13-99.64	81.82%	48.22-97.72
> 3.600	85.71%	42.13-99.64	90.91%	58.72-99.77
> 4.050	71.43%	29.04-96.33	90.91%	58.72-99.77
> 4.700	71.43%	29.04-96.33	100%	71.51-100.0
> 7.100	57.14%	18.41-90.10	100%	71.51-100.0
> 9.850	42.86%	9.899-81.59	100%	71.51-100.0
> 10.75	28.57%	3.669-70.96	100%	71.51-100.0
> 11.35	14.29%	0.3610-57.87	100%	71.51-100.0
> 11.35	14.29%	0.3610-57.87	100%	76.84-100.0

A procalcitonin cut-off value of 0.055 ng/mL had good diagnostic accuracy for detecting severe ulcerative colitis, as demonstrated by the sensitivity (100%; 95%CI: 59.04-100.0) and specificity (100%; 95%CI: 71.51-100.0) (Table 2). Moreover, at a cut-off level of C-reactive protein  $> 3.6$  mg/dL, the sensitivity for detecting severe ulcerative colitis was 85.71% (95%CI: 42.13-99.64), and the specificity was 90.91% (95%CI: 58.72-99.77).

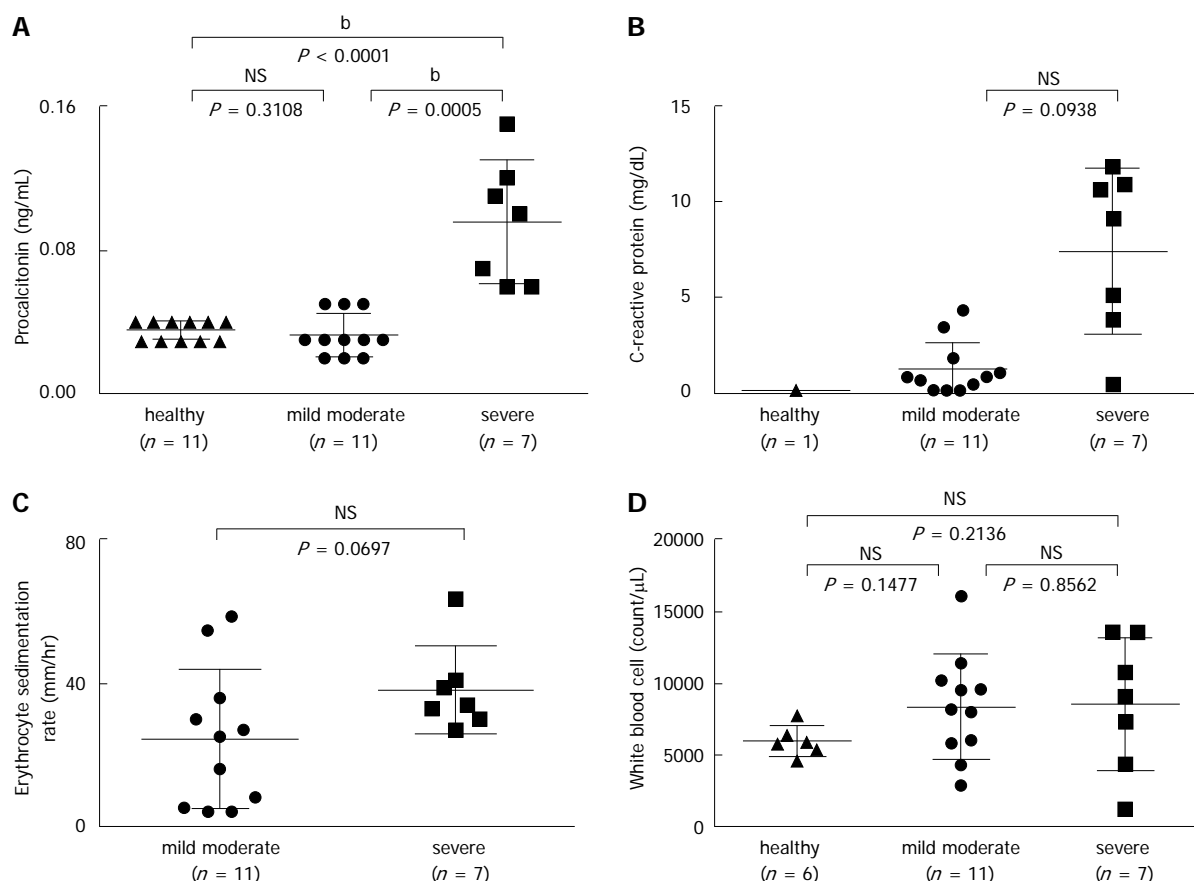
0.0938, 0.0697, and 0.8562, respectively) (Figure 1B-D).

### Correlation of serum PCT and CRP levels with UC activity

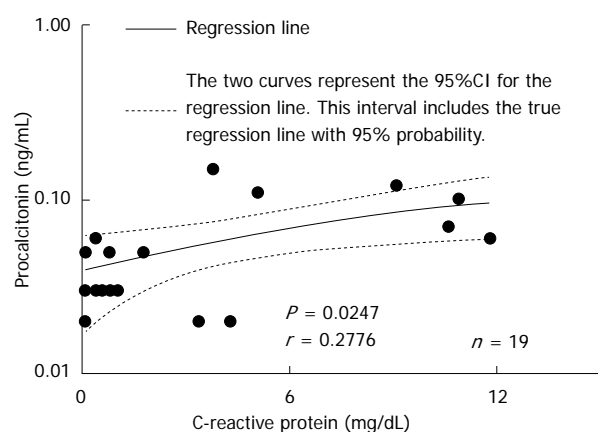
Among mild-to-moderate UC patients, 3 displayed 0.05 ng/mL serum PCT, which was above normal; however, another 8 exhibited normal levels. Interestingly, serum PCT levels were  $> 0.06$  ng/mL in all 7 patients with severe UC. In addition, the levels in all healthy volunteers were  $< 0.04$  ng/mL, which was defined as normal at the SRL. A PCT cut-off value of 0.055 ng/mL had good diagnostic accuracy for detecting severe UC, as demonstrated by the sensitivity (100%; 95%CI: 59.04-100.0) and specificity (100%; 95%CI: 71.51-100.0) (Table 2). Moreover, at a cut-off level of CRP  $> 3.6$  mg/dL, the sensitivity for detecting severe UC was 85.71% (95%CI: 42.13-99.64), and the specificity was 90.91% (95%CI: 58.72-99.77) (Table 2). In addition, there was a significant correlation between PCT and CRP levels ( $r = 0.2776$ ,  $P = 0.0247$ ) (Figure 2). Taken together, these results suggest that serum PCT is superior as a detection marker of severe UC compared with CRP.

### Serum PCT and CRP levels in patients with Mayo endoscopic subscore of 2 or 3 points

Regarding Truelove and Witts' scoring, it could be diffi-



**Figure 1** Scatter dot plot of biological markers in mild-to-moderate and severe ulcerative colitis patients. A: Procalcitonin (PCT) levels; B: C-reactive protein levels (CRP); C: The erythrocyte sedimentation rate (ESR); D: The total white blood cell (WBC) count were examined in healthy volunteers or mild-to-moderate and severe ulcerative colitis patients. Serum PCT levels in healthy volunteers were used as a control. Based on our laboratory reference, the normal limits for the other inflammatory variables were a CRP level of 0.0-0.3 mg/dL, a WBC count of 3300-8600 count/ $\mu$ L, and an ESR of 2-19 mm/h. The results are expressed as the mean  $\pm$  SD. <sup>b</sup> $P < 0.01$  vs healthy or mild-to-moderate group. NS: Not significant.



**Figure 2** Correlation of serum procalcitonin levels with C-reactive protein levels in patients with ulcerative colitis. The two curves represent the 95%CI for the regression line. This interval includes the true regression line, with a 95% probability. There was a significant correlation between procalcitonin (ng/mL) and C-reactive protein (mg/dL) levels ( $r = 0.2776$ ,  $P = 0.0247$ ).

cult to use a scale of 1-3. Therefore, 14 patients with UC were also examined for their Mayo endoscopic score and serum PCT and CRP levels after total colonoscopy in this study. As only 1 patient had an endoscopic subscore

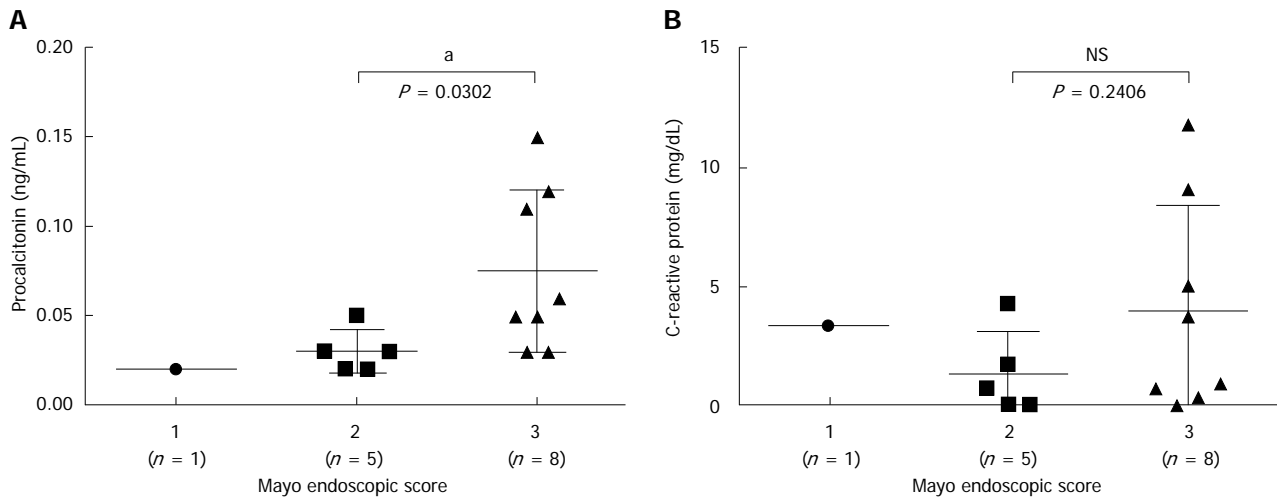
of 1 point, we assessed serum PCT and CRP levels in patients with a subscore of 2 ( $n = 5$ ) or 3 ( $n = 8$ ) points. There was no significant difference in serum CRP levels between patients with a subscore of 2 or 3 points ( $P = 0.2406$ ), whereas patients with a subscore of 3 points displayed significantly increased levels of serum PCT ( $0.075 \pm 0.043$  ng/mL) compared with patients with a subscore of 2 points ( $0.03 \pm 0.011$  ng/mL) ( $P = 0.0302$ ) (Figure 3).

## DISCUSSION

This study demonstrates that serum PCT levels are correlated with clinical, biological, or endoscopic disease activity in patients with UC. Moreover, serum PCT levels have superior diagnostic accuracy for detecting severe UC in comparison with CRP levels, the ESR, and the WBC count.

The assessment of disease activity in patients with UC has been performed using clinical and endoscopic indices. Biological markers are a noninvasive way of objectively measuring inflammation and can play an adjunctive or primary role in the assessment of disease activity. The currently used UC biomarkers, such as CRP, the ESR, and WBC and platelet counts, have low sensitivity and





**Figure 3** Serum procalcitonin and C-reactive protein levels in patients with Mayo endoscopic subscore of 2 or 3 points. A: Procalcitonin; B: C-reactive protein levels were examined in patients with a Mayo endoscopic subscore of 1 ( $n = 1$ ), 2 ( $n = 5$ ), or 3 ( $n = 8$ ) points. The results are expressed as the mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  vs score 2 group. NS: Not significant.

often do not reliably assess clinical activity<sup>[2,15]</sup>. It has been reported that although both PCT and CRP are acute-phase reactant proteins, PCT is a more specific marker of bacterial infections<sup>[16]</sup>. Moreover, PCT is an excellent marker of bacterial infection in patients with sepsis and its related conditions<sup>[9,10]</sup>. There is growing evidence that colonic luminal bacteria contribute to clinical activity in UC<sup>[17]</sup>, suggesting that serum PCT levels may be an important biomarker for detecting active UC; however, little is known about whether serum PCT is a reliable marker for the diagnosis of active UC. To date, four studies have evaluated PCT levels in adult patients with UC<sup>[18-21]</sup>. Among the four studies, only one<sup>[21]</sup> evaluated the correlation between serum PCT levels and Truelove and Witts' severity index or a simple clinical colitis activity index (SCCAI)  $> 5$ <sup>[22]</sup>; however, this report demonstrated conflicting results when evaluating serum PCT as a biological marker of UC activity. Our present study is the first to show that serum PCT levels in patients with UC are significantly correlated with Truelove and Witts' severity index and a Mayo endoscopic subscore. Importantly, serum PCT levels in patients with a Mayo endoscopic subscore of 3 points are significantly higher than in patients with a subscore of 2 points. PCT levels have been evaluated in chronic inflammatory and autoimmune conditions, such as pulmonary Wegener's granulomatosis<sup>[11]</sup> and systemic lupus erythematosus/systemic antineutrophil cytoplasmic antibody-associated vasculitis<sup>[12]</sup>. Moreover, PCT levels have been associated with bacterial infection<sup>[9,10]</sup>. Severe UC patients are characterized by bacterial invasion due to a defensive deficiency of the mucosa; thus, it is possible that PCT levels are correlated with disease activity in UC. In our study, serum PCT levels were analyzed by ECLIA, which showed good reproducibility, linearity, and functional sensitivity compared with previously reported immunofluorescent assays<sup>[23]</sup>. The superior detection of serum PCT in our study may be explained, at least in part, by differences in methodology. In contrast, serum

CRP levels in severe UC and in patients with a Mayo endoscopic subscore of 3 points were not significantly higher than in mild-to-moderate UC or in patients with a subscore of 2 points, respectively. It has been reported that local and systemic administration of steroids has the potential to decrease CRP levels<sup>[24-27]</sup>, whereas PCT levels appear to be unaltered<sup>[24,25]</sup>. In the current study, there was no significant difference in concomitant medication use, such as steroid use, between mild-to-moderate and severe UC. Therefore, it is suggested that PCT levels are correlated with disease activity in UC.

In conclusion, our results suggest that serum PCT levels are useful in clinical practice to assess disease activity in patients with UC. The limitation of our study is the relatively small sample size that was evaluated. Thus, further studies are needed to evaluate the clinical significance of serum PCT in a large sample of UC patients. Moreover, a definitive approach is needed to assess serum PCT levels as a marker for managing severe UC patients after treatment.

## COMMENTS

### Background

Biological markers have been investigated in the United States for the assessment of disease activity. Among traditional biological markers, C-reactive protein (CRP) has been shown to be a good marker for predicting the course and outcome of several diseases. However, only 51% of ulcerative colitis (UC) patients with active disease based on colonoscopy have elevated CRP levels, suggesting that CRP levels add to clinical assessment's ability to predict active mucosal inflammation in UC patients.

### Research frontiers

Traditional biological markers, such as CRP, often do not reliably assess clinical activity in UC patients. In the assessment of clinical activity in UC, the research hotspot is the identification of excellent biomarkers.

### Innovations and breakthroughs

It has been reported that although procalcitonin (PCT) is an excellent specific marker of bacterial infections in patients with sepsis and its related conditions. Moreover, colonic luminal bacteria contribute to clinical activity in UC. Thus, serum PCT levels may be an important biomarker for detecting active UC. This

prospective study aimed to assess the correlation of serum PCT levels with UC activity. Authors showed that serum PCT levels in patients with UC were significantly correlated with Truelove and Witts' severity index and the Mayo endoscopic subscore.

### Applications

The study's results suggest that serum PCT is an excellent biological marker for assessing the activity of UC.

### Terminology

UC and Crohn's disease are idiopathic inflammatory bowel diseases that are generally complicated by systemic or local infection. Clinical UC activity is difficult to assess objectively because of several subjective components. PCT is the precursor of the calcium homeostasis hormone calcitonin, which is found in thyroid C cells and pulmonary endocrine cells.

### Peer review

The strength of this study is a well-executed study. The statistics are appropriate, and the manuscript is well written. This study demonstrates that serum PCT levels are useful in clinical practice to assess disease activity in patients with UC. However, the limitation of the study is the relatively small sample size evaluated. The study recommends a future study on the results of treatment based on PCT levels in a large sample.

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## Safety and utility of capsule endoscopy for infants and young children

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### Abstract

**AIM:** To assess the safety and utility of capsule endoscopy (CE) for children who are unable to swallow the capsule endoscope.

**METHODS:** The medical records of all of the children who underwent CE between 2010 and 2012 were retrospectively reviewed. The patients were divided into 2 groups: group A included patients who were unable to swallow the capsule endoscope, and group B included patients who were able to swallow it. For the patients who were unable to swallow the capsule endoscope, it was placed in the duodenum endoscopically. The small bowel transit time, endoscopic diagnosis and complications of the 2 groups were compared.

**RESULTS:** During the study period, 28 CE procedures

were performed in 26 patients. Group A included 11 patients with a median age of 2 years (range 10 mo-9 years), and group B included 15 patients with a median age of 12 years (range 8 years-16 years). The lightest child in the study weighed 7.9 kg. The detection rates did not differ between the 2 groups. The median small bowel transit time was 401 min (range 264-734 min) in group A and 227 min (range 56-512 min) in group B ( $P = 0.0078$ ). No serious complications, including capsule retention, occurred. No significant mucosal trauma occurred in the pharynx, esophagus, stomach or duodenum when the capsule was introduced using an endoscope.

**CONCLUSION:** CE is a safe and useful procedure for infants and young children who are unable to swallow the capsule endoscope.

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**Key words:** Capsule endoscopy; Retention; Infants; Children; Small bowel transit time; Complications

**Core tip:** We retrospectively reviewed the medical records of all children who underwent capsule endoscopy (CE) and compared the results of the patients who were unable to swallow the capsule (group A) with those of the patients who were able to swallow the capsule (group B). Although the mean small bowel transit time was significantly longer in group A, there were no significant differences between the 2 groups in the frequency of lesion detection or in the occurrence of adverse events. Capsule retention was not observed in either group. Thus, CE is a safe and useful procedure for infants and young children.

Oikawa-Kawamoto M, Sogo T, Yamaguchi T, Tsunoda T, Kondo T, Komatsu H, Inui A, Fujisawa T. Safety and utility of capsule



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## INTRODUCTION

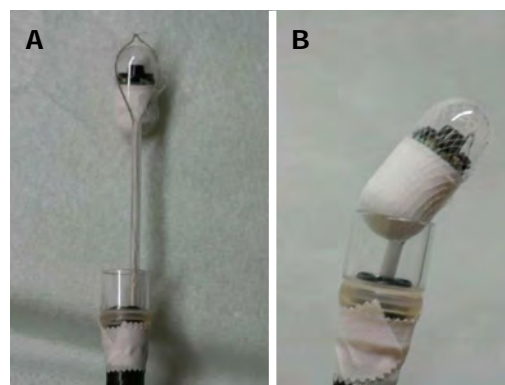
The United States Food and Drug Administration (FDA) approved the use of capsule endoscopy (CE) for the evaluation of small bowel diseases in adults in 2001 and in patients 10 to 18 years of age in January 2004. In September 2009, CE was approved by the FDA for use in children 2 years of age and older<sup>[1]</sup>.

CE provides a unique opportunity to visualize the entire small bowel, not only in adults but also in children. When the patient cannot swallow the endoscopic capsule, it can be safely introduced into the duodenum using various techniques or an introducing device<sup>[2-4]</sup>. Based on recent reports, the use of CE in the pediatric population is increasing<sup>[5-7]</sup>. However, its use in infants and young children who are unable to swallow the endoscopic capsule remains limited<sup>[8-12]</sup>, and the safety and utility of CE in this age group are relatively unknown. The aim of this study was to clarify the safety and utility of CE in infants and young children who were unable to swallow the capsule endoscope, including patients younger than 1 year of age.

## MATERIALS AND METHODS

All of the CE procedures performed at the Children's Center for Health and Development at Saiseikai Yokohama City Tobu Hospital from August 2010 through March 2012 were retrospectively reviewed. Informed consent was obtained from the guardians of the children who underwent CE. The patients were divided into 2 groups: group A included patients who were unable to swallow the endoscopic capsule, and group B included patients who were able to swallow the capsule. We compared the 2 groups in terms of small bowel transit time, complications and endoscopic findings and diagnosis. The small bowel transit time was calculated from the first duodenal image to the first image of the cecum. Capsule retention was defined as a capsule endoscope remaining in the digestive tract for a minimum of 2 wk or requiring directed intervention or therapy to aid its passage<sup>[13]</sup>.

Prior to the CE procedures, patients who were suspected of having a small bowel stricture were screened by small bowel X-ray in an attempt to decrease the risk of capsule retention. A standard PillCam<sup>®</sup> 2 (Given Imaging, Ltd., Yokneam, Israel) was used to perform all of the CE procedures. The PillCam<sup>®</sup> SB2 capsule is 11 mm × 26 mm in size and weighs 2.9 g. Its battery allows more than 8 h of work (usually approximately 13 h), during which the capsule photographs 2 images per second. All of the patients fasted overnight for at least 8 h prior to the delivery of the capsule. The patients were allowed to drink clear liquids 2 h after the procedure and to consume a



**Figure 1** A capsule endoscope in a net device for foreign body extraction; B: A capsule endoscope in a net device for foreign body extraction; the device has been retracted and is seated firmly against a clear plastic hood attached to the tip of the upper gastrointestinal endoscope.

light meal 4 h after the procedure.

If a patient was unable to swallow the capsule, it was placed into the duodenum endoscopically. These patients were anesthetized intravenously with midazolam and ketamine. A plastic hood was attached to the tip of a gastrointestinal endoscope (XQ260, Olympus, Tokyo, Japan). A net device for foreign body extraction was then introduced into the operative channel, and the capsule was placed into the net, pulled into the plastic hood and fixed. To avoid posterior pharyngeal damage during the introduction of the endoscope, a slight angle was created between the long axis of the capsule endoscope and that of the plastic hood (Figure 1). The pediatric endoscopist introduced the capsule into the stomach while observing both the real-time viewer of the endoscopic capsule and the video monitor of the endoscope. The endoscopic capsule was released into the stomach, caught with a polypectomy snare and delivered into the proximal portion of the duodenum (Figure 2).

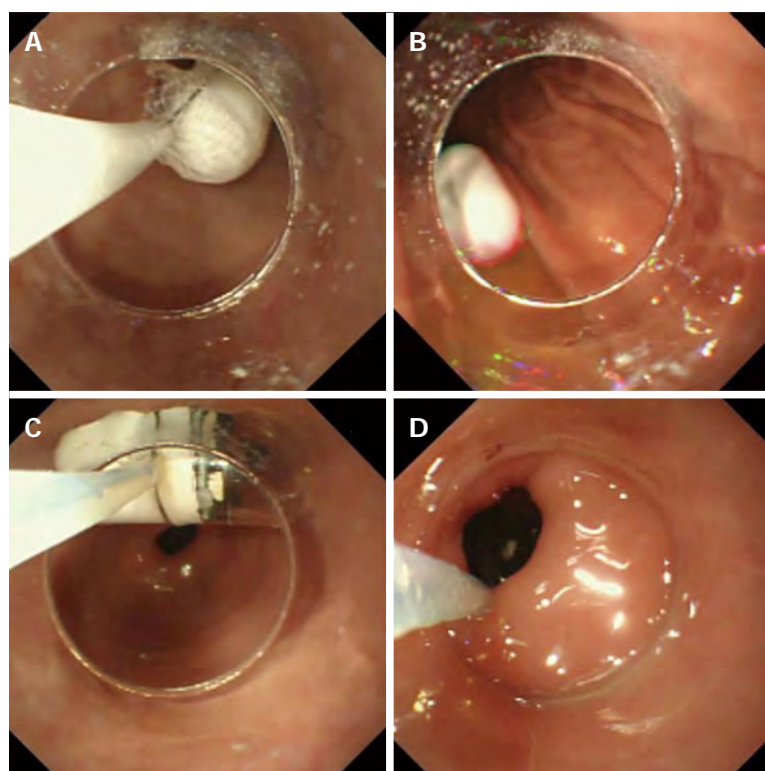
## Statistical analysis

The results are reported as percentages or, in the case of continuous variables, as medians and ranges. The Mann-Whitney *U* test was used to evaluate differences in continuous variables between the 2 groups, and the chi-square test was used for dichotomous variables. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

Twenty-eight CE procedures were performed in pediatric patients during the study period, August 2010 to March 2012. Thirteen males and 13 females underwent CE; the median age of the patients was 9.5 years (range 10 mo-16 years).

Group A included 11 patients (median age 2 years, range 10 mo-9 years), and group B included 15 patients (median age 12 years, range 8 years-16 years) (Table 1). The age distribution of the patients in each group is shown in Table 2. The lightest child, a group A patient,



**Figure 2** Capsule endoscope. A: The capsule endoscope was delivered into the stomach with endoscopic assistance; B: The capsule endoscope was released into the stomach; C: The capsule endoscope was caught by the polypectomy snare; D: The capsule endoscope was introduced into the duodenal bulb with the snare and was then pushed with the plastic hood into the proximal duodenum.

**Table 1** Patient demographics, transit times and complications

	Group A ( <i>n</i> = 11)	Group B ( <i>n</i> = 15)	<i>P</i> value
Median age (yr)	2 (0.8-9)	12 (8-16)	0.0006
Male:female	5:6	8:7	NS
Median weight (kg)	14 (7.9-24.1)	41.8 (21.8-52.9)	0.0003
Median gastric transit time (min)	10 (1-313)	32 (4-446)	0.0169
Median small bowel transit time (min)	401 (264-734)	227 (56-512)	0.0078
Number of retentions	0	0	NS
Number of complications	0	0	NS

The numbers in parentheses represent ranges. NS: Not significant.

weighed 7.9 kg. Ten of the 11 procedures performed in group A used endoscopy to place the capsule endoscope into the duodenum. For a 9-year-old boy in group A, a net device for foreign body extraction was used to pass the capsule through the pharynx and into the stomach without endoscopy.

Of the 28 CE procedures, 26 were completed (92.9%) and 2 were not completed (7.1%). In one case, it took 9 h for a capsule endoscope placed in the stomach of a 2-year-old girl to pass through the stomach; the battery's power became depleted before the entire small bowel could be observed. Therefore, the next day, we delivered the capsule into the duodenum using an endoscopic guide and successfully observed the entire small bowel without any problems. In another case, we misidentified the location of the capsule and terminated the examination before the capsule reached the cecum. The procedure was not repeated in the latter patient.

**Table 2** Age distribution of the patients

Age at examination, yr	Cases ( <i>n</i> )	
	Group A	Group B
< 1	2	0
1	2	0
2	2	0
3	1	0
4	0	0
5	1	0
6	1	0
7	1	0
8	0	1
9	1	2
10	0	1
11	0	3
12	0	1
13	0	0
14	0	5
15	0	1
16	0	1

The median gastric transit time in group B was 32 min (range 4-446 min). The median small bowel transit time in group A (401 min, range 264-734 min) was significantly longer than that in group B (227 min, range 56-512 min) (Table 1).

The indications and findings of CE are summarized in Table 3. The CE examinations were performed for the following reasons: anemia or obscure gastrointestinal bleeding (OGIB) (*n* = 2), small bowel polyps with juvenile polyposis (*n* = 1), chronic or recurrent abdominal pain of unknown etiology (*n* = 8), evaluation of small bowel lesions associated with intractable Henoch-Schönlein purpura (HSP) (*n* = 3), recurrent vomiting (*n* = 1),

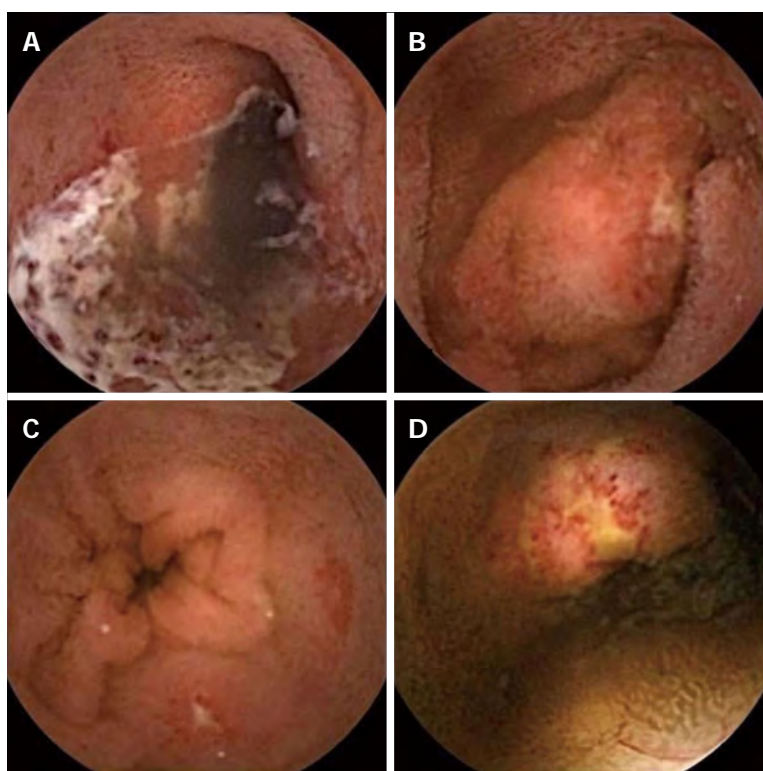


Figure 3 Three-year-old girl with Henoch-Schonlein purpura. A: Extended ulcer; B, C and D: Multiple erosions and ulcers.

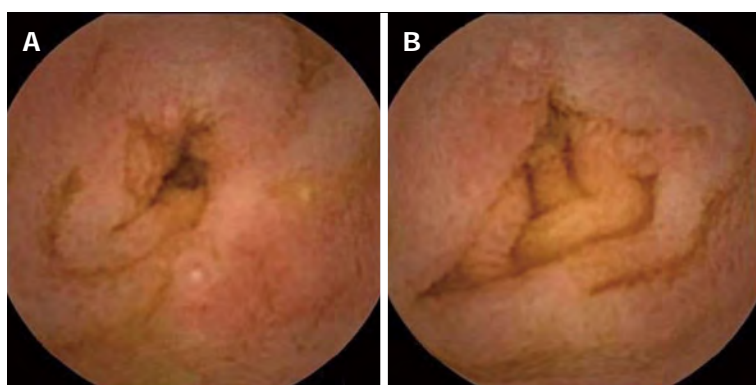


Figure 4 Ten-month-old girl with allergic gastroenteropathy (A and B) caused by the ingestion of cow's milk.

diarrhea or steatorrhea ( $n = 3$ ), ulcerative colitis ( $n = 4$ ) and suspicion of Crohn's disease ( $n = 4$ ). Three patients with ulcerative colitis underwent CE to rule out Crohn's disease, and 1 underwent CE to evaluate small bowel lesions caused by ulcerative colitis and portal hypertension due to primary sclerosing cholangitis. The most common indication in group B was abdominal pain (7 patients, 53.8%); among the patients in group A, the indications were more diverse.

Seven of the 26 capsule procedures (26.9%) were interpreted as normal. CE resulted in a new diagnosis for 6 patients (23.1%): 3 in group A (27.3%) and 3 in group B (20.0%). The CE small bowel findings were not sufficiently specific to permit a diagnosis in 12 patients (46.2%), 5 of in group A (45.5%) and 7 in group B (46.7%). The CE findings resulted in a change in therapy

for 1 patient (6.6%) in group B. There was no significant difference in the incidence of abnormal findings between the 2 groups.

Of the 2 cases in which OGIB or anemia was investigated, CE identified angiodysplasia in both. The patient with juvenile polyposis was found to have no abnormal lesions in the small intestine. Of the 8 procedures performed to investigate abdominal pain, CE identified erosion in 1, duodenal ulcers in 1 and petechiae suggestive of HSP in 2. In a 3-year-old girl with intractable steroid-dependent HSP, CE revealed multiple erosions, extended ulcers and bleeding (Figure 3). In the child examined for recurrent vomiting, CE identified ulcers and aphthae (Figure 4). Of the 3 procedures in which diarrhea or steatorrhea were investigated, CE identified ulcers and erosion in 1. Of the 4 children with ulcerative colitis, 1



**Table 3** Capsule endoscopy findings and outcomes *n* (%)

	<i>n</i>	Normal	New diagnosis	Suggestive of specific small bowel findings	Nonspecific small bowel findings	Change in therapy
Group A	11	4 (36.4)	3 (27.3)	5 (45.5)	2 (18.2)	0 (0.0)
Group B	15	3 (20.0)	3 (20.0)	7 (46.7)	5 (33.3)	1 (6.6)

exhibited erosion and mucosal redness; 1 had a longitudinal ulcer and round ulcers; and 2 had mucosal edema. Of the 4 procedures undertaken for suspicion of Crohn's disease, 1 revealed a linear ulcer; 1 showed a round region of redness; 1 revealed erosions with hematin; and 1 demonstrated white villi and erosion.

No significant mucosal trauma to the pharynx, esophagus, stomach or duodenum occurred when the capsule was introduced using an endoscope. No further complications were noted; specifically, no capsule retention occurred.

## DISCUSSION

CE is an endoscopic technique that offers an extremely safe approach for investigating small bowel pathology in adults. Although the US FDA has approved the use of CE in children 2 years of age and older, Japan's Ministry of Health, Labor and Welfare has not officially approved CE for infants or young children who cannot swallow the capsule endoscope. Several techniques and devices for introducing the capsule endoscope into the duodenum have been described<sup>[2-4,14,15]</sup>, and reports of the use of CE in children who are unable to swallow the capsule have been increasing<sup>[2,3,11,12]</sup>. Fritscher-Ravens *et al*<sup>[8]</sup> conducted a multicenter study to evaluate the feasibility and proper technique for using CE to identify small intestinal pathology in children < 8 years of age. No instances of severe complications or capsule retention occurred in the 85 examinations in that study, in which the smallest child weighed merely 10 kg. To our knowledge, the smallest child (an 8-mo-old) in whom the use of this technique has been reported weighed 9 kg<sup>[12]</sup>.

In this study, we used a net device for foreign body extraction to introduce the capsule and a plastic hood adaptor to hold and stabilize the alignment of the capsule during its passage through the pharynx into the esophagus. It proved difficult to pass the capsule into the duodenum using a net device according to the method described by Barth *et al*<sup>[21]</sup>; therefore, we used a polypectomy snare to pass the capsule beyond the pylorus. Using this technique, we successfully performed CE in children who were unable to swallow the capsule. These children included 4 infants weighing < 10 kg, the lightest of whom weighed 7.9 kg and the youngest of whom was 10 mo old. The smallest child in group A weighed 7.9 kg; to our knowledge, this child is the smallest patient to be included in a study of CE.

Aparicio *et al*<sup>[16]</sup> reported that the patient's age, sex, diabetes mellitus and body position after swallowing the capsule endoscope did not influence the gastric transit

time or small intestinal transit time. In contrast, Fireman *et al*<sup>[17]</sup> reported age- and pathology-related effects on the small bowel transit time of the capsule endoscope. It has been unclear what factors influence small bowel transit time in children. We compared the small bowel transit time between group A, children who could not swallow the endoscopic capsule, and group B, children who could swallow the capsule, and found a significant difference between the 2 groups. This is the first report to compare small bowel transit times between 2 such groups. Small bowel transit time is thought to be influenced by the sphincter of the ileocecal valve, the length and luminal diameter of the small bowel and digestive peristalsis. Post-mortem studies have revealed that the capsule endoscope can traverse the pylorus and ileocecal valve in infants 1 year of age<sup>[8]</sup>, suggesting that the size of the ileocecal valve may not be the primary factor influencing small bowel transit time during CE in children 1 year of age and older. Growth in intestinal length continues during early postnatal life; from approximately 1 year of age (75 cm body length) onward, this growth slows but continues to progress linearly with age until adulthood<sup>[18]</sup>. A study employing the lactulose hydrogen breath test showed that there was no correlation between age and transit time<sup>[19]</sup>, although a test using solids might produce different results. We examined the relationships between small bowel transit time and age and body weight and found no significant relationships between these parameters. We demonstrated that small bowel transit time was significantly longer in group A than in group B. We hypothesize that the depression of digestive peristalsis caused by the anesthetic agents used during the placement of the capsule may have influenced the small bowel transit time.

CE is generally considered more sensitive than radiological and standard endoscopic modalities for the detection of small bowel lesions in children, especially for the distribution of Crohn's disease lesions<sup>[20,21]</sup>, the source of OGIB and the presence of polyps<sup>[8,22,23]</sup>. In our study, the source of bleeding was identified in all of the patients who underwent CE for OGIB or anemia. However, there was a low detection rate among the patients with juvenile polyposis or abdominal pain.

A major concern of both investigators and ethics review boards is the possibility of respiratory complications and/or CE retention in infants and young children. General endotracheal anesthesia was not required for the endoscopic placement of the capsule endoscope in any of the patients in group A, and no respiratory complications occurred in this group. There may be a risk of airway obstruction by the capsule or the endoscope during capsule introduction without general endotracheal anesthesia in infants and young children. The patient's blood oxygen saturation, blood pressure, heart rate, electrocardiogram and respiratory rate must be monitored, and oxygen should be supplied via nasal cannula during capsule introduction in all cases. Our technique considerably reduces the risk of airway obstruction because it allows the accurate determination of the location of the CE: the



use of the clear plastic hood permits the observation of both the real-time viewer of the capsule endoscope and the monitor of the endoscope.

A systematic review of CE studies, primarily in adults, reported retention rates of 1.4%, 1.2%, 2.6% and 2.1% for overall, OGIB, Crohn's disease and neoplastic lesions, respectively<sup>[13]</sup>. A meta-analysis indicated that the CE retention rate in the pediatric population was 2.6% (stomach, 0.5%; small bowel, 1.9%), and pediatric patients showed similar risks of retention in OGIB, Crohn's disease and polyp cases, with rates of 1.6% ( $n = 2/125$ ), 2.5% ( $n = 9/359$ ) and 1.3% ( $n = 1/75$ ), respectively<sup>[24]</sup>. In a European multicenter study, Fritscher-Ravens *et al*<sup>[8]</sup> reported that no capsule retention was observed in 83 children younger than 8 years of age. These reports suggest that the retention rate is related to the indication for the procedure rather than to the age of the patient and that capsule retention is relatively uncommon in both adults and children. Atay *et al*<sup>[25]</sup> reported that the "red flags" for potential capsule retention included inflammatory bowel disease (IBD) (5.2% retention risk), previous small bowel follow-through demonstrating small bowel Crohn's disease (37.5% retention risk) and body mass index < 5th percentile with known IBD (43% retention risk). To reduce the risk of capsule retention, small bowel follow-through should be performed prior to CE in all cases of suspected Crohn's disease, especially in cases of growth failure. In our study, all of the patients with suspicion of Crohn's disease underwent small bowel follow-through before CE, and no capsule retention occurred. If capsule retention were to occur, we would attempt to remove the capsule by double balloon endoscopy or by surgery if double balloon endoscopy could not be performed. Although the use of patency capsules in children has been reported<sup>[26]</sup>, we did not use patency capsules prior to CE because such capsules are unavailable in Japan. Furthermore, the number of patients in this study was small, and any comparison of 2 groups with significant differences in age and weight would likely be of questionable validity. Because the number of pediatric patients who undergo endoscopy is far smaller than the number of adult patients who undergo this procedure, there are few data regarding the safety and utility of CE in infants and young children. We believe that our findings will encourage pediatric endoscopists to perform CE more frequently in infants and young children who cannot swallow capsules.

In conclusion, CE examinations of infants and young children who were unable to swallow the endoscopic capsule were performed without adverse events. In infants and young children, as in adults, CE is a useful and safe diagnostic method, especially in cases of gastrointestinal bleeding or anemia.

## COMMENTS

### Background

The United States Food and Drug Administration (FDA) approved capsule endoscopy (CE) for the evaluation of small-bowel diseases in adults in 2001 and

for patients 10 to 18 years of age in January 2004. In September 2009, CE was approved by the FDA for use in children 2 years of age and older. However, the safety and utility of the use of CE in infants and young children who cannot swallow the capsule are unclear.

### Research frontiers

The smallest child (an 8-mo-old) in whom the use of CE was previously reported weighed 9 kg. In this report, authors used a net device for foreign body extraction and a plastic hood adaptor to hold the capsule and stabilize its alignment during its passage through the pharynx into the esophagus. Using this technique, authors successfully performed CE in children who were unable to swallow the capsule, including an infant weighing 7.9 kg.

### Innovations and breakthroughs

Authors compared the small bowel transit time between group A, consisting of children who could not swallow the endoscopic capsule, and group B, consisting of children who could swallow the capsule, revealing a significant difference between the 2 groups. This is the first report to compare the small bowel transit times in 2 such groups.

### Applications

The capsule was endoscopically placed into the duodena of the patients who were unable to swallow the capsule. Thus, CE is a safe and useful procedure for infants and younger children who are unable to swallow the capsule endoscope.

### Peer review

This is a valuable retrospective observational study. The study is very well thought out and should be useful to the readers in the Pediatric Group. If the retention of the capsule were to occur, what intervention or action would be undertaken by the clinician?

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## Association of visceral obesity and early colorectal neoplasia

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VAT and subcutaneous adipose tissue area as a reference group.

**RESULTS:** The body mass index (BMI), total cholesterol, fasting glucose and VAT areas were significantly different among normal, adenoma and CRC groups. The VAT area was  $120.6 \pm 49.0 \text{ cm}^2$  in normal controls,  $130.6 \pm 58.4 \text{ cm}^2$  in adenoma group and  $117.6 \pm 51.6 \text{ cm}^2$  in CRC group ( $P = 0.002$ ). In univariate analysis, increased BMI was a risk factor for CRC compared to control ( $P = 0.025$ ). However, VAT area was not a risk factor for CRC compared to control. In multivariate analysis that adjusted for smoking, alcohol consumption and subcutaneous adipose tissue area, VAT area was inversely related to CRC, compared to the adenoma (OR = 0.53, 95%CI: 0.31-0.92, highest quartile vs lowest quartile).

**CONCLUSION:** Our study shows that visceral obesity is not a risk factor for early CRC. Visceral obesity might influence the normal-adenoma sequence but not the adenoma-early carcinoma sequence.

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### Abstract

**AIM:** To examine whether visceral adipose tissue (VAT) serves as a risk factor for colorectal adenoma-early colorectal cancer (CRC) sequence.

**METHODS:** A retrospective case-control study was conducted with 153 patients with stage I CRC, age/sex-matched 554 patients with colorectal adenoma and 557 normal controls. All subjects underwent various laboratory tests, abdominal fat computed tomography (CT), and colonoscopy. VAT was defined as an intra-abdominal adipose tissue area measured by CT scan. Adipose tissue area was measured at the level of the umbilicus from CT scan. We used the lowest quartile of

**Key words:** Adipose tissue; Visceral fat; Obesity; Colorectal cancer; Colorectal adenoma; Abdominal computed tomography

**Core tip:** This study showed that visceral obesity is not a risk factor for early colorectal cancer, although it is an independent risk factor for colorectal adenoma in previous studies. Therefore, these data suggest that visceral obesity might influence the normal-adenoma sequence but not the adenoma-early carcinoma sequence.

Choe EK, Kim D, Kim HJ, Park KJ. Association of visceral obesity and early colorectal neoplasia. *World J Gastroenterol* 2013; 19(45): 8349-8356 Available from: URL: <http://www.wjg->

## INTRODUCTION

The prevalence of obesity has increased over the past few decades<sup>[1]</sup>, and the prevalence of colorectal neoplasia has also increased in Asian countries, particularly in South Korea<sup>[2]</sup>. There is mounting evidence linking obesity to colorectal neoplasia<sup>[3]</sup>. Data from recent studies, including our previous research, have suggested that among the diverse methods that are used to define obesity, the visceral adipose tissue (VAT) area is a strong indicator of obesity as a risk factor for developing colorectal adenoma<sup>[4-7]</sup>. The natural course of adenoma is well known as the “adenoma-carcinoma sequence”<sup>[8]</sup>. Therefore, the role of VAT in developing colorectal cancer (CRC) should be interpreted at each step of this sequence. Several studies have assessed the association between the VAT area and CRC. For example, in a Japanese study<sup>[9]</sup>, VAT was positively associated with developing early stage CRC but not adenoma. However, in this study, the number of CRC patients was small, and the control subjects were selected from the subjects with negative screening results but without colonoscopic exams, which may have resulted in including patients with colorectal adenomas. In a Turkish study<sup>[10]</sup>, the VAT area did not differ between the CRC patients and controls. This study involved also a small number of cases, and the controls were consecutively collected, which means that they were not matched by age or gender.

The relationship between VAT and the colorectal adenoma-cancer sequence has not yet been completely documented. The aim of this study was to examine whether VAT serves as a risk factor for the colorectal adenoma-early CRC sequence.

## MATERIALS AND METHODS

### Study population

A retrospective case-controlled study was conducted to compare stage I, early CRC patients with an age- and sex-matched colorectal adenoma group and normal controls. From October 2004 to December 2008, 1206 CRC patients underwent colectomy at Seoul National University Hospital by an identical main operator. For preoperative evaluation, all patients had colonoscopic examination and abdominal computed tomography (CT) scan. To minimize the wasting effect of cancer itself, we included only the stage I CRC patients in our study. The cancer stage was determined using the American Joint Committee on Cancer criteria<sup>[11]</sup>. Stage I CRC includes the T1N0 and T2N0 on tumor node metastasis stages. There were 153 eligible patients after the following exclusion criteria were applied: preoperative neoadjuvant chemoradiation therapy, a history of colonic disease such as CRC, inflammatory bowel disease and a family history of CRC.

The colon adenoma patients and normal controls were recruited at Seoul National University Hospital Healthcare System Gangnam Center during a routine health check-up over a similar time period. The inclusion criteria were those who underwent abdominal fat CT scan and colonoscopy on the same day. Patients with colorectal adenoma and normal controls (subjects with normal colonoscopic findings) followed the study design as previously described<sup>[4]</sup>. The following exclusion criteria were applied to the colorectal adenoma and normal control groups: a history of colonic disease, such as colorectal adenomatous polyps, cancer, inflammatory bowel disease, and bowel resection; a colonic examination including sigmoidoscopy, colonoscopy, or barium enema in the previous 10 years, a family history of CRC.

To adjust for age and sex, which are considered important confounders in CRC and adenoma studies, we formed three age- and sex-matched groups: the early CRC group, the adenoma group and the control group.

### Ethics statement

The Institutional Review Board of Seoul National University Hospital approved the study protocol (IRB number H-1208-001-420), and the study was conducted in accord with the Helsinki Declaration. Informed consent was waived by the board.

### Clinical and laboratory evaluations

All subjects were questioned regarding smoking, alcohol consumption, diabetes mellitus, hypertension, and medication histories. Height, weight and blood pressure were measured by trained nurses using a standardized protocol. Venous samples were drawn after an overnight 12-h fast to check serum total cholesterol and fasting glucose. All biochemical tests were performed using an automatic analyzer within the Department of Laboratory Medicine at Seoul National University Hospital.

### Definitions and exposure measurements

Current smoking was defined as smoking at least one cigarette per day for the previous 12 mo, and alcohol consumption was defined as drinking over 140 g of alcohol per week. Hypertension was defined as a blood pressure of > 140/90 mmHg or taking an anti-hypertensive medication. Diabetes mellitus was defined as a fasting glucose of > 126 mg/dL or taking a diabetes mellitus medication. Body mass index (BMI) was calculated from the measured weight and height.

### Measurement of adipose tissue areas by CT

All of the CRC patients had preoperative abdominal CT for staging work-up. The adipose tissue area was measured at the level of the umbilicus using a 16-detector row CT scanner (Somatom Sensation 16, Siemens medical Solutions, Forchheim, Germany), as previously described<sup>[12]</sup>. We defined VAT as an intra-abdominal adipose tissue area confined by the parietal peritoneum, excluding the paraspinal muscles and the vertebral column. Subcutaneous



**Table 1** Comparison of the baseline characteristics of the colorectal cancer, adenoma, and control groups *n* (%)

Characteristics	Control ( <i>n</i> = 557)	Adenoma ( <i>n</i> = 554)	Cancer ( <i>n</i> = 153)	<i>P</i> value
Age (yr)	59.1 ± 8.7	59.1 ± 8.2	60.3 ± 9.1	0.278
Male	370 (66.4)	372 (67.1)	100 (65.4)	0.910
Alcohol consumption	77 (13.8)	86 (15.5)	31 (20.3)	0.146
Current smoking	86 (15.4)	106 (19.1)	28 (18.3)	0.255
Hypertension	195 (35.0)	217 (39.2)	63 (41.2)	0.222
Diabetes	70 (12.6)	70 (12.6)	19 (12.4)	0.997
Body mass index (kg/m <sup>2</sup> )	23.80 ± 2.48	24.34 ± 2.56	24.25 ± 2.51	0.001
≤ 22.9	194 (34.8)	150 (27.1)	46 (30.1)	0.011
23.0-24.9	197 (35.4)	188 (33.9)	51 (33.3)	
25.0-29.9	161 (28.9)	202 (36.5)	55 (35.9)	
≥ 30.0	5 (0.9)	14 (2.5)	1 (0.7)	
Total cholesterol (mg/dL)	197.7 ± 35.5	193.1 ± 35.4	179.9 ± 45.5	< 0.001
Fasting glucose (mg/dL)	100.7 ± 21.2	100.7 ± 20.0	107.3 ± 26.4	0.002
Total adipose tissue area (cm <sup>2</sup> )	262.4 ± 81.3	281.3 ± 90.1	264.1 ± 86.0	0.001
Visceral adipose tissue area (cm <sup>2</sup> )	120.6 ± 49.0	130.6 ± 58.4	117.6 ± 51.6	0.002
Subcutaneous adipose tissue area (cm <sup>2</sup> )	141.9 ± 55.0	150.7 ± 59.8	146.5 ± 56.6	0.040

Results are expressed as mean ± SD.

adipose tissue (SAT) areas were defined as adipose tissue areas external to the abdomen and the back muscles<sup>[4]</sup>. As there are no standard values for the definition of a normal amount of abdominal adipose tissue, we used the lowest quartile of SAT and VAT area in this study as a reference group. Same method was applied to the normal and adenoma groups from their abdominal fat CT. To minimize interpersonal variation, all measurements were performed by one experienced nurse who was blinded to the clinical and laboratory details of subject.

#### Colonoscopy and the detection of colorectal adenoma

The colonoscopies were performed by experienced gastroenterologists. The colonoscopists were blinded to the clinical findings and adipose tissue amounts. The bowel preparations were performed using four liters of colon-lyte solution. Histopathologically, colorectal carcinoma was defined as colorectal adenocarcinoma, regardless of amount of the mucinous component or differentiation. Colorectal adenoma was defined as a colorectal adenoma, regardless of the grading and the amount of villous component.

#### Statistical analysis

The continuous variables are expressed as the mean ± SD. The  $\chi^2$  test or Student's *t* test and an analysis of variance (ANOVA) for independent samples were used to assess the differences in risk factors among the groups. The effect of obesity, as measured by SAT or VAT area, were estimated by calculating an OR and a 95%CI using conditional logistic regression analysis. In addition to the risk factors that were determined to be significant in a univariate analysis, we included additional variables that are known or reportedly have an association with CRC, such as smoking and alcohol consumption, for the multiple conditional logistic regression model to identify independent risk factors for CRC. All of the statistical analyses were performed using SPSS 19.0 (SPSS, Chi-

cago, IL, United States) and SAS 9.2 (SAS institute, Cary, NC, United States). *P* < 0.05 was considered statistically significant.

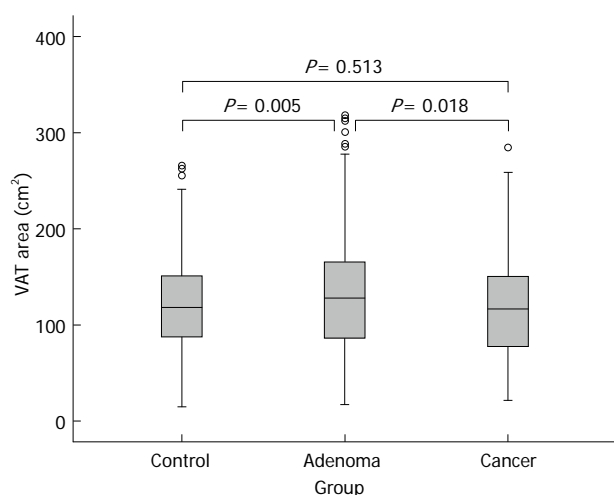
## RESULTS

#### Characteristics of participants

Of 1206 colorectal patients who had colectomies, 153 stage I patients met the inclusion criteria and were enrolled in the final analysis. The patients were matched by age and sex to produce 554 adenoma group members and 557 normal controls. The mean age of all 1264 subjects was 59.2 ± 8.5 years, and the male to female ratio was 842:422. The BMI, total cholesterol and fasting glucose levels, total adipose tissue, VAT and SAT areas were significantly different among the three groups. The VAT area was 120.6 ± 49.0 cm<sup>2</sup> in normal controls, 130.6 ± 58.4 cm<sup>2</sup> in the adenoma group, and 117.6 ± 51.6 cm<sup>2</sup> in the early CRC group (*P* = 0.002). There were no differences in age, gender, smoking history, alcohol consumption, hypertension or diabetes (Table 1). The VAT areas in colorectal carcinoma patients were significantly lower than in the adenoma patients (*P* = 0.02), but not significantly lower than in the control group (*P* = 0.51). The VAT areas in colorectal adenoma patients were significantly higher than in the control group (*P* = 0.005) (Figure 1).

#### Adiposity in stage I CRC vs normal group

In a prior paper from our research group, the VAT area was an independent risk factor for colorectal adenoma compared to normal controls<sup>[4]</sup>. In this study, according to a univariate analysis, the VAT and SAT area were not risk factors for early CRC. However, BMI was found to be significantly associated with the presence of CRC (*P* = 0.021). Conditional logistic regression models adjusting for smoking and alcohol consumption showed that CRC was not associated with VAT area. Including the SAT



**Figure 1** Comparison of visceral adipose tissue area (cm<sup>2</sup>) among patients with colorectal carcinoma, those with colorectal adenoma, and controls. Visceral adipose tissue (VAT) area in colorectal carcinoma patients was significantly lower than in adenoma patients ( $P = 0.018$ ), but it was not significantly lower than in control group ( $P = 0.513$ ).

area in the multivariate analysis provided the same result. Increased BMI, as a surrogate marker for general obesity, was an independent risk factor for CRC ( $P$  for trend = 0.025) (Table 2).

#### Adiposity in stage I CRC vs adenoma group

In the univariate analysis, VAT area, SAT area and BMI were not risk factors for early CRC compared to colorectal adenoma. The conditional logistic regression models (adjusted for smoking and alcohol consumption) showed that overall obesity, which is indicated by BMI, was not different between the adenoma and CRC groups. Contrary to expectations, an inverse relationship of the VAT area to early CRC was observed, compared with colorectal adenoma (OR = 0.53, 95%CI: 0.31-0.92,  $P = 0.02$ , highest quartile *vs* lowest quartile of the VAT area) (Table 3).

## DISCUSSION

In this study of 153 stage I CRC patients, age- and sex-matched 554 colorectal adenoma patients and 557 normal controls, we found that visceral obesity was not a risk factor for early CRC. Overall obesity (measured by BMI) was a significant risk factor for CRC compared with normal controls. This was consistent with previous findings<sup>[13]</sup>.

Recently, the prevalence of obesity has increased rapidly and is becoming a major public health problem. At present, more than 300 million people worldwide are obese<sup>[14]</sup>. There is mounting evidence relating obesity to colorectal neoplasia. However, the relationship between obesity and colorectal neoplasia differs depending on the methodology used to measure obesity. Whole body fat area is distributed into two main compartments with different metabolic characteristics: SAT and VAT<sup>[15]</sup>. BMI and body weight reflects the whole body fat amount.

Waist circumference (WC) and waist to hip ratio (WHR) implies the visceral fat area. Visceral obesity is also referred to as abdominal or central obesity. In several reports, BMI and body weight were not consistently related to the development of colorectal neoplasia, an association which is strongly influenced by gender<sup>[6,10,16-18]</sup>. WC and WHR have shown a stronger positive relationship<sup>[18-21]</sup>; however, several studies of WC and WHR suggested a greater risk for CRC in men than women, thus providing inconclusive results regarding their relationship<sup>[22,23]</sup>. The visceral fat area can be assessed most objectively by CT scanning at the umbilical level<sup>[12]</sup>. Therefore, our study assessed the relationship of visceral obesity with colorectal adenoma and cancer based on CT analysis instead of WC and WHR. The technique we used for visceral adipose tissue area measurements in CT scan has been standardized and validated in previous studies<sup>[24-28]</sup>. Note that many studies have related visceral obesity to colorectal adenoma<sup>[7,17,19,29,30]</sup>. Our institute reported a similar result<sup>[4]</sup>. Results assessing the relationship between abdominal obesity and colorectal neoplasia are inconclusive. Studies have proven positive<sup>[9]</sup>, negative<sup>[10]</sup> or no association<sup>[31]</sup>. In the paper showing a positive relationship, the number of case subjects with CRC was small, and the authors did not confirm with colonoscopy that the control groups were polyp-free<sup>[31]</sup>. In the study showing an inverse relationship, the number of cases was small, and the authors included overall CRC stages, which resulted in the weight loss over the course of cancer development influencing the result. Studies that evaluate the effect of abdominal obesity in a large number CRC cases and define the control and adenoma groups clearly by colonoscopic examination are sparse. Colorectal neoplasia follows the “adenoma-carcinoma sequence”<sup>[8]</sup>, which is characterized by the progression from precancerous adenoma to carcinoma. Because adenomatous polyps are common and only a small portion progress to cancer<sup>[32]</sup>, the association of visceral obesity with colorectal neoplasia should be explored at each step. However, studies assessing their association with cancer (separately) are sparse. In our study, we assessed the effect of visceral obesity on the “normal to cancer” and “adenoma to cancer” progression. Contrary to the effect of visceral obesity on the “normal to colorectal adenoma sequence”, visceral obesity was not a risk factor for developing CRC. Every step of the “adenoma-carcinoma sequence” is affected by multiple factors<sup>[33-37]</sup>, such as genetic instability, cell cycle, apoptosis and other environmental factors, including inflammatory cells and dietary carcinogens. Therefore, the association between visceral fat and these multiple factors may be step-specific over the progression of this sequence. The role of visceral fat in the progression of the “adenoma-carcinoma sequence” is complex and has not been thoroughly explored. From the previous research and our paper, it appears that visceral obesity is a risk factor for colorectal adenoma formation but does not have an additional effect on its further progress to colorectal carcinoma.

**Table 2** Body measure index, adipose tissue area and the risk of colorectal cancer *vs* control

	Univariate		Multivariate 1 <sup>1</sup>		Multivariate 2 <sup>2</sup>	
	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value
BMI						
Quartile I	1	0.021 <sup>3</sup>	1	0.025 <sup>3</sup>		
Quartile II	0.82 (0.49-1.40)	0.471	0.81 (0.48-1.38)	0.446		
Quartile III	1.33 (0.81-2.20)	0.262	1.32 (0.80-2.19)	0.279		
Quartile IV	1.61 (0.97-2.66)	0.066	1.58 (0.95-2.63)	0.077		
VAT						
Quartile I	1	0.478 <sup>3</sup>	1	0.501 <sup>3</sup>	1	0.378 <sup>3</sup>
Quartile II	0.80 (0.49-1.31)	0.376	0.79 (0.48-1.29)	0.341	0.73 (0.43-1.23)	0.235
Quartile III	0.65 (0.40-1.07)	0.089	0.65 (0.40-1.07)	0.089	0.59 (0.35-1.02)	0.057
Quartile IV	0.92 (0.56-1.53)	0.758	0.93 (0.56-1.54)	0.776	0.82 (0.47-1.46)	0.507
SAT						
Quartile I	1	0.716 <sup>3</sup>	1	0.770 <sup>3</sup>	1	0.522 <sup>3</sup>
Quartile II	1.10 (0.67-1.82)	0.708	1.10 (0.66-1.82)	0.726	1.25 (0.73-2.13)	0.426
Quartile III	1.10 (0.62-1.68)	0.940	1.00 (0.60-1.66)	0.993	1.15 (0.66-1.99)	0.623
Quartile IV	1.14 (0.68-1.89)	0.625	1.12 (0.67-1.87)	0.664	1.31 (0.73-2.34)	0.362

<sup>1</sup>Multivariate model 1 was adjusted for current smoking status, and alcohol consumption; <sup>2</sup>Multivariate model 2 was adjusted for subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), in addition. Since body mass index (BMI) showed co-linearity to adipose tissue amount, it was not included in the analysis; <sup>3</sup>P value for test of trend of odds.

**Table 3** Body measure index, adipose tissue area and the risk of colorectal cancer *vs* colorectal adenoma

	Univariate		Multivariate 1 <sup>1</sup>		Multivariate 2 <sup>2</sup>	
	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value
BMI						
Quartile I	1	0.937 <sup>3</sup>	1	0.887 <sup>3</sup>		
Quartile II	0.87 (0.51-1.48)	0.600	0.86 (0.50-1.48)	0.595		
Quartile III	0.96 (0.58-1.58)	0.875	0.96 (0.58-1.58)	0.857		
Quartile IV	0.95 (0.58-1.55)	0.824	0.93 (0.57-1.53)	0.777		
VAT						
Quartile I	1	0.032 <sup>3</sup>	1	0.024 <sup>3</sup>	1	0.028 <sup>3</sup>
Quartile II	0.74 (0.45-1.21)	0.227	0.74 (0.45-1.20)	0.224	0.70 (0.42-1.18)	0.182
Quartile III	0.71 (0.43-1.17)	0.182	0.68 (0.41-1.13)	0.141	0.65 (0.38-1.12)	0.118
Quartile IV	0.57 (0.35-0.94)	0.028	0.56 (0.34-0.92)	0.023	0.53 (0.31-0.92)	0.024
SAT						
Quartile I	1	0.550 <sup>3</sup>	1	0.556 <sup>3</sup>	1	0.729 <sup>3</sup>
Quartile II	0.90 (0.55-1.49)	0.686	0.91 (0.55-1.50)	0.697	1.08 (0.63-1.83)	0.786
Quartile III	1.03 (0.62-1.71)	0.898	1.02 (0.62-1.70)	0.933	1.26 (0.73-2.17)	0.407
Quartile IV	0.82 (0.49-1.35)	0.427	0.82 (0.50-1.36)	0.443	1.09 (0.62-1.91)	0.362

<sup>1</sup>Multivariate model 1 was adjusted for current smoking status, and alcohol consumption; <sup>2</sup>Multivariate model 2 was adjusted for subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), in addition. Since body mass index (BMI) showed co-linearity to adipose tissue amount, it was not included in the analysis; <sup>3</sup>P value for test of trend of odds.

Contrary to Western population, carcinogenesis is different in the Asian population, which develops CRC more often directly in normal colonic mucosa without adenoma stage. Our results might be compatible to this *de novo* pathway hypothesis. Further studies were needed to prove this hypothesis<sup>[38]</sup>.

Diabetes, one of the factors comprising metabolic syndrome, is also considered a risk factor for CRC. Some studies have shown positive<sup>[39-41]</sup> association between diabetes and CRC while others have shown inconclusive<sup>[39,41,42]</sup>. There are reports that the association is affected by ethnicity, gender and the duration of treatment<sup>[43,44]</sup>. In our study, diabetes showed no relationship with CRC. Recent study demonstrated that colon cancer risk is increased in diabetic men before diabetes onset<sup>[45]</sup>. Diabetes did not alter colon cancer risk in men or women after

clinical diabetes onset<sup>[45]</sup>. Because we only included patients with early CRC, we thought abnormal glucose metabolism, including impaired fasting glucose and diabetes all together, was a risk factor for early CRC. Multivariable analysis showed impaired fasting glucose/diabetes is an independent risk factor of early colon cancer (data was not shown). Future studies with objective measurement of diabetes (insulin level, C-peptide) and incorporating ethnicity, gender and medication difference might help elucidate the risk of CRC in diabetes.

Our study has some superiority over previous studies. Although several studies have assessed the effect of visceral obesity on colorectal neoplasia using CT scanning, few studies that analyzed its respective effect on the normal to adenoma and the adenoma to carcinoma sequences<sup>[7,9,10,19,31,46]</sup>. In our study, we included only the

stage I CRC patients to minimize the wasting characteristics of the cancer itself. Because we perform more than 500 cases CRC surgery in a year, the total number of early CRC cases that were included was sufficiently larger. In our study, we benefited from utilizing a control group that was selected by performing a complete; therefore, definite comparisons among the control, adenoma and CRC groups were feasible.

Several limitations of the study also warrant mentioning. First, because this study had a cross-sectional design, we could not evaluate the effect of the duration of visceral obesity and could not consider recent weight changes. Second, we did not adjust healthcare, socioeconomic status, dietary factors or physical activity factors, which affect the visceral fat amount and the CRC development. Third, as the control and adenoma groups were selected from the health screening population, there might be a socioeconomic difference in the CRC groups. However, several variables such as diabetes, hypertension, smoking, and alcohol consumption were not different from three groups. Fourth, information of previous adenoma before surgery was not available, which might affect the development of CRC. Fifth, since we did not analyze the genetic data in each group, *de novo* pathway, which is comprise some portion for CRC development<sup>[47]</sup>, was not taken into consideration to explain CRC development. Sixth, volumetric measure of VAT was more accurate than visceral adipose tissue area measurement<sup>[48,49]</sup>. However, the technique we used for VAT area measurements has been standardized and validated in previous studies<sup>[24-28]</sup>. Finally, since our study was designed retrospectively, we could not take into consideration in the relationship of metabolic syndromes for analysis because data such as triglyceride, high-density lipoprotein-cholesterol and insulin level, were not available. In addition, we did not have data such as WC or WHR in patients with CRC due to our retrospective study design.

In conclusion, although visceral obesity is an independent risk factor for colorectal adenoma in previous studies, it is not a risk factor for early CRC. Visceral obesity might not influence the adenoma-early carcinoma sequence, but it does influence the normal-adenoma sequence. In addition, role of visceral obesity which might be explained *de novo* pathway for CRC development in Asian population need to be studied in future.

## COMMENTS

### Background

Recently, several studies have addressed the association between colorectal adenoma and visceral adipose tissue (VAT) area as measured by abdominal computed tomography (CT). In addition, it has been well documented that most colorectal cancers (CRC) stem from colorectal adenoma in a process referred to as the adenoma-carcinoma sequence. However, the relationship between VAT and early CRC has not been thoroughly documented.

### Research frontiers

Although several studies have assessed the effect of visceral obesity on colorectal neoplasia using CT scanning, few studies that analyzed its respective effect on the normal to adenoma and the adenoma to carcinoma sequences. The authors investigated the role of VAT in developing CRC at each step of this sequence

## Innovations and breakthroughs

This study showed that visceral obesity is not a risk factor for early CRC, although it is an independent risk factor for colorectal adenoma in previous studies. Therefore, these data suggest that visceral obesity might influence the normal-adenoma sequence but not the adenoma-early carcinoma sequence. All of these observations are original and provide new information between visceral obesity and colorectal neoplasia above and beyond CT-measured visceral adiposity.

## Applications

The role of visceral fat in the progression of the "adenoma-carcinoma sequence" is complex and has not been thoroughly explored. From the previous research and our paper, it appears that visceral obesity is a risk factor for colorectal adenoma formation but does not have an additional effect on its further progress to colorectal carcinoma. These findings will be valuable data to understand the role of adipose tissue in the progression of "adenoma-carcinoma sequence".

## Terminology

VAT is defined as an intra-abdominal adipose tissue area confined by the peritoneum, excluding the paraspinal muscles and the vertebral column. Subcutaneous adipose tissue areas were defined as adipose tissue areas external to the abdomen and the back muscles.

## Peer review

Although there are some limitations due to its retrospective study design, this article is a nice and well written case controlled study showing the role of adipose tissue in the progression of colorectal adenoma-carcinoma sequence.

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## Added value of hepatobiliary phase gadoxetic acid-enhanced MRI for diagnosing hepatocellular carcinoma in high-risk patients

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### Abstract

**AIM:** To determine the added value of hepatobiliary phase (HBP) gadoxetic acid-enhanced magnetic resonance imaging (MRI) in evaluating hepatic nodules in high-risk patients.

**METHODS:** The institutional review board approved this retrospective study and waived the requirement for informed consent. This study included 100 patients at high risk for hepatocellular carcinoma (HCC) and 105 hepatic nodules that were larger than 1 cm. A blind review of two MR image sets was performed in a random order: set 1, unenhanced (T1- and T2-weighted) and dynamic images; and set 2, unenhanced, dynamic 20-min and HBP images. The diagnostic accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were compared for the two image sets. Univariate and multivariate analyses were performed on the MR characteristics utilized to diagnose HCC.

**RESULTS:** A total of 105 hepatic nodules were identified in 100 patients. Fifty-nine nodules were confirmed to be HCC. The diameter of the 59 HCCs ranged from 1 to 12 cm (mean: 1.9 cm). The remaining 46 nodules were benign (28 were of hepatocyte origin, nine were hepatic cysts, seven were hemangiomas, one was chronic inflammation, and one was focal fat infiltration). The diagnostic accuracy significantly increased with the addition of HBP images, from 88.7% in set 1 to 95.5% in set 2 ( $P = 0.002$ ). In set 1 *vs* set 2, the sensitivity and NPV increased from 79.7% to 93.2% and from 78.9% to 91.8%, respectively, whereas the specificity and PPV were not significantly different. The hypointensity on the HBP images was the most sensitive (93.2%), and typical arterial enhancement followed by washout was the most specific (97.8%). The multivariate analysis revealed that typical arterial enhancement followed by washout, hyperintensity on T2-weighted images, and hypointensity on HBP images were statistically significant MRI findings that could diagnose HCC ( $P < 0.05$ ).

**CONCLUSION:** The addition of HBP gadoxetic acid-enhanced MRI statistically improved the diagnostic accuracy in HCCs larger than 1 cm. Typical arterial enhancement followed by washout and hypointensity on HBP images are useful for diagnosing HCC.

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**Key words:** Magnetic resonance imaging; Liver; Gadaxetic acid; Hepatobiliary phase; Hepatocellular carcinoma

**Core tip:** This study demonstrated the added value of hepatobiliary phase gadoxetic acid-enhanced magnetic resonance imaging (MRI) for diagnosing hepatocellular carcinoma, based on the changes in contrast uptake

on hepatobiliary phase images during hepatocarcinogenesis. The pitfalls of interpreting hepatobiliary phase MRI are important to recognize in obtaining the most accurate results.

Phongkitkarun S, Limsamutpetch K, Tannaphai P, Jatchavala J. Added value of hepatobiliary phase gadoxetic acid-enhanced MRI for diagnosing hepatocellular carcinoma in high-risk patients. *World J Gastroenterol* 2013; 19(45): 8357-8365 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8357.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8357>

## INTRODUCTION

Liver cancer is the second most frequent cause of death in men and the sixth leading cause of death in women worldwide. Southeast Asia is one of the regions with the greatest incidences of liver cancer, and hepatocellular carcinoma (HCC), associated with the hepatitis B virus, accounts for the majority of the cases<sup>[1,2]</sup>.

The therapeutic approach for HCC depends on the accurate diagnosis and identification of HCC lesions, *i.e.*, the number, size, and location of the lesions. In practice, the high prevalence of benign lesions in cirrhotic livers and the variability in HCC imaging characteristics, which depend on differentiation of the state of the disease, render the detection of HCC difficult. Magnetic resonance imaging (MRI), particularly contrast-enhanced dynamic MRI, plays an important role in accurately diagnosing HCC<sup>[3]</sup>. A study by Forner *et al.*<sup>[4]</sup> reported that the sensitivity and specificity were as high as 62% and 97%, respectively, for detecting HCC with conventional contrast-enhanced dynamic MRI. Nevertheless, undetectable HCC lesions pose a serious issue.

A recent molecular study reported that organic anion transporting polypeptide 8 (OATP8) is expressed in hepatocytes and that OATP8 expression significantly decreases during the multistep process of hepatocarcinogenesis<sup>[5]</sup>. A newly developed liver-specific contrast agent, gadoxetic acid, is taken up by hepatocytes *via* OATP8 in an *in vitro* study<sup>[6]</sup>. Therefore, the use of liver-specific contrast-enhanced MRI might provide superior detection and characterization of HCC<sup>[7-9]</sup>.

Gadoxetic acid, or gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA), has the properties of being both an extracellular gadolinium chelate and a hepatobiliary agent, thus enabling dynamic perfusion imaging, delayed hepatocyte uptake, and biliary excretion. A recent study demonstrated that, compared with conventional contrast-enhanced dynamic and T2-weighted MRI, Gd-EOB-DTPA-enhanced hepatobiliary phase MRI improved HCC detection<sup>[3]</sup>. However, there are no supportive data for such a benefit in the Thai population, which has a high prevalence of HCC with different risk factors. Because of differences in the population and the nature of the disease, we performed a study to determine the added value of Gd-EOB-DTPA-enhanced

**Table 1** Demographic characteristics of the study population (*n* = 100) *n* (%)

Variables	Results
Age (mean ± SD; range, yr)	59.5 ± 11.4 (range 27-89)
Sex (male:female)	71:29
Risk factors for HCC	
Chronic HBV infection	20 (20)
Chronic HCV infection	2 (2)
Hepatitis B-related cirrhosis	45 (45)
Hepatitis C-related cirrhosis	16 (16)
Alcoholic cirrhosis	9 (9)
Nonalcoholic steatohepatitis	6 (6)
Only high serum AFP levels	2 (2)
Child-Pugh score	
A	94 (94)
B	5 (5)
C	1 (1)
Alpha-fetoprotein (mean ± SD; range, ng/mL)	111.6 ± 935.1 (range 0.98-8973)
< 20	84 (84)
20-200	5 (5)
> 200	3 (3)
Total bilirubin (mean ± SD; range, mg/dL)	1.43 ± 2.5 (range 0.2-17.1)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein.

hepatobiliary phase MRI for diagnosing HCC in high-risk patients.

## MATERIALS AND METHODS

### Patient selection and reference standards

This retrospective study was approved by our institutional review board, which waived the informed consent requirement. Between January 2010 and July 2010, 100 consecutive patients (mean age: 59.5 years old; range: 27-89 years), consisting of 71 men (mean age: 59.4 years; range: 27-89 years) and 29 women (mean age: 59.6 years old; range: 36-81 years), were registered as high-risk patients with HCC and underwent Gd-EOB-DTPA-enhanced MRI. The high-risk patients included those with chronic viral hepatitis infections (HBV or HCV infection), liver cirrhosis, or elevated serum alpha-fetoprotein (AFP) levels. Among the 100 patients, 76 had liver cirrhosis. Forty-five patients had hepatitis B-related cirrhosis, 16 had hepatitis C-related cirrhosis, nine had alcoholic cirrhosis, six had nonalcoholic steatohepatitis, 22 had a chronic hepatitis infection (20 with chronic hepatitis B and two with chronic hepatitis C), and the remaining two patients only presented with abnormal serum AFP. The Child-Pugh score was calculated for all of the patients within 60 d of the MRI scan (Table 1).

The reference standard for diagnosing HCC was classified as follows: based on pathologically proven HCC (by surgical specimen or percutaneous biopsy); by lipiodol staining after transhepatic arterial chemoembolization (TACE); or on the basis of progression of the disease on follow-up computed tomography (CT) or MRI performed at least 6 mo after the initial imaging.



**Table 2** 1.5-T and 3.0-T magnetic resonance pulse sequence parameters

Parameters	Double-echo T1-weighted gradient echo		Respiratory-triggered fast-spin T2-weighted		T1-weighted Gd-EOB-DTPA-enhanced	
	1.5-T	3.0-T	1.5-T	3.0-T	1.5-T	3.0-T
Matrix	256 × 192	228 × 136	320 × 224	320 × 188	256 × 192	180 × 163
Section thickness (mm)	6-8	6	4	6	2-3	4
Intersection gap (mm)	2	2	1	2	-	-
TR (ms)	180-220	150-250	2000-4000	2500-3500	4.2	2.8
TE (ms)	2.3-4.6	1.15-2.3	60	65-85	2	1.4
Flip angle (degrees)	80	70	90	120	12	10
Reduction factor	2	2	2	2	2	2

Gd-EOB-DTPA: Gadolinium ethoxybenzyl diethylenetriaminepentaacetic.

### MRI methods

All of the MRI studies were performed with a 1.5-T system (Signa HDxt; GE Healthcare, Milwaukee, Wisconsin, United States) and a 3.0-T system (Intera Achieva; Philips Medical Systems, Best, The Netherlands) with phased-array coils. All of the images were obtained on the axial plane. The field of view (32-40 cm × 32-40 cm) was adjusted for each patient. The MR protocol consisted of a double-echo T1-weighted gradient-echo sequence (in-phase and opposed-phase), a respiratory-triggered fast-spin T2-weighted sequence, and a contrast-enhanced dynamic sequence. The parameters for all of the sequences are presented in Table 2. For the contrast-enhanced dynamic MR images, Gd-EOB-DTPA was administered at 0.025 mmol per kilogram of body weight at 2 mL per second, followed by a 10-mL saline flush. After administering the contrast, early arterial phase (25-30 s), late arterial phase (45-50 s), portal venous phase (65-70 s), equilibrium phase (5 min), and additional hepatobiliary phase (after 20 min) images were obtained.

### MR image analysis

Two sets of MR images were reviewed by an experienced gastrointestinal radiologist (S.P.) in a random order. The observer was blinded to the patient history, the findings from other imaging modalities, treatments, outcomes, and the final diagnoses. Set 1 of the MR images contained unenhanced (pre-contrast T1- and T2-weighted images) and Gd-EOB-DTPA-enhanced dynamic images (arterial, portal venous, and 5-min equilibrium phases); set 2 consisted of unenhanced, Gd-EOB-DTPA-enhanced dynamic, and 20-min hepatobiliary phase images. The MR images in set 2 were obtained at least 1 mo after finishing the reviews of all of the MR images in set 1. A diagnosis of HCC met at least one of the following criteria: (1) a liver nodule 1 cm or larger, with increased enhancement on arterial phase images and washout on portal venous or equilibrium phase images<sup>[10]</sup>; or (2) a hypervascular liver nodule 1 cm or larger, with no uptake on 20-min hepatobiliary phase Gd-EOB-DTPA-enhanced MR images<sup>[11]</sup>.

The observer recorded the possibility of HCC for each dominant lesion (defined as 1 cm or larger) using a four-point confidence rating scale: (1) unlikely to be HCC; (2) a concerning nodule; (3) probably HCC; and (4) definitely HCC. A score of 0 was recorded when the observer did not find any dominant lesions. Lesions

that received a score of 3 or 4 were classified as positive for HCC in later analyses. The size and location of each lesion were clearly documented using liver segmental anatomy, defined by Couinaud classification and MR characteristics (in-phase, opposed-phase, T2-weighted, arterial phase, portal venous phase, 5-min equilibrium phase, and 20-min hepatobiliary phase images, which were only obtained in set 2). All of the MR images were reviewed with Picture Archiving and Communications System (PACS) and Digital Imaging and Communications in Medicine Conformance (Synapse, version 3.2.0, FUJIFILM Medical Systems United States's Synapse® PACS system, Stamford, Connecticut, United States).

### Statistical analysis

Statistical analyses were performed using statistical software (SPSS, version 17.0.1, SPSS, Chicago, Illinois, United States). Descriptive statistics were calculated for all of the clinical variables and for those evaluated on the MR images. For continuous data (age, AFP level, total bilirubin level, and lesion size), the means, SDs, and ranges were calculated. Descriptive statistics, such as raw numbers and percentages, were determined for categorical data. An alternative free-response receiver operating characteristic analysis was performed on the confidence rating scale of the observer. The area under the alternative free-response receiver operating characteristic curve ( $A_z$ ) was utilized to determine the diagnostic accuracy of detecting HCC in each image set and according to lesion size (1-2 cm or larger than 2 cm). Sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) were calculated for the two MR image sets.

The relationships between each MRI variable and the presence of an HCC lesion were tested *via* univariate analysis. Significant variables identified by the univariate analysis were integrated into the logistic regression model for the multivariate analysis. The MRI variables with significant associations in the multivariate analysis were regarded as significant predictors of the presence of HCC. All of the statistical tests were two-tailed, and a *P* value of < 0.05 indicated statistical significance.

## RESULTS

A total of 105 hepatic nodules were identified in 100 patients. Fifty-nine nodules were confirmed to be HCC by

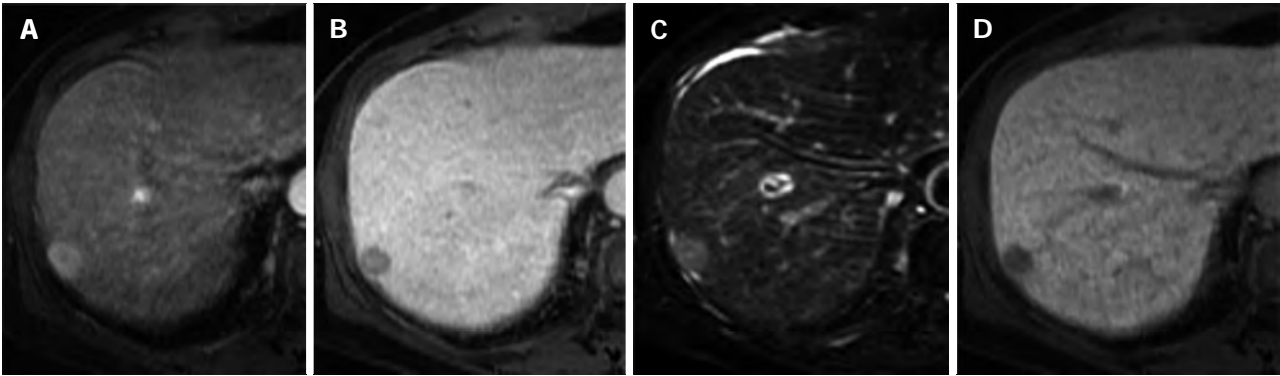


Figure 1 Magnetic resonance images from a 77-year-old woman with hepatitis B virus cirrhosis. A liver nodule at segment VII was surgically confirmed as hepatocellular carcinoma. Gadolinium ethoxybenzyl diethylenetriaminepentaacetic -enhanced magnetic resonance (MR) arterial phase images depicting a 1.9-cm arterial enhanced nodule (A) with rapid washout on the equilibrium phase image (B). C: Hyperintensity on a T2-weighted MR image; D: Hypointensity on a 20-min hepatobiliary phase image.

Table 3 Diagnostic accuracy of diagnosing hepatocellular carcinoma using conventional dynamic magnetic resonance imaging or conventional dynamic magnetic resonance imaging with hepatobiliary phase images			
	Conventional dynamic MRI	Conventional dynamic MRI+ HBP	P value
All lesions	88.7%	95.5%	0.002
Lesions 1-2 cm	86.7%	94.0%	0.008
Lesions > 2 cm	94.4%	100%	0.145

MRI: Magnetic resonance imaging; HBP: Hepatobiliary phase.

Table 4 Sensitivity, specificity, and positive and negative predictive values for diagnosing hepatocellular carcinoma using conventional dynamic magnetic resonance imaging or conventional dynamic magnetic resonance imaging with hepatobiliary phase images				
	Conventional dynamic MRI	95%CI	Conventional dynamic MRI+ HBP	95%CI
Sensitivity	79.7%	67.2-89.0	93.2%	83.5-98.1
Specificity	97.8%	85.5-99.9	97.8%	88.5-99.9
Positive predictive value	97.9%	88.9-99.9	98.2%	90.4-100.0
Negative predictive value	78.9%	66.1-88.6	91.8%	80.4-97.7

MRI: Magnetic resonance imaging; HBP: Hepatobiliary phase.

pathologic examination of surgical resection or percutaneous biopsy specimens ( $n = 23$ ), by lipiodol staining after TACE ( $n = 30$ ), or on the basis of disease progression on follow-up CT or MRI at least 6 mo after the initial imaging ( $n = 6$ ; mean: 11.9 mo). The diameter of the 59 HCCs ranged from 1 to 12 cm (mean: 1.9 cm). The remaining 46 nodules were benign (28 were of hepatocyte origin, nine were hepatic cysts, seven were hemangiomas, one was chronic inflammation, and one was focal fat infiltration). Four of the 46 nodules were pathologically confirmed as benign lesions (two were regenerative nodules, one was a cirrhotic nodule, and one was a hemangioma), and 42 nodules were considered benign because they decreased or remained unchanged in size on the last follow-up CT, or MRI (mean: 22.1 mo).

The diagnostic accuracy of diagnosing HCC in the two image sets is presented in Table 3. The diagnostic accuracy of the combination of unenhanced, Gd-EOB-DTPA-enhanced dynamic, and 20-min hepatobiliary phase images (set 2, 95.5%) was significantly greater than

that of the unenhanced and Gd-EOB-DTPA-enhanced dynamic images (set 1, 88.7%) ( $P = 0.002$ ; Figure 1). For 1- to 2-cm lesions, the diagnostic accuracy significantly improved from 86.7% in set 1 to 94.0% in set 2 ( $P = 0.008$ ), whereas there was no statistical improvement for lesions > 2 cm (94.4% in set 1 and 100% in set 2;  $P = 0.145$ ).

The sensitivity, specificity, PPVs, and NPVs for the two image sets are presented in Table 4. The addition of 20-min hepatobiliary phase images increased the sensitivity from 79.7% (95%CI: 67.2-89) to 93.2% (95%CI: 83.5-98.1). The specificity of both sets was 97.8% (95%CI: 85.5-99.9). The addition of 20-min hepatobiliary phase images increased the positive and NPVs from 97.9% (95%CI: 88.9-99.9) to 98.2% (95%CI: 90.4-100) and from 78.9% (95%CI: 66.1-88.6) to 91.8% (95%CI: 80.4-97.7), respectively.

The sensitivity, specificity, PPVs, and NPVs of each MRI characteristic utilized to diagnose HCC were as follows: (1) fat metamorphosis: 16.9% (95%CI: 8.4-29), 95.7% (95%CI: 85.2-99.5), 83.3% (95%CI: 51.6-97.9),

**Table 5** Sensitivity, specificity, and positive and negative predictive values for diagnosing hepatocellular carcinoma based on magnetic resonance imaging findings after excluding cysts and hemangiomas ( $n = 89$ )

MRI findings	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Fat metamorphosis	16.9 (8.4-29.0)	93.3 (77.9-99.2)	83.3 (51.6-97.9)	36.4 (25.7-48.1)
Hyperintensity on T2-weighted images	66.1 (52.6-77.9)	93.3 (77.9-99.2)	95.1 (83.5-99.4)	58.3 (43.2-72.4)
Arterial enhancement	88.1 (77.1-95.1)	70.0 (50.6-85.3)	85.2 (73.8-93.0)	75.0 (55.1-89.3)
Arterial enhancement and washout on venous or equilibrium phase	79.7 (67.2-89.0)	96.7 (82.8-99.9)	97.9 (88.9-99.9)	70.7 (54.5-83.9)
Hypointensity on 20-min hepatobiliary phase	93.2 (83.5-98.1)	53.3 (34.3-71.7)	79.7 (68.3-88.4)	80.0 (56.3-94.3)
Arterial enhancement and no/partial uptake on 20-min hepatobiliary phase	83.1 (71.0-91.6)	93.3 (77.9-99.2)	96.1 (86.5-99.5)	73.7 (56.9-86.6)

MRI: Magnetic resonance imaging.

**Table 6** Results of the univariate analysis of the magnetic resonance imaging findings for the diagnosis of hepatocellular carcinoma  $n$  (%)

MRI findings	All lesions ( $n = 105$ )			Lesions excluding cysts and hemangiomas ( $n = 89$ )		
	HCC ( $n = 59$ )	Not HCC ( $n = 46$ )	$P$ value	HCC ( $n = 59$ )	Not HCC ( $n = 30$ )	$P$ value
Fat metamorphosis	10 (16.9)	2 (4.3)	0.061	10 (16.9)	2 (6.7)	0.190
Hyperintensity on T2-weighted images	39 (66.1)	18 (39.1)	0.007	39 (66.1)	2 (6.7)	< 0.001
Arterial enhancement	52 (88.1)	15 (32.6)	< 0.001	52 (88.1)	9 (30)	< 0.001
Arterial enhancement and washout on venous or equilibrium phase images	47 (79.7)	1 (2.2)	< 0.001	47 (79.7)	1 (3.3)	< 0.001
Hypointensity on 20-min hepatobiliary phase images	55 (93.2)	30 (65.2)	0.001	55 (93.2)	14 (46.7)	< 0.001
Arterial enhancement and no/partial uptake on 20-min hepatobiliary phase images	49 (83.1)	8 (17.4)	< 0.001	49 (83.1)	2 (6.7)	< 0.001

HCC: Hepatocellular carcinoma. MRI: Magnetic resonance imaging.

**Table 7** Results of the multivariate analysis of the magnetic resonance imaging findings for diagnosing hepatocellular carcinoma

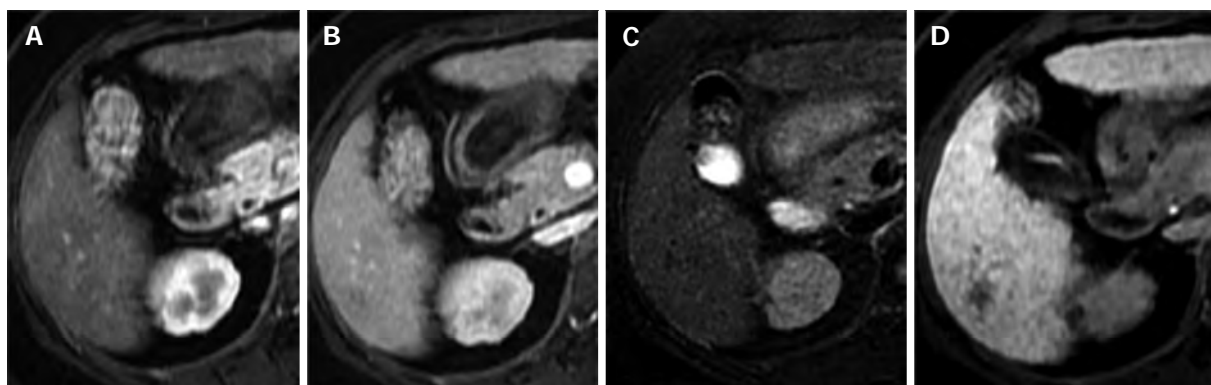
MRI findings	All lesions ( $n = 105$ )			Lesions excluding cysts and hemangiomas ( $n = 89$ )		
	OR	95%CI	$P$ value	OR	95%CI	$P$ value
Hyperintensity on T2-weighted images	1.1	0.3-4.3	0.86	12.6	1.8-87.8	0.01
Arterial enhancement	1.8	0.4-7.0	0.42	3.5	0.5-22.8	0.18
Arterial enhancement and washout on venous or equilibrium phase images	102.0	10.3-1012.4	< 0.001	16.9	1.3-221.1	0.03
Hypointensity on 20-min hepatobiliary phase images	1.8	0.4-8.6	0.48	6.7	1.1-41.9	0.04

MRI: Magnetic resonance imaging.

and 47.3% (95%CI: 36.9-57.9); (2) hyperintensity on T2-weighted images: 66.1% (52.6-77.9), 60.9% (45.4-74.9), 68.4% (54.8-80.1), and 58.3% (43.2-72.4); (3) arterial enhancement: 88.1% (77.1-95.1), 67.4% (52.0-80.5), 77.6% (65.8-86.9), and 81.6% (65.7-92.3); (4) arterial enhancement and washout on venous or equilibrium phase images: 79.7% (67.2-89.0), 97.8% (88.5-99.9), 97.9% (88.9-99.9), and 78.9% (66.1-88.6); (5) hypointensity on 20-min hepatobiliary phase images: 93.2% (83.5-98.1), 34.8% (21.4-50.2), 64.7% (53.6-74.8), and 80% (56.3-94.3); and (6) arterial enhancement and no or partial uptake on 20-min hepatobiliary phase images: 83.1% (71.0-91.6), 82.6% (68.6-92.2), 86% (74.2-93.7), and 79.2% (65.0-89.5). The MRI finding with the greatest sensitivity (93.2%) for detecting HCC was hypointensity on the 20-min hepatobiliary phase images, and the greatest specificity (97.8%) for detecting HCC occurred with typical arterial enhancement and washout on venous or

equilibrium phase images. Excluding cysts and hemangiomas, the specificity of diagnosing HCC was greater for hyperintense T2-weighted images and for hypointensities on hepatobiliary phase images (Table 5).

Table 6 summarizes the results of the univariate analysis of the ability of the MRI findings in the two groups to diagnose HCC, as well as the results after excluding cysts and hemangiomas. Although almost all of the MRI features were statistically significant in the univariate analysis, the multivariate analysis revealed that only the arterial enhancement and washout on venous or equilibrium phase images were statistically significant MRI findings ( $P < 0.001$ ; OR = 102.0; 95%CI: 10.3-1012.4; Table 7). Furthermore, typical arterial enhancement followed by washout on venous or equilibrium phases, hyperintensity on T2-weighted images, and hypointensity on 20-min hepatobiliary phase images were statistically significant MRI findings, after excluding cysts and hemangiomas.



**Figure 2** Magnetic resonance images from a 59-year-old woman with hepatocellular carcinoma at hepatic segment VI. This patient underwent transhepatic arterial chemoembolization, and the 6-wk follow-up computerized tomography scan revealed lipiodol staining in the lesion. A and B: Arterial phase and portal venous phase images; C: T2-weighted magnetic resonance image with no definite focal lesion; D: The hepatobiliary phase image revealed a 1.0-cm discrete nodule that was not visible on the dynamic or T2-weighted images.

## DISCUSSION

Our study demonstrated that adding hepatobiliary phase Gd-EOB-DTPA-enhanced MRI significantly improved the accuracy of diagnosing HCC. The significant improvement was particularly apparent for small lesions (1–2 cm in diameter). No significant differences were observed for lesions larger than 2 cm in diameter. This finding could be explained by most large HCCs being unequivocally diagnosed based on clearly enhanced characteristics using conventional dynamic MRI. Smaller HCCs sometimes exhibit atypical enhancement characteristics. Therefore, the inclusion of the hepatobiliary phase provided more information for including or excluding the diagnosis of HCC for small nodules. However, other important information for diagnosing HCC was still based on conventional dynamic MRI.

Sensitivity increased with the addition of hepatobiliary phase images, whereas specificity did not change. The inclusion of hepatobiliary phase images allowed subtle abnormalities present in other sequences to be visualized more clearly. The results of our study agreed with those of recent reports in terms of the improvement of diagnostic performance, the characterization of HCC, and the dismissal of pseudolesions<sup>[3,10,12–14]</sup>. In our study, adding the hepatobiliary phase images increased lesion detection by eight HCCs (13.5%), compared with conventional dynamic MRI (Figure 2).

Our study also highlighted other characteristic MRI findings that were involved in diagnosing HCC. Fat metamorphosis, which can be seen with chemical shift GRE imaging, was present in 10 of 59 HCCs (16.9%) with high specificity (93.3%). Two of the 46 benign lesions (4.3%) with fat components were histologically confirmed to be regenerative nodules in the background of cirrhosis and focal fat infiltration in normal livers. The results were similar to those in the study by Martín *et al.*<sup>[15]</sup>. The presence of various fat alterations might be a significant morphological marker for malignant transformation from adenomatous hyperplasia to HCC, although it occurs rarely and can be present in benign hepatic nodules

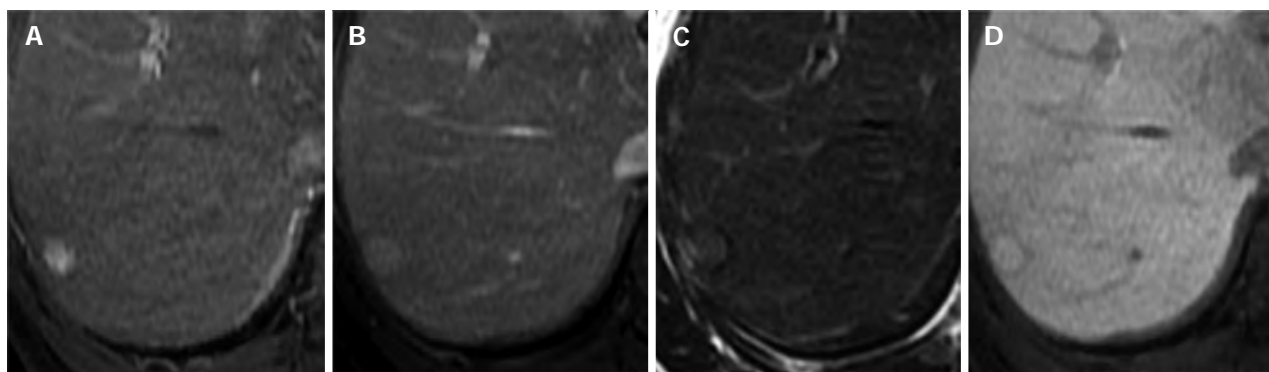
in cirrhotic patients<sup>[15,16]</sup>.

A recent study demonstrated that T2-weighted images did not provide additional diagnostic value in detecting and characterizing focal lesions. The heterogeneity and hyperintense fibrotic septa and bridges in cirrhotic liver parenchyma can obscure moderately hyperintense HCCs on T2-weighted images, and 42%–53% of HCCs can also be isointense to hypointense on T2-weighted images<sup>[17]</sup>. In this study, however, the specificity improved with the addition of T2-weighted images after excluding cysts and hemangiomas, and T2-weighted images had the greatest sensitivity among all of the tests, with an odds ratio statistically different from 1 for differentiating HCC from benign lesions larger than 1 cm; these results were similar to those of previous reports<sup>[18,19]</sup>.

In 2010, the American Association for the Study of Liver Diseases guidelines for 1- to 2-cm HCCs were changed to require the visualization of a typical enhancement pattern using only one contrast-enhanced imaging technique<sup>[10]</sup>. In our series, a typical enhancement pattern was observed in 47 of 59 HCC lesions (79.7%) and in one of 30 benign lesions (3.3%); this benign lesion was determined by tissue biopsy to be a regenerating nodule. In our study, 12 of 59 HCC lesions (20.3%) had no typical arterial enhancement or washout on portal venous or equilibrium phase images, and these lesions were classified as well-differentiated HCCs (Figure 3); one had a motion artifact that resulted in a technical error. A recent study by Witjes *et al.*<sup>[20]</sup> demonstrated a strong association between the presence of washout on dynamic MRI and moderately to poorly differentiated HCC.

The majority of the HCCs in our study (55 lesions, 93.2%) had no uptake or partial uptake on the hepatobiliary phase images. Only four HCC lesions (6.8%) were iso- or hyperintense on the hepatobiliary phase images. Fortunately, three-quarters were scored as probably HCC based on the presence of a hypointense capsule, even without the typical enhancement pattern (Figure 3). Two lesions were histologically confirmed to be well-differentiated HCCs. The diminished obviousness of these well-differentiated lesions on hepatobiliary phase





**Figure 3** Magnetic resonance images from a 54-year-old man with a 1.4-cm hepatocellular carcinoma with liver-specific contrast uptake at segment VII. This nodule was confirmed by tissue biopsy to be a well-differentiated hepatocellular carcinoma. Arterial enhanced nodule (A) without washout on the portal venous (B) phase images; C: A slightly hyperintense nodule with a hypointense capsule on a T2-weighted magnetic resonance image; D: Hepatobiliary phase image illustrating the hyperintense nodule.

images might have been related to residual hepatocyte activity within the lesions, enabling the delayed uptake of the contrast material<sup>[3]</sup>. Another possible cause of diminished obviousness on the hepatobiliary phase images was impaired hepatic function or hyperbilirubinemia, which can affect the hepatocyte uptake of the contrast agent in the liver parenchyma<sup>[21]</sup>. However, all of the false-negative cases in our study that demonstrated iso- or hyperintensity on the hepatobiliary phase images had normal bilirubin levels. A recent study by Kim *et al*<sup>[22]</sup> reported that 10% of HCCs were hyperintense on hepatobiliary phase images, and most of the hyperintense HCCs were either well differentiated or moderately differentiated.

A variety of benign hepatic lesions can appear hypointense on hepatobiliary-phase MRI because of the following causes: (1) a lack of functional hepatocytes in the lesion; (2) damage to the functional hepatocytes from infection or inflammation; and (3) impairment of the biliary function of the lesion<sup>[23]</sup>. In this study, hepatic cysts and hemangiomas accounted for most of the benign hypointense lesions on hepatobiliary phase images. To reduce false-positive findings, hepatobiliary phase imaging should be considered an adjunct method, rather than the sole method, for diagnosing HCC. By excluding benign hepatic cysts and hemangiomas, the arterial enhancement with hypointensity on hepatobiliary phase images had high diagnostic accuracy, comparable to the typical enhancement pattern for HCC (sensitivity: 83.1% *vs* 79.7%; specificity: 93.3% *vs* 96.7%). However, false-positive cases accounted for one (3.3%) typical enhancement pattern and two (6.7%) arterial enhancement patterns with hypointensity on hepatobiliary phase images. These cases were hypothesized to be dysplastic nodules that had stable appearances on imaging.

Several limitations of this study should be addressed. First, the retrospective nature introduced the possibility of selection bias, although we attempted to avoid bias by recruiting consecutively registered patients who met the inclusion criteria. Second, not all of the lesions were confirmed pathologically, which could have introduced verification bias. Histopathology was obtained for 23 of 59

(39%) HCC lesions and four of 46 (8.7%) benign lesions. However, we attempted to avoid this factor by extending the follow-up period to confirm the benign diagnoses (mean: 22.2 mo). Additionally, lesions smaller than 1 cm in diameter were not included in the study. Third, because we had only one observer scoring the images, testing inter-observer reliability was not feasible, and observer bias could have occurred. Finally, our findings might not reflect a direct comparison between imaging with an extracellular contrast agent and a liver-specific hepatobiliary agent. Hepatobiliary enhancement can begin as early as the first pass (the portal venous phase); therefore, it can be difficult to define clearly which phase is the most important for diagnosing HCC: the contrast-enhanced dynamic images or the hepatobiliary phase images.

In a conclusions, The addition of hepatobiliary phase imaging after the intravenous injection of Gd-EOB-DTPA significantly improved diagnostic accuracy in detecting HCC in high-risk patients. Typical arterial enhancement followed by washout, hyperintensity on T2-weighted images, and hypointensity on 20-min hepatobiliary phase images were useful for diagnosing HCCs larger than 1 cm.

## COMMENTS

### Background

Dynamic imaging plays an important role in diagnosis of hepatocellular carcinoma (HCC), which is based on typical enhancement characteristics. For years, rapid arterial enhancement and rapid washout pattern in dynamic contrast study is accepted as one of diagnostic criteria without tissue pathology. However, there are still limitations in term of sensitivity and specificity, particular in small lesions or during early phase of hepatocarcinogenesis. The newly developed hepatocyte-specific contrast [gadolinium ethoxybenzyl diethylenetriaminepentaacetic (Gd-EOB-DTPA)] that has ability to evaluate both enhancement characteristics and hepatocyte function could have a potential in improving diagnostic accuracy.

### Research frontiers

The change of organic anion transporting polypeptide 8 expression in hepatocytes during the multistep process of hepatocarcinogenesis could be imaged by a newly developed hepatocyte-specific contrast agent, gadoxetic acid, on dynamic magnetic resonance imaging (MRI). The use of hepatocyte-specific contrast-enhanced MRI provides superior detection and characterization of HCC.

### Innovations and breakthroughs

Adding hepatobiliary phase Gd-EOB-DTPA-enhanced MRI significantly improved the accuracy of diagnosing HCC. The significant improvement is apparent for small lesions (1-2 cm in diameter), for including or excluding the diagnosis of HCC, particular when the nodules exhibit atypical enhancement characteristics. The specificity can be improved with the addition of T2-weighted images after excluding cysts and hemangiomas during interpretation.

### Applications

A typical enhancement pattern on dynamic imaging for diagnosing HCC has limited sensitivity, particular for small lesions. Therefore, the addition of hepatobiliary phase imaging after the intravenous injection of Gd-EOB-DTPA will improve diagnostic accuracy in detecting HCC. However, a variety of benign hepatic lesions can appear hypointense on hepatobiliary-phase MRI. To get the best diagnostic accuracy, the hepatobiliary phase imaging should be interpreted in together with early dynamic phases and T2-weighted images.

### Peer review

Gadoxetic acid (Gd-EOB-DTPA)-enhanced MRI of the liver has certain advantages over other imaging modalities in the detection and characterization of HCC in the high-risk liver. Hepatobiliary phase images obtained after Gd-EOB-DTPA-enhanced MRI imaging may improve diagnosis of HCC and assist in surgical planning. This study aims to the added value of hepatobiliary phase Gadoxetic acid-enhanced MRI for diagnosis of HCC by the fact that there would be the changes of hepatocyte-specific contrast uptake on hepatobiliary phase during hepatocarcinogenesis and the interpretation's pitfalls of hepatobiliary phase MRI is also important to know for getting the most accurate result.

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## Application of quantitative estimates of fecal hemoglobin concentration for risk prediction of colorectal neoplasia

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### Abstract

**AIM:** To determine the role of the fecal immunochemical test (FIT), used to evaluate fecal hemoglobin concentration, in the prediction of histological grade and risk of colorectal tumors.

**METHODS:** We enrolled 17881 individuals who attended the two-step colorectal cancer screening program in a single hospital between January 2010 and October 2011. Colonoscopy was recommended to the participants with an FIT of  $\geq 12$  ngHb/mL buffer. We classified colorectal lesions as cancer (C), advanced adenoma (AA), adenoma (A), and others (O) by their

colonoscopic and histological findings. Multiple linear regression analysis adjusted for age and gender was used to determine the association between the FIT results and colorectal tumor grade. The risk of adenomatous neoplasia was estimated by calculating the positive predictive values for different FIT concentrations.

**RESULTS:** The positive rate of the FIT was 10.9% (1948/17881). The attendance rate for colonoscopy was 63.1% (1229/1948). The number of false positive results was 23. Of these 1229 cases, the numbers of O, A, AA, and C were 759, 221, 201, and 48, respectively. Regression analysis revealed a positive association between histological grade and FIT concentration ( $\beta = 0.088$ ,  $P < 0.01$ ). A significant log-linear relationship was found between the concentration and positive predictive value of the FIT for predicting colorectal tumors ( $R^2 > 0.95$ ,  $P < 0.001$ ).

**CONCLUSION:** Higher FIT concentrations are associated with more advanced histological grades. Risk prediction for colorectal neoplasia based on individual FIT concentrations is significant and may help to improve the performance of screening programs.

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**Key words:** Colorectal cancer; Fecal immunochemical test; Screening; Risk prediction; Performance

**Core tip:** The fecal immunochemical test (FIT) for hemoglobin is specific for detecting colorectal lesions. With adjustment for age and gender of 17 881 attendees at a colorectal cancer screening program in a single hospital, we demonstrated that higher FIT concentrations were associated with more advanced histological grades ( $\beta = 0.088$ ,  $P < 0.01$ ). A significant log-linear relationship was found between the FIT concentration and positive predictive value of the FIT for predicting



colorectal tumors ( $R^2 > 0.95$ ,  $P < 0.001$ ). Risk stratification for colorectal neoplasia based on individual FIT concentration may help to improve the performance of screening programs.

Liao CS, Lin YM, Chang HC, Chen YH, Chong LW, Chen CH, Lin YS, Yang KC, Shih CH. Application of quantitative estimates of fecal hemoglobin concentration for risk prediction of colorectal neoplasia. *World J Gastroenterol* 2013; 19(45): 8366-8372 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8366.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8366>

## INTRODUCTION

Colorectal cancer (CRC) accounts for the highest number of newly diagnosed cancer cases in Taiwan<sup>[1]</sup>. Most CRC develops *via* the well-known adenoma-carcinoma sequence that averages 10-15 years for progression and enables clinicians to detect neoplasms at the precancerous or early stages through adequate screening of the average-risk population<sup>[2]</sup>.

Several population-based studies suggest that screening for colorectal neoplasms using the fecal occult blood test can reduce mortality<sup>[3,4]</sup>. Occult blood may be measured using either the guaiac fecal occult blood test or the fecal immunochemical test (FIT). The FIT has several advantages. For example, the FIT can provide a measurable value of the fecal hemoglobin concentration and can predict colorectal bleeding more specifically<sup>[5-7]</sup>. Notably, the quantitative FIT value may be converted using a defined cut-off value into a “negative” or “positive” qualitative result, which facilitates subsequent management. Despite its widespread use, little is known about the clinical significance and application of the quantitative value of the FIT. Recently, Omata *et al.*<sup>[8]</sup> reported that the performance of the FIT for detecting CRC could be promising by choosing an optimal cut-off value of the quantitative FIT concentration<sup>[8]</sup>. Chen *et al.*<sup>[9]</sup> investigated the association between baseline fecal hemoglobin concentration and the risk of incident colorectal neoplasia using a cut-off fecal hemoglobin concentration of 100 ng/mL to classify attendees as negative or positive. In subjects with a negative FIT, the adjusted hazard ratios (HRs) increased from 1.43 for a baseline fecal hemoglobin concentration of 20-39 ng/mL, to 3.41 for a baseline concentration of 80-99 ng/mL (*t* test  $P < 0.0001$ ), relative to 1-19 ng/mL. They concluded that quantitative fecal hemoglobin concentration at baseline could predict subsequent risk of incident colorectal neoplasia<sup>[9]</sup>. These important findings suggest that quantitative FIT could be applied more widely in clinical practice.

The Taiwan Bureau of National Health Insurance began population-based CRC detection programs in 2006 for average-risk individuals aged between 50 and 69 years. A two-step approach was applied: a biennial 1-d FIT was

used as a screening tool; subjects with a “positive” FIT underwent follow-up endoscopy to detect asymptomatic CRC. The effectiveness of the program appeared encouraging as a significant number of CRCs were detected in the early stages. However, a substantial portion of the participants did not adhere to the screening protocol because they were asymptomatic and/or worried about the discomfort and risk associated with colonoscopy. In addition, some physicians and health providers were less inclined to recommend feces-based CRC screening to patients. These factors may limit the outcomes of the project<sup>[10-12]</sup>. To improve the performance of CRC screening, a more personalized approach on the basis of risk stratification may be helpful<sup>[13]</sup>. Here, we evaluated the quality of colonoscopy in the screening program and determined the association between the FIT and colorectal neoplasia with the aim of determining whether the risk of CRC could be deduced from the quantitative value of the FIT.

## MATERIALS AND METHODS

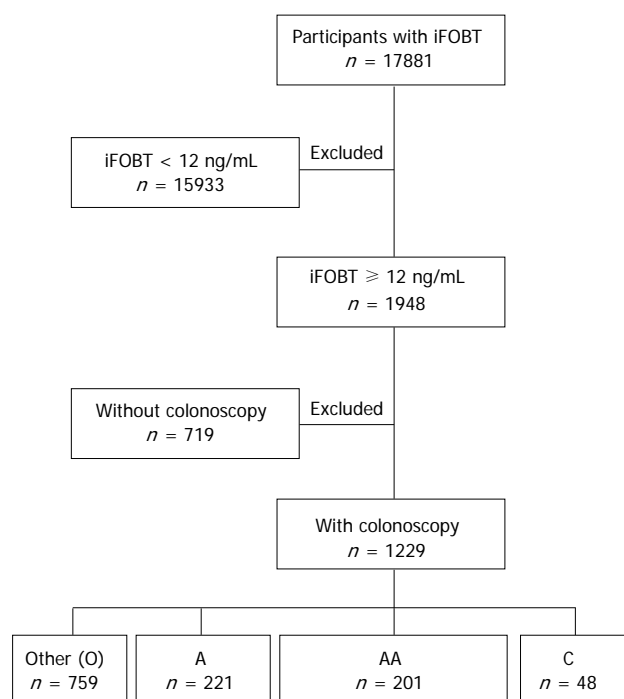
### Subjects and feces analysis

We enrolled individuals who attended the national colorectal cancer screening program in a single medical center from January 2010 through October 2011. The participants were interviewed by a cancer screening specialist nurse during a face-to-face meeting to explain the purpose of CRC screening, the procedure for feces collection, and to sign the consent form. They were asked to provide one sample of feces in a stool container (Kyowa, Tokyo, Japan) to enable an accurate and quantitative evaluation by decreasing the variations in feces sampling levels. Storage of the samples was suggested at 4 °C and they were to be returned to the hospital within 3 d. Feces specimens were analyzed on a fully automated analyzer. The reagents, calibrators and controls for the samples were prepared according to the manufacturer's guidance (Kyowa, Tokyo, Japan). The FIT measurements were converted to the proposed standardized reporting units:  $2.5 \text{ (ngHb/mL buffer)} = 1 \text{ (}\mu\text{gHb/g feces)}$ <sup>[14,15]</sup>.

Colonoscopy within 2 months was recommended to the subjects if their FIT was  $\geq 12 \text{ ngHb/mL buffer}$ . If a polyp with a diameter  $> 0.5 \text{ cm}$  was observed, polypectomy or biopsy was performed to obtain histological results. The Hospital Ethics Committee approved this study.

### Quality indicators

Several quality indicators were chosen according to the National Health Service bowel cancer screening program guideline to investigate the performance of the screening program<sup>[16]</sup>. The colonoscopic attendance rate was determined as: No. of colonoscopies/No. of positive FITs. Cecal intubation rate was defined as: No. of photographic documentations of the cecal landmark/No. of colonoscopies. The adenoma detection rate was calculated as: no. of cases with adenomatous tumors/No. of colonos-



**Figure 1** Flowchart of study participants recruited from the two-step colorectal cancer screening program. O: Other; A: Adenoma; AA: Advanced adenoma; C: Cancer.

copies. The cancer detection rate was calculated as: no. of cases with cancer/No. of colonoscopies. The incidence of complications was calculated as: No. of cases with major bleeding or perforation/No. of colonoscopies).

### Cancer stage

The cancer stage was recorded according to the American Joint Committee on Cancer 7<sup>th</sup> edition. To determine whether cancer detected by screening (CS) was at an earlier stage compared with the hospital cancer registry (CR), the stage migration was calculated as the difference in the proportion of tumors at each stage between CS and CR.

### Classifications used in the study

The colorectal findings were classified as cancer (C), advanced adenoma (AA), adenoma (A), and other (O) on the basis of their endoscopic and histological findings. Advanced adenoma was defined by the presence of any of the following characteristics: adenoma with a villous component, high-grade dysplasia, and polyp size  $\geq 1.0$  cm. If more than two lesions were found in one examination, we grouped the cases according to the most advanced findings.

The FIT data of all participants with colonoscopies were recorded. We transformed the continuous *F* value (ngHb/mL buffer) into ordinal scales: 12.1-25.0, 25.1-50.0, 50.1-100.0, 100.1-200.0, 200.1-400.0, 400.1-800.0, and  $> 800.0$ . To determine the associations between age, gender, FIT concentration and tumor grade, these non-normally distributed data were compared among the groups using the Kruskal-Wallis test. Subsequently, a multiple linear regression analysis adjusted for

**Table 1** Quality measures of the colorectal cancer screening program in this study

Quality indicator	Outcomes	<sup>1</sup> Standard	Quality
Colonoscopy attendance rate	63.10%	$\geq 85\%$	Inadequate
Cecum intubation rate	98.10%	$\geq 90\%$	Adequate
Polyp detection rate	57.20%	-	-
Adenoma detection rate	35.70%	$\geq 35\%$	Adequate
Cancer detection rate	2.7‰ screened by FIT 3.9‰ screening colonoscopies	$\geq 2\%$ screened by fecal occult blood test $\geq 11\%$ screening colonoscopies	Auditable outcome
Major bleeding	0.24%	$< 1\%$	Adequate
Perforation	0	$< 1\%$	Adequate

<sup>1</sup>NHS BCSP quality assurance guidelines for colonoscopy 2011. NHS: National Health Service; BCSP: bowel cancer screening program.

age and gender was used to determine whether the FIT concentration was independently associated with the histologic grade of colorectal tumors.

### Statistical analysis

To determine the risk of colorectal neoplasia with regard to the FIT concentration, we evaluated the positive predictive value (PPV) of the FIT concentration (ngHb/mL buffer) at different cut-off concentrations (12, 25, 50, 100, 200, 400, 800 and 1000) for detecting A + AA + C, AA + C, and C. After log(2) transformation of the FIT results, a simple linear regression analysis was performed to determine the relationship. All statistical analyses were conducted using the software SPSS 19.0 to compare the differences among groups.

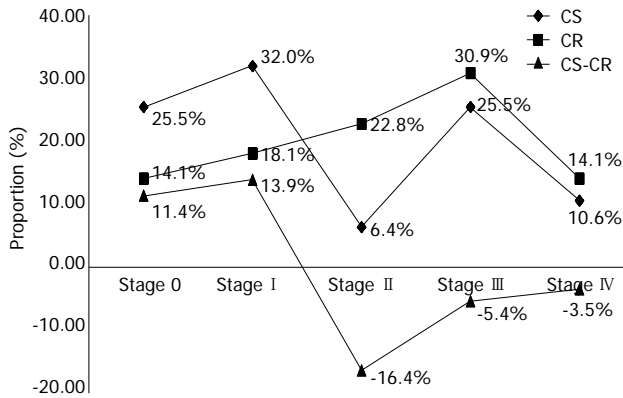
## RESULTS

### Baseline characteristics and performance of the CRC screening program

A flowchart of the study participants is shown in Figure 1. A total of 17881 participants underwent the FIT for colorectal cancer screening. The positive rate of the FIT was 10.9% (1948/17881). The number of false positive results was 23. The indicators for the quality of colonoscopy are listed in Table 1. The attendance rate for colonoscopy was 63.1% (1229/1948). The cecum-reaching rate was 98.1% (1206/1229). The polyp detection rate was 57.2% (703/1229). The adenoma detection rate was 35.7% (422/1181). The cancer detection rate was 3.9% (48/1229). The incidence of complications was 0.24% (3 cases of bleeding requiring hemostasis intervention and no perforations). The other endoscopic findings included normal, hemorrhoids, colitis, ulcers, diverticulum, and submucosal lesions.

### Cancer stage migration

The stage migration of colorectal cancer is shown in Figure 2. Of the 48 malignancies, 1 was a lymphoma and 47



**Figure 2** Cancer stage difference between the colorectal cancer screening program and cancer registry database. The cancer stage (X-axis) and proportion (Y-axis) of screening-detected cancers (CS) and the hospital cancer registry (CR) database. The stage migration was calculated as the difference in cancer proportions between the two databases (CS-CR).

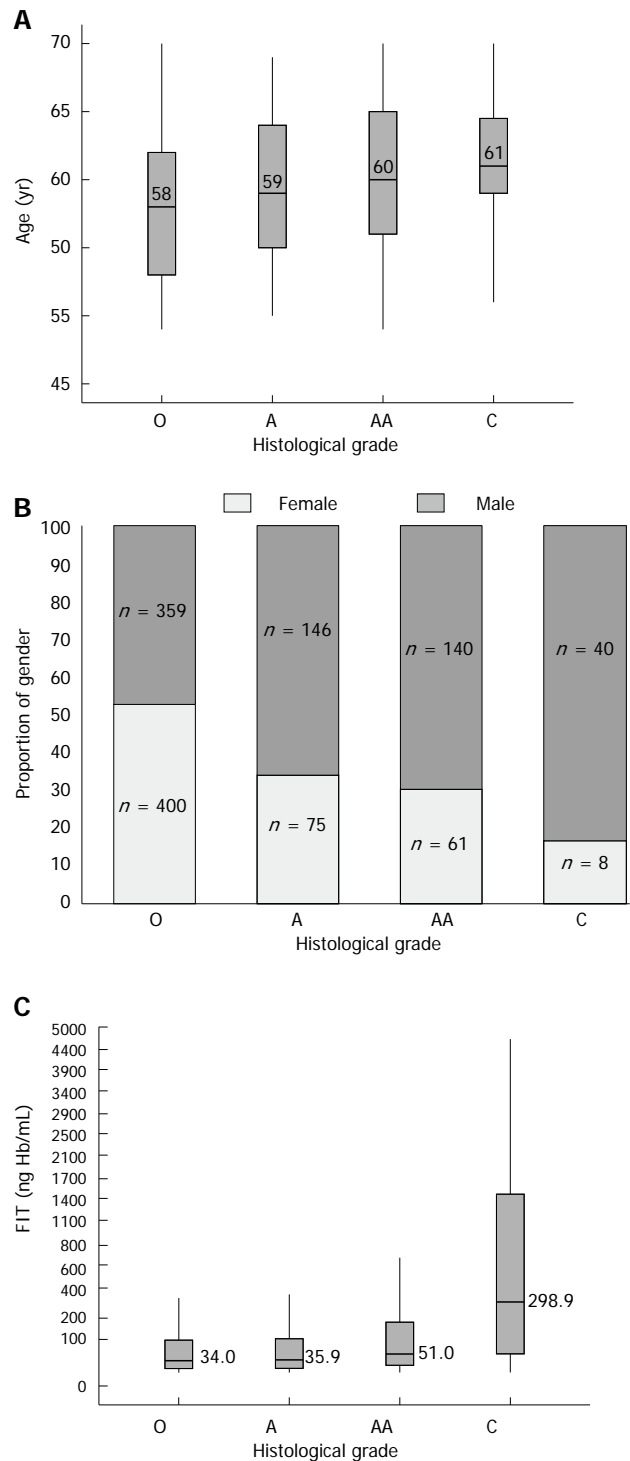
were adenocarcinomas. Of the 47 cases, the proportions of stages 0, I, II, III, and IV were 25.5%, 31.9%, 6.4%, 25.5%, and 10.6%, respectively. In the hospital cancer registration database, the proportions of stages 0, I, II, III, and IV were 14.1%, 18.1%, 22.8%, 30.9%, and 14.1%, respectively. Therefore, the corresponding differences for stages 0, I, II, III, and IV were 11.4%, 13.9%, -16.4%, -5.4%, and -3.5%, respectively, indicating that cancers tended to be detected at an earlier stage by screening.

#### Age, gender, and FIT in association with histologic grade of colorectal tumors

The patient numbers in the 4 groups O, A, AA, and C were 759, 221, 201, and 48, respectively. Figure 3 shows the differences in age, gender, and FIT concentrations in these groups. The median age of the 1229 patients was 59 years. The median ages of patients with O, A, AA, and C were 58, 59, 60 and 61 years, respectively. Male patients accounted for 55.7% of the 1229 patients. The percentages of men with O, A, AA, and C were 47.3%, 66.1%, 69.7% and 83.3%, respectively. The median FIT concentration (ngHb/mL buffer) in the 1229 cases was 37.5. The median FIT values in patients with O, A, AA, and C were 34.0, 35.9, 51.0 and 298.9, respectively. Comparing the distributions of age, gender, and FIT concentration among the groups, the histological grade of the colorectal tumors increased with increased age, FIT level and male gender (Kruskal-Wallis test, all  $P < 0.001$ ). Subsequent multiple linear regression analysis revealed a significant association between ordinal scales of the FIT and histological grade of colorectal tumors after adjusting for age and gender ( $\beta = 0.088$ ,  $P < 0.001$ ) (Table 2).

#### Risk prediction for colorectal tumors based on the FIT

The PPVs for detecting A + AA + C, AA + C, and C were elevated from 38.2%-58.7%, 20.3%-42.7% and 4.0%-21.3%, respectively, as the FIT concentration (ngHb/mL buffer) increased from 12 to 1000. After log(2) transformation of the FIT values, a linear relation-



**Figure 3** Age, gender, and fecal immunochemical test concentration in association with histological grade of colorectal tumor. A: Age; B: gender; C: FIT. The differences in age, gender, and FIT concentrations (Y-axis) in the different histological groups (X-axis). (Kruskal-Wallis test, all  $P < 0.001$ ). FIT: fecal immunochemical test; O: Other; A: Adenoma; AA: Advanced adenoma; C: Cancer.

ship between quantitative level and PPV of the FIT for predicting colorectal adenomatous polyps and malignancies was observed (all  $R^2 > 0.95$ ,  $P < 0.001$ ). The linear relationship reached a plateau when the FIT concentration was  $> 800$  (Figure 4).

**Table 2** Age, gender, and fecal immunochemical test concentration in association with histological grade of colorectal tumor: multiple linear regression analysis

<sup>1</sup> Multiple linear regression model		Beta (95%CI)	P value
Age (yr)	50-69	0.021 (0.012-0.030)	< 0.001
Gender	Female: 0, Male: 1	0.342 (0.245-0.44)	< 0.001
<sup>2</sup> FIT (ngHb/mL)	Scale 1: 12.1-25	0.088 (0.062-0.114)	< 0.001
	Scale 2: 25.1-50		
	Scale 3: 50.1-100		
	Scale 4: 100.1-200		
	Scale 5: 200.1-400		
	Scale 6: 400.1-800		
	Scale 7: > 800		

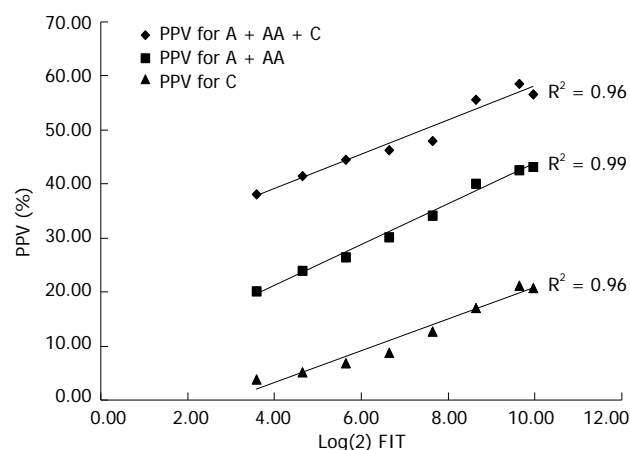
<sup>1</sup>The model revealed a significant association between histological grade of colorectal tumors and three independent variables: age, gender and FIT concentrations; <sup>2</sup>We converted the continuous FIT value into an ordinal variable after a log(2) transformation of the FIT concentrations. FIT: Fecal immunochemical test.

## DISCUSSION

According to these results, the performance of the two-step CRC screening strategy in our study appeared satisfactory as most of the quality indicators were adequate<sup>[16]</sup>, and a stage migration phenomenon of the detected cancer was observed in the screening population. However, the following issues remain to be clarified: (1) whether colonoscopy should be recommended in asymptomatic subjects only on the basis of a “positive” feces test; (2) whether subjects with “positive” feces tests, but different FIT concentrations have the same risk of significant tumors; and (3) how to improve the performance of the CRC screening program.

Compared with the hospital cancer registry, we observed a stage migration phenomenon in detected cancer in the screening program. The transition zone was between stages I and II reflecting early cancer detection (Figure 2). This result was similar to several cohort studies which showed that feces-based screening programs may offer better prognosis and a reduced CRC mortality rate due to early diagnosis<sup>[17,18]</sup>. Whether early detection translates to improved overall survival may be disputed. CRC occurs predominantly in elderly patients, who may have comorbid conditions. CRC screening mainly identifies slower-growing lesions, and as most symptomatic populations have more advanced stages, they will not attend the screening program. Therefore, the lead-time bias and length bias cannot be neglected in the migration phenomenon<sup>[19-21]</sup>. Even so, the CRC screening program was still effective in detecting precancerous lesions and preventing tumor progression to more advanced stages. Taking these advantages together, colonoscopy should be recommended in individuals in the screening population with a positive feces test.

Consistent with previous reports, our research showed that the FIT concentration increases as disease becomes more serious, from non-significant findings to non-advanced adenomas to advanced adenomas to cancer<sup>[6,22,23]</sup>. This trend is independent after adjustment for age and



**Figure 4** Fecal immunochemical test concentration in the prediction of colorectal neoplasia: linear regression analysis. The association between FIT concentration with log(2) transformation (X-axis) and PPV (Y-axis). (all  $R^2 > 0.95$ ,  $P < 0.001$ ). FIT: Fecal immunochemical test; O: Other; A: Adenoma; AA: Advanced adenoma; C: Cancer; PPV: Positive predictive value.

gender which are important influencing factors<sup>[24]</sup>. The biological explanation for this association is complex. In summary, a more advanced tumor may be larger and more friable, may have increased vascularity in the stroma, and may have significant inflammation in the surrounding tissues<sup>[25,26]</sup>. These changes in the characteristics and environment of the tumor may result in significant occult bleeding and an increased FIT concentration. These interesting findings suggest that interpretation of the FIT should be extended beyond “negative” or “positive” and should be tailored according to concentration.

Colonoscopy compliance in participants who were positive for the FIT was 63.1% in this study. This inadequate performance has been frequently reported in other studies. The reasons for non-adherence to the protocol may be due to lack of awareness in the patient or inadequate recommendation by the physicians<sup>[27]</sup>. The present study demonstrated a dose-dependent relationship between the FIT level and the risk of colorectal tumors. This relationship between the FIT level and colorectal neoplasia could be used to offer more personalized care in risk stratification and management<sup>[7,8,28]</sup>. As we increased the FIT cut-off values, the trend in predicting advanced colorectal neoplasia increased from 20.3% to 42.7% (Figure 4). These data allow both participants and physicians to be aware of the possibility of occult tumors at each FIT concentration. Accordingly, patients may be more willing to undergo colonoscopy if they are aware of the risk of advanced neoplasia according to the FIT concentration.

There are some limitations in this study. First, the study design was retrospective. Second, the lifestyles, BMI, and family history of colorectal cancer patients, which could be weighted risk factors for colorectal tumors, were incomplete and not included in the present study due to recall bias and missing records. Subsequent prospective studies will be needed to determine the validity of risk prediction for advanced colorectal neoplasia



based on the FIT.

In summary, the adenoma detection rate and cancer stage migration support the effectiveness of the FIT as the first step in the current screening strategy. In addition, higher FIT concentrations were associated with more advanced colorectal neoplasia in the average-risk population. Risk prediction for colorectal neoplasia based on the FIT was significant and may help to improve the performance of the colorectal cancer screening program.

## COMMENTS

### Background

The fecal immunochemical test (FIT) for hemoglobin is specific for detecting colorectal lesions. Screening for colorectal neoplasms using the fecal occult blood test can reduce mortality.

### Research frontiers

Despite its widespread use, little is known about the clinical significance and application of the quantitative value of the FIT.

### Innovations and breakthroughs

This study used a linear regression model to determine the associations between key variables (age, gender and FIT concentration) and the pathology of colorectal neoplasia. The result demonstrated a dose-dependent relationship between the FIT level and the risk of colorectal tumors.

### Applications

FIT concentrations are associated with histological grades and can improve risk prediction for colorectal neoplasia in screening programs.

### Peer review

The paper contains interesting results. Authors reported a linear relationship between quantitative level and positive predictive value of FIT for predicting colorectal adenomatous polyps and cancers. Furthermore, this study includes a very large cohort of individuals and it is therefore possible to assume that the results actually reflect the trend of the general population.

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## Antiviral drug resistance increases hepatocellular carcinoma: A prospective decompensated cirrhosis cohort study

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### Abstract

**AIM:** To study the clinical outcome of antiviral therapy in hepatitis B-related decompensated cirrhotic patients.

**METHODS:** Three hundred and twelve patients with decompensated hepatitis B cirrhosis were evaluated in a prospective cohort. With two years of follow-up, 198 patients in the group receiving antiviral therapy with nucleos(t)ide analogues and 39 patients in the control

group without antiviral treatment were analysed.

**RESULTS:** Among the antiviral treatment patients, 162 had a complete virological response (CVR), and 36 were drug-resistant (DR). The two-year cumulative incidence of hepatocellular carcinoma (HCC) in the DR patients (30.6%) was significantly higher than that in both the CVR patients (4.3%) and the control group (10.3%) ( $P < 0.001$ ). Among the DR patients in particular, the incidence of HCC was 55.6% (5/9) in those who failed rescue therapy, which was extremely high. The rtA181T mutation was closely associated with rescue therapy failure ( $P = 0.006$ ). The Child-Pugh scores of the CVR group were significantly decreased compared with the baseline ( $8.9 \pm 2.3$  vs  $6.0 \pm 1.3$ ,  $P = 0.043$ ).

**CONCLUSION:** This study showed that antiviral drug resistance increased the risk of HCC in decompensated hepatitis B-related cirrhotic patients, especially in those who failed rescue therapy.

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**Key words:** Hepatitis B; Decompensated cirrhosis; Nucleos(t)ide analogues; Hepatocellular carcinoma; Drug resistance

**Core tip:** This study was performed to analyse the clinical data of 312 patients with decompensated hepatitis B cirrhosis in a prospective cohort. These data showed that complete virological response could improve the clinical outcome in decompensated hepatitis B cirrhotic patients. However, clinicians should be aware of the high risk of hepatocellular carcinoma and liver failure in antiviral drug-resistant patients.

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DX, Ding HG. Antiviral drug resistance increases hepatocellular carcinoma: A prospective decompensated cirrhosis cohort study. *World J Gastroenterol* 2013; 19(45): 8373-8381 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8373.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8373>

## INTRODUCTION

Chronic hepatitis B virus (HBV) infection, the main aetiology of liver cirrhosis and hepatocellular carcinoma (HCC), remains a major public health problem worldwide, especially in China<sup>[1-3]</sup>. Among these patients, the annual incidence of HCC is 2%-5%<sup>[3-5]</sup>. The most effective method to prevent HCC is to control HBV infection through vaccination<sup>[5,6]</sup>. In patients already infected with HBV, antiviral therapy remains the best strategy to prevent liver cirrhosis and HCC<sup>[6-9]</sup>. Major progress in the treatment of chronic hepatitis B has recently been made during the last decade with the development of antiviral drugs, especially nucleos(t)ide analogues (NUCs)<sup>[10-12]</sup>. Some data supporting the benefit of antiviral therapy on the prevention of HCC in chronic hepatitis B patients have been reported in several randomised controlled trials<sup>[12-15]</sup>. Nonetheless, antiviral drug resistance is important in determining the success of long-term therapy for chronic hepatitis B patients<sup>[16,17]</sup>. Based on recent clinical data, the development of resistance to NUCs is associated with an exacerbation of liver disease, including the development of cirrhosis and HCC<sup>[16]</sup>. In addition, the risk of HCC remains high in HBV-related cirrhosis patients who are treatment-naïve for NUCs<sup>[17]</sup>. Decompensated cirrhosis is the end stage of the disease and is characterised by high mortality and an extremely high risk of HCC. In HBV-related decompensated cirrhotic patients (DCPs), antiviral therapy using NUCs is recommended according to the 2005 global guidelines<sup>[18-20]</sup>. However, clinical data regarding the incidence of HCC in HBV-related DCPs with NUC antiviral therapies are limited. Therefore, the aim of the current study was to evaluate the two year outcomes in HBV-related decompensated cirrhotic, treatment-naïve patients using NUCs in a real life practical prospective cohort.

## MATERIALS AND METHODS

### Patients

The patients were enrolled due to HBV-related decompensated cirrhosis in a clinical practice between January 2008 and July 2012 at the Beijing Youan Hospital Affiliated Capital Medical University. Four hundred and seventy-six patients with hepatitis B-related decompensated cirrhosis were screened. Three hundred and twelve patients fulfilled the inclusion criteria and were prospectively enrolled in this study. The study protocol was approved by the Ethical Committee at Beijing Youan Hospital of Capital Medical University. All the patients provided written informed consent before being included in the study.

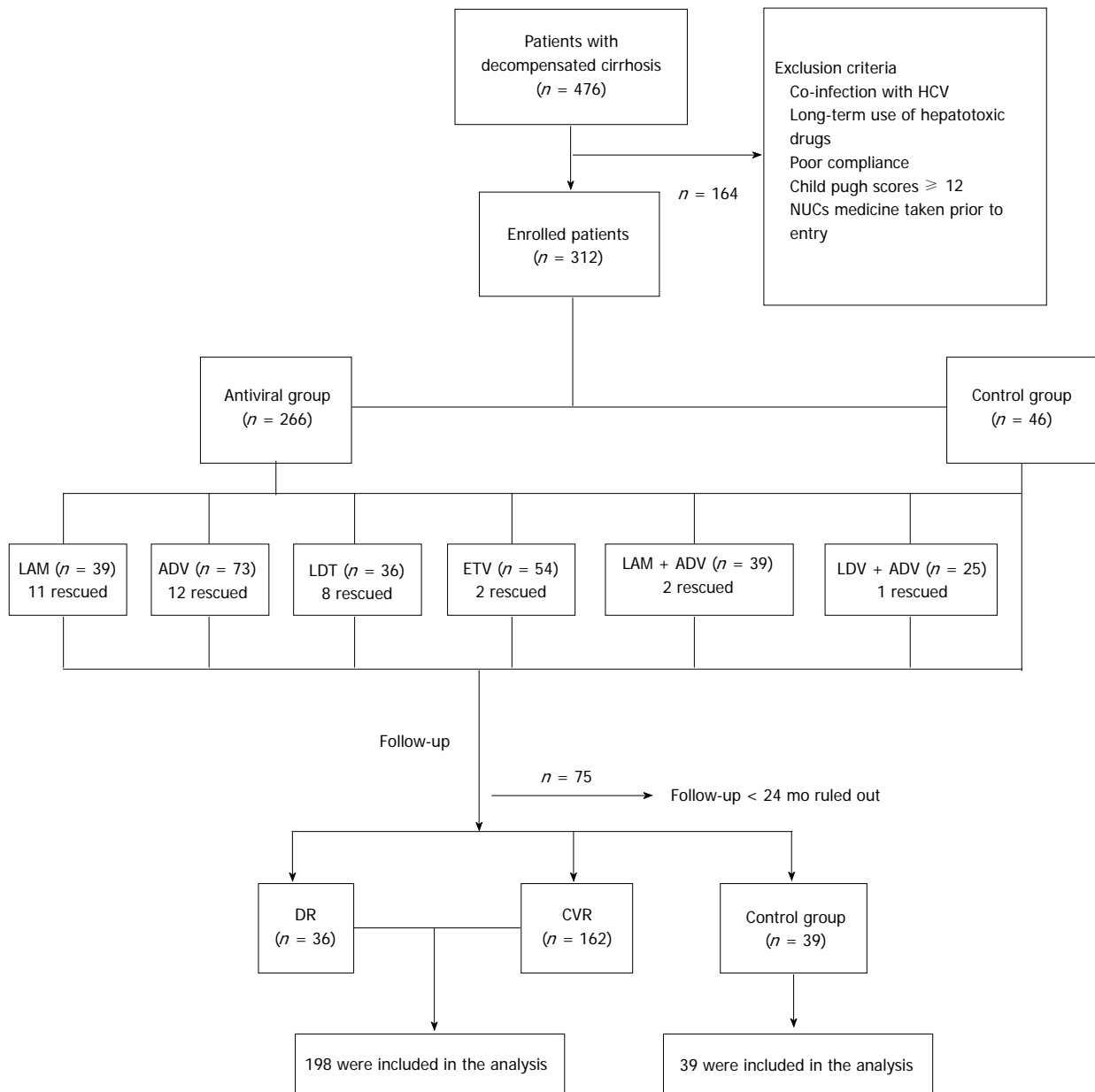
Clinical parameters (gender, age, family history of hepatitis B, alcohol abuse, Child-Pugh scores, HBeAg positive, HBV DNA load, renal function, and presence of ascites, encephalopathy, or variceal bleeding) were recorded. A diagnostic work-up for decompensated liver cirrhosis was performed, including clinical manifestations, physical examination, and laboratory tests, according to the criteria suggested by the Chinese Medical Association in 2005 for liver diseases<sup>[18]</sup>. The inclusion criteria were as follows: (1) chronic hepatitis B history and/or signs; (2) abnormal liver function accompanied by portal hypertension, such as ascites, encephalopathy, and esophageal or gastric variceal bleeding; (3) B-ultrasound scanning (LOGIQ9; GE Company, Fairfield, United States) and computerised tomography (CT; GE HISPEED DXI; GE Company) results consistent with the signs of liver cirrhosis without images of liver cancer; and (4) no NUC medications taken prior to entry. The exclusion criteria were as follows: (1) co-infection with HAV, HCV, HDV, HEV, and HIV as determined by an electrochemical luminescence method for the detection of the HAV and HCV antibodies and by an enzyme-linked immunosorbent assay for the HDV, HEV, and HIV antibodies; CMV and EBV IgM-positive patients as determined by an enzyme-linked immunosorbent assay for the detection of CMV and EBV IgM; (2) use of hepatotoxic drugs, including long-term analgesics and antipyretics, antibiotics, anti-lipidemic drugs, hypoglycaemic drugs, and herbal medicines; (3) HCC or metastatic liver cancer; (4) poor compliance and uncontrolled serious cardiovascular, respiratory, digestive, and nervous system diseases; (5) pregnant or lactating; and (6) Child-Pugh score  $\geq 12$ . Alcohol abuse was defined in this study as follows: (1) alcohol abuse  $> 5$  years, (2) drinking the ethanol equivalent of  $> 40$  g/d for men or 20 g/d for women, or (3) heavy drinking in the most recent 2 wk equivalent to ethanol  $> 80$  g/d.

In addition, 10 mL of venous blood was collected and centrifuged at 4 °C. All serum samples were stored at -80 °C in the Medical Bioinformation Research Center Biobank (Beijing, China).

### Antiviral therapy

In the HBV-related DCPs in this cohort, the antiviral therapy included lamivudine (LAM; 100 mg/d;  $n = 39$ ), adefovir (ADV; 10 mg/d;  $n = 73$ ), telbivudine (LDT; 600 mg/d;  $n = 36$ ), and entecavir (ETV; 0.5 mg/d;  $n = 54$ ) monotherapies or combinations of LAM (100 mg/d) and ADV (10 mg/d; LAM + ADV;  $n = 39$ ) or of LDT (600 mg/d) and ADV (10 mg/d; LDT + ADV;  $n = 25$ ) according to clinical real-life practices. The control group consisted of patients who declined antiviral therapy ( $n = 46$ ). All patients were followed every 3 mo, and their virologic, biochemical, and clinical parameters were obtained. Among these patients, 75 were excluded because they had follow-up  $< 24$  mo or were lost to follow-up; thus, 198 patients in the antiviral therapy group and 39 in the control group were included in the analysis. The antiviral therapy patients were subdivided into a drug-resistant





**Figure 1** Flow chart of enrolled patients. NUCs: Nucleos(t)ide analogues; HCC: Hepatocellular carcinoma; DR: Drug-resistant; CVR: Complete virologic response; LAM: Lamivudine; ADV: Adefovir; LDT: Telbivudine; ETV: Entecavir; HCV: Hepatitis C virus.

(DR) group ( $n = 36$ ) and a complete virologic response (CVR) group ( $n = 162$ ) based on whether they showed drug resistance before HCC was diagnosed (Figure 1). Rescue therapy for the drug-resistant patients was performed according to the guidelines for hepatitis B treatment<sup>[2]</sup> (Table 1).

#### Detection of HBV

Serum hepatitis B markers were detected by an electrochemiluminescence immunoassay using a Roche E170 modular immunoassay analyser (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. The serum HBV-DNA was quantified using a real-time polymerase chain reaction (PCR, FQ-PCR Kit; DaAn Gene Co., ShenZhen, China) and a GeneAmp 5700 Sequence Detection System (PE Applied Biosys-

tems, Boston, United States). The lower limit of HBV DNA detection was 500 copies/mL. CVR was defined as HBV-DNA being undetectable and persistently negative during the antiviral therapy for two years. Successful rescue therapy was defined as undetectable HBV-DNA 12 wk after changing antiviral drugs.

#### DR mutation detection by HBV polymerase

DR was defined as the conversion of HBV DNA to positive (virologic rebound) or the detection by sequence analysis of mutations known to be related to drug resistance during the NUC treatment. Serum HBV DNA was extracted according to the instructions of a commercial kit (Qiagen Blood Kit, Dusseldorf, Germany), using Platinum Taq DNA polymerase high fidelity (Invitrogen, CA, United States) for nested PCR amplification.

**Table 1** Rescue therapy strategies in drug resistance patients

DR patients	Rescue therapy prescriptions	HBN DNA after rescue therapy 12 w	
		< 500 copies/mL (n)	> 500 copies/mL (n)
LAM (n = 11)	+ ADV (n = 6) → ETV + ADV (n = 5)	4 3	2 2
ADV (n = 12)	+ ETV (n = 5) + LAM (n = 7)	4 6	1 1
LDT (n = 8)	+ ADV (n = 6) → ETV + ADV (n = 2)	4 2	2 0
ETV (n = 2)	+ ADV (n = 2)	2	0
LAM+ADV (n = 2)	→ ETV + ADV (n = 2)	1	1
LDT+ADV (n = 1)	→ ETV + ADV (n = 1)	1	0

+: Add-on; →: Altered drug. DR: Drug resistance; LAM: Lamivudine; ADV: Adefovir; LDT: Telbivudine; ETV: Entecavir; HBV: Hepatitis B virus.

The first-round primer sequences were as follows: P54, 5'-TYCCTGCTGGTGGCT CCAGTTC-3' (nt54-75) and P1287, 5'-CATACTGCGGAATCCTAGCG-3' (nt1267-1287). The second-round primer sequences were as follows: P253, 5'-CTCGTGGTGGACTTCTCTC-3' (nt253-271) and P1000, 5'-GCAAANCCCM AAA GRC-CCAC-3' (nt1000-1019). The primers were synthesised by the Yingjun Biotechnology Company (Shanghai, China). The PCR products were sequenced with an ABI 3730XL sequencer using the bi-directional method. The sequencing results were spliced and corrected by Contig Express and Bioedit software. The spliced and corrected results of the sequencing were submitted to the Stanford University webpage for antiviral resistance and genotypic resistance location analysis (<http://www.hiv-grade.de/hbvgrade/deployed/>).

### Assessment of liver and renal functions

The parameters of the liver and renal biochemical profiles, such as alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, blood urea nitrogen, and creatinine (Cr) levels, were tested with an Olympus automatic biochemical analyser (Olympus AU640, Tokyo, Japan). The prothrombin time (PT) was measured using a blood coagulation analyser (AcL Top; Beckman Coulter, CA, United States). The Child-Pugh scores were calculated according to the parameters.

### HCC screening

The incidence of HCC was monitored by serum alpha fetoprotein (AFP) and liver ultrasonography every 3 mo in the HBV-related DCPs. HCC was diagnosed according to the criteria suggested by the Chinese Anticancer Association in 2001<sup>[21]</sup>. In brief, the diagnostic criteria for HCC were as follows: (1) AFP > 400 ng/mL and posi-

**Table 2** Clinical characterizations of the enrolled patients at baseline *n* (%)

	DR (n = 36)	CVR (n = 162)	Control (n = 39)
Gender <i>n</i> (male/female)	23/13	109/53	24/15
Age, mean ± SD (yr)	56.9 ± 14.6	58.4 ± 11.2	52.2 ± 10.8
Family history of HCC	6 (16.7)	21 (12.9)	7 (17.9)
Alcohol abuse	4 (11.1)	22 (13.5)	10 (10.2)
HBeAg positive	20 (55.6)	95 (58.6)	22 (56.4)
Child-Pugh Score, mean ± SD	9.0 ± 2.8	8.6 ± 2.3	7.9 ± 2.1
qHBV DNA (log), mean ± SD	5.4 ± 2.3	4.9 ± 2.5	4.9 ± 1.7
PTINR, mean ± SD	1.98 ± 0.87	2.24 ± 1.29	2.03 ± 1.16
BUN, mean ± SD (mmol/L)	8.3 ± 3.7	9.1 ± 4.2	8.0 ± 2.5
Cr, mean ± SD (μmol/L)	87.6 ± 39.3	77.4 ± 45.8	74.8 ± 20.4
Ascites	21 (58.3)	90 (55.6)	20 (51.3)
Encephalopathy	0 (0)	2 (1.2)	1 (2.6)
Variceal bleeding	17 (47.2)	85 (52.4)	18 (46.1)

SD: Standard deviation; HCC: Hepatocellular carcinoma; DR: Drug-resistant; CVR: Complete virologic response; BUN: Blood urea nitrogen; Cr: Creatinine; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

tive B-ultrasound and CT findings or (2) serum AFP < 400 ng/mL, positive B-ultrasound and CT findings, and pathological findings for HCC in a liver biopsy specimen. The serum AFP levels were tested by electrochemiluminescence (Abbott Ltd, IL, United States).

### Study endpoints

The study endpoints were considered to be patient death, liver transplantation, or diagnosis with HCC.

### Statistical analysis

Parametric data were expressed as the mean with SD when a normal distribution was assumed. The statistical analysis was conducted using SPSS (version 16.0; SPSS, Inc., Chicago, IL, United States). Logistic regression analysis was performed to evaluate the association of variables with liver failure, death, and HCC. The Kaplan-Meier method was used to estimate the cumulative incidence of HCC, liver failure, and cumulative survival and compared with the log-rank test. Categorical variables were analysed using the Fisher test. Differences between paired and unpaired samples were determined with the non-parametric Wilcoxon paired sample test, the Mann-Whitney *U* test, or the Kruskal-Wallis test. All tests were two-sided, and a *P* value < 0.05 was considered statistically significant.

## RESULTS

### Clinical characteristics

No differences in the gender, age, family history of hepatitis B or HCC, alcohol abuse, Child-Pugh score, HBeAg positivity, HBV DNA load, renal function, or the presence of ascites, encephalopathy, or variceal bleeding existed among each group at baseline (*P* > 0.05; Table 2). The Child-Pugh score of the CVR group was significantly decreased at 2 years, compared with the baseline ( $8.9 \pm 2.3$  vs  $6.0 \pm 1.3$ , *P* = 0.043). In the control group and

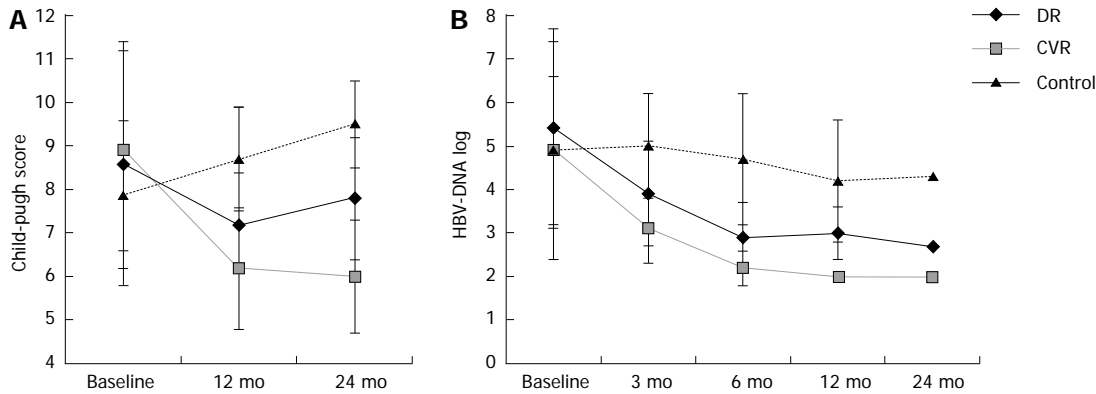


Figure 2 Child-Pugh score (A) and sequential change of serum hepatitis B virus DNA level (B) in the course of antiviral therapy over 2 years.

DR patients, however, the Child-Pugh score showed an increasing trend at 2 years without significance ( $P > 0.05$ ). Compared with the DR and control groups, the serum HBV DNA level during the course of antiviral therapy over 2 years in the CVR group declined sharply and was maintained at an undetectable level (Figure 2).

### Incidence of HCC

The two-year cumulative incidence of HCC in the DR patients (30.6%) was significantly higher than that in both the CVR patients (4.3%) and control group (10.3%) ( $P < 0.001$ ). Among these DR patients, the incidence of HCC was 55.6% (5/9) in those who failed rescue therapy, which was extremely high. The Kaplan-Meier curves representing the incidence of HCC in the DR, CVR, and control groups are shown in Figure 3A and B. The 2-year cumulative incidence of HCC in the DR group was higher than in the CVR (RR = 7.1; 95%CI: 2.2-16.1;  $P < 0.001$ ) and control groups (RR = 3.0; 95%CI: 1.3-9.5;  $P = 0.042$ ). However, there was no difference in the two-year cumulative HCC incidence between the patients who received antiviral therapy and the controls ( $P = 0.805$ ). The mutations associated with HBV polymerase-related antiviral drug resistance are shown in Table 3. The rtA181T mutation was closely associated with rescue therapy failure ( $P = 0.006$ ). The mutation combination rate in the subgroup of failed rescue therapy was markedly higher than that in the subgroup of successful rescue therapy (66.7% *vs* 11.1%,  $P = 0.004$ ).

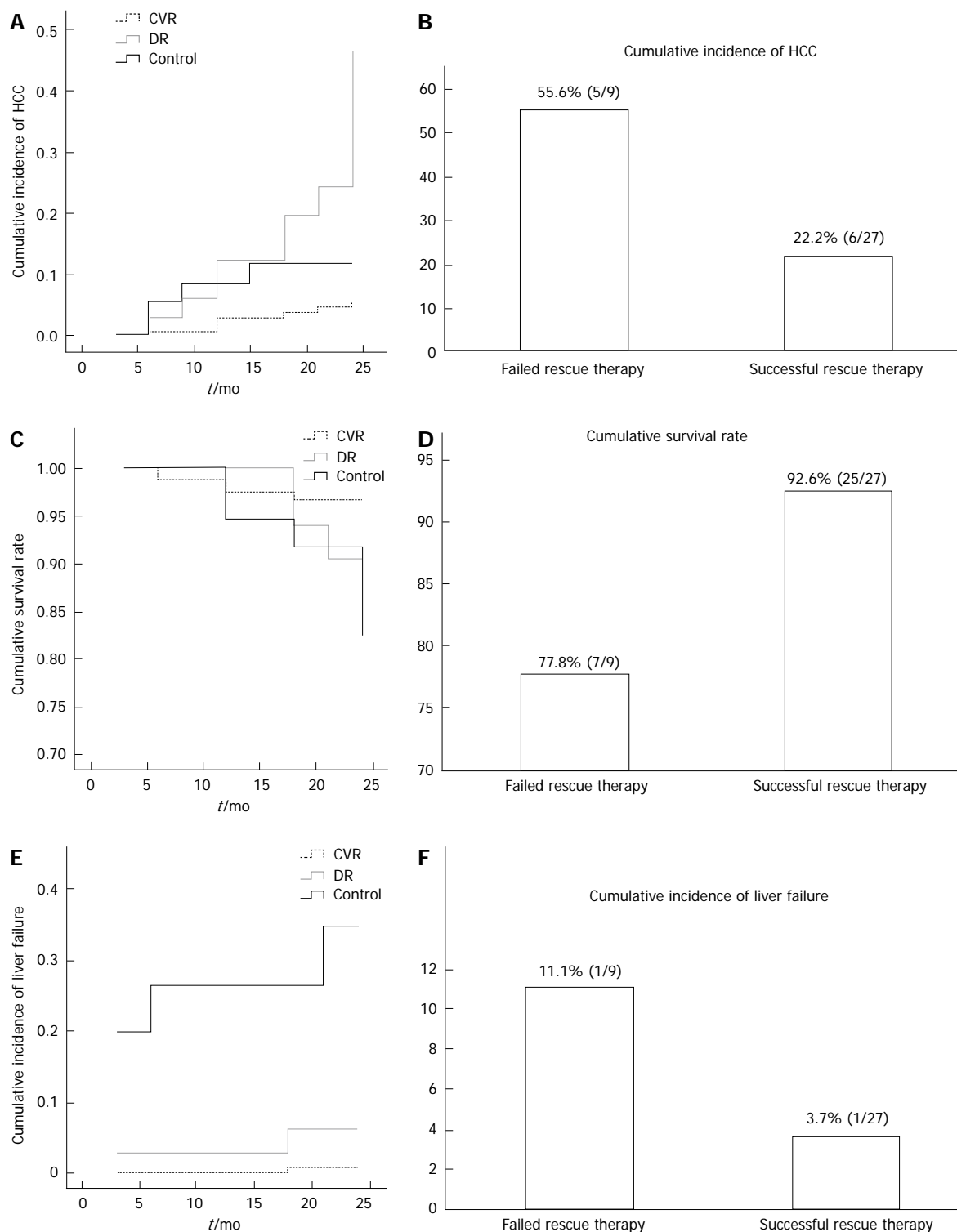
### Prognosis

The cumulative survival rates in the DR, CVR and control groups were 88.9% (32/36), 96.9% (157/162), and 84.6% (33/39), respectively. No significant difference was observed in the two year cumulative survival between the DR and CVR groups ( $P = 0.059$ ) (Figure 3C). There was no significant difference between the subgroups of failed rescue therapy and successful rescue therapy (77.8% *vs* 92.6%,  $P = 0.221$ ) (Figure 3D). The cumulative incidence of liver failure in the DR, CVR and control groups was 5.6% (2/36), 0.6% (1/162), and 28.2% (11/39), respectively. The two-year cumulative incidence of liver failure in the CVR group was significantly lower than those in

the DR (RR = 9.0, 95%CI: 2.8-34.5,  $P < 0.001$ ) and control (RR = 45.69, 95%CI: 13.8-96.4,  $P < 0.001$ ) groups (Figure 3E). However, there was no significant differences between the subgroups of failed rescue therapy and successful rescue therapy (11.1% *vs* 3.7%,  $P = 0.401$ ) (Figure 3F). These results suggested that the CVR patients had a good prognosis.

## DISCUSSION

HBV infection remains a global public health problem. The geographic distribution of the rates of chronic HBV infection and HCC are strikingly parallel. The incidence and mortality of HBV-related cirrhosis and HCC, however, have increased significantly<sup>[1-3]</sup>. The clinical evidence clearly shows that the differences in clinical outcomes after HBV infection might be related to the HBV DNA level, antiviral treatment response, and immune activation<sup>[22-24]</sup>. All guidelines for the prevention and treatment of chronic hepatitis B in China and in other countries state that the main aim of the treatment for chronic hepatitis B is to reduce the incidence and death rate of cirrhosis and liver cancer, to prolong life, and to improve the quality of life<sup>[15,25]</sup>. For patients with liver cirrhosis, especially those in the decompensation period, the clinical outcomes after antiviral therapy with nucleoside analogues are unclear<sup>[8,26,27]</sup>. NUCs are effective drugs for the suppression of HBV reproduction, and compliance is good amongst most chronic hepatitis B patients, especially in those with HBV-related cirrhosis. Failure of the antiviral treatment and drug resistance are serious factors for liver disease progression and for increases in the incidence of liver cancer<sup>[16,28]</sup>. Bae *et al*<sup>[24]</sup> reported that 58.2% of patients showed complete antiviral responses using LAM in decompensated HBV-related cirrhosis, which can improve the clinical prognosis. A single randomised double-blind controlled trial of LAM in patients with HBeAg and/or high serum HBV DNA levels showed that antiviral therapy prevented disease progression and reduced the incidence of HCC<sup>[29]</sup>. Several studies have shown that the failure of the antiviral treatment of chronic hepatitis B or drug resistance increases the risk of liver cirrhosis and liver cancer and also causes the progression of



**Figure 3** Kaplan-Meier curve. Kaplan-Meier curve for incidence of hepatocellular carcinoma (HCC) in drug resistance (DR), complete virologic response (CVR) and control group was shown (A). The two-year cumulative incidence of HCC was extremely higher in rescue therapy failure group (B). However, no significant difference was seen in two years cumulative survival between DR group and CVR group as well as between the subgroup of failed rescue therapy and successful rescue therapy (C and D). The cumulative incidence of liver failure was higher in the control group than in the DR, CVR (E). However, there was no significant difference between failed rescue therapy and successful rescue therapy subgroups (F).

liver disease<sup>[30-33]</sup>. Recently, a beneficial effect of antiviral therapy on the risk of HCC was also shown in cohort studies and meta-analyses, particularly among responders. A greater effect was observed in patients who achieved a sustained virologic response, whereas the benefit in non-

responders was unclear<sup>[12,17,22]</sup>. In this study, we found that antiviral therapy patients with a CVR response may show significantly improved liver function. The Child-Pugh score in the CVR group was significantly decreased over 2 years. In the drug-resistance group, however, the



**Table 3 Comparison of drug-resistant mutations between successful and failed rescue therapy *n* (%)**

Mutations	Successful rescue therapy ( <i>n</i> = 27)	Failed rescue therapy ( <i>n</i> = 9)	<i>P</i> value
rtM204V			0.443
+	1 (3.7)	1 (11.1)	
-	26 (96.3)	8 (88.9)	
rtM204I			0.333
+	4 (14.8)	3 (33.3)	
-	23 (85.2)	6 (66.7)	
rtA181T			0.006
+	2 (7.4)	5 (55.6)	
-	25 (92.6)	4 (44.4)	
rtL180M			0.255
+	2 (7.4)	2 (22.2)	
-	25 (92.6)	7 (77.8)	
rtL80I			0.057
+	0 (0)	2 (22.2)	
-	27 (100)	7 (77.8)	
rtN236T			0.057
+	0 (0)	2 (22.2)	
-	27 (100)	7 (77.8)	
rtT184I			0.250
+	0 (0)	1 (11.1)	
-	27 (100)	8 (88.9)	
rtS202G			1.000
+	1 (3.7)	0 (0)	
-	26 (96.3)	9 (100)	
rtA181T + rtM204I			0.443
+	1 (3.7)	1 (11.1)	
-	26 (96.3)	8 (88.9)	
rtA181T + rtL180M			1.000
+	1 (3.7)	0 (0)	
-	26 (96.3)	9 (100)	
rtM204V + rtL180M			1.000
+	1 (3.7)	0 (0)	
-	26 (96.3)	9 (100)	
rtA181T + rtN236T			0.057
+	0 (0)	2 (22.2)	
-	27 (100)	7 (77.8)	
rtA181T + rtL80I			0.250
+	0 (0)	1 (11.1)	
-	27 (100)	8 (88.9)	
rtA181T + rtM204I + rtT184I			0.250
+	0 (0)	1 (11.1)	
-	27 (100)	8 (88.9)	
rtM204I + rtL180M + rtL80I			0.250
+	0 (0)	1 (11.1)	
-	27 (100)	8 (88.9)	

+: DR mutations positive; -: DR mutations negative; DR: Drug resistance.

Child-Pugh score increased. Interestingly, the incidence of HCC was high at 30.6% in the DR patients, especially in the failed rescue therapy patients, whereas the incidence of HCC was 4.3% in the CVR group and 10.3% in the control group. Therefore, the rescue therapies were very important for the DR patients. Kim *et al*<sup>[34]</sup> and Ha *et al*<sup>[35]</sup> recently reported that the adefovir add-on treatment in patients with LAM-resistant CHB suppressed HBV replication more effectively than ETV or ADV monotherapy. Therefore, NUC combination therapy as a rescue therapy could be a better prescription for NUC-

resistant HBV patients in this cohort study. In this study, the rtA181T mutation was found to be closely associated with rescue therapy failure, which may be related to the high incidence of HCC in patients with antiviral drug-resistance. Yeh *et al*<sup>[16]</sup> reported that the emergence of the rtA181T/sW172 mutation in the LAM-resistant patients increased the risk of HCC development during the subsequent courses of antiviral therapy. In this cohort study, we also found that the 2-year cumulative incidence of HCC was 22.2% in the successful rescue therapy group and 55.6% in the failed rescue therapy group among the DR patients. Therefore, we conclude that effective antiviral therapies might have benefits and a good prognosis for decompensated HBV-related cirrhosis. However, we observed an increased incidence of HCC in the patients with antiviral drug-resistance. The aetiology for carcinogenesis is still being studied.

Therefore, the maintenance of virologically persistent remission is important for the reduction of HCC risk in DCPs. More effective and more affordable antiviral therapies are needed for patients with HBV-related decompensated cirrhosis to ensure that more patients could benefit from the treatment and more HCCs could be prevented, which might have a major impact on the global incidence of HCC.

In conclusion, a complete virological response should reduce the incidence of HCC and improve the clinical outcomes in decompensated hepatitis B cirrhotic patients. However, clinicians should be aware of the extremely high risk of HCC and liver failure in antiviral drug-resistant patients or in those not treated with antiviral therapy.

## COMMENTS

### Background

The decompensated cirrhosis is the end stage of the disease and is characterised by high mortality and an extremely high risk of hepatocellular carcinoma (HCC). In HBV-related decompensated cirrhotic patients (DCPs), antiviral therapy using nucleos(t)ide analogues (NUCs) is recommended according to the 2005 global guidelines. The antiviral therapy remains the best strategy to prevent liver cirrhosis and HCC in chronic hepatitis B patients. However, clinical data regarding the incidence of HCC in HBV-related DCPs with NUCs antiviral therapies are limited.

### Research frontiers

Some cohort studies and meta-analyses have shown a beneficial effect of antiviral therapy on the risk of HCC, particularly among responders. A greater effect was observed in patients who achieved a sustained virologic response, whereas the benefit in non-responders is unclear, especially in those DCPs.

### Innovations and breakthroughs

This study revealed that, in decompensated cirrhotic patients, a complete virologic response (CVR) should reduce the incidence of hepatocellular carcinoma and improve the clinical outcomes in decompensated hepatitis B cirrhotic patients. However, in those with antiviral drug resistance the risk of HCC increased, especially in those who failed rescue therapy.

### Applications

From this study, it is suggested that the maintenance of virologically persistent remission is important for the reduction of HCC risk in DCPs. The clinicians should be aware of the extremely high risk of HCC and liver failure in antiviral drug-resistant patients. Therefore, more effective and more affordable antiviral therapies are needed for patients with HBV-related decompensated cirrhosis to ensure that more patients could benefit from the treatment and more HCC could be prevented.

## Terminology

A CVR was defined as HBV-DNA being undetectable and persistently negative during the antiviral therapy for two years.

## Peer review

This is a large single centre prospective cohort study based on real life clinical practice aimed to evaluate the relationship between drug-resistance and the development of HCC in hepatitis B related decompensated cirrhotic patients. This study gave interesting and partially innovative results for the clinician.

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## Primary adenosquamous carcinoma of the esophagus

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### Abstract

**AIM:** To investigate the clinical characteristics, diagnosis, treatment, and prognosis of primary adenosquamous carcinoma (ASC) of the esophagus.

**METHODS:** A total of 4015 patients with esophageal carcinoma underwent surgical resection between January 1995 and June 2012 at the Cancer Hospital of Shantou University Medical College. In 37 cases, the histological diagnosis was primary ASC. Clinical data were retrospectively analyzed from these 37 patients, who underwent transthoracic esophagectomy with lymphadenectomy. The  $\chi^2$  or Fisher's exact test was used to compare the clinicopathological features between patients with ASC and those with squamous cell carcinoma (SCC). The Kaplan-Meier and Log-Rank methods were used to estimate and compare survival

rates. A Cox proportional hazard regression model was used to identify independent prognostic factors.

**RESULTS:** Primary esophageal ASC accounted for 0.92% of all primary esophageal carcinoma cases (37/4015). The clinical manifestations were identical to those of other types of esophageal cancer. All of the 24 patients who underwent preoperative endoscopic biopsy were misdiagnosed with SCC. The median survival time (MST) was 21.0 mo (95%CI: 12.6-29.4), and the 1-, 3-, and 5-year overall survival rates were 67.5%, 29.4%, and 22.9%, respectively. In multivariate analysis, only adjuvant radiotherapy (HR = 0.317, 95%CI: 0.114-0.885,  $P = 0.028$ ) was found to be an independent prognostic factor. The MST for ASC patients was significantly lower than that for SCC patients [21.0 mo (95%CI: 12.6-29.4) vs 46.0 mo (95%CI: 40.8-51.2),  $P = 0.001$ ]. In subgroup analyses, the MST for ASC patients was similar to that for poorly differentiated SCC patients.

**CONCLUSION:** Primary esophageal ASC is a rare disease that is prone to be misdiagnosed by endoscopic biopsy. The prognosis is poorer than esophageal SCC but similar to that for poorly differentiated SCC patients.

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**Key words:** Adenosquamous carcinoma; Diagnosis; Esophagus; Prognosis; Treatment

**Core tip:** Primary adenosquamous carcinoma (ASC) of the esophagus is an uncommon malignant esophageal neoplasm containing coexisting elements of infiltrating squamous cell carcinoma and adenocarcinoma. The biological behavior and response to therapies of this disease have not been well studied. In the current study, we reported the largest ever single-center patient cohort undergoing surgical resection for pri-



mary esophageal ASC, and we investigated the clinical characteristics, diagnosis, treatment, and prognosis in these patients. These data will give us a better understanding of this disease and help select the proper strategy for therapy.

Chen SB, Weng HR, Wang G, Yang JS, Yang WP, Liu DT, Chen YP, Zhang H. Primary adenosquamous carcinoma of the esophagus. *World J Gastroenterol* 2013; 19(45): 8382-8390 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8382.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8382>

## INTRODUCTION

Primary adenosquamous carcinoma (ASC) of the esophagus is an uncommon malignant esophageal neoplasm containing coexisting elements of infiltrating squamous cell carcinoma (SCC) and adenocarcinoma (AC)<sup>[1]</sup>. To our knowledge, no more than 40 cases of primary esophageal ASC have been published in the English literature over the past 20 years<sup>[1-11]</sup>. Most of the previous studies concerning esophageal ASC were case reports, while only one series of 18 cases was previously reported by Yachida *et al*<sup>[1]</sup>. The biological behavior and response to therapies of this disease have not been well studied. In the current study, we present data of 37 patients with primary esophageal ASC who underwent surgical resection with systematic lymphadenectomy from a single cancer center. We investigate the clinical characteristics, diagnosis, treatment, and prognosis in these patients, and we further analyze the factors that are associated with survival.

## MATERIALS AND METHODS

This study was undertaken at the Cancer Hospital of Shantou University Medical College and was approved by the Ethics Committee of that hospital. A total of 4015 patients with esophageal carcinoma underwent surgical resection between January 1995 and June 2012. In 37 cases (0.92%, 37/4015), the histological diagnosis was primary ASC. The clinical records of these 37 patients were analyzed retrospectively.

### Patient selection

The medical history was obtained from all patients, who then underwent a physical examination. A chest radiograph, barium meal, contrast-enhanced computed tomography scan of the chest and abdomen, complete blood count, blood biochemistry analyses, and liver and renal function evaluations were also performed. Twenty-four patients underwent esophagoscopy biopsy before surgery. The remaining 13 patients from the early period of the study did not undergo an endoscopic biopsy when typical features of esophageal cancer were confirmed by clinical manifestations, esophagographic examinations and computed tomography scan of the chest. All histological

specimens were re-examined by expert pathologists.

### Histopathology

All resection specimens, including the lymph nodes, were assessed by two expert pathologists (Wu MY and Tian DP). Specimen analysis was performed in a standardized fashion with prospective documentation of all of the assessed parameters. Tumors were classified as ASC when the features of SCC and AC could be identified by light microscopy, with each accounting for at least 20% of the area in the sections of the deepest portion of tumor penetration. Patients with mucoepidermoid carcinomas were not included in this series.

### Follow-up

Follow-up was performed every 3 mo for the first year, every 6 mo for the second year and every 6-12 mo thereafter. During each follow-up visit, the patients received a clinical evaluation, blood biochemistry examination, including that of tumor markers (SCC antigen, carcinoembryonic antigen), ultrasonography, and X-ray examination. Computed tomography was performed every year. Endoscopic examinations were performed when necessary. Follow-up was continued up to January 2013 or until death, if this occurred earlier. The mean follow-up was 33.5 mo (range: 1-158 mo). One patient was lost to follow-up (2.7%).

Local and regional recurrences were defined, respectively, as recurrence at the anastomosis or at any site within the operative field. Distant metastases were defined as tumor growth outside the operative field. A diagnosis of recurrent disease was made when pathologically or radiologically confirmed.

### Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL). The  $\chi^2$  or Fisher's exact test was used to compare the clinicopathological features between patients with ASC and SCC. Overall survival time was calculated from the date of operation to the date of death or the most recent follow-up. Univariate analysis of survival was performed using the Kaplan-Meier method to estimate survival probabilities in patient subgroups, with the entry factors of gender, age ( $\leq 60$  years *vs*  $> 60$  years), location, length of the primary lesion ( $\leq 4$  cm *vs*  $> 4$  cm), macroscopic tumor type, pT category, pN category, operation (radical/palliative), and radiotherapy (yes/no). The log-rank test was used to assess differences in survival between groups. Factors identified at a significance level of  $P < 0.2$  in the univariate analysis were selected for inclusion in a multivariate Cox proportional hazard regression model. All of the performed statistical tests were two-sided, and a  $P$  value less than 0.05 was considered to be statistically significant. The treatment results in this group of patients were compared with those in patients with different histological grades of SCC who were treated during the same period.

**Table 1** Clinicopathologic features of the 37 patients with esophageal adenosquamous carcinoma

Gender	Age (yr)	Location	Length (cm)	Macroscopic tumor type	pT	pN	Operation	Treatment	Result	Last follow up (mo)
M	40	Lt	7	Medullary	4a	1	Radical	S + R	D	4
M	51	Mt	7	Medullary	3	2	Radical	S	D	19
F	74	Mt	8	Polypoid	4a	1	Radical	S	A	158
M	47	Mt	5	Medullary	3	1	Radical	S + R	A	114
M	64	Mt	7	Medullary	4a	0	Radical	S	D	2
F	51	Mt	3	Medullary	3	0	Radical	S	D	74
M	61	Ut	4	Medullary	1b	0	Radical	S	D	45
M	42	Ut	4	Sclerotic	4b	0	Palliative	S	D	20
M	50	Mt	3	Medullary	3	0	Radical	S	D	42
M	56	Mt	6	Ulcerative	3	0	Radical	S	D	21
M	60	Mt	3	Sclerotic	3	0	Radical	S + R	D1	140
M	63	Lt	4	Medullary	3	1	Radical	S	D	14
M	65	Mt	4	Sclerotic	3	0	Radical	S	D	26
M	67	Mt	10	Intraluminal	2	0	Radical	S	D	1
M	72	Mt	4	Medullary	3	1	Radical	S	D	11
M	56	Mt	4	Medullary	3	1	Radical	S	D	15
M	78	Mt	4	Medullary	2	1	Radical	S	D	12
F	66	Lt	2	Ulcerative	2	1	Radical	S + R	D	28
M	50	Mt	7	Medullary	4a	2	Radical	S + R	D	11
M	40	Mt	5	Medullary	1b	0	Radical	S	D	30
M	53	Mt	6	Medullary	3	1	Radical	S	D	25
F	54	Mt	4	Medullary	3	0	Radical	S	D	5
M	62	Mt	4	Medullary	3	3	Palliative	S + C	D	5
M	63	Mt	4	Medullary	3	0	Radical	S	A	85
M	63	Ut	6	Medullary	3	0	Radical	S	D	19
M	41	Mt	6	Medullary	3	1	Radical	S + R	A	80
M	47	Mt	6	Medullary	3	0	Radical	S	D	9
M	57	Mt	3	Polypoid	2	0	Radical	S	A	60
M	53	Mt	6	Medullary	3	3	Radical	S	D	10
M	65	Mt	3	Medullary	1b	0	Radical	S	D	33
M	56	Mt	3	Medullary	3	1	Radical	S + R	D	32
F	45	Mt	3	Medullary	3	0	Radical	S + R	L	16
M	63	Mt	7	Medullary	3	1	Radical	S + R	A	34
M	63	Lt	2	Medullary	1b	3	Radical	S	D	6
M	65	Mt	5	Medullary	3	1	Radical	S + R	D	20
F	73	Mt	5	Medullary	3	1	Radical	S	A	11
M	73	Lt	10	Medullary	4a	2	Palliative	S	D	2

F: Female; M: Male; Ut: Upper third of thoracic esophagus; Mt: Middle third of thoracic esophagus; Lt: Lower third of thoracic esophagus; S: Surgery; R: Radiotherapy; C: Chemotherapy; A: Alive; D: Die of tumor recurrence; D1: Die of myocardial infarction.

## RESULTS

### Patient characteristics

The clinicopathological features of the 37 primary esophageal ASC patients are shown in Table 1. This study group contained 31 men and 6 women, ranging in age from 40 to 78 years (median: 60 years). The primary lesions were most often found in the middle third of the thoracic esophagus and had a median length of 4.0 cm (2.0-10.0 cm). The clinical manifestations were identical to those of other types of esophageal cancer, with dysphagia, retrosternal pain, and loss of body weight being the main presenting symptoms.

None of the 37 patients underwent chemotherapy or radiotherapy before surgery, and none had prior malignant disease or distant metastases on routine examination before surgery.

### Treatment

All 37 patients underwent transthoracic esophagectomy

with two-field lymphadenectomy (the mediastinal and perigastric lymph nodes), including 34 cases of radical resection and three cases of palliative resection. A total of 480 lymph nodes were removed, and 69 had metastases. Twenty of the 37 patients (54.1%) proved postoperatively to have histologically confirmed lymph node metastases. According to the seventh edition of the American Joint Committee on Cancer staging system for esophageal cancer, there were fourteen stage pN1 cases, three stage pN2 cases, and three stage pN3 cases. Postoperative complications included pneumonia, pneumothorax, and esophagogastric anastomotic leak in each of two cases. No patients died during treatment in hospital and 30 d after surgery.

Adjuvant therapies were routinely recommended to patients with a locally advanced tumor or mediastinal lymph node metastases. However, not all patients complied with this recommendation. Twenty-four patients who underwent a radical operation were treated by surgery alone, and ten patients who underwent a radi-

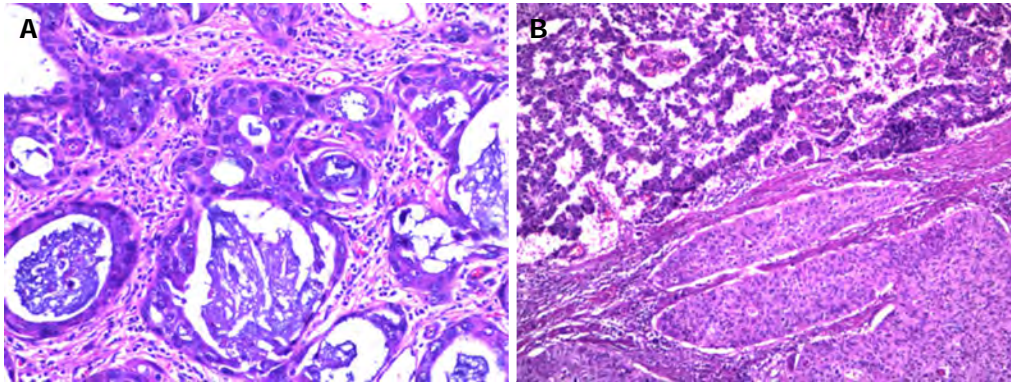


Figure 1 Adenosquamous carcinoma of the esophagus. Adenocarcinoma and squamous cell carcinoma could be intermingled (A: Hematoxylin and eosin staining,  $\times 200$  magnification) or have a fairly clear boundary (B: Hematoxylin and eosin staining,  $\times 100$  magnification).

cal operation were treated by surgery plus postoperative radiotherapy. None of the 34 patients who underwent a radical operation received adjuvant chemotherapy. One of the 3 patients who underwent a palliative operation received adjuvant chemotherapy (Cisplatin plus 5-Fu for two cycles) and died 5 mo after surgery. One palliatively operated patient died 2 mo after surgery and did not receive adjuvant therapy. The other palliatively operated patient did not receive adjuvant therapy for economic reasons and survived for 20 mo. Therapeutic radiation was delivered using 6 or 8 MV photons. A total dose of 50 Gy was delivered in 2 Gy fractions 5 d/wk.

### Pathology

Grossly, ASC was indistinguishable from pure SCC. The macroscopic tumor was classified as intraluminal type in one case, polyploid type in two cases, ulcerative type in two cases, sclerotic type in three cases, and medullary type in 29 cases. Microscopically, ASC was characterized by a mixture of AC and SCC features (Figure 1). SCC could have different stages of squamous differentiation with or without intercellular bridges, individual cell keratinization, and cancer pearls. AC contained tubular or glandular structures with focal to abundant intracellular or extracellular mucin. The AC and SCC could be intermingled or have a fairly clear boundary. No intermediate cells such as the type observed in mucoepidermoid carcinoma were evident in ASC. In most tumors, SCC dominated in the mucosa, while AC was identified in the deeper portion of the tumor.

Twenty-four patients underwent esophagoscopy biopsy before surgery, and all of them were misdiagnosed with SCC.

### Survival and prognostic factors

By January 2013, with a mean follow-up of 33.5 mo (range: 1–158 mo), 29 patients had died, seven were still alive, and one was lost to follow-up.

The median survival time (MST) of the 37 patients was 21.0 mo (95%CI: 12.6–29.4), and the 1-, 3-, and 5-year overall survival rates were 67.5%, 29.4%, and 22.9%, re-

spectively. One patient died of myocardial infarction 140 mo after the surgery.

Univariate analysis and multivariate analysis were performed to assess the relationship between the clinicopathological features and the prognosis of ASC patients. The variables related to survival in univariate analysis are shown in Table 2. Only location, macroscopic tumor type, pN category, and operation (radical/palliative) affected the overall survival ( $P < 0.05$ ). Although the MST at 32.0 mo for the patients who received adjuvant radiotherapy was longer than that at 19.0 mo for the patients who did not, the difference was not significant ( $P = 0.066$ ). In multivariate analysis, adjuvant radiotherapy ( $P = 0.028$ ) was found to be an independent factor for the prediction of prognosis (Table 3). Patients who received adjuvant radiotherapy had a relatively better survival.

Finally, we assessed the prognosis between esophageal ASC and different histological grades of SCC. Three thousand seven hundred eighty five of 4015 esophageal carcinoma patients were histologically diagnosed with SCC. Of these patients, 3439 did not receive neoadjuvant therapy, including 1011 cases of well-differentiated SCC, 2007 cases of moderately differentiated SCC, and 421 cases of poorly differentiated SCC. All patients underwent transthoracic esophagectomy with two-field lymphadenectomy (the mediastinal and perigastric lymph nodes). One hundred twenty patients were lost to follow-up (3.5%). The clinicopathological features of patients with ASC or different histological grades of SCC are shown in Table 4. All of the factors were balanced between patients with ASC and SCC. The MST at 21.0 mo for ASC patients was significantly lower than that at 46.0 mo for SCC patients ( $P = 0.001$ ) (Figure 2). We further sub-classified SCC into different histological grades and compared the prognosis with esophageal ASC. The MSTs for well-differentiated SCC, moderately differentiated SCC, and poorly differentiated SCC were 148.0, 39.0, and 22.0 mo, respectively (Table 5). The MST for ASC patients was significantly lower than that for well- ( $P < 0.001$ ) and moderately differentiated SCC patients ( $P = 0.004$ ), but there was no significant difference in the MST

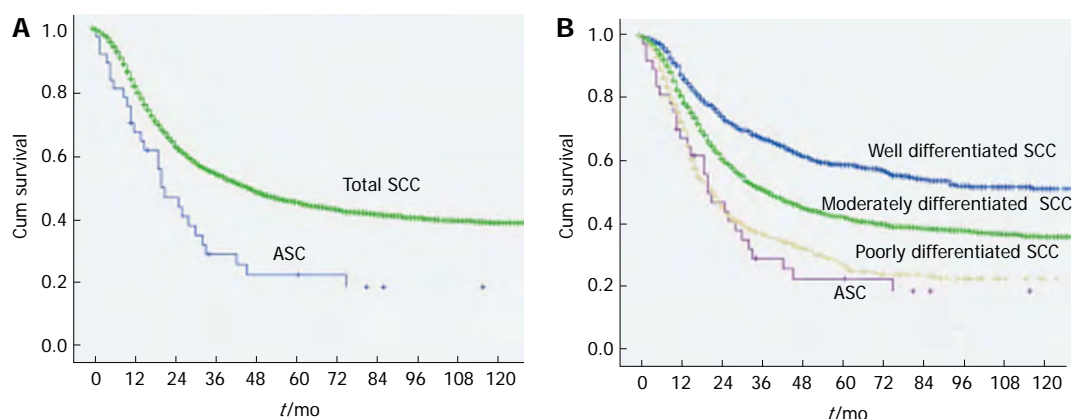


Figure 2 Comparison of Kaplan-Meier curves for patients with esophageal adenosquamous carcinoma and those with squamous cell carcinoma. A: Kaplan-Meier curves for esophageal adenosquamous carcinoma (ASC) patients and total esophageal squamous cell carcinoma (SCC) patients. The survival difference was significant ( $P = 0.001$ ); B: Kaplan-Meier curves for patients with esophageal ASC and those with different histological grades of SCC (between ASC and well-differentiated SCC,  $P < 0.001$ ; between ASC and moderately differentiated SCC,  $P = 0.004$ ; between ASC and poorly differentiated SCC,  $P = 0.536$ ).

**Table 2** Univariate analysis of the prognosis of the 37 patients with esophageal adenosquamous carcinoma

	Number of patients	Survival rate			MST (mo, 95%CI)	P value
		1-yr	3-yr	5-yr		
Gender						0.302
Male	31	64.5%	25.8%	18.4%	20.0 (13.5-26.5)	
Female	6	83.3%	55.6%	55.6%	74.0 (0.0-149.4)	
Age (yr)						0.448
≤ 60	19	73.7%	34.2%	28.5%	25.0 (11.6-38.4)	
> 60	18	60.6%	24.2%	16.2%	19.0 (8.6-29.4)	
Location						0.031
Ut	3	100.0%	33.3%	0.0%	20.0 (18.4-21.6)	
Mt	29	68.8%	34.5%	30.2%	25.0 (12.8-37.2)	
Lt	5	40.0%	0.0%	0.0%	6.0 (1.7-10.3)	
Length						0.630
≤ 4 cm	19	73.7%	34.4%	23.0%	28.0 (12.0-44.0)	
> 4 cm	18	61.1%	24.4%	24.4%	19.0 (7.4-30.6)	
Macroscopic tumor type						< 0.001
Medullary	29	61.9%	27.3%	18.2%	19.0 (11.7-26.3)	
Intraluminal	1	0.0%	0.0%	0.0%	1.0	
Polyploid	2	100.0%	100.0%	100.0%	-	
Ulcerative	2	100.0%	0.0%	0.0%	21.0	
Sclerotic	3	100.0%	33.3%	33.3%	26.0 (16.4-35.6)	
pT category						0.573
pT1	4	75.0%	25.0%	0.0%	30.0 (3.5-56.5)	
pT2	4	50.0%	25.0%	25.0%	12.0 (0.0-38.5)	
pT3	23	78.3%	34.5%	28.8%	21.0 (12.4-29.6)	
pT4	6	33.3%	16.7%	16.7%	4.0 (0.0-14.8)	
pN category						0.002
pN0	17	76.5%	38.2%	25.5%	30.0 (12.8-47.2)	
pN1	14	77.9%	31.2%	31.2%	25.0 (9.9-40.1)	
pN2	3	33.3%	0.0%	0.0%	11.0 (0.0-25.4)	
pN3	3	0.0%	0.0%	0.0%	6.0 (4.4-7.6)	
Adjuvant radiotherapy						0.066
No	27	62.7%	23.5%	15.7%	19.0 (11.7-26.3)	
Yes	10	80.0%	45.7%	45.7%	32.0	
Operation						0.025
Radical	34	70.5%	32.2%	25.0%	25.0 (15.4-34.6)	
Palliative	3	33.3%	0.0%	0.0%	5.0 (0.2-9.8)	

Ut: Upper third of thoracic esophagus; Mt: Middle third of thoracic esophagus; Lt: Lower third of thoracic esophagus; MST: Median survival time.

between ASC patients and poorly differentiated SCC patients ( $P = 0.536$ ) (Figure 2).

Twenty patients had complete recurrence and metastasis data. The first failure sites in these 20 cases included

local/regional recurrences in six cases, local/regional recurrences with distant metastases in four cases, and distant metastases in 10 cases. The mean time from treatment to failure was 14.0 (2.0-65.0) mo. Metastases were



**Table 3** Multivariate Cox regression analysis for the prognosis of the 37 patients with esophageal adenosquamous carcinoma

Prognostic factor	Hazard ratio	95%CI	P value
Location	2.177	0.797-5.951	0.129
Macroscopic tumor type	1.002	0.633-1.585	0.995
pN category	1.656	0.956-2.871	0.072
Adjuvant radiotherapy	0.317	0.114-0.885	0.028
Operation	3.718	0.782-17.679	0.099

**Table 4** Comparison of clinicopathological variables between patients with esophageal adenosquamous carcinoma and those with squamous cell carcinoma

Variable	ASC	SCC				P value
		Well differentiated (n = 1011)	Moderately differentiated (n = 2007)	Poorly differentiated (n = 421)	Total (n = 3439)	
Gender						0.191
Male	31	748	1473	313	2534	
Female	6	263	534	108	905	
Age (yr)						0.056
≤ 60	19	672	1347	270	2289	
> 60	18	339	660	151	1150	
Location						0.647
Ut	3	124	284	54	462	
Mt	29	746	1426	303	2475	
Lt	5	141	297	64	502	
Tumor length						0.182
≤ 4 cm	19	397	799	191	1387	
> 4 cm	18	614	1208	230	2052	
pT category						0.409
pTis	0	10	17	5	32	
pT1a	0	18	35	10	63	
pT1b	4	39	88	22	149	
pT2	4	202	350	80	632	
pT3	23	562	1135	232	1929	
pT4a	5	139	278	68	485	
pT4b	1	41	84	24	149	
pN category						0.187
pN0	17	587	1049	212	1848	
pN1	14	235	530	110	875	
pN2	3	151	328	69	548	
pN3	3	38	100	30	168	
Adjuvant radiotherapy						1.000
No	27	729	1465	292	2486	
Yes	10	282	542	129	953	
Operation						0.755
Radical	34	945	1851	385	3181	
Palliative	3	66	156	36	258	

ASC: Adenosquamous carcinoma; SCC: Squamous cell carcinoma; Ut: Upper third of thoracic esophagus; Mt: Middle third of thoracic esophagus; Lt: Lower third of thoracic esophagus.

**Table 5** Comparison of prognosis between patients with adenosquamous carcinoma and those with squamous cell carcinoma

	Cases	1-yr OS	3-yr OS	5-yr OS	MST (mo, 95%CI)
Well differentiated SCC	1011	87.5%	67.1%	58.9%	148.0
Moderately differentiated SCC	2007	81.0%	51.5%	42.5%	39.0 (34.7-43.3)
Poorly differentiated SCC	421	72.6%	37.3%	27.4%	22.0 (18.3-25.7)
Total SCC	3439	81.8%	54.3%	45.6%	46.0 (40.8-51.2)
ASC	37	67.5%	29.4%	22.9%	21.0 (12.6-29.4)

ASC: Adenosquamous carcinoma; SCC: Squamous cell carcinoma; OS: Overall survival rates; MST: Median survival time.

detected in the lung in two cases, the brain in two cases, the liver in two cases, the bone in three cases, and distant lymph nodes in five cases.

## DISCUSSION

ASC is a malignant tumor that has granular and squa-

mous histological components. According to the guidelines for clinical and pathological studies of carcinomas of the esophagus established by the Japan Esophageal Society, ASC of the esophagus is defined as having at least 20% of the SCC and AC features on routine microscopic examination using hematoxylin and eosin staining<sup>[12]</sup>. Primary esophageal ASC was relatively uncommon, and in our study, it accounted for only 0.92% of all cases of primary esophageal carcinoma. Due to the low incidence of esophageal ASC, the biological behavior and response to therapies of this disease have not been well studied.

We report the largest ever single-center patient cohort undergoing surgical resection for primary esophageal ASC, having investigated the clinical characteristics, diagnosis, treatment, and prognosis in these patients. Most of the patients were male with a median age of 60 years. The primary lesions were most often found in the middle third of the thoracic esophagus, and progressive dysphagia, retrosternal pain, and loss of body weight were the main presenting symptoms, which were identical to those of other types of esophageal carcinoma.

The origin of primary esophageal ASC remains obscure. It has been suggested that both components of an ASC originate from the same clone<sup>[11]</sup>. Some authors believe that this type of tumor arises from esophageal gland cells or ductal cells<sup>[10]</sup>. However, it has also been speculated that ASC first develops as SCC and that glandular differentiation subsequently occurs in the tumor cell populations<sup>[1,8]</sup>. In our study group, most of the tumors exhibited carcinoma *in situ* differentiating in the mucosa adjacent to the tumors, while no cellular atypia or transition to tumor cells was found in the underlying esophageal glands or their ducts. These features indicated that esophageal ASC could originate from the covering squamous epithelium.

Because the clinical features of primary esophageal ASC are basically identical to those of other types of esophageal cancer, the diagnosis of this disease is dependent on histopathological examination. Esophagoscopy biopsy is the most frequently used method for diagnosis before treatment. However, in our study, all 24 patients who underwent esophagoscopy biopsy before surgery were misdiagnosed with SCC. In the postoperative pathological examinations, we found that SCC dominated in the mucosa in most tumors, while AC was identified in the deeper portion of the tumor. This difference could have contributed to the high misdiagnosis rate of esophageal ASC due to the small volume of the biopsy specimen. Deeper biopsies that include a greater tissue volume and are performed under an endoscope could help improve the diagnostic accuracy.

No standard treatment for esophageal ASC has been established because its incidence is low. Few studies have addressed the treatment of esophageal ASC. At present, the treatment for this condition is similar to that for other types of esophageal carcinoma because of the difficulty in establishing the definitive diagnosis preop-

eratively. Surgical resection is still an important method for resectable esophageal carcinoma with neoadjuvant chemoradiotherapy for a locally advanced disease<sup>[13-16]</sup>. In the current study, none of the 37 patients received neoadjuvant therapy, while 10 patients were treated by surgery plus postoperative radiotherapy. We found that adjuvant radiotherapy was an independent prognostic factor in multivariate analysis ( $P = 0.028$ ). Patients who received adjuvant radiotherapy had a relatively better survival. The role of postoperative radiotherapy is still debated in esophageal cancer, and most previous studies have revealed no survival benefit with postoperative radiotherapy for esophageal SCC and AC<sup>[17,18]</sup>. In our study, although postoperative radiotherapy was found to be predictive of survival in the multivariate analysis, because the number of patients in this study was small, we think that more data should be collected to confirm this finding. Recently, neoadjuvant chemoradiotherapy had been increasingly used for the treatment of esophageal cancer patients, and it has shown a significant survival benefit<sup>[13-16]</sup>. It is suggested that further research should be conducted to establish the value of this new therapeutic approach for patients with esophageal ASC.

ASC has been confirmed to be more aggressive than “pure” AC and SCC in many other tumors, with frequent lymph node metastasis and poor prognosis<sup>[19-27]</sup>. Some case reports of esophageal ASC have also indicated that it has a highly aggressive biological behavior<sup>[5,7]</sup>. Another study of 18 cases reported by Yachida *et al.*<sup>[1]</sup> found that esophageal ASC had a better prognosis than conventional SCC and AC, but their finding is likely due to the smaller size and lower stage of the tumors in that study. In our study, although the 54.1% rate of lymph node metastases was higher than that of esophageal SCC (46.3%, 1591/3439), no significant difference was observed ( $P > 0.05$ ). Regarding the prognosis, the MST at 21.0 mo for ASC patients was significantly lower than that at 46.0 mo for the SCC patients in our study ( $P = 0.001$ ). In the subgroup analyses, the MST for ASC patients was significantly lower than that for well- and moderately differentiated SCC patients ( $P < 0.001$ ) but was similar to that for poorly differentiated SCC patients ( $P = 0.536$ ). The poor prognosis of esophageal ASC could be caused by its aggressive biological behavior.

In summary, primary esophageal ASC is a rare disease that is prone to be misdiagnosed by endoscopic biopsy. The prognosis for patients undergoing surgical resection is poorer than that for most esophageal SCC patients but similar to that for poorly differentiated SCC patients. Further investigations are required to determine the biological behavior of this tumor and develop new therapeutic modalities to achieve further improvements in the clinical outcome.

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## COMMENTS

## Background

Primary adenosquamous carcinoma (ASC) of the esophagus is an uncommon malignant esophageal neoplasm containing coexisting elements of infiltrating squamous cell carcinoma (SCC) and adenocarcinoma (AC). The biological behavior and response to therapies of this disease have not been well-studied due to its low incidence.

## Research frontiers

No more than 40 cases of primary esophageal ASC have been published in the English literature over the past 20 years. Most of the previous studies concerning esophageal ASC were case reports. The authors reported the largest ever single-center patient cohort of 37 cases undergoing surgical resection for primary esophageal ASC, and investigated the clinical characteristics, diagnosis, treatment, and prognosis.

## Innovations and breakthroughs

This study was the largest that was conducted by a single institution to report the clinical characteristics, diagnosis, treatment, and prognosis of esophageal ASC. For the first time, the authors determined that esophageal ASC was prone to be misdiagnosed by endoscopic biopsy and that the prognosis for patients undergoing surgical resection is poorer than that for most esophageal SCC patients but similar to that for poorly differentiated SCC patients.

## Applications

This is the largest study of primary esophageal ASC, and it will give the authors a better understanding of this disease and help the selection of the proper therapy strategy.

## Terminology

Esophageal ASC is an uncommon malignant esophageal neoplasm containing coexisting elements of infiltrating SCC and AC. According to the guidelines for clinical and pathological studies of carcinomas of the esophagus that were established by the Japan Esophageal Society, ASC of the esophagus is defined as having at least 20% each of the SCC and AC elements on routine microscopic examination using hematoxylin and eosin staining.

## Peer review

This is a well written and thorough coverage of this important topic. The authors investigated the clinical characteristics, diagnosis, treatment, and prognosis of 37 cases of ASC of the esophagus from 4015 esophageal carcinoma patients who received surgical resection between January 1995 and June 2012. They concluded that the prognosis of esophageal ASC is poorer than SCC. The paper is of interest for gastroenterologists.

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## Investigation of relationships among gastroesophageal reflux disease subtypes using narrow band imaging magnifying endoscopy

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### Abstract

**AIM:** To investigate the relationships among subtypes of gastroesophageal reflux disease (GERD) using narrow band imaging (NBI) magnifying endoscopy.

**METHODS:** A reflux disease questionnaire was used to screen 120 patients representing the three subtypes of GERD ( $n = 40$  for each subtypes): nonerosive reflux disease (NERD), reflux esophagitis (RE) and Barrett's esophagus (BE). NBI magnifying endoscopic procedure was performed on the patients as well as on 40 healthy controls. The demographic and clinical characteristics,

and NBI magnifying endoscopic features, were recorded and compared among the groups. Targeted biopsy and histopathological examination were conducted if there were any abnormalities. SPSS 18.0 software was used for all statistical analysis.

**RESULTS:** Compared with healthy controls, a significantly higher proportion of GERD patients had increased number of intrapapillary capillary loops (IPCLs) (78.3% vs 20%,  $P < 0.05$ ), presence of microerosions (41.7% vs 0%,  $P < 0.05$ ), and a non-round pit pattern below the squamocolumnar junction (88.3% vs 30%,  $P < 0.05$ ). The maximum ( $228 \pm 4.8$  vs  $144 \pm 4.7$ ,  $P < 0.05$ ), minimum ( $171 \pm 3.8$  vs  $103 \pm 4.4$ ,  $P < 0.05$ ), and average ( $199 \pm 3.9$  vs  $119 \pm 3.9$ ,  $P < 0.05$ ) numbers of IPCLs/field were also significantly greater in GERD patients. However, comparison among groups of the three subtypes showed no significant differences or any linear trend, except that microerosions were present in 60% of the RE patients, but in only 35% and 30% of the NERD and BE patients, respectively ( $P < 0.05$ ).

**CONCLUSION:** Patients with GERD, irrespective of subtype, have similar micro changes in the distal esophagus. The three forms of the disease are probably independent of each other.

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**Key words:** Gastroesophageal reflux; Gastroesophageal reflux disease; Intrapapillary capillary loops; Magnifying endoscopy; Narrow band imaging

**Core tip:** Gastroesophageal reflux disease (GERD) has been diagnosed with conventional endoscopy and 24-h esophageal pH monitoring. There are three forms of GERD: nonerosive reflux disease (NERD), reflux esophagitis (RE) and Barrett's esophagus (BE). However,

whether GERD is a spectrum of diseases or a “tripartite” disease remains unclear. Using narrow band imaging magnifying endoscopy, we observed no significant differences in the lower esophagus among patients with the three forms of GERD. There was also no increasing trend from NERD, RE to BE, indicating that these subtypes might be independent of each other. Thus, GERD is a “tripartite” disease, rather than a spectrum of diseases.

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## INTRODUCTION

The montreal definition<sup>[1]</sup>, an evidence-based global consensus definition, has demonstrated that gastroesophageal reflux disease (GERD) is a common chronic disorder that develops when the reflux of stomach contents causes troublesome symptoms and/or complications. The prevalence of GERD has been reported to range from 10% to 48% in Asia, slightly lower than that in Western countries<sup>[2-4]</sup>, and is increasing year by year<sup>[5-8]</sup>. GERD affects seriously patients' quality of life and poses heavy economic burdens on individuals as well as society because the lack of effective treatment. Unfortunately, the natural history of GERD has not been fully illustrated. Based on the findings of conventional endoscopy and histopathological examination, GERD is generally categorized into three progressive stages: nonerosive reflux disease (NERD), reflux esophagitis (RE), and Barrett's esophagus (BE). Patients with GERD, according to the current model of GERD as a spectrum disease, could potentially progress from mild NERD toward RE, BE, and then neoplasia<sup>[9]</sup>. This concept may in fact help the planning of diagnostic and therapeutic approaches, as well as the allocation of financial resources. Nevertheless, some researchers disagree, and propose that GERD is a “tripartite” disease. By dividing GERD into the three unique subtypes, physicians or surgeons may concentrate on the specific mechanisms that lead to the development of a subtype of GERD, leading to improvements in the specific therapeutic modalities that benefit the patients with a particular subtype.

Narrow band imaging (NBI) can better capture the microstructures of the superficial mucosa. Recent studies have shown that NBI can reveal subtle changes of esophageal superficial mucosa in GERD patients<sup>[10-15]</sup>. However, NBI endoscopy has not yet been used to study the microvascular differences among patients with NERD, RE and BE.

The present study aimed to explore the relationships among the three subtypes of GERD by observing the

subtle vascular changes detected with NBI magnifying endoscopy. Our study might provide a basis for further diagnosis and treatment of GERD.

## MATERIALS AND METHODS

### Subjects

Patients with reflux-associated symptoms, who were treated in our hospital between June 2010 and May 2012, were recruited in this study. All the patients were required to fulfill the reflux disease questionnaire (RDQ) concerning their reflux-associated symptoms to decide whether or not NBI magnifying endoscopy would be performed. The inclusion criteria were: aged between 18 and 70 years; ability to provide written informed consent; RDQ score of no less than 12; reflux-related symptom duration of no less than 3 mo; and effective treatment with proton pump inhibitors (PPIs). The exclusion criteria included: evidence of cancer or mass lesion in the upper alimentary tract, such as esophageal varices and gastric lesion; severe gastroparesis; history of drug use 4 wk before the study, including PPIs, nonsteroidal anti-inflammatory drugs, aspirin, antibiotics, antacid, mucosal protective agents, calcium channel blocks and acetylcholine or histamine receptor antagonists; and prior history of upper alimentary surgery, hemorrhage or severe uncontrolled systematic dysfunction. The healthy control group comprised subjects without any gastroesophageal reflux (GER) symptoms and with no positive findings confirmed by conventional endoscopy.

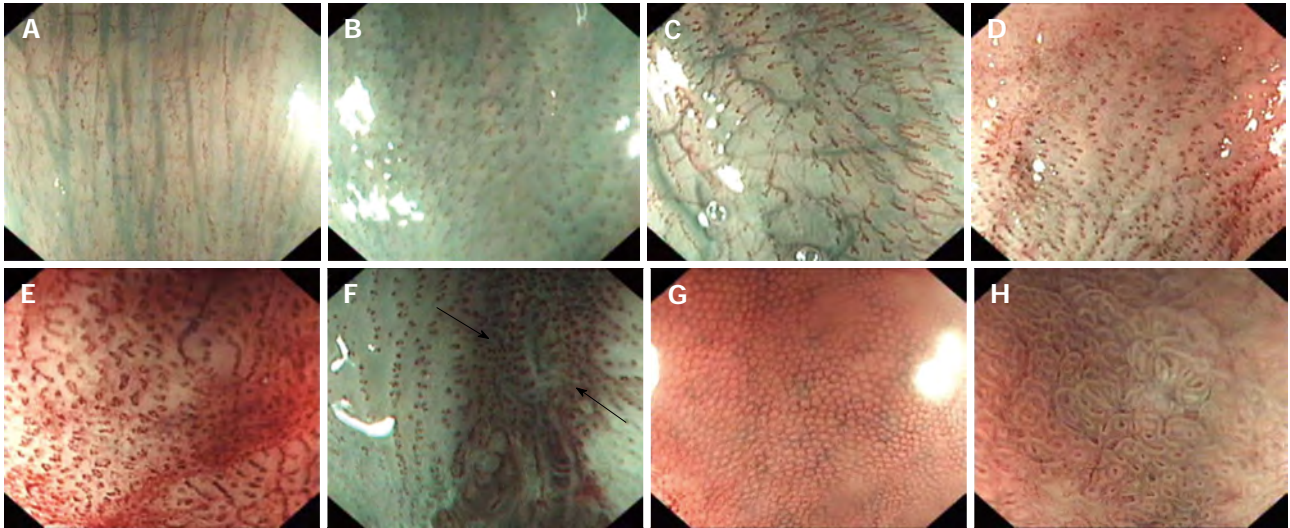
Written informed consent was obtained from all the subjects before endoscopy. The study protocol form was prepared according to the Declaration of Helsinki and approved by the Ethical Institutional Board of the hospital (No. 2010091).

### Research facility

The NBI magnifying endoscope (Olympus EVLS LU-CERA CV-260SL GIF-H260Z) used in this study was purchased from Olympus Medical Systems Corp. (Tokyo, Japan), and permitted a magnification of alimentary mucosa up to 80-fold by zoom and was compatible with an NBI light source in addition to the conventional white light source.

### Endoscopic procedure

After oral administration of 20 mL (1.0%) dimethicone and 10 mL (0.1 g) oropharyngeal anesthesia agent of dyclonine hydrochloride mucilage, all subjects underwent endoscopic procedure by the same experienced endoscopist who was blind to the GER symptoms. A complete evaluation of the upper alimentary tract was performed under the conventional mode. Then, the distal 5 cm of the esophagus, the squamocolumnar junction (SCJ) and cardia were reexamined carefully with the NBI light source under the maximum magnification of 80-fold. The key point was observation of the superficial vasculature. Images with the microstructure features in these locations were collected during the endoscopy, together



**Figure 1** Evaluation indicators of narrow band imaging magnifying mode ( $\times 80$ ). A: Normal intrapapillary capillary loops (IPCLs); B: Increment of IPCLs; C: Prolongation of IPCLs; D: Dilation of IPCLs; E: Tortuosity of IPCLs; F: Microerosion (indicated by arrows); G: Round pit pattern; H: Non-round pit pattern.

with the standardized four-quadrant images from the distal esophagus above the SCJ. An experienced investigator blinded to the GER symptoms evaluated the images.

#### Diagnostic criteria

The diagnostic criteria of NERD were: one or more of the typical reflux-associated symptoms of heartburn, regurgitation and retrosternal pain as chief complaints<sup>[1]</sup>; absence of mucosal breaks at conventional endoscopy; and effective treatment response to PPIs.

Diagnosis of RE followed the Los Angeles classification<sup>[16]</sup>.

BE was diagnosed when conversion of normal esophageal squamous epithelium to specialized intestinal metaplastic epithelium was confirmed by histopathological examination<sup>[1]</sup>.

Criteria of healthy controls included: no symptoms, no prior history of GERD, RDQ score of 0, and no visible mucosal breaks at conventional endoscopy. If microerosions were detected during the NBI magnifying endoscopy, 24-h pH monitoring was used to exclude asymptomatic GERD.

#### Evaluation indicators of NBI magnifying mode

Intrapapillary capillary loops (IPCLs) arise from the sub-mucosal drainage vein and go into the esophageal papillary. Assessment of IPCLs is important in the diagnosis of esophageal disorders. Histopathological examination has revealed that normal IPCLs exist in the esophageal submucosa, and are usually shown as dot-like structures with regular intervals of about 100  $\mu\text{m}$ . The IPCLs in the esophageal mucosa can be readily identified with the help of NBI magnifying endoscopy, under which their morphology is seen to be dynamic and can be affected by neoplasia and inflammation. The morphological changes of IPCLs were defined as follows: (1) Normal IPCLs (Figure 1A): hairpin-like structures with small diameters; (2) Increment (Figure 1B): an increase in the number of

IPCLs in individual fields; (3) Prolongation (Figure 1C): a change in the pattern characterized by increased lengths of individual IPCLs; (4) Dilation (Figure 1D): a change in the pattern characterized by increased sizes or calibers of individual IPCLs; (5) Tortuosity (Figure 1E): presence of corkscrewing or the twisted nature of individual IPCLs; (6) Another indicator was microerosion (Figure 1F): mucosal breaks not visible under conventional endoscopy but visible under NBI magnifying endoscopy; (7) Mucosal pit pattern under SCJ was the third indicator. Taking the Endo Classification Criteria as a reference<sup>[17]</sup>, the pit pattern was classified into two categories: round (Figure 1G) and non-round (including straight, oval, tubular and villous pit patterns) (Figure 1H); and (8) Photoshop software (Photoshop CS5 v 8.0; Adobe Inc., United States) was used to edit the image files.

#### Histopathological examination

Targeted biopsy was conducted when there was any mucosal lesion, such as erosion. Biopsy samples above and below the SCJ in four quadrants were also taken from all subjects. If esophageal metaplasia was suspected, biopsy sampling was performed in a systematic manner, *i.e.*, four-quadrant biopsies were obtained every 2 cm throughout the affected segment<sup>[18]</sup>. Histopathological examination was then performed on all the biopsy tissues.

#### Statistical analysis

The statistical software SPSS (SPSS PASW Statistics v18 Multilingual-EQUiNOX; SPSS Inc., United States) was used for all statistical analyses. The two-sample *t* test and analysis of variance (ANOVA) were performed on appropriate continuous quantitative variables that were normally distributed to determine whether significant differences existed among groups. Continuous quantitative variables that were not normally distributed were tested using the Wilcoxon test. Categorical variables were analyzed by the  $\chi^2$  test. Whenever the validity of  $\chi^2$  was



**Table 1** Demographic and clinical characteristics of gastroesophageal reflux disease patients and healthy controls *n* (%)

	HC	GERD			
		GERD	NERD	RE	BE
Number of subjects	40	120	40	40	40
Gender (male: female)	16:24	66:54	12:28	32:8	22:18
Mean age, yr (SD)	47.7 ± 2.20	52.9 ± 1.09	52.2 ± 1.72	51.4 ± 1.73	55.1 ± 2.17
Mean BMI (SD)	22.3 ± 0.55	23.6 ± 0.36	23.5 ± 0.56	24.2 ± 0.72	23.1 ± 0.60
Mean courses, mo (SD)	-	26 ± 32.3	33 ± 5.4	24 ± 4.4	21 ± 5.4
Scores of RDQ (SD)	-	16 ± 5.2	18 ± 0.8	15 ± 0.7	16 ± 0.5
No. of patients with bile regurgitation	6 (15)	22 (18.3)	10 (25)	12 (30)	0 (0)
No. of patients with hiatus hernia	18 (45)	72 (60)	18 (45)	26 (65)	28 (70)

GERD: Gastroesophageal reflux disease; RE: Reflux esophagitis; BE: Barrett's esophagus; NERD: Nonerosive reflux disease; HC: Healthy controls; BMI: Body mass index; RDQ: Reflux Disease Questionnaire.

**Table 2** Narrow band imaging magnifying endoscopic findings in gastroesophageal reflux disease patients and healthy controls *n* (%)

NBI magnification findings	HC	GERD			
		GERD	NERD	RE	BE
Patients with abnormality	28 (70)	118 (98.3) <sup>a</sup>	40 (100)	38 (95)	40 (100)
IPCLs increased	8 (20)	94 (78.3) <sup>a</sup>	32 (80)	32 (80)	30 (75)
IPCLs prolonged	14 (35)	62 (51.7)	24 (60)	18 (45)	20 (50)
IPCLs dilated	8 (20)	34 (28.3)	10 (25)	12 (30)	12 (30)
IPCLs tortuous	0 (0)	2 (1.7)	0 (0)	0 (0)	2 (5)
Microerosions	0 (0)	50 (41.7) <sup>a</sup>	14 (35) <sup>b</sup>	24 (60)	12 (30) <sup>b</sup>
Round pit pattern below SCJ	28 (70)	14 (11.7)	4 (10)	8 (20)	2 (5)
Non-round pit pattern below SCJ	12 (30)	106 (88.3) <sup>a</sup>	36 (90)	32 (80)	38 (95)

<sup>a</sup>*P* < 0.05 *vs* healthy control; <sup>b</sup>*P* < 0.05 *vs* RE patients. NBI: Narrow band imaging; GERD: Gastroesophageal reflux disease; RE: Reflux esophagitis; BE: Barrett's esophagus; NERD: Nonerosive reflux disease; HC: Healthy controls; IPCLs: Intrapapillary capillary loops; SCJ: Squamocolumnar junction.

called in question, Fisher's exact test was used instead. The probability of error (alpha) (*P*) was set at 0.05. *P* < 0.05 was considered statistically significant.

## RESULTS

### Characteristics of the subjects

A total of 157 patients were screened, of whom 145 were eligible for inclusion (40 NERD, 54 RE and 51 BE). To keep a balance among the groups, we selected 40 RE and 40 BE patients randomly. Altogether, we had 120 (66 male, 54 female) patients and 40 healthy controls (16 male, 24 female) included in the final analysis. Among these 120 GERD patients, there were 40 NERD, 40 RE and 40 BE patients. There were no significant differences between healthy controls and GERD patients in terms of gender, age, body mass index (BMI), prevalence of hiatus hernia, and prevalence of bile regurgitation. As for inter-subtype comparison, NERD patients were more likely to be female (*P* < 0.05), while most of the RE patients were male (*P* < 0.05). Patients with BE were less likely to demonstrate bile regurgitation (*P* < 0.05). In addition, the differences were not significant with respect to age, BMI, course, scores of RDQ and prevalence of hiatus hernia. The demographic and clinical characteristics, and conventional observations of the subjects are shown in Table 1.

### Evaluation indicators with NBI magnifying endoscopy mode

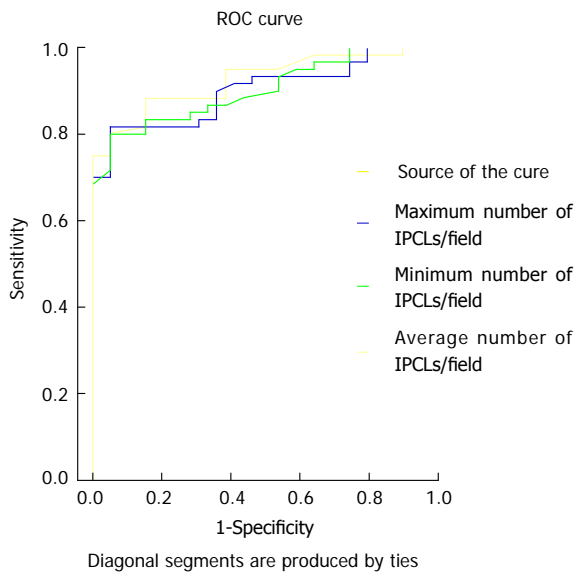
The majority of GERD patients presented abnormalities

under NBI magnifying endoscopy. Increment of IPCLs appeared in a significantly higher proportion of GERD patients than in healthy controls (*P* < 0.05). Microerosion above and non-round pit pattern below the SCJ were also seen more frequently in patients with GERD (*P* < 0.05). However, there was no significant difference in the presence of prolonged, dilated, or tortuous IPCLs between patients and controls. The comparison among groups of different GERD subtypes revealed no evident difference in the number of patients presenting with abnormalities. No statistical differences were found in the increased, prolonged, dilated or tortuous IPCLs in the distal esophagus, as well as in the distributions of the mucosal pit patterns below the SCJ. As expected, RE patients were more likely to demonstrate microerosions. The NBI magnifying endoscopic features are shown in Table 2.

Qualitative analysis of the IPCLs increment was also performed. The numbers of IPCLs/field in the standardized four-quadrant images from the distal esophagus were counted manually in each subject. The results showed that the maximum, minimum and average numbers of IPCLs/field were significantly higher in GERD patients than in healthy controls (*P* < 0.05). There was no significant difference among the three subtype groups. More importantly, there was no linear increasing trend from NERD to RE to BE. The quantitative data are presented in Table 3.

Receiver operating characteristic curves were plotted for the maximum, minimum and average numbers





**Figure 2** Receiver operating characteristic curves analysis. Receiver operating characteristic (ROC) curves were drawn based on the maximum, minimum and average numbers of IPCLs/field in gastroesophageal reflux disease patients and healthy controls. IPCLs: Intrapapillary capillary loops.

of IPCLs/field in GERD patients to obtain the cut-off values with the best sensitivity and specificity for diagnosis, namely, diagnostic threshold. The best sensitivity and specificity for GERD were 81.7% and 95.0% at a maximum IPCLs/field count of 185, 80% and 95% at a minimum IPCLs/field count of 135, and 80% and 95% at an average IPCLs/field count of 162 (Figure 2). The areas under the curves were 0.900, 0.902 and 0.925, respectively.

## DISCUSSION

GERD is considered to result from a long period of GER that causes troublesome symptoms and/or complications<sup>[1]</sup>. It has traditionally been approached as a spectrum of diseases with the same pathophysiological mechanisms. Based on conventional endoscopy and histopathological examination, GERD is generally categorized into three progressive stages: NERD, RE and BE. This understanding of GERD has a profound impact on its treatment, which focuses on esophageal mucosal injury<sup>[19]</sup>. Nevertheless, some researchers believe that GERD is a “tripartite” disease, rather than the model above. They take the three forms of GERD as being independent of each other, which may have their own mechanisms and should be approached with specific therapeutic modalities.

NBI, based upon the optical phenomenon that the depth of light penetrating into tissues depends on its wavelength, can capture the more detailed microstructure of the superficial mucosa. Some researchers have already used NBI magnifying endoscopy to observe the superficial mucosal changes in NERD patients, which cannot be observed under conventional endoscopy. For patients with RE and BE, micro changes have also been revealed

**Table 3** Intrapapillary capillary loops/field in gastroesophageal reflux disease patients and healthy controls

IPCLs/field	HC	GERD			
		GERD	NERD	RE	BE
Maximum (SD)	144 ± 4.7	228 ± 4.8	229 ± 7.8	233 ± 8.2	220 ± 9.3
Minimum (SD)	103 ± 4.4	171 ± 3.8	162 ± 5.6	178 ± 6.7	174 ± 7.1
Average (SD)	119 ± 3.9	199 ± 3.9	193 ± 5.7	208 ± 6.8	195 ± 7.7

GERD: Gastroesophageal reflux disease; RE: Reflux esophagitis; BE: Barrett's esophagus; NERD: Nonerosive reflux disease; HC: Healthy controls; IPCLs: Intrapapillary capillary loops.

with NBI magnifying endoscopy, in addition to the macro changes visualized by conventional endoscopy.

The microscopic IPCLs in the esophageal mucosa can be readily identified with the help of NBI magnifying endoscopy. A pilot feasibility trial conducted by Sharma *et al.*<sup>[11]</sup> illustrated the clinical utility of NBI magnifying endoscopy, which presents a significant improvement over standard endoscopy in the diagnosis of GERD. Their results indicated that increased number and dilatation of IPCLs may be the best predictors for the diagnosis of GERD. Normal IPCLs appear to be hairpin-shaped and small in diameter, while there are evident changes in the morphology and arrangement of IPCLs in GERD patients<sup>[10,12-15]</sup>. However, the micro vascular differences among NERD, RE and BE patients have not been investigated before.

The demographic and clinical characteristics of the subjects in our study are consistent with former epidemiological data in China. The increased number of IPCLs identified in the GERD patients would be helpful for the diagnosis of the disease. There were no remarkable differences in the micro structural changes among patients with the three subtypes of GERD. Additionally, there is no trend of development from NERD to RE and then BE. As pointed out by some researchers, the micro changes of esophageal mucosa, such as increment of IPCLs and microerosion, could truly represent the regurgitation-induced damage, and the assessment of differences in micro mucosal structure and vascular architecture could provide useful information for predicting histopathological findings<sup>[11,20-22]</sup>. Therefore, our statistics imply that GERD may be a “tripartite” disease resulting from GER, rather than a spectrum of diseases.

NERD, RE and BE, as three independent forms of GERD, may have their respective pathogenesis, clinical manifestations and complications. As Fass *et al.*<sup>[19]</sup> and Fass<sup>[23]</sup> have proposed, genetic factors may play a role in determining the phenotypes of GERD. Correspondingly, the diseases may require different therapeutic approaches and will have different prognoses and natural histories. There has already been clinical evidence for the independence of the three GERD forms. RE can be diagnosed with endoscopy and BE with endoscopy together with biopsy. However, conventional endoscopy is merely an exclusive examination for NERD. As to the 24 h pH monitoring, the symptomatic severity and frequency

between NERD and RE are not significantly different, and they affect the patients' quality of life similarly. Martinez *et al*<sup>[24]</sup> noted that the esophageal acid exposure of patients with NERD, RE and BE were different, and the reflux characteristics and symptom patterns suggested heterogeneity among their patients. In addition, the therapeutic efficacy in patients with different forms of GERD is different. A systematic review showed that a higher proportion of RE patients, compared with patients with NERD, achieved sufficient heartburn relief after the use of PPIs, and the therapeutic efficacy of NERD was poorer than that of RE<sup>[25]</sup>. The reason for the difference between NERD and RE patients in response to PPIs is still unclear. Limited data has indicated that NERD rarely moves on to RE with the prolonged course, relapse in cured RE patients after drug withdrawal often displays esophageal mucosal erosion, and BE is usually discovered during the first endoscopy examination instead of being developed from NERD or RE<sup>[19,26]</sup>. However, there have not been adequate follow-up studies to show the natural history of GERD.

It remains controversial whether GERD is a spectrum of diseases or not, and the relationships among the three forms of GERD has not yet been clarified. The montreal definition<sup>[1]</sup>, which takes RE as an esophageal complication of GERD, has shifted the attention from esophageal mucosal lesion to reflux symptoms, and may influence the cognition of the subtypes of GERD. More epidemiological investigation, prospective follow-up research and clinical observation are needed to clarify this issue.

Despite the value of our study, some limitations exist. This is a single-center investigation with a limited number of patients. A multicenter study with a larger sample size will be more reliable. In addition, NBI magnifying endoscopy has not yet been widely used. The inspection area under the magnifying mode is very small, which makes it time-consuming to examine the entire distal esophagus.

If the view that NERD, RE and BE are relatively independent forms of GERD is confirmed, the therapeutic strategy for each form could be adjusted according to the specific mechanism of that form, increasing efficacy. Therefore, determination of the internal relationships among NERD, RE and BE has profound significance in clinical practice.

## COMMENTS

### Background

Gastroesophageal reflux diseases (GERD), with the subtypes of nonerosive reflux disease (NERD), reflux esophagitis (RE) and Barrett's esophagus (BE), has been studied for many years. Conventional endoscopy and 24-h esophageal pH monitoring are common diagnostic approaches for GERD. However, it has not been clarified whether GERD is a spectrum of diseases or a "tripartite" disease.

### Research frontiers

Narrow band imaging (NBI) can capture the more detailed microstructure of the superficial mucosa. Some recent studies have shown that NBI magnifying endoscopy can reveal subtle changes of esophageal superficial mucosa in GERD patients. However, the microvascular differences among NERD, RE and BE

patients have not been investigated before using this new technology.

### Innovations and breakthroughs

It has been reported that patients with GERD could potentially progress from mild NERD toward RE, then BE, and finally neoplasia, which indicates that GERD may be a spectrum of diseases. However, some researchers disagree. They regard GERD a "tripartite" disease. The relationships among the three forms of GERD have not been fully investigated. The present study aimed to explore the relationships among the three subtypes of GERD by observing the subtle vascular changes detected by NBI magnifying endoscopy. The authors study might provide a basis for further diagnosis and treatment of GERD.

### Applications

By dividing GERD into three unique subtypes, physicians or surgeons may concentrate on the specific mechanisms leading to the development of a subtype of GERD and improve the specific therapeutic modalities that benefit the patients with subtype of disease.

### Terminology

Normal intrapapillary capillary loops (IPCLs) exist in the esophageal submucosa, and are dot-like structures, arranged at regular intervals of about 100  $\mu\text{m}$ . The microscopic IPCLs in the esophageal mucosa can be readily identified with the help of NBI magnifying endoscopy. Under magnifying endoscopy, they appear hairpin-shaped and small in diameter. Their morphology is dynamic and could be affected by neoplasia and inflammation.

### Peer review

In this study, the authors conducted research on the relationships among the three common forms of GERD using NBI magnifying endoscopy to observe the micro changes in the patients. All efforts to establish classifications in this very frequent disease are welcome. NBI facilitates diagnosis and includes new imaging areas. The results demonstrated that the GERD patients had similar micro changes in the distal esophagus, and they indicated that the disease might be a model of "tripartite" disease, rather than a spectrum of diseases.

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## Neutrophil-lymphocyte ratio predicts the prognosis of patients with hepatocellular carcinoma after liver transplantation

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### Abstract

**AIM:** To determine whether an elevated neutrophil-lymphocyte ratio (NLR) is negatively associated with tumor recurrence in patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) after liver transplantation (LT), and to determine the optimal predictive NLR cut-off value.

**METHODS:** The data of HCC patients who had undergone LT came from the China Liver Transplant Registry database. We collected data from 326 liver cancer patients who had undergone LT at our medical center. We divided the patients into groups based on their NLRs (3, 4 or 5). We then compared the clinicopathological data and long-time survival between these groups. Meanwhile, we used receiver operating characteristic analy-

sis to determine the optimal NLR cut-off.

**RESULTS:** Of 280 HCC patients included in this study, 263 were HBV positive. Patients with an NLR < 3 and patients with an NLR  $\geq$  3 but < 4 showed no significant differences in overall survival (OS) ( $P = 0.212$ ) or disease-free survival (DFS) ( $P = 0.601$ ). Patients with an NLR  $\geq$  4 but < 5 and patients with an NLR  $\geq$  5 also showed no significant differences in OS ( $P = 0.208$ ) or DFS ( $P = 0.618$ ). The 1-, 3- and 5-year OS rates of patients with an NLR < 4 vs an NLR  $\geq$  4 were 87.8%, 63.8% and 61.5% vs 73.9%, 36.7% and 30.3%, respectively ( $P < 0.001$ ). The 1-, 3- and 5-year DFS rates of patients with an NLR < 4 vs NLR  $\geq$  4 were 83.9%, 62.9% and 60.7% vs 64.9%, 30.1% and 30.1%, respectively ( $P < 0.001$ ). Univariate and multivariate analyses demonstrated that three factors, including NLR  $\geq$  4 ( $P = 0.002$ ), were significant predictors of tumor recurrence in HCC patients after LT.

**CONCLUSION:** A preoperative elevated NLR significantly increased the risk for tumor recurrence in HCC patients after LT.

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**Key words:** Hepatocellular carcinoma; Liver transplantation; Inflammatory reaction; Neutrophil-lymphocyte ratio; Hepatitis B virus

**Core tip:** Inflammation has been linked to the biological characteristics of tumors. The neutrophil-lymphocyte ratio (NLR) is a simple biomarker of inflammation. Several studies have reported that a preoperative elevated NLR (in peripheral blood) is negatively associated with the prognosis of patients with hepatocellular carcinoma (HCC) after liver transplantation (LT). However, the ideal cut-off value is controversial, with studies citing both



3 and 5 as the appropriate cut-off NLR. In this study, we report 326 HCC patients who had undergone LT at our center. We identify NLR = 4 as the cut-off point for predicting the prognosis of HCC patients after LT.

Xiao GQ, Liu C, Liu DL, Yang JY, Yan LN. Neutrophil-lymphocyte ratio predicts prognosis of patients with hepatocellular carcinoma after liver transplantation. *World J Gastroenterol* 2013; 19(45): 8398-8407 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i45/8398.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8398>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and is the third leading cause of cancer-related death. Every year, more than 500000 people are diagnosed with HCC, and most of these patients are in developing countries<sup>[1]</sup>. Liver transplantation (LT) is the ideal choice for HCC patients<sup>[2]</sup> because it completely removes tumors in the liver and also improves hepatic function. However, the outcome of HCC patients after LT was unsatisfactory, owing to a high tumor recurrence, until Mazzaferro *et al*<sup>[3]</sup> proposed the Milan criteria. Since the Milan criteria were adopted, the outcome of HCC after LT has significantly improved. Several LT centers have confirmed the satisfactory outcome of HCC patients within the Milan criteria after LT<sup>[4-7]</sup>. However, a large proportion of patients fall outside the Milan criteria when they are diagnosed with liver cancer. Thus, the LT criteria for HCC should be revised so that more people can become candidates for LT. Over the past 10 years, many centers have attempted to establish more suitable criteria for selecting HCC patients<sup>[8-12]</sup>. Yao *et al*<sup>[8]</sup> presented the University of California San Francisco (UCSF) criteria in 2001. The Milan and UCSF criteria are based on tumor number, tumor size and macro-vascular invasion, which are estimated by preoperative imaging.

However, preoperative radiological imaging is inaccurate, especially for patients with liver cirrhosis. Micro-vascular invasion and histological grade cannot be detected by imaging, and these two important factors greatly influence the recurrence of HCC after LT. Some studies have reported a recurrence rate of HCC after LT of nearly 15%-20% in patients who were within Milan or UCSF criteria<sup>[13,14]</sup>. This condition prompted us to identify better predictors of the recurrence of HCC after LT.

Several studies have investigated the effect of inflammation on carcinogenesis because the cytokines and mediators released by inflammatory cells can promote angiogenesis and tumor cell metastasis<sup>[15-17]</sup>. Several inflammatory markers, such as C reactive protein, have been suggested as surrogates for biological characteristics in some types of tumors<sup>[18,19]</sup>. The neutrophil-lymphocyte ratio (NLR) is a simple biomarker of inflammation, and an elevated NLR has been linked to several malignan-

cies<sup>[20-22]</sup>. Halazun *et al*<sup>[23]</sup> reported that patients with colorectal liver metastases with an elevated NLR had higher rates of recurrence after partial hepatic resection than patients with normal NLRs. Furthermore, studies have also shown that an elevated NLR has a negative impact on the prognosis of HCC patients after LT. However, these different studies have employed NLRs of 3, 4 and 5 as the cut-offs<sup>[24-28]</sup>, and the NLR cut-offs are not unified. Our study aimed to calculate the optimal preoperative cut-off NLR for predicting the prognosis of HCC patients after LT.

## MATERIALS AND METHODS

### *Patient selection and intra- and post-operative treatment*

The data of HCC patients who had undergone LT came from the China Liver Transplant Registry database. We collected data from 326 liver cancer patients who had undergone LT at our medical center from August 2000 to January 2011. Preoperative demographic, clinical and laboratory data were recorded for these patients. A systemic plain/enhanced computed tomography (CT) scan or a magnetic resonance imaging scan was employed within one week before the surgery. Pathology was considered as the definite diagnosis for HCC. Pathological data were considered as the standard for tumor characteristics. Moreover, micro-vascular invasion and tumor differentiation were also assessed by pathology.

Blood cell testing is part of the routine work-up for HCC patients who have undergone LT. The absolute value of white blood cells and the differential counts were recorded within one week before surgery. The NLR was calculated by dividing the neutrophil count by the lymphocyte count. Patients with missing blood records; patients who had preoperative sepsis, hypersplenism, massive alimentary tract bleeding, hepatitis C virus (HCV) infection, cholangiocarcinoma or other neoplasms; and pediatric patients were excluded from this study.

The patients received LT at least one month after they had received preoperative adjuvant therapy when their blood test became normal. LT was performed using standard techniques without the use of veno-venous bypass, and a "piggy back" was used when necessary. After surgery, an immune-suppression regimen, including corticosteroids, cyclosporine or tacrolimus with or without azathioprine and mycophenolate, was administered. The steroids were withdrawn after 3-6 mo of post-operative treatment<sup>[29]</sup>. The e antigen status and HBV-DNA of several patients were positive, and the HBV-positive patients received anti-viral drugs, such as lamivudine, adefovir, telbivudine and entecavir, prior to and after transplantation<sup>[30]</sup>.

### *Follow-up*

After surgery, the patients underwent follow-up procedures. Plain/enhanced CT scans and  $\alpha$ -fetoprotein (AFP) tests were performed every month for the first 6 mo. The above examinations were performed every 2 mo for the second 6 mo. In the following years, the patients received

**Table 1** Comparison of demographic and clinicopathological data of patients with hepatocellular carcinoma classified by different neutrophil-lymphocyte ratios

Variable	NLR < 3 ( <i>n</i> = 105)	3 ≤ NLR < 4 ( <i>n</i> = 61)	4 ≤ NLR < 5 ( <i>n</i> = 56)	NLR ≥ 5 ( <i>n</i> = 58)	<i>P</i> (2-tailed)
Gender (F/M)	16/89	4/57	7/49	4/54	0.235
Age, yr (mean)	47.0	46.4	45.6	46.8	0.529
Age, yr (≥ 60/< 60)	13/92	5/56	6/50	7/51	0.859
Child-Pugh class (A/B/C)	59/39/7	31/26/4	31/21/4	25/22/11	0.083
BMI (mean)	23.3	22.3	22.2	22.9	0.899
AFP, g/L (< 400/≥ 400)	53/52	30/31	19/37	31/27	0.145
Preoperative adjuvant therapy (Y/N)	47/58	27/34	22/34	31/27	0.492
Tumor (≤ 3/> 3), <i>n</i>	90/15	49/12	41/15	39/19	0.036 <sup>2</sup>
Largest tumor size, cm (≤ 5/5-9/> 9)	50/32/23	29/17/15	20/19/17	21/14/23	0.168
Total tumor size, cm (≤ 5/5-9/> 9)	41/31/33	24/16/21	13/17/26	15/10/33	0.257
Macro-vascular invasion (Y/N)	20/85	13/48	20/36	21/37	0.028 <sup>2</sup>
Micro-vascular invasion (Y/N)	44/61	28/33	33/23	35/23	0.059
Differentiation (1-2/3-4)	44/17 <sup>1</sup>	26/14 <sup>1</sup>	29/15 <sup>1</sup>	32/15 <sup>1</sup>	0.866
HBV infection (-/+)	5/100	3/58	1/55	8/50	0.064
Donor (living/deceased)	30/75	13/48	16/40	7/51	0.083

<sup>1</sup>Differentiation was reported for 192 patients; <sup>2</sup>Significant *P* value. NLR: Neutrophil-lymphocyte ratios; BMI: Body mass index; AFP:  $\alpha$ -fetoprotein; HBV: Hepatitis B virus.

examinations every 3-6 mo or when necessary. Suspicious lesions in the liver or lung were biopsied. Bone pain and progression of growth were observed. The date of tumor recurrence was regarded as the time that the AFP level began to rise once tumor recurrence had been confirmed.

### Ethics

This study was approved by the Institutional Review Board of West China Hospital of Sichuan University in Sichuan Province. Written informed consent was obtained according to the Declaration of Helsinki of the World Medical Association.

### Statistical analysis

SPSS v17.0 and MedCalc v11.3.0.0 were used to analyze the data. Receiver operating characteristic (ROC) analysis was used to determine the NLR cut-off value. Independent sample *t* test, Pearson's  $\chi^2$  test and Fisher's exact test were used to analyze the differences among HCC patients classified by different NLR values. Kaplan-Meier survival analysis was used to analyze overall survival (OS) and disease-free survival (DFS). Univariate analysis was performed to estimate the hazard ratio of the clinicopathological factors for the risk of tumor recurrence. The factors that had a significant impact on the outcome of HCC patients after LT were selected into multivariate Cox proportional hazards regression analysis to assess the hazard ratio for the risk of tumor recurrence in HCC patients after LT. The confidence interval quoted area was 95%, and significant differences were defined as *P* < 0.05.

## RESULTS

### Patient demographics and outcomes

Of 326 HCC patients who had undergone LT at our

medical center from August 2000 to January 2011, 46 were excluded from the study: 10 for missing blood records, 2 pediatric patients, 3 for preoperative sepsis, 10 for hypersplenism, 2 for massive alimentary tract bleeding, 1 HCV-positive patient and 18 for the diagnosis of cholangiocarcinoma or other neoplasms by pathology. Thus, 280 patients were included in this study. Of these patients, 263 (93.9%) were HBV positive. The carcinogenic factor of 17 HCC patients may have been alcohol because they had a history of alcohol abuse. Among 280 patients, there were 31 (11.1%) women and 249 (88.9%) men. The mean age of the patients who had received LT was 46.5 years (range: 20.5-69.1 years, SD: 9.6 years). The median waiting times for living donor and deceased donor LT were 0.9 and 1.6 mo, respectively. The mean follow-up time was 2.63 years (range: 1.1-12.0 years). A total of 120 people died during follow-up. The 1-, 3- and 5-year OS rates of the patients in our study were 82.2%, 52.6% and 48.5%, respectively, and the 1-, 3- and 5-year DFS rates were 76.1%, 50.3% and 47.8%, respectively.

### Comparison of variables between patients with different NLRs

Several studies have considered NLRs of 3, 4 and 5 as the cut-off points to predict the prognosis of HCC patients after LT<sup>[24-28]</sup>. Thus, we divided the HCC patients who had received LT at our hospital based on these three NLR cut-offs. There were 105 patients with an NLR < 3, 61 patients with NLRs between 3 and 4, 56 patients with NLRs between 4 and 5 and 58 patients with an NLR ≥ 5. We compared the demographic and clinicopathological data of HCC patients after LT. HCC patients classified based on their NLRs showed significant differences in tumor number > 3 (*P* = 0.036) and macro-vascular invasion (*P* = 0.028). There were no significant differences in the other variables among the groups with different NLRs (Table 1).

**Table 2** Univariate analysis of the effects of clinicopathological factors on the disease-free survival of patients with hepatocellular carcinoma who underwent liver transplantation

Variable	$\chi^2$	P value	HR	95%CI
Gender (F/M)	0.001	0.973	0.990	0.558-1.759
Age, yr ( $\geq 60$ / $< 60$ )	4.573	0.032 <sup>1</sup>	0.477	0.242-0.940
Child-Pugh class (A/B/C)				
A	-	-	-	-
B	0.291	0.590	1.106	0.766-1.598
C	0.287	0.592	0.827	0.412-1.658
AFP, g/L ( $< 400$ / $\geq 400$ )	6.673	0.010 <sup>1</sup>	1.600	1.120-2.287
NLR ( $< 4$ / $\geq 4$ )	24.251	$< 0.001$ <sup>1</sup>	2.424	1.704-3.440
Preoperative adjuvant therapy (Y/N)	0.019	0.890	1.025	0.721-1.457
Tumor No. ( $\leq 3$ / $> 3$ )	23.518	$< 0.001$ <sup>1</sup>	2.524	1.736-3.670
Largest tumor size, cm				
$\leq 5$	-	-	-	-
5-9	11.105	0.001 <sup>1</sup>	2.195	1.382-3.487
$> 9$	36.829	$< 0.001$ <sup>1</sup>	3.894	2.510-6.041
Total tumor size, cm				
$\leq 5$	-	-	-	-
5-9	6.590	0.010	2.123	1.195-3.771
$> 9$	45.107	$< 0.001$ <sup>1</sup>	5.553	3.358-9.115
Macro-vascular invasion (Y/N)	33.195	$< 0.001$ <sup>1</sup>	2.904	2.021-4.174
Micro-vascular invasion (Y/N)	31.135	$< 0.001$ <sup>1</sup>	2.910	1.999-4.234
Differentiation (1-2/3-4)	3.136	0.077	1.435	0.962-2.140
HBV infection (Y/N)	0.012	0.912	1.048	0.461-2.382
Donor (living/deceased)	0.480	0.488	1.163	0.759-1.780

<sup>1</sup>Significant P value. NLR: Neutrophil-lymphocyte ratios; AFP:  $\alpha$ -fetoprotein; HBV: Hepatitis B virus.

### Outcome of different HCC categories divided by NLRs

Of the 105 patients with an NLR  $< 3$ , 12 died and 15 had tumor recurrence within 1 year. For these patients, the 1-, 3- and 5-year OS rates were 88.6%, 65.8% and 65.8%, respectively, and the 1-, 3- and 5-year DFS rates were 85.4%, 63.7% and 62.0%, respectively. For the 61 patients with NLRs between 3 and 4, their OS and DFS were not significantly different compared with patients having an NLR  $< 3$  ( $P = 0.212$  and  $P = 0.601$ , respectively). There were 56 patients with an NLR  $\geq 4$  but  $< 5$  and 58 patients with an NLR  $\geq 5$ . The outcome of these two categories was not significantly different, as shown in Figure 1. However, the outcome of patients with an NLR  $\geq 3$  but  $< 4$  and an NLR  $\geq 4$  but  $< 5$  revealed significant differences: the 1-, 3- and 5-year OS rates of patients with an NLR  $\geq 3$  but  $< 4$  *vs* an NLR  $\geq 4$  but  $< 5$  were 86.4%, 57.3% and 54.3% *vs* 79.7%, 35.5% and 31.1%, respectively ( $P = 0.026$ ). The 1-, 3- and 5-year DFS rates of patients with an NLR  $\geq 3$  but  $< 4$  *vs* an NLR  $\geq 4$  but  $< 5$  were 81.2%, 61.6% and 58.6% *vs* 68.9%, 31.2% and 31.2%, respectively ( $P = 0.005$ ) (Figure 1).

In addition, we used ROC curve analysis to determine the optimal NLR cut-off for HCC patients who received LT. The area under the ROC curve was 0.670 (Figure 2). When the NLR was 4.0634, the sensitivity was 56.3%, the specificity was 75.0%, and the sensitivity and specificity were highest. Therefore, we considered NLR = 4 as the cut-off. Patients with an NLR less than 3 and patients with an NLR  $\geq 3$  but  $< 4$  were combined into one cat-

**Table 3** Multivariate analysis of the effects of clinicopathological factors on the disease-free survival of patients with hepatocellular carcinoma who underwent liver transplantation

Variable	$\chi^2$	P value	HR	95%CI
Age, yr ( $\geq 60$ / $< 60$ )	2.731	0.098	0.561	0.283-1.113
AFP, g/L ( $< 400$ / $\geq 400$ )	0.397	0.529	1.128	0.776-1.640
NLR ( $< 4$ / $\geq 4$ )	9.260	0.002 <sup>1</sup>	1.758	1.222-2.527
Tumor No. ( $\leq 3$ / $> 3$ )	1.450	0.229	1.301	0.848-1.997
Largest tumor size, $> 5$ cm	1.761	0.185	1.378	0.858-2.214
Total tumor size, $> 9$ cm	16.939	$< 0.001$ <sup>1</sup>	2.725	1.691-3.393
Macro-vascular invasion (Y/N)	11.168	$< 0.001$ <sup>1</sup>	2.013	1.336-3.035
Micro-vascular invasion (Y/N)	3.085	0.071	1.597	1.001-2.546

<sup>1</sup>Significant P value. NLR: Neutrophil-lymphocyte ratios; AFP:  $\alpha$ -fetoprotein.

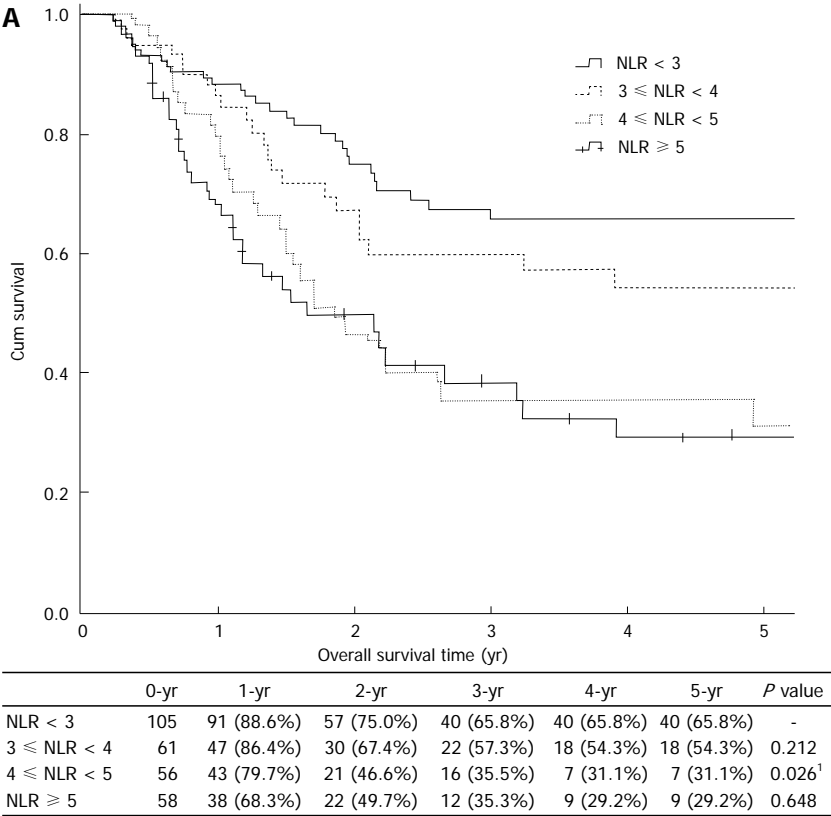
egory, while the patients with an NLR  $\geq 4$  were considered as one category. We then compared the outcome of these two categories. As shown in Figure 3, the OS and DFS of these two categories were significantly different.

### Predictors of prognosis of HCC patients after LT

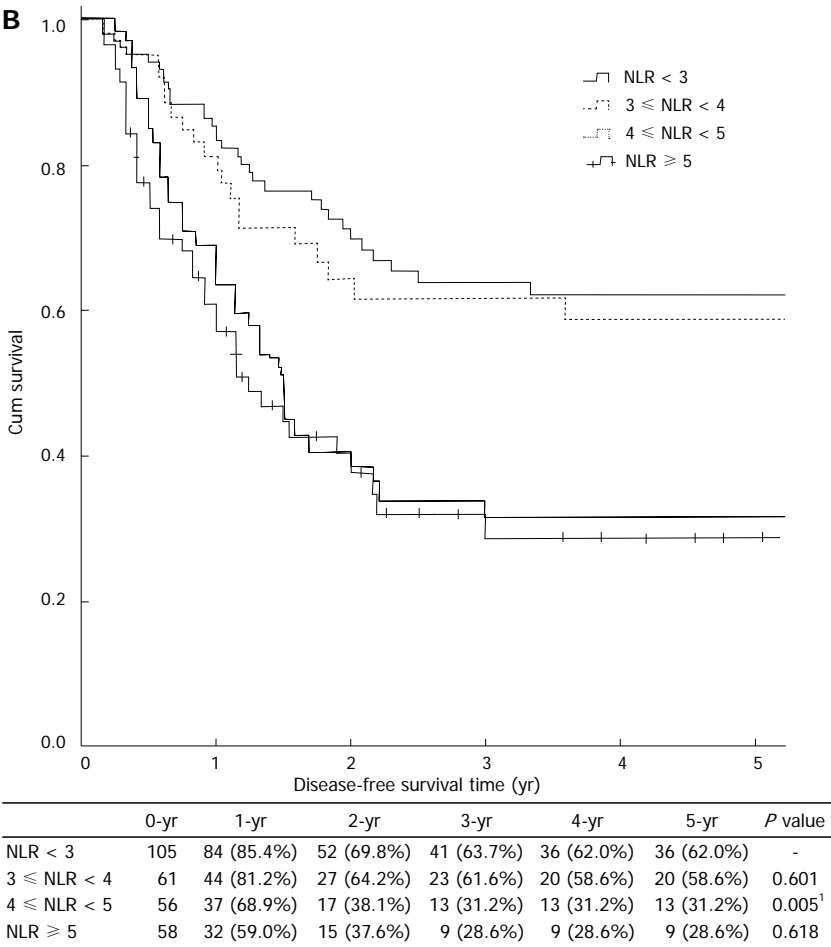
The lists in Table 2 show 8 significant factors that affects the DFS of HCC patients after LT by univariate analysis: age  $\geq 60$  years, AFP  $\geq 400$  g/L, NLR  $\geq 4$ , tumor number  $> 3$ , largest tumor size more than 5 cm, total tumor size more than 9 cm, macro-vascular invasion and micro-vascular invasion. The significant predictors were then utilized for a multivariate proportional hazard regression analysis. The result revealed that NLR  $\geq 4$  ( $P = 0.002$ , HR = 1.758, 95%CI: 1.222-2.527), total tumor size  $> 9$  cm ( $P < 0.001$ , HR = 2.725, 95%CI: 1.691-3.393) and macro-vascular invasion ( $P < 0.001$ , HR = 2.013, 95%CI: 1.336-3.035) were independent predictors of DFS of HCC patients after LT (Table 3). We also performed univariate analysis and multivariate proportional hazard regression analysis to analyze the factors that affect the OS of HCC patients who underwent LT. The results showed that NLR  $\geq 4$  ( $P = 0.006$ , HR = 1.695, 95%CI: 1.164-2.466), total tumor size  $> 9$  cm ( $P < 0.001$ , HR = 4.114, 95%CI: 2.438-6.940) and macro-vascular invasion ( $P < 0.001$ , HR = 2.049, 95%CI: 1.364-3.078) were independent predictors of OS of HCC patients after LT.

## DISCUSSION

Since the Milan criteria were proposed by Mazzaferro *et al.*<sup>[3]</sup> in 1996, many liver transplantation centers worldwide have reported excellent results after LT for patients with HCC who fall within the Milan criteria<sup>[6,29,31]</sup>. However, most HCC patients are outside the Milan criteria. To let those people receive corresponding treatment, revised LT criteria for HCC patients need to be established. Therefore, Yao *et al.*<sup>[8]</sup> presented revised criteria and demonstrated that the outcomes of patients with HCC after LT outside the Milan criteria but within UCSF had no significant difference compared with the outcomes of patients within the Milan criteria. This result has been confirmed



<sup>1</sup>Significant P value



<sup>1</sup>Significant P value

**Figure 1** Kaplan-Meier survival analysis curve. A: The overall survival for patients with hepatocellular carcinoma undergoing liver transplantation classified by different neutrophil-lymphocyte ratios (NLRs); B: The disease-free survival for patients with hepatocellular carcinoma undergoing liver transplantation by different NLRs.



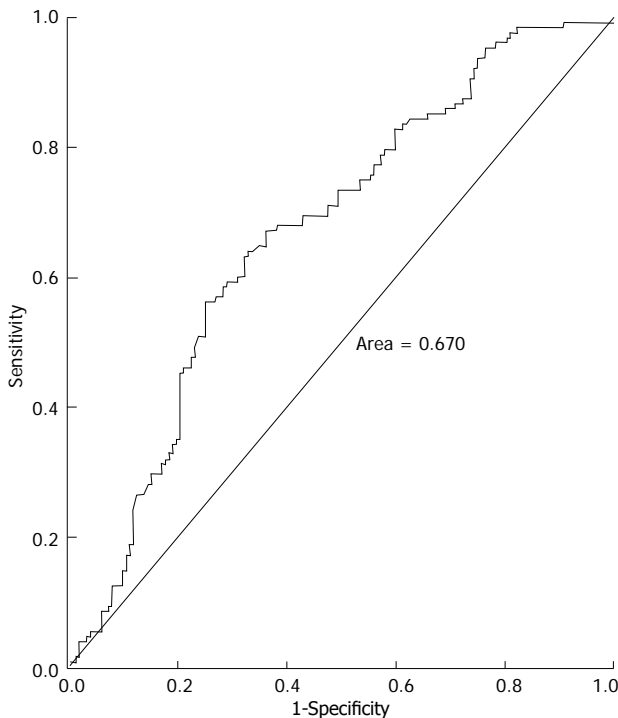


Figure 2 Receiver operating characteristic curve for the neutrophil-lymphocyte ratio cut-off value to predict tumor recurrence of hepatocellular carcinoma patients after liver transplantation.

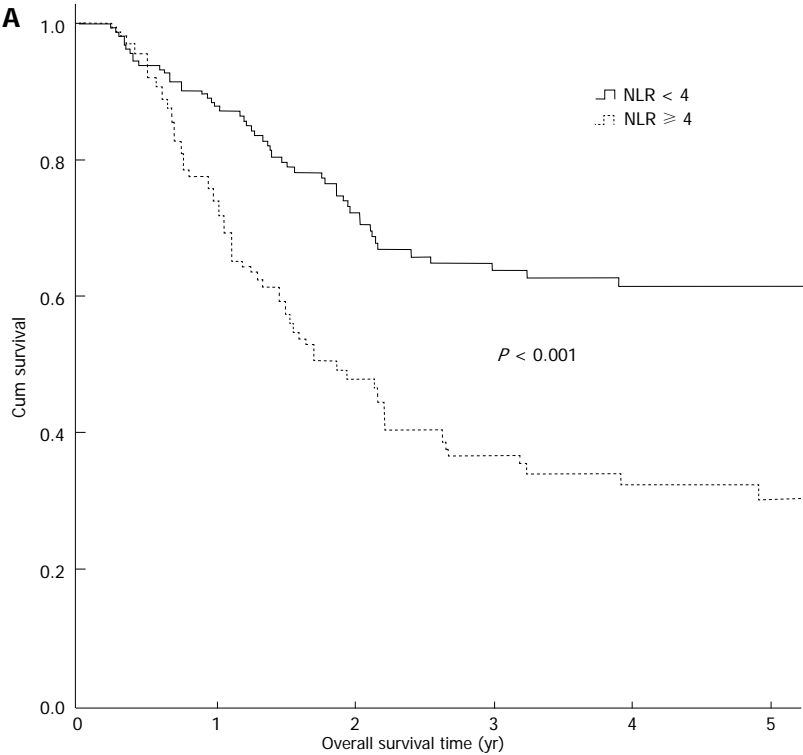
by many LT centers<sup>[9,32]</sup>. With the revision of the LT criteria for HCC, another issue arises: organ shortages. Therefore, it is necessary to judge the biological characteristics of the tumor to exclude patients with negative tumor behavior. These patients will likely respond poorly to LT despite being within the Milan or UCSF criteria. Tumor size and number, as assessed by preoperative radiology, are used as surrogate markers of tumor biology. Some studies have reported that tumor size is related to the risk of recurrence and vascular invasion<sup>[13,33]</sup>. However, we found tumor number > 3 and largest tumor size > 5 cm were not independent predictors of the OS and DFS of patients with HCC after LT. Nevertheless, total tumor size > 9 cm was an independent factor that predicted OS and DFS (Table 2). In view of the inaccuracy of preoperative tumor assessments and the inconsistency of the effect of tumor number and size on the prognosis of HCC patients after LT, new non-invasive surrogates are needed to predict the outcome of HCC patients after partial hepatic resection or LT.

Several studies have reported that inflammation plays an important role in the development of malignant disease<sup>[15,17]</sup>. NLR was first linked to liver malignancy by Halazun *et al*<sup>[23]</sup>. NLR is a simple marker of inflammation and can be obtained easily by routine blood testing. However, the cut-off values of NLR are not unified. We found that the outcomes of patients with an NLR < 3 and patients with an NLR ≥ 3 but < 4 were not significantly different, nor were the outcomes of patients with an NLR ≥ 4 but < 5 and patients with an NLR ≥ 5. In

our study, ROC analysis demonstrated that the sensitivity and specificity were highest when the NLR was 4.0634. Therefore, we considered patients with an NLR < 4 as one group and patients with an NLR ≥ 4 as another group. We then compared the outcomes of these two groups. We observed a marked and significant difference between these two groups in OS and DFS. Therefore, we consider NLR ≥ 4 as an elevated ratio. NLR ≥ 4 was also recognized as elevated by Shimada *et al*<sup>[21]</sup> in patients with gastric cancer. Halazun *et al*<sup>[25]</sup> considered NLR ≥ 5 to be elevated, and they reported the 1-, 3- and 5-year DFS of patients with an elevated NLR *vs* a normal NLR as 62%, 28% and 28% *vs* 88%, 74% and 64%, respectively ( $P = 0.001$ ).

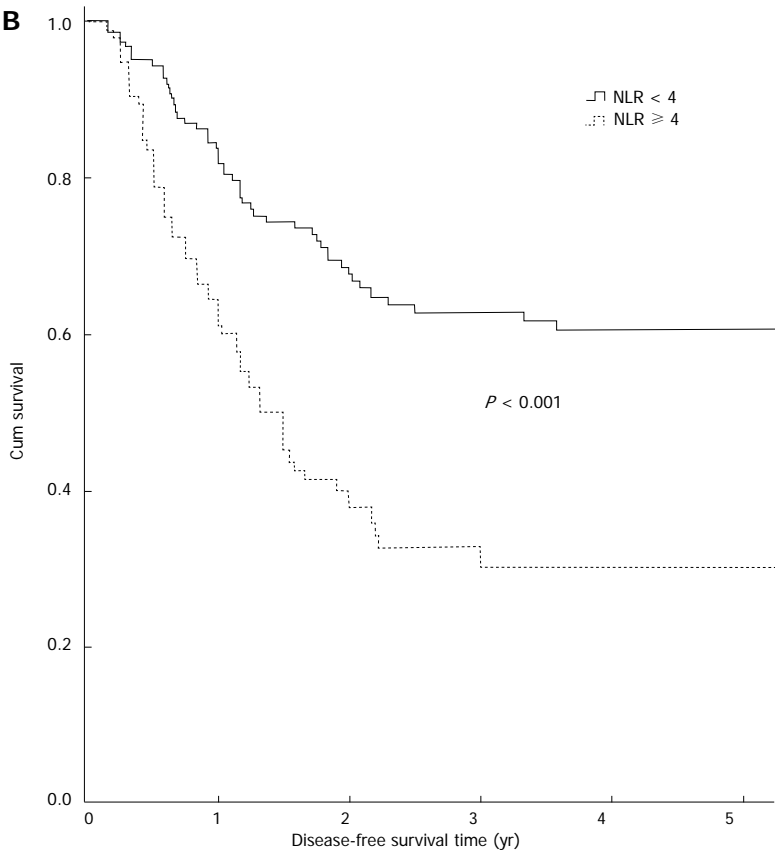
Although HCC patients with an elevated NLR have a poor prognosis, the mechanism through which the NLR affects tumor recurrence remains undefined. There are several hypotheses regarding the link between elevated NLR and tumor recurrence. First, neutrophils are the major source of vascular endothelial growth factor (VEGF), which promotes tumor angiogenesis and metastasis<sup>[34-37]</sup>. High levels of VEGF expression have been correlated with tumor recurrence in HCC<sup>[38]</sup>. Furthermore, some studies have reported that patients with elevated VEGF expression have increased vascular density in their tumor nodules<sup>[34,39]</sup>. Generally, the white blood cell counts of these patients were within the normal range, so patients with a higher NLR had higher neutrophil counts and higher VEGF expression. Second, the human immune system mostly depends on lymphocytes. However, lymphocyte counts are greatly reduced in patients with elevated NLRs, who are left unable to defend against the tumor malignancy. Several studies have demonstrated that patients with few lymphocytes infiltrating into the tumor margin have poor outcomes after treatment<sup>[39,40]</sup>. Patients with elevated NLRs have relative neutrophilia and lymphocytopenia, leading to an imbalance in the inflammatory cascade and immune response to malignant tumors. In this type of micro-environment, tumors proliferate and metastasize more easily. It remains unclear whether neutrophils or lymphocytes play a more important role in tumor recurrence of HCC after LT, and the mechanism has not been explored clearly. It is necessary to perform more clinical and basic studies.

Although univariate analysis of this study demonstrated that age ≥ 60 years, AFP ≥ 400 g/L, tumor number > 3, largest tumor size > 5 cm and micro-vascular invasion were preoperative predictors of DFS, none of these variables were independent factors for predicting tumor recurrence of HCC after LT. Some studies have reported tumor nodules > 3 as an independent predictor of tumor recurrence<sup>[9,24,41]</sup>. However, our results showed that the long-term survival of HCC patients with tumor nodules < 3 and those with tumor nodules > 3 was unchanged after LT, and our result is in agreement with the results of several other studies<sup>[25,42]</sup>. In this study, NLR ≥ 4 ( $P = 0.002$ , HR = 1.758, 95%CI: 1.222-2.527), total tumor size > 9 cm ( $P < 0.001$ , HR = 2.725, 95%CI: 1.691-3.393) and



	0-yr	1-yr	2-yr	3-yr	4-yr	5-yr	P value
NLR < 4	166	137 (87.8%)	85 (72.3%)	63 (63.8%)	51 (61.5%)	51 (61.5%)	-
NLR ≥ 4	114	81 (73.9%)	41 (48.0%)	29 (36.7%)	21 (32.4%)	15 (30.3%)	< 0.001 <sup>1</sup>

<sup>1</sup>Significant P value



	0-yr	1-yr	2-yr	3-yr	4-yr	5-yr	P value
NLR < 4	166	127 (83.9%)	76 (67.7%)	64 (62.9%)	55 (60.7%)	55 (60.7%)	-
NLR ≥ 4	114	70 (64.9%)	32 (37.9%)	22 (30.1%)	22 (30.1%)	22 (30.1%)	< 0.001 <sup>1</sup>

<sup>1</sup>Significant P value

**Figure 3** Kaplan-Meier survival analysis curve. A: The overall survival for patients with hepatocellular carcinoma undergoing liver transplantation classified by the cut-off neutrophil-lymphocyte ratio (NLR) of 4; B: The disease-free survival for patients with hepatocellular carcinoma undergoing liver transplantation by the cut-off NLR of 4.

macro-vascular invasion ( $P < 0.001$ , HR = 2.013, 95%CI: 1.336-3.035) were independent factors that predict the prognosis of HCC patients after LT. HCC patients with a higher preoperative NLR had a higher tumor recurrence rate than those with a normal NLR after LT.

There are many limitations to this study. First, we know that the preoperative NLR is affected by many factors, such as unidentified sepsis, weight loss, massive hemorrhage and instrumental error, which make the NLR inaccurate. In addition, the majority of patients enrolled in our study had HBV infection, which may bias the result because hepatitis C is the most common cause of HCC in developed countries. Moreover, this is a retrospective study, and the number of patients included in our study is relatively small. More multi-center and prospective studies are needed to confirm and update the findings demonstrated in this study.

In summary, we have found that HCC patients with an elevated preoperative NLR have poorer OS and DFS after LT. This biomarker allows us to preoperatively identify patients with a high NLR, who have a poor prognosis and adverse tumor biology.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Several studies have investigated the effect of inflammation on carcinogenesis because cytokines and mediators released by inflammatory cells can promote angiogenesis and tumor cell metastasis. Several inflammatory markers, such as C reactive protein, have been suggested as surrogates for biological characteristics in some types of tumors. The neutrophil-lymphocyte ratio (NLR) is a simple biomarker of inflammation, and an elevated NLR has been linked to several malignancies.

### Research frontiers

Halazun *et al* reported that patients with colorectal liver metastases with an elevated NLR had higher rates of recurrence after partial hepatic resection than patients with normal NLRs. Furthermore, studies have also shown that an elevated NLR has a negative impact on the prognosis of hepatocellular carcinoma (HCC) patients after liver transplantation (LT).

### Innovations and breakthroughs

Several studies have employed NLRs of 3, 4 and 5 as the cut-offs, and the NLR cut-offs are not unified. The study aims to calculate the optimal preoperative NLR cut-off for predicting the prognosis of HCC patients after LT.

### Applications

A preoperative elevated NLR significantly increased the risk for tumor recurrence in HCC patients after LT. This biomarker allows them to preoperatively identify patients with a high NLR, who have a poor prognosis and adverse tumor biology.

### Terminology

The NLR was calculated by dividing the neutrophil count by the lymphocyte count in peripheral blood.

### Peer review

This is a very interesting paper on a prognostic factor for liver cancer recurrence after transplantation. The authors stated that NLR is a strong prognostic factor for outcome of liver transplantation for HCC from their experience. This manuscript is easy to understand and well organized.

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## Proton pump inhibitor for non-erosive reflux disease: A meta-analysis

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### Abstract

**AIM:** To evaluate the efficacy, safety and influential factors of proton pump inhibitor (PPI) treatment for non-erosive reflux disease (NERD).

**METHODS:** PubMed, MEDLINE, EMBASE and the Cochrane Library were searched up to April 2013 to identify eligible randomized controlled trials (RCTs) that probed into the efficacy, safety and influential factors of PPI treatment for NERD. The rates of symptomatic relief and adverse events were measured as the outcomes. After RCT selection, assessment and data collection, the pooled RRs and 95%CI were calculated. This meta-analysis was performed using the Stata 12.0 software (Stata Corporation, College Station, Texas, United States). The level of evidence was estimated by the Grading of Recommendations Assessment, Development and Evaluation system.

**RESULTS:** Seventeen RCTs including 6072 patients

met the inclusion criteria. The results of the meta-analysis showed that PPI treatment was significantly superior to H<sub>2</sub> receptor antagonists (H<sub>2</sub>RA) treatment (RR = 1.629, 95%CI: 1.422-1.867,  $P = 0.000$ ) and placebo (RR = 1.903, 95%CI: 1.573-2.302,  $P = 0.000$ ) for the symptomatic relief of NERD. However, there were no obvious differences between PPI and H<sub>2</sub>RA (RR = 0.928, 95%CI: 0.776-1.110,  $P = 0.414$ ) or PPI and the placebo (RR = 1.000, 95%CI: 0.896-1.116,  $P = 0.997$ ) regarding the rate of adverse events. The overall rate of symptomatic relief of PPI against NERD was 51.4% (95%CI: 0.433-0.595,  $P = 0.000$ ), and relief was influenced by hiatal hernia ( $P = 0.030$ ). The adverse rate of PPI against NERD was 21.0% (95%CI: 0.152-0.208,  $P = 0.000$ ), and was affected by hiatal hernia ( $P = 0.081$ ) and drinking ( $P = 0.053$ ).

**CONCLUSION:** PPI overmatched H<sub>2</sub>RA on symptomatic relief rate but not on adverse rate for NERD. Its relief rate and adverse rate were influenced by hiatal hernia and drinking.

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**Key words:** Proton pump inhibitor; Non-erosive reflux disease; Symptomatic relief; Adverse event; Meta-analysis

**Core tip:** As a kind of powerful and effective acid-suppressive drugs, proton pump inhibitor (PPI) has been used for patients with non-erosive reflux disease (NERD), but its efficacy, safety and their influential factors are inconclusive. We performed this systematic review and meta-analysis of randomized controlled trials to assess its efficacy, safety and influential factors. Based on the results of the meta-analysis, we conclude that PPI has a higher symptomatic relief rate and roughly the same adverse rate for NERD. Hiatal hernia and drinking could influence symptomatic relief rate and adverse rate of PPI on NERD.

Zhang JX, Ji MY, Song J, Lei HB, Qiu S, Wang J, Ai MH, Wang J, Lv XG, Yang ZR, Dong WG. Proton pump inhibitor for non-erosive reflux disease: A meta-analysis. *World J Gastroenterol* 2013; 19(45): 8408-8419 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i45/8408.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8408>

## INTRODUCTION

Non-erosive reflux disease (NERD) is a heterogeneous group of disorders, which present with the typical gastroesophageal reflux symptoms of heartburn, regurgitation or both in the absence of visible esophageal injury upon endoscopy<sup>[1-3]</sup>. Patients with NERD are more likely to be female, young, thin, and without hiatal hernias<sup>[4-6]</sup>, and over time, the regurgitation of gastric juice associated with NERD can have significant and comparable negative effects on their quality of life that correlate with heartburn severity<sup>[7-9]</sup>. To improve these patients' quality of life, provide a rapid relief of symptoms and reduce the severity and number of recurrent episodes<sup>[10-12]</sup>, acid-suppressive drugs have been used to combat NERD.

Proton pump inhibitors (PPI) are a type of acid-suppressive drugs that inhibit the secretion of gastric acid by restraining the exchange of  $H^+-K^+$ <sup>[13,14]</sup>. Due to their powerful inhibition of the secretion of gastric acid, PPIs have been widely used to treat gastroesophageal diseases that result from too much acid, including gastroesophageal reflux disease, gastritis and gastric and duodenal ulcers<sup>[15-18]</sup>. However, the efficacy, safety and influential factors of PPI use remain inconclusive, especially for NERD<sup>[19,20]</sup>.

Although two papers<sup>[21,22]</sup> have previously discussed the efficacy and influential factors of PPI use against NERD, neither paper used randomized controlled trials (RCTs) as the source of their data or used  $H_2$  receptor antagonists ( $H_2RA$ ) or placebos as control groups. Meanwhile, the clinical safety of PPIs was not addressed by the authors of these two papers. In view of the importance of understanding their clinical implications, we determined that the quality of the previous two papers was insufficient and performed the present meta-analysis.

## MATERIALS AND METHODS

### Search strategy

We conducted a computer-aided search for RCTs which probed into the efficacy, safety and influential factors of PPI for NERD. Source databases were PubMed (1966 to April 2013), the Cochrane Library (1997 to April 2013), MEDLINE (1966 to April 2013) and EMBASE (1985 to April 2013). The medical subject headings which were used in retrieving citation were: non-erosive reflux disease or NERD, proton pump inhibitors or PPI or esomeprazole or pantoprazole or omeprazole or rabeprazole or lansoprazole. We also searched the references in retrieved articles manually in order to prevent missing relevant

publications.

### Study selection

The titles and abstracts were independently screened by two reviewers (Zhang JX and Song J), and studies were chosen for the meta-analysis if they fit the following criteria: (1) randomized controlled trials; (2) comparing PPI with other acid-suppressive drugs or placebo; and (3) probing into the efficacy, safety and influential factors of PPI on the symptomatic relief of NERD. We did not consider the restriction on language of publication. Exclusion criteria were: (1) no human subjects in the study; (2) without control group; (3) comparing a PPI with another one; (4) incomplete outcome data; (5) selective reporting; and (6) duplicate publication.

### Data extraction and quality assessment

Independently, three reviewers (Qiu S, Ai MH and Wang J) extracted data including the following items: first author, year of publication, country, type of publication, study duration, age, gender, medication duration, drug dose, follow-up time, methods of treatment, *H. pylori* infection and primary outcomes. Based on the adequate sequence generation, allocation concealment, blinding, incomplete outcome data addressed, free of selective reporting, free from baseline imbalance, sample size calculation and free from sources of funding bias, the risk of bias was evaluated in detail. Each quality component was judged as high, unclear, or low. On the basis of each separate component, the quality of the trials was assessed. When difference appeared, a forth reviewer (Lei HB) joined in the discussion.

### Statistical analysis

We treated the rates of symptomatic relief of PPI *vs* placebo and PPI *vs*  $H_2RA$  as the primary endpoints and the rates of adverse events as the secondary endpoints. Meanwhile, factors influencing rates of symptomatic relief and adverse events of PPI against NERD were analyzed. The RRs, to summary statistics in meta-analysis, were strongly recommended for dichotomous data. So we used Stata12.0 to calculate RR for the rates of symptomatic relief and the rates of adverse events in this meta-analysis. When the *P* value was less than 0.05, it was considered significant. The data was pooled according to the Mantel-Haenszel fixed-effects model and the DerSimonian and Laird random-effects model. The differences were shown as pooled RRs and 95%CI between different groups. The statistical heterogeneity among trials was assessed by the  $\chi^2$  test and  $I^2$  test. The percentage of the variability in the estimates of effect, caused by heterogeneity but not chance, was described by  $I^2$  test. When the values were greater than 50%, it was considered having substantial heterogeneity. If there was no statistically significant heterogeneity, the fixed-effects model was chosen. According to the drug dose and therapeutic duration, subgroup analysis was performed.

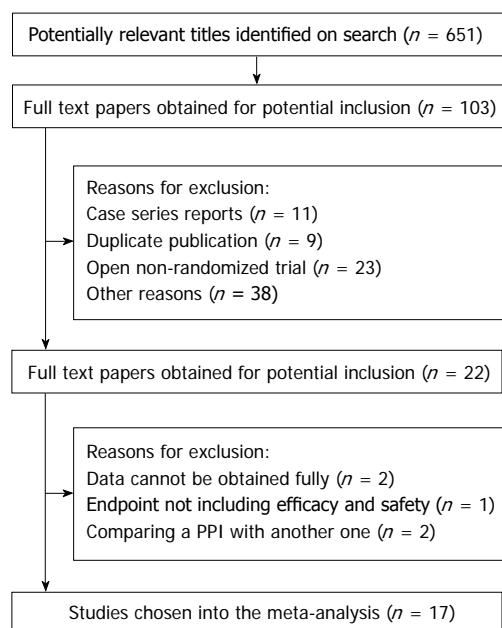


Figure 1 Screening process of studies. PPI: Proton pump inhibitor.

### Risk of bias and publication bias

We assessed the risks of bias according to assessment of study quality in Cochrane Handbook 4.2.2. Egger's test and Begg's test were used to check the publication bias, and  $P < 0.05$  indicated that there was a risk of bias.

### Sensitivity analysis

Sensitivity analysis was performed to identify the studies which influence the result obviously.

### Meta-regression analysis

Meta-regression analysis was performed to study the relationship between covariates and the outcomes and to find the source of heterogeneity.

### Assessment of quality evidence

Grade system was applied to assess the quality of these outcomes.

## RESULTS

### Study identification and selection

Following the searching strategy, we initially acquired 651 studies. Having discarded the studies of repetitive publication and that did not meet the criteria apparently, and after reading the titles and abstracts, there were 51 studies left. To search and read the full text, 34 studies which were not RCTs or without control groups were abandoned and 17 RCTs<sup>[23-39]</sup> were left finally. The screening process of studies is shown in Figure 1.

### Study characteristics

For the 17 RCTs, 5 were single-center studies and 12 were multi-center studies. In the RCTs, there were 6072 patients with 3937 patients in the combination group and

the 2135 patients in PPI alone group. The details of these studies are listed in Table 1.

### PPI vs H<sub>2</sub>RA on the rate of symptomatic relief

Seven studies<sup>[24,26,29,30,32,34,39]</sup>, which involved 1882 patients, compared PPI with H<sub>2</sub>RA on the rate of symptomatic relief of NERD. There are 935 patients who received PPI and 947 patients who received H<sub>2</sub>RA. Heterogeneity analysis showed that there was obviously statistical heterogeneity among these studies ( $I^2 = 42.4\%$ ,  $P = 0.096$ ). Sensitivity analysis indicated that one study<sup>[32]</sup> influenced the result apparently, and after excluding this study, the heterogeneity disappeared ( $I^2 = 0.1\%$ ,  $P = 0.422$ ). The result showed that PPI was significantly superior to H<sub>2</sub>RA on the rate of symptomatic relief of NERD (RR = 1.629, 95%CI: 1.422-1.867,  $P = 0.000$ ), (Figure 2A).

In the subgroup analysis of short duration (PPI 158/372, placebo 112/384,  $I^2 = 0\%$ ,  $P = 0.640$ ), PPI advanced over H<sub>2</sub>RA (RR = 1.521, 95%CI: 1.303-1.775,  $P = 0.000$ ). In the subgroup analysis of long duration (PPI 90/186, placebo 43/184,  $I^2 = 0\%$ ,  $P = 0.737$ ), similar result was found (RR = 2.063, 95%CI: 1.544-2.756,  $P = 0.000$ ).

In the subgroup analysis of low dose (PPI 130/308, placebo 78/307,  $I^2 = 0\%$ ,  $P = 0.422$ ), PPI significantly overmatched H<sub>2</sub>RA (RR = 1.656, 95%CI: 1.320-2.078,  $P = 0.000$ ). In the subgroup analysis of high dose (PPI 220/526, placebo 141/537,  $I^2 = 23.5\%$ ,  $P = 0.365$ ), PPI was also superior to H<sub>2</sub>RA (RR = 1.614, 95%CI: 1.361-1.914,  $P = 0.000$ ).

In the subgroup analysis of lansoprazole (PPI 227/585, placebo 141/584,  $I^2 = 0\%$ ,  $P = 0.603$ ), PPI advanced over H<sub>2</sub>RA (RR = 1.866, 95%CI: 1.435-2.448,  $P = 0.000$ ). But compared with groups of omeprazole (PPI 41/67, placebo 31/64,  $I^2 = 0\%$ ,  $P = 0.434$ ), there were no statistical differences ( $P = 0.149$ ).

### PPI vs placebo on the rate of symptomatic relief

There were 11 studies<sup>[23,25,27,28,31,33-38]</sup> which compared PPI with placebo on the rate of symptomatic relief of NERD. In the 5416 patients of the 11 trials, there are 3287 patients who received PPI and 2129 patients received placebo. Heterogeneity analysis showed that there was obviously statistical heterogeneity among these studies ( $I^2 = 84.3\%$ ,  $P = 0.000$ ). Sensitivity analysis did not find studies that influenced the result obviously. The result showed that PPI was significantly superior to placebo on the rate of symptomatic relief of NERD (RR = 1.903, 95%CI: 1.573-2.302,  $P = 0.000$ ), (Figure 2B).

In the subgroup analysis of long duration (PPI 407/855, placebo 114/315,  $I^2 = 65.4\%$ ,  $P = 0.034$ ), PPI advanced over placebo (RR = 1.442, 95%CI: 1.034-2.010,  $P = 0.031$ ). In short duration (PPI 1139/2432, placebo 459/1241,  $I^2 = 78.6\%$ ,  $P = 0.000$ ), similar result was also found (RR = 2.029, 95%CI: 1.665-2.473,  $P = 0.000$ ).

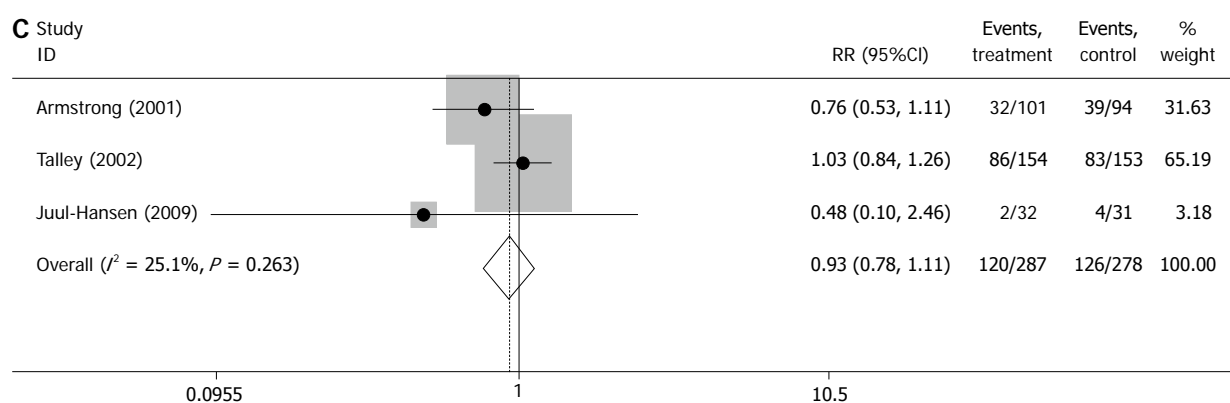
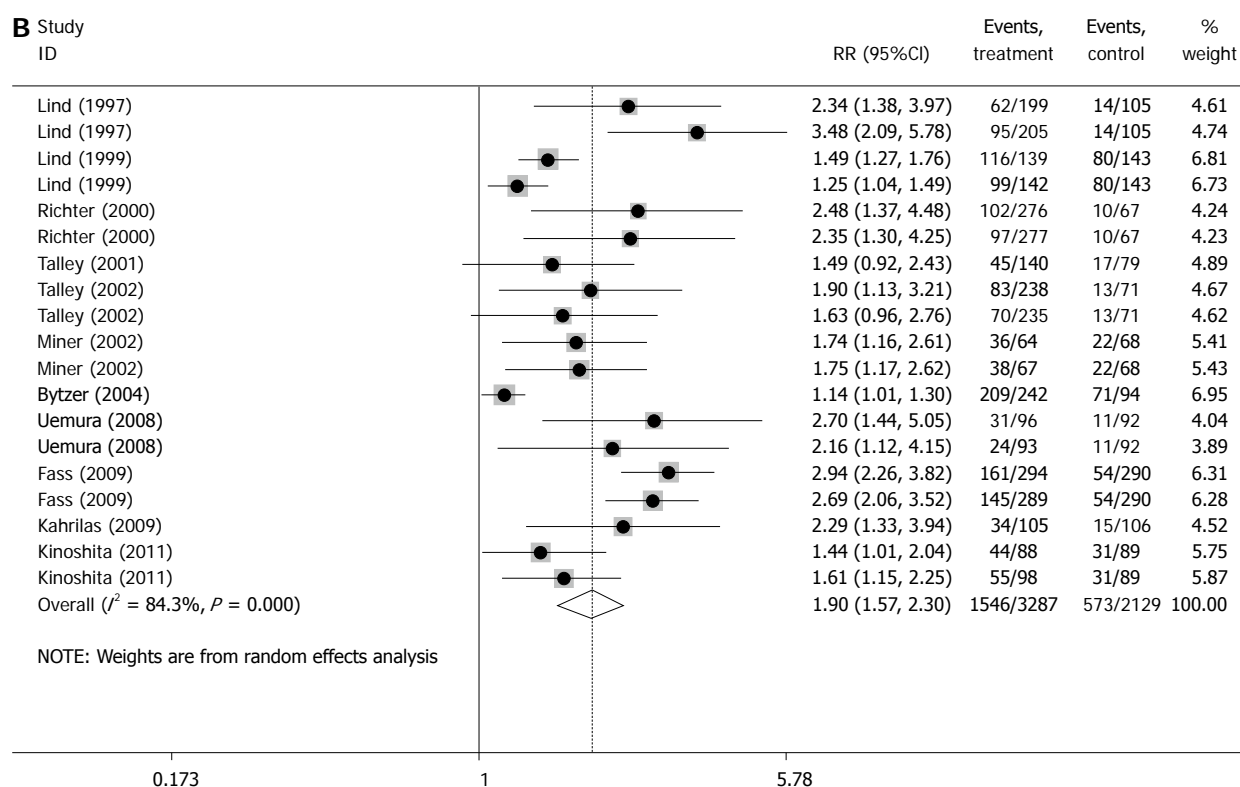
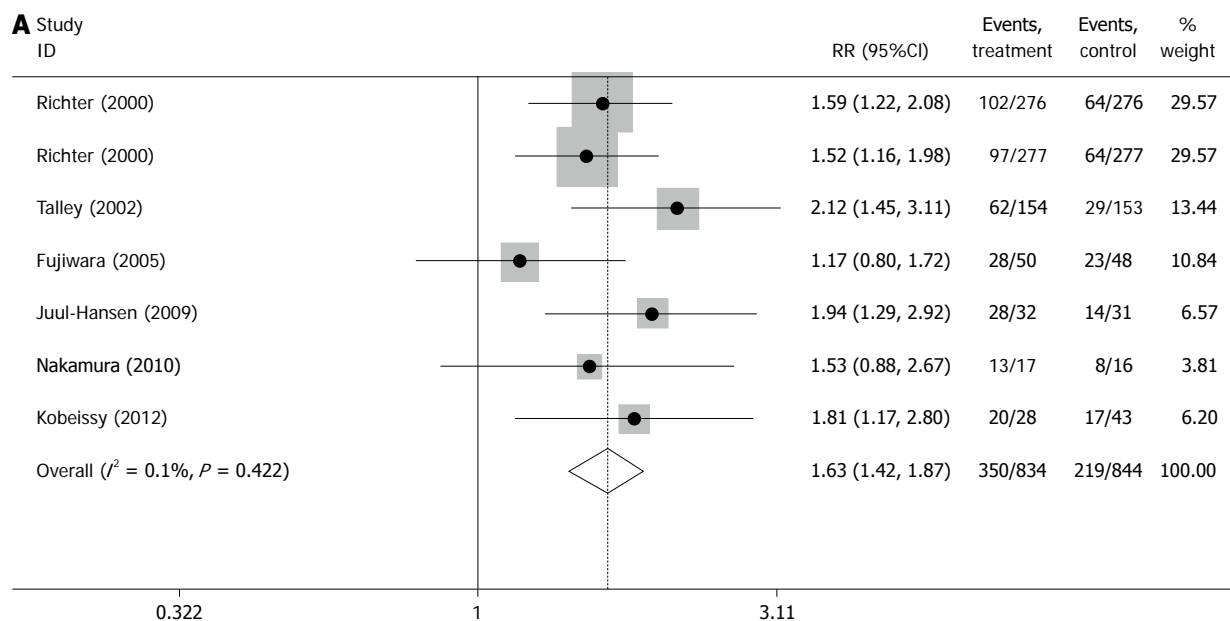
In the subgroup analysis of high dose (PPI 486/1098, placebo 131/718,  $I^2 = 0\%$ ,  $P = 0.506$ ), PPI significantly overmatched placebo (RR = 2.664, 95%CI: 2.251-3.154,

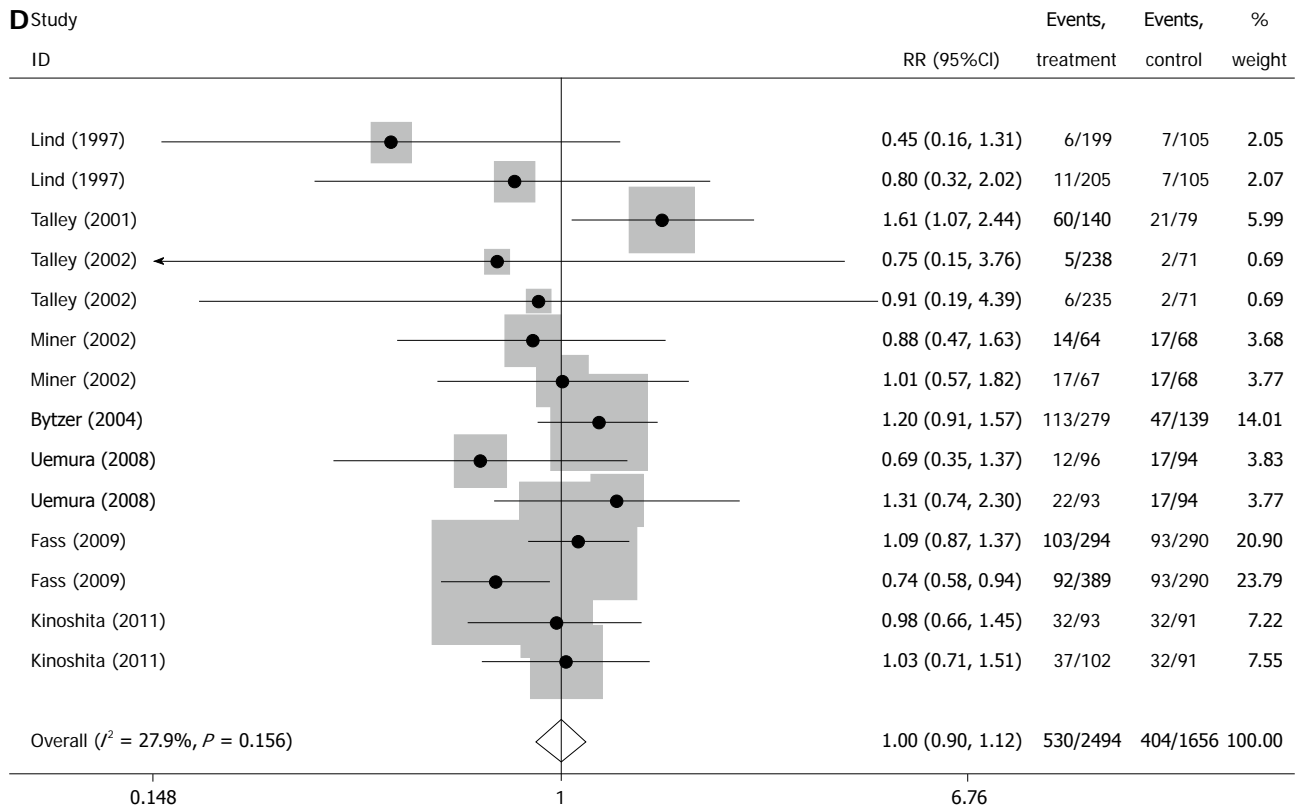
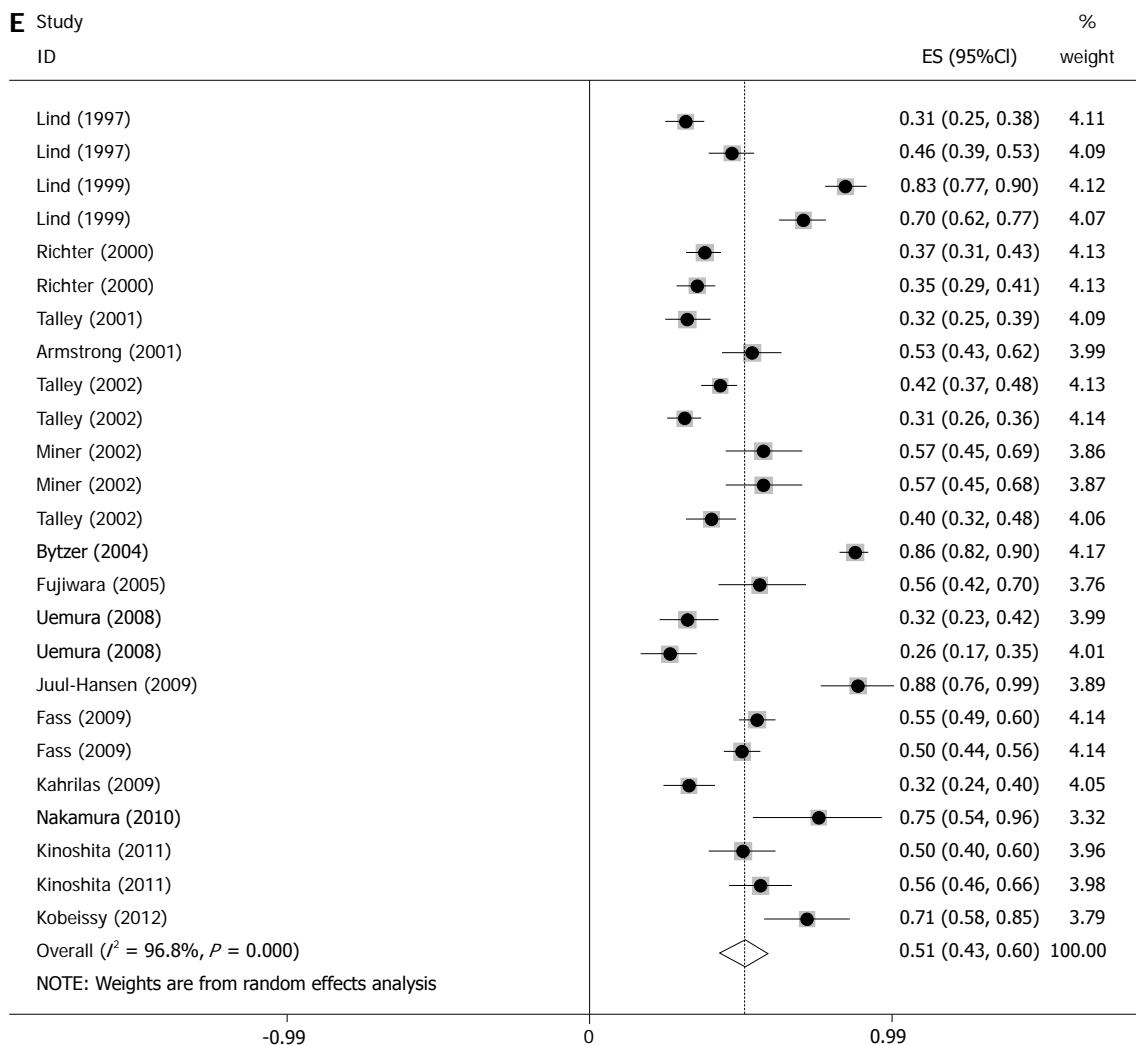


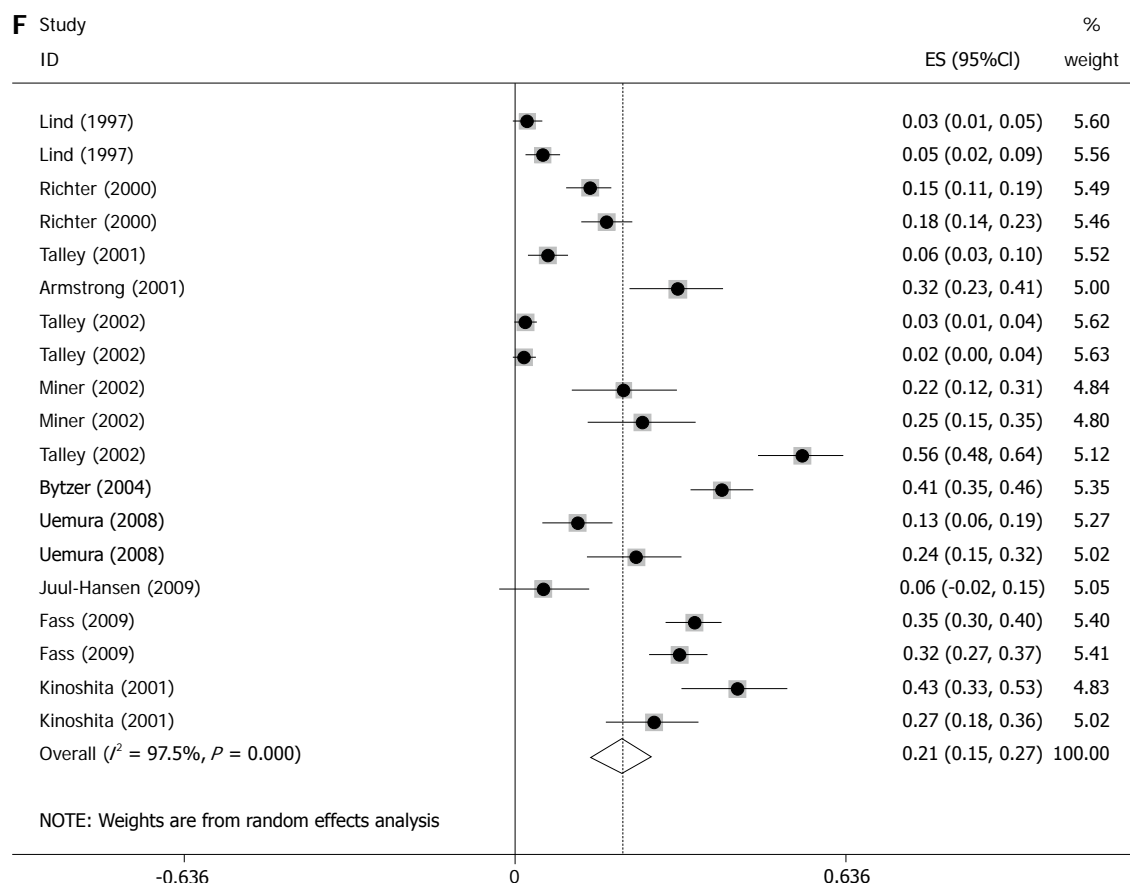
Table 1 Details of these studies *n* (%)

First author	Country	Year	Study design	Arms of treatment	Age (yr)	Gender (M/F)	<i>n</i>	BMI	<i>Helicobacter pylori</i> infection	Hiatal hernia	Smokers	Alcohol users	Therapeutic duration	Adverse events	Effective
Talley <i>et al</i> <sup>[21]</sup>	United Kingdom	2002	RCT	Esomeprazole 20 mg Esomeprazole 40 mg placebo	48.0 48.4 48.2	135/158 135/147 58/88	293 282 146	- - -	90 101 57	89 92 39	- - -	-	6 mo	5 6 2	101 (34.6) 84 (29.7) 30 (17.8)
Juul-Hansen <i>et al</i> <sup>[24]</sup>	Norway	2009	RCT	Lansoprazole 15 mg Ranitidine 75 mg	47.5 48.0	11/21 12/19	32 31	26.4 25.2	6 6	10 6	8 8	-	6 mo	2 4	28 (87.5) 14 (45.2)
Kinoshita <i>et al</i> <sup>[25]</sup>	Japan	2011	RCT	Rabeprazole 5 mg Rabeprazole 10 mg placebo	46.3 46.8 49.7	38/55 50/51 40/51	93 101 91	22.4 23.2 23.2	35 42 44	- - -	- - -	-	4 wk	32 37 32	36 (35.0) 44 (44.0) 19 (21.0)
Kobeissy <i>et al</i> <sup>[26]</sup>	Lebanon	2012	RCT	Rabeprazole 20 mg Ranitidine 300 mg	45.4 45.1	16/28 16/23	44 39	- -	- -	- -	- -	-	4 wk	- -	- 17 (43.6)
Talley <i>et al</i> <sup>[27]</sup>	Australia	2001	RCT	Esomeprazole 20 mg placebo	49.0 49.0	94/76 98/74	170 172	- -	64 57	65 75	- -	-	6 mo	11 13	146 (85.0) 83 (48.0)
Fass <i>et al</i> <sup>[28]</sup>	United States	2009	RCT	Dexlansoprazole 30 mg Dexlansoprazole 60 mg placebo	47.6 47.5 47.6	84/231 106/209 84/233	315 315 317	29.0 29.6 29.1	95 90 89	- - -	72 57 52	162 181 182	4 wk	6 8 1	173 (54.9) 157 (50.0) 59 (18.5)
Fujiwara <i>et al</i> <sup>[29]</sup>	Japan	2005	RCT	Omeprazole 20 mg Famotidine 20 mg	55.0 56.6	20/30 23/25	50 48	22.8 22.2	22 22	14 11	- -	18 11	4 wk	- -	- 36 (75.0)
Nakamura <i>et al</i> <sup>[30]</sup>	Japan	2010	RCT	Omeprazole 20 mg Roxitidine 75 mg	51.4 48.6	8/9 9/7	17 16	- -	5 7	9 8	- -	-	8 wk	0 0	13 (75.0) 11 (70.6)
Miner <i>et al</i> <sup>[31]</sup>	United States	2002	RCT	Rabeprazole 10 mg Rabeprazole 20 mg placebo	44.4 45.5 46.1	28/37 25/33 24/26	65 68 70	30.9 30.3 30.2	17 24 28	- - -	22 21 22	29 26 30	4 wk	14 17 18	19 (29.3) 19 (28.3) 2 (3.4)
Armstrong <i>et al</i> <sup>[32]</sup>	Canada	2001	RCT	Pantoprazole 40 mg Nizatidine 150 mg	47.1 47.6	57/49 51/51	106 102	- -	16 20	- -	66 64	71 67	4 wk	32 39	41 (52.8) 33 (42.9)
Lind <i>et al</i> <sup>[33]</sup>	Sweden	1999	RCT	Omeprazole 10 mg Omeprazole 20 mg placebo	52.0 51.0 48.0	53/86 65/77 61/82	139 142 143	- - -	- - -	76 72 74	24 43 57	88 85 97	4 wk	- - -	116 (83.5) 99 (69.7) 80 (55.9)
Richter <i>et al</i> <sup>[34]</sup>	United States	2000	RCT	Lansoprazole 15 mg Lansoprazole 30 mg Ranitidine 150 mg placebo	44.9 45.1 45.2 46.3	115/161 122/155 108/170 30/37	276 277 278 67	- - - -	66 66 63 9	- - -	75 88 69 19	132 129 126 35	8 wk	- - -	37 (13.4) 97 (35.0) 64 (23.0) 10 (14.7)
Bytzer <i>et al</i> <sup>[12]</sup>	Denmark	2004	RCT	Rabeprazole 10 mg placebo	47.0 48.0	123/156 57/82	279 139	26.0 27.0	100 52	- -	- -	-	6 mo	113 47	241 (86.4) 94 (67.6)
Kahrilas <i>et al</i> <sup>[35]</sup>	United States	2009	RCT	Rabeprazole 20 mg placebo	43.1 44.0	40/89 45/87	129 132	29.3 30.0	42 40	- -	35 36	58 51	4 wk	- -	42 (32.4) 5 (3.8)
Lind <i>et al</i> <sup>[33]</sup>	Sweden	1997	RCT	Omeprazole 10 mg Omeprazole 20 mg placebo	49.0 50.0 51.0	89/110 66/139 51/54	199 205 105	- - -	- - -	- -	60 46 22	133 121 64	4 wk	6 11 7	98 (49.2) 125 (61.0) 25 (23.8)
Uemura <i>et al</i> <sup>[36]</sup>	Japan	2008	RCT	Omeprazole 10 mg Omeprazole 20 mg placebo	44.4 43.8 42.4	47/49 53/40 53/49	96 93 92	- - -	37 49 32	5 9 2	- -	-	4 wk	12 22 17	44 (45.8) 43 (46.2) 22 (23.9)
Talley <i>et al</i> <sup>[27]</sup>	Australia	2002	RCT	Pantoprazole 20 mg Ranitidine 150 mg	53.0 52.0	78/76 83/70	154 153	28.9 28.4	- -	- -	65 61	28 30	6 mo	86 83	109 (71.0) 86 (56.0)

RCT: Randomized controlled trials; M: Male; F: Female; BMI: Body mass index.



**D** Study**E** Study



**Figure 2 Forest plot.** A: Comparison of proton pump inhibitor (PPI) vs H<sub>2</sub> receptor antagonists (H<sub>2</sub>RA) on the rate of symptomatic relief; B: Comparison of PPI vs placebo on the rate of symptomatic relief; C: Comparison of PPI vs H<sub>2</sub>RA on the rate of adverse events; D: Comparison of PPI vs placebo on the rate of adverse events; E: Overall efficacy of PPI against non-erosive reflux disease (NERD); F: Adverse rate of PPI against NERD.

$P = 0.000$ ). In low dose (PPI 1060/2189, placebo 442/1411,  $I^2 = 75.1\%$ ,  $P = 0.000$ ), PPI was significantly superior to placebo (RR = 1.726; 95%CI: 1.451-2.054,  $P = 0.000$ ).

In the subgroup analysis of lansoprazole (PPI 505/1136, placebo 128/714,  $I^2 = 0\%$ ,  $P = 0.879$ ), pantoprazole (PPI 252/649, placebo 88/320,  $I^2 = 0\%$ ,  $P = 0.844$ ), omeprazole (PPI 427/874, placebo 210/680,  $I^2 = 81.4\%$ ,  $P = 0.000$ ) and rabeprazole (PPI 317/478, placebo 130/336,  $I^2 = 81.3\%$ ,  $P = 0.001$ ), PPI advanced over placebo ( $P = 0.000$ ).

#### PPI vs H<sub>2</sub>RA on the rate of adverse events

Three studies<sup>[24,32,39]</sup>, which involved 565 patients, compared PPI with H<sub>2</sub>RA on the rate of adverse events of NERD. There were 287 patients who received PPI and 278 patients who received H<sub>2</sub>RA. Because there was no obviously statistical heterogeneity among these studies ( $I^2 = 25.1\%$ ,  $P = 0.263$ ), fixed-effects model was chosen to perform the meta-analysis. The result showed that there was no significantly difference between PPI and H<sub>2</sub>RA on the rate of adverse events of NERD (RR = 0.928; 95%CI: 0.776-1.110,  $P = 0.414$ , Figure 2C).

#### PPI vs placebo on the rate of adverse events

There were eight studies<sup>[23,25,27,28,31,36]</sup> which compared PPI

with placebo on the rate of adverse events of NERD. Among the 4150 patients, 2494 patients received PPI and 1656 patients received placebo. Because there was no obviously statistical heterogeneity among these studies ( $I^2 = 27.9\%$ ,  $P = 0.156$ ), fixed-effects model was chosen to perform the meta-analysis. The result showed that there was no significant difference between PPI and placebo on the rate of adverse events of NERD (RR = 1.000; 95%CI: 0.896-1.116,  $P = 0.997$ ), (Figure 2D).

In the subgroup analysis of long duration (PPI 184/892, placebo 72/360,  $I^2 = 8.5\%$ ,  $P = 0.364$ ), there was no significant difference between PPI and placebo (RR = 0.921, 95%CI: 0.812-1.046,  $P = 0.206$ ). In short duration (PPI 346/1602, placebo 332/1296,  $I^2 = 0\%$ ,  $P = 0.565$ ), PPI was significantly superior to placebo (RR = 1.290, 95%CI: 1.032-1.613,  $P = 0.025$ ).

In the subgroup analysis of high dose (PPI 316/1661, placebo 252/1068,  $I^2 = 45.6\%$ ,  $P = 0.075$ ), there was no obvious difference between PPI and placebo (RR = 0.999, 95%CI: 0.868-1.150,  $P = 0.988$ ). In low dose (PPI 214/833, placebo 152/588,  $I^2 = 2.9\%$ ,  $P = 0.398$ ), no significant difference was found either (OR = 1.002, 95%CI: 0.841-1.195,  $P = 0.979$ ).

In the subgroup analysis of lansoprazole (PPI 308/962, placebo 233/719,  $I^2 = 75.4\%$ ,  $P = 0.017$ ), pantoprazole (PPI 80/668, placebo 68/224,  $I^2 = 0\%$ ,  $P =$



**Table 2 Results of univariate meta-regression analysis exploring factors influencing efficacy and adverse rate of proton pump inhibitor for non-erosive reflux disease**

Factors	Efficacy		Adverse rate	
	Coefficient	P value	Coefficient	P value
Age	0.0177315	0.170	0.0075041	0.665
Gender	-0.2213605	0.186	0.0209630	0.894
BMI	-0.0127987	0.484	-0.0044024	0.808
n	-0.0005643	0.154	-0.0001338	0.733
<i>Helicobacter pylori</i> infection	-0.6007750	0.217	0.1326016	0.736
Hiatal hernia	0.9702707	0.030	-0.4392244	0.081
Smoking	-0.2591453	0.528	0.5030517	0.245
Drinking	0.3296374	0.303	-0.6776039	0.053
Therapeutic duration	0.0008200	0.857	-0.0015192	0.722
Dose	-0.0026090	0.414	0.0003684	0.897

BMI: Body mass index.

0.981), omeprazole (PPI 51/593, placebo 48/398,  $I^2 = 23.5\%$ ,  $P = 0.270$ ) and rabeprazole (PPI 31/131, placebo 34/136,  $I^2 = 0\%$ ,  $P = 0.732$ ), the significant difference was also not found ( $P > 0.05$ ).

#### Overall efficacy of PPI against NERD and its influential factors

All the 17 studies<sup>[23-39]</sup> provided the data of the efficacy of PPI against NERD and its influential factors. Heterogeneity analysis showed that there was obviously statistical heterogeneity among these studies ( $I^2 = 96.8\%$ ,  $P = 0.000$ ). The result showed that the overall rate of symptomatic relief of PPI against NERD was 51.4% (95%CI: 0.433-0.595,  $P = 0.000$ ), (Figure 2E).

In the subgroup analysis of long duration, the effective rate of PPI against NERD was 51.4% (95%CI: 0.433-0.595,  $P = 0.000$ ). In short duration, the rate was 51.5% (95%CI: 0.432-0.598,  $P = 0.000$ ).

In the subgroup analysis of high dose, the effective rate of PPI against NERD was 48.4% (95%CI: 0.404-0.564,  $P = 0.000$ ). In low dose, the rate was 56.3% (95%CI: 0.395-0.732,  $P = 0.000$ ).

In the subgroup analysis of lansoprazole, the effective rate of PPI against NERD was 52.1% (95%CI: 0.392-0.650,  $P = 0.000$ ). In that of pantoprazole, omeprazole and rabeprazole, the effective rate were 44.7% (95%CI: 0.369-0.526,  $P = 0.000$ ), 52.1% (95%CI: 0.355-0.688,  $P = 0.000$ ) and 60.8% (95%CI: 0.367-0.849,  $P = 0.000$ ), respectively.

Univariate meta-regression analysis found that the rate of hiatal hernia ( $P = 0.030$ ) was associated with the rate of symptomatic relief of PPI against NERD, but not with others.

#### Overall safety of PPI against NERD and its influential factors

Twelve studies<sup>[23-25,27,28,31,32,34,37-39]</sup> provided the data of the rate of adverse events of PPI against NERD and its influential factors. Heterogeneity analysis showed that there

was obviously statistical heterogeneity among these studies ( $I^2 = 97.5\%$ ,  $P = 0.000$ ). Sensitivity analysis indicated that no study influenced the result apparently. The result showed that the adverse rate of PPI against NERD was 21.0% (95%CI: 0.152-0.208,  $P = 0.000$ ), (Figure 2F).

In the subgroup analysis of long duration, the adverse rate of PPI against NERD was 18.0% (95%CI: 0.094-0.265,  $P = 0.000$ ). In short duration, the rate was 23.3% (95%CI: 0.145-0.322,  $P = 0.000$ ).

In the subgroup analysis of high dose, the adverse rate of PPI against NERD was 21.1% (95%CI: 0.152-0.268,  $P = 0.000$ ). In low dose, the rate was 20.8% (95%CI: 0.100-0.317,  $P = 0.000$ ).

In the subgroup analysis of lansoprazole, the adverse rate of PPI against NERD was 21.5% (95%CI: 0.121-0.309,  $P = 0.000$ ). In that of pantoprazole, omeprazole and rabeprazole, the effective rate respectively were 26.2% (95%CI: 0.150-0.375,  $P = 0.000$ ), 9.8% (95%CI: 0.036-0.161,  $P = 0.002$ ) and 29.5% (95%CI: 0.165-0.426,  $P = 0.000$ ).

Univariate meta-regression analysis found that the rate of hiatal hernia ( $P = 0.081$ ) and drinking ( $P = 0.053$ ) were associated with the rate of adverse events of PPI against NERD, but not with the other factors (Table 2).

#### Sensitivity analysis

In the analysis of PPI *vs* H<sub>2</sub>RA on the rate of symptomatic relief, sensitivity analysis indicated that one study<sup>[32]</sup> influenced the result apparently, and after excluding the this study, the heterogeneity disappeared ( $I^2 = 0.1\%$ ,  $P = 0.422$ ). And in other analysis, there was no study which influenced the results.

#### Risk of bias and publication bias

Three studies<sup>[26,28,32]</sup> performed adequate sequence generation with the others unclear. No study carried out allocation concealment. Two studies<sup>[24,26]</sup> were open-label trials without blinding of participants and personnel and 11 studies<sup>[23,25,27,28,31-36,39]</sup> mentioned blinding of participants and personnel. All the studies had complete data, without selective reporting and other bias. According to the Egger's test and Begg's test, we did not find obvious publication bias in the outcome of PPI *vs* H<sub>2</sub>RA on the rate of symptomatic relief (Egger's test:  $P = 0.711$  and Begg's test:  $P = 0.646$ ), PPI *vs* H<sub>2</sub>RA on the rate of adverse events (Egger's test:  $P = 1.000$  and Begg's test:  $P = 0.374$ ) and PPI *vs* placebo on the rate of adverse events (Egger's test:  $P = 0.125$  and Begg's test:  $P = 0.552$ ). But in the outcome of PPI *vs* placebo on the rate of symptomatic relief, the potential publication bias may exist (Egger's test:  $P = 0.010$  and Begg's test:  $P = 0.013$ ). A language bias, inflated estimates by a flawed methodologic design in smaller studies, and/or a lack of publication of small trials with opposite results may be the causes.

#### Quality of evidence

Following the classification of the Grading of Recommendations Assessment, Development and Evaluation,

**Table 3** Quality of outcomes according to Grade system

Outcome	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Quality of evidence
PPI vs H <sub>2</sub> RA on the rate of symptomatic relief	RCT	Serious <sup>1</sup>	No	No	No	Serious <sup>4</sup>	Low
Long-duration subgroup	RCT	Serious <sup>2</sup>	No	No	No	Serious <sup>4</sup>	Low
Short-duration subgroup	RCT	Serious <sup>2</sup>	No	No	No	Serious <sup>4</sup>	Low
High-dose subgroup	RCT	Serious <sup>2</sup>	No	No	No	Serious <sup>4</sup>	Low
Low-dose subgroup	RCT	Serious <sup>2</sup>	No	No	No	Serious <sup>4</sup>	Low
PPI vs placebo on the rate of symptomatic relief	RCT	No	Series <sup>3</sup>	No	No	No	Moderate
Long-duration subgroup	RCT	No	Series <sup>3</sup>	No	No	No	Moderate
Short-duration subgroup	RCT	No	No	No	No	No	High
High-dose subgroup	RCT	No	No	No	No	No	High
Low-dose subgroup	RCT	No	Series <sup>3</sup>	No	No	No	Moderate
PPI vs H <sub>2</sub> RA on the rate of adverse events	RCT	Serious <sup>2</sup>	No	No	No	Serious <sup>4</sup>	Low
PPI vs placebo on the rate of adverse events	RCT	No	Series <sup>3</sup>	No	No	No	Moderate
Long-duration subgroup	RCT	No	No	No	No	No	High
Short-duration subgroup	RCT	No	Series <sup>3</sup>	No	No	No	Moderate
High-dose subgroup	RCT	No	Series <sup>3</sup>	No	No	No	Moderate
Low-dose subgroup	RCT	No	No	No	No	No	High
Overall efficacy of PPI against NERD	RCT	Serious <sup>1</sup>	Series <sup>3</sup>	No	No	No	Low
Long-duration subgroup	RCT	Serious <sup>2</sup>	Series <sup>3</sup>	No	No	No	Low
Short-duration subgroup	RCT	Serious <sup>2</sup>	No	No	No	No	Moderate
High-dose subgroup	RCT	Serious <sup>2</sup>	Series <sup>3</sup>	No	No	No	Low
Low-dose subgroup	RCT	Serious <sup>2</sup>	No	No	No	No	Moderate
Overall safety of PPI against NERD	RCT	Serious <sup>2</sup>	Series <sup>3</sup>	No	No	Serious <sup>4</sup>	Low
Long-duration subgroup	RCT	Serious <sup>2</sup>	Series <sup>3</sup>	No	No	Serious <sup>4</sup>	Low
Short-duration subgroup	RCT	No	No	No	No	Serious <sup>4</sup>	High
High-dose subgroup	RCT	No	Series <sup>3</sup>	No	No	Serious <sup>4</sup>	Moderate
Low-dose subgroup	RCT	Serious <sup>2</sup>	No	No	No	Serious <sup>4</sup>	Moderate

<sup>1</sup>Allocation concealment and blind method were not offered which resulted in very serious bias; <sup>2</sup>Allocation concealment and blind method were not offered which resulted in very serious bias mild bias; <sup>3</sup>The assessing standard of outcomes maybe contribute to the heterogeneity; <sup>4</sup>Publication bias may be existed. RCT: Randomized controlled trials; NERD: Non-erosive reflux disease; PPI: Proton pump inhibitor; H<sub>2</sub>RA: H<sub>2</sub> receptor antagonists.

the quality of evidences and their causes are shown in Table 3.

## DISCUSSION

PPIs have been widely used to treat NERD, but their efficacy, safety and influential factors are unclear. Our meta-analysis, including 17 well-designed randomized controlled trials, 12 of which were multi-center and 5 of which were single-center, had systematically and comprehensively evaluated the evidence concerning the efficacy, safety and influential factors of PPIs against NERD.

The first major finding revealed by this comprehensive approach was that the activity of PPIs is obviously superior to that of H<sub>2</sub>RA in its efficacy and safety against NERD. Because heartburn, the main symptom of patients with NERD, results from erosion due to gastric acid reflux into the esophagus, acid-suppressive drugs, including PPI and H<sub>2</sub>RA, have been deemed effective treatments for NERD<sup>[40,41]</sup>. After a meal, gastrin secretion stimulates the release of histamine by enterochromaffin-like cells, which binds to histamine H<sub>2</sub> receptors, leading to acid release via the hydrogen potassium ATPase (H<sup>+</sup>-K<sup>+</sup>-ATPase) pump<sup>[42]</sup>. Compared to the mechanism of H<sub>2</sub>RA, which acts against one of the three histamine-H<sub>2</sub>

receptors, PPI acts against the H<sup>+</sup>-K<sup>+</sup>-ATPase<sup>[43]</sup>. To control for the influences of different dose and therapeutic duration, we performed a subgroup analysis. This analysis showed that PPI treatment against NERD was superior to H<sub>2</sub>RA and placebo regardless of the dose or duration. However, only after short durations was PPI treatment safer than placebo.

The second major finding of this meta-analysis was that the overall rate of symptomatic relief of PPI against NERD was 51.4%; this value was influenced by the presence of a hiatal hernia. Compared with the approximate 50% symptomatic relief rate of PPI against ERD<sup>[44,45]</sup>, the 51.4% rate of PPI against NERD is fairly high. PPIs with a high dose, long duration and from a new generation should be more effective than those with a low dose, short duration and from an older generation; however, according to our subgroup analysis, there were no obvious differences among different doses, durations and PPI types. PPI enacts its role by binding to the binding sites of the saturable enzyme H<sup>+</sup>-K<sup>+</sup>-ATPase; therefore, an excessively high blood concentration of PPI is not only unable to increase but even decreases the acid suppression effect of the enzyme. Univariate meta-regression analysis found that the rate of hiatal hernia was associated with the rate of the symptomatic relief of PPI use

against NERD. One role of the gastroesophageal junction is to minimize gastroesophageal reflux; hiatal hernias, which are protrusions (or herniations) of the upper part of the stomach into the thorax through a tear or weakness in the diaphragm, can cause reflux and reduce the clear effects of the esophagus<sup>[46]</sup>. Due to their effects on gastroesophageal reflux and the normal function of the esophagus, the presence of hiatal hernias may influence the symptomatic relief rate of PPIs against NERD.

The third major finding of this meta-analysis was that the adverse rate of PPI treatment against NERD was 21.0%; this value was affected by hiatal hernia and drinking. PPI use was not, however, without shortcomings. Primary adverse events, typically in the order of 1%-5%, included headache, diarrhea, constipation, nausea, and rash<sup>[47]</sup>. Long-term PPI use was able to cause diminished acid secretion and reduced somatostatin release, resulting in enterochromaffin-like cell hyperplasia and hypergastrinemia<sup>[48,49]</sup>. As indicated by univariate meta-regression analysis, the adverse rate of PPI use for NERD was influenced by hiatal hernia and drinking. The mechanism through which hiatal hernia influences the adverse rate of PPI for NERD is uncertain, but the reason might be that hiatal hernias cause reflux, stimulating the nausea-inducing receptors in the esophageal and throat, as well as other adverse events. In addition, the metabolism of PPI generates two different CYP isoforms in the liver, which are responsible for the majority of their biotransformation due to their susceptibility to ethyl alcohol (CYP2C19 and CYP3A4)<sup>[50-52]</sup>. Thus, as drinking increases the blood concentration of ethyl alcohol, adverse events due to the reduced biotransformation of CYP2C19 and CYP3A4 and an increased blood concentration of PPI may arise.

There are a few shortcomings in our meta-analysis that should be mentioned. First, the analytical results are influenced by the reviewers, although we attempted to overcome this drawback. Second, a few differences may exist due to the various assessments of the efficacy and safety of PPI against NERD. Third, the evaluation index resulted from subjective feelings, which may influence the authenticity of these studies.

In conclusion, our meta-analysis showed that PPI is more effective than H<sub>2</sub>RA or placebo for the treatment of NERD. However, there was no significant difference between the safeties of PPI and H<sub>2</sub>RA or placebo. In addition, the effective rate of PPI for NERD was associated with hiatal hernia, while the adverse rate was associated with hiatal hernia and drinking. In the clinic, it is necessary to choose a PPI with a suitable dose, therapeutic duration and type for different NERD patients. More multi-center, high-quality randomized controlled trials with larger samples and longer term of follow-up visits are desirable.

## COMMENTS

### Background

Patients with non-erosive reflux disease (NERD) suffer from heartburn due to gastric acid in the reflux content. Acid-suppressive drugs, especially proton

pump inhibitor (PPI), have been used widely to manage NERD.

### Research frontiers

Though PPI has been used for patients with NERD for years, however, sufficient and convictive evidences concerning its efficacy and safety are lacking and whether its efficacy and safety are influenced by other factors remains unclear.

### Innovations and breakthroughs

The meta-analysis and systematic review was conducted according to Cochrane Handbook. The rates of symptomatic relief of PPI vs placebo and PPI vs H<sub>2</sub> receptor antagonists (H<sub>2</sub>RA) were treated as the primary endpoint and the rates of adverse events as the secondary endpoint. Meanwhile, factors influencing rates of symptomatic relief and adverse events of PPI against NERD are analyzed.

### Applications

This meta-analysis indicated that PPI overmatched H<sub>2</sub>RA on symptomatic relief rate but not on adverse rate for NERD. The rate of symptomatic relief of PPI against NERD was influenced by hiatal hernia and the adverse rate was affected by hiatal hernia and drinking.

### Peer review

This is a well written, sufficiently interesting original article in which the authors reviewed the efficacy, safety and their influential factors of PPI against NERD.

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## Bile leakage test in liver resection: A systematic review and meta-analysis

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### Abstract

**AIM:** To assess systematically the safety and efficacy of bile leakage test in liver resection.

**METHODS :** Randomized controlled trials and controlled clinical trials involving the bile leakage test were included in a systematic literature search. Two authors independently assessed the studies for inclusion and extracted the data. A meta-analysis was conducted to estimate postoperative bile leakage, intraoperative positive bile leakage, and complications. We used either the fixed-effects or random-effects model.

**RESULTS:** Eight studies involving a total of 1253 patients were included and they all involved the bile leakage test in liver resection. The bile leakage test group was associated with a significant reduction in bile leakage compared with the non-bile leakage test group (RR = 0.39, 95%CI: 0.23-0.67;  $I^2 = 3\%$ ). The white test had superiority for detection of intraoperative bile leakage compared with the saline solution test (RR =

2.38, 95%CI: 1.24-4.56,  $P = 0.009$ ). No significant intergroup differences were observed in total number of complications, ileus, liver failure, intraperitoneal hemorrhage, pulmonary disorder, abdominal infection, and wound infection.

**CONCLUSION:** The bile leakage test reduced postoperative bile leakage and did not increase incidence of complications. Fat emulsion is the best choice of solution for the test.

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**Key words:** Bile leakage test; Bile leakage; Liver resection; Postoperative complications; Meta-analysis

**Core tip:** Bile leakage is a common complication after hepatic resection and seriously affects postoperative quality of life. The bile leakage test was introduced to prevent bile leakage after liver resection. Many studies have evaluated the feasibility, safety and efficacy of the bile leakage test, however, the clinical significance of this technique remains inconsistent. We conducted a systematic review and showed that the bile leakage test reduced the incidence of postoperative bile leakage and did not increase the incidence of complications. In addition, fat emulsion may be the best choice of solution for the bile leakage test.

Wang HQ, Yang J, Yang JY, Yan LN. Bile Leakage test in liver resection: A systematic review and meta-analysis. *World J Gastroenterol* 2013; 19(45): 8420-8426 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8420.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8420>

### INTRODUCTION

With the refinement of surgical techniques and periop-

erative care in liver surgery, the postoperative morbidity and mortality have markedly decreased. However, the incidence of bile leakage has not changed over the past few decades, ranging from 4.0% to 9.8% in recent studies<sup>[1-7]</sup>. Biliary complications remain a common cause of major morbidity after hepatic resection<sup>[1]</sup>. The presence of bile in the peritoneal cavity may impair the normal host defense mechanisms and predispose to the development of sepsis, liver failure and mortality<sup>[2,3]</sup>. Therefore, many methods have been introduced to prevent bile leakage after liver transection, including intraoperative cholangiography<sup>[8]</sup>, spreading fibrin glue on the transected liver surface<sup>[9]</sup>, assessing bile duct patency by injecting air under ultrasonographic monitoring<sup>[10]</sup>, and the bile leakage test. The latter is a common approach to reduce postoperative bile leakage<sup>[11]</sup>. With this technique, after cholecystectomy and liver parenchymatous division, a catheter is inserted through the cystic duct into the common bile duct and the distal common bile duct is occluded. A solution, such as isotonic sodium, fat emulsion, indocyanine green or methylene blue, is slowly injected into the biliary tree and a clinical judgment is then made as to whether a bile leak is present on the transected surface of the liver. If so, the bile leakage site will be closed steadily beforehand<sup>[11]</sup>. Using this technique, some studies<sup>[2,11-14]</sup> have identified intraoperatively additional potential bile leakage points in 19.7%-80.8% of the patients. The bile leakage test proved to be useful for preventing postoperative bile leakage in several studies, however, other studies suggested no advantage in using the bile leakage test. Moreover, the additional operation associated with the test may also result in a risk of postoperative complications. Some studies have suggested excessive bile duct pressure from the bile leakage test could cause cholangiovenous reflux and cholangitis<sup>[15,16]</sup>.

Many randomized controlled trials (RCTs) and controlled clinical trials have evaluated the feasibility, safety and efficacy of the bile leakage test, however, the clinical significance of this technique remains inconsistent. To date, we have been unable to identify any meta-analysis that assessed the role of the bile leakage test. We conducted a systematic review and meta-analysis to evaluate the safety and efficacy of the test in liver resection.

## MATERIALS AND METHODS

We conducted the meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions<sup>[17]</sup> and preferred reporting items for systematic reviews and meta-analysis<sup>[18]</sup>.

### Systematic literature search

A systematic literature search was independently conducted by two authors. We systematically searched the Cochrane Central Register of Controlled Trials, Embase, Science Citation Index (Web of Knowledge), PubMed and Chinese Biomedical Literature Database up to June 7, 2013. The search strategies were as follows: (biliary leakage OR bile leakage OR bile fistula OR biliary fistula

OR bile leakage test OR biliary complication) AND (hepatic resection OR hepatectomy (MeSH terms) OR liver resection). The literature search was performed with restriction in language to English or Chinese and RCTs or controlled clinical trials. After completing all searches, we merged the search results using Endnote X3 (reference management software) and removed duplicated records. Two independent authors scanned the title and abstract of every record identified by the searches for inclusion. If compliance with inclusion criteria was not clear from the abstract, we retrieved full texts for further assessment.

### Inclusion and exclusion criteria

**Types of studies:** RCTs and controlled clinical trials were considered for this review.

**Types of participants:** Patients who were about to undergo selective liver resection for any disease were included in our study, irrespective of age, sex, tumor size and nodule numbers. Trials in which patients required living donor liver transplantation were excluded. Trials in which patients required the bile leakage test without liver resection were excluded.

**Types of interventions:** We included trials comparing patients with and without the bile leakage test undergoing hepatectomy, or trials comparing bile leakage with different methods.

**Types of outcome measures:** Primary outcomes: postoperative bile leakage, and intraoperative positive bile leakage; secondary outcomes: operation time, blood loss, postoperative complications, postoperative hospital stay, and duration of drainage.

### Data collection and analysis

**Selection of studies:** Any disagreement during study selection and data extraction was resolved by discussion and referral to a third author for adjudication.

**Data extraction:** Two authors extracted data on a standard form that included population characteristics, intraoperative parameters, and information about the outcome measures in each trial. In the case of missing data, we contacted the original investigators to request further information.

**Quality assessment:** Two authors assessed the methodological quality of the trials independently. The Jadad score<sup>[19,20]</sup> was used to assess the quality of RCTs, with a cumulative score of  $> 3$  indicating high quality. The Newcastle-Ottawa scale<sup>[21,22]</sup> was used to assess the quality of non-randomized studies, with a score  $\geq 5$  indicating high quality.

### Statistical analysis

We pooled the synchronized extraction results as estimates of overall treatment effects in a meta-analysis

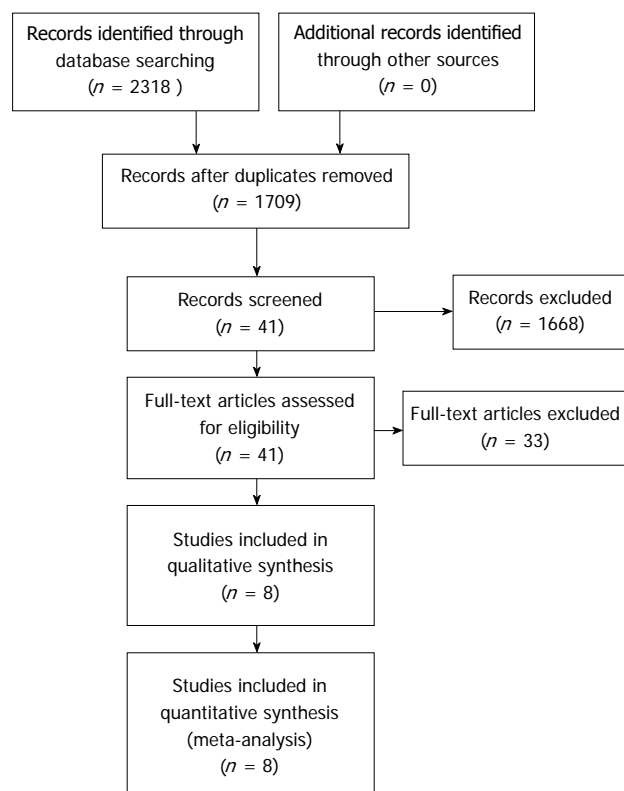


Figure 1 Flow diagram of the study selection process.

using Review Manager for Windows version 5.0. The estimated effect measures were RR for dichotomous data and weighted mean difference (WMD) for continuous data; both reported with 95%CI. We checked all results for clinical and statistical heterogeneity. Clinical heterogeneity was evaluated by assessing study populations and interventions, definition of outcome measures, concomitant treatment, and perioperative management. Heterogeneity was explored by  $\chi^2$  test with significance setting at  $P = 0.10$ , and  $I^2$  statistics were used for the evaluation of statistical heterogeneity ( $I^2 \geq 50\%$  indicating presence of heterogeneity)<sup>[23]</sup>. We used a fixed-effects model to synthesize data when heterogeneity was absent, otherwise a random-effects model was used for synthesizing data. Data are presented as forest plots and the funnel plot was used to assess publication bias.

## RESULTS

### Description of the included studies

A total of 2318 articles were retrieved through the search strategy. Eight studies<sup>[2,11-13,24-27]</sup> including 1253 patients matched the inclusion criteria (Figure 1). Details on the included studies are shown in Table 1. Five studies<sup>[2,11-13,24]</sup> compared patients with and without the bile leakage test, including 1032 patients (505 in the bile leakage test group and 527 in the control group). Two trials<sup>[25,27]</sup> compared a bile leakage test using indocyanine green solution with fluorescent imaging (ICGF Test)

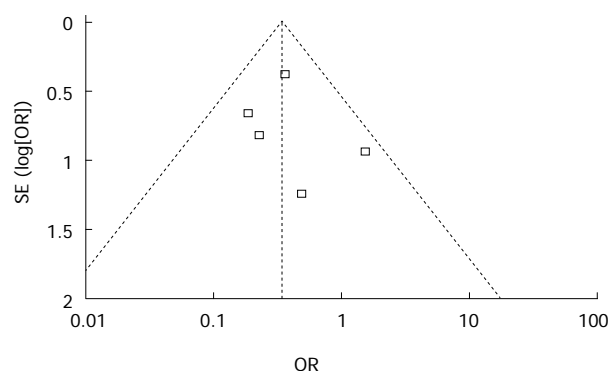


Figure 2 Funnel plot of studies included in the meta-analysis of bile leakage test group vs non-bile leakage test group.

with a bile leakage test without fluorescent imaging (N-ICGF Test), including 161 patients (79 in the trial group and 82 in the control group). One study<sup>[26]</sup> compared a bile leakage test using fat emulsion (white test; injecting a fat emulsion solution through the cystic duct) with a test using saline solution, including 60 patients (30 in the white test group and 30 in the saline solution test group). These studies were published between 2000 and 2012. One trial<sup>[24]</sup> only enrolled liver donor patients and another<sup>[13]</sup> enrolled patients with tumor or hepatolithiasis; all remaining trials enrolled patients with a tumor. The quality assessment of RCTs and controlled clinical trials is displayed in Tables 2 and 3.

### Bile leakage test group vs non-bile leakage test group

**Bile leakage:** Information on postoperative bile leakage was available for all five trials<sup>[2,11-13,24]</sup>. Funnel plot (Figure 2) did not demonstrate strong asymmetry. Meta-analysis indicated that the bile leakage test group had less postoperative bile leakage than the non-bile leakage test group (Figure 3) (RR = 0.39, 95%CI: 0.23-0.67,  $P = 0.39$ ,  $I^2 = 3\%$ ). Clinical heterogeneity analysis found that one study<sup>[2]</sup> including a large sample of 616 patients was a retrospective study and the others were prospective. Meta-analysis of the other four trials also showed higher incidence of bile leakage in the non-bile leakage test group (RR = 0.32, 95%CI: 0.15-0.70,  $P = 0.29$ ,  $I^2 = 20\%$ ).

**Other complications:** Four trials<sup>[11-13,24]</sup> reported the incidence of total complications, however, only data from three trials<sup>[11-13]</sup> could be used for analysis, and one trial<sup>[24]</sup> did not provide sufficient information on complications. There was no significant difference in the incidence of total complications (RR = 0.84, 95%CI: 0.63-1.13,  $P = 0.37$ ,  $I^2 = 0\%$ ). No significant heterogeneity was observed. Other complications such as wound infection, ileus, liver insufficiency, intraperitoneal hemorrhage, pulmonary disorder, and hepatic failure were reported and analyzed. Wound infection (RR = 1.83, 95%CI: 0.58-5.78,  $P = 0.94$ ,  $I^2 = 0\%$ ), pulmonary disorder (RR = 1.03, 95%CI: 0.54-1.96,  $P = 0.87$ ,  $I^2 = 0\%$ ), and abdominal infection (RR = 1.47, 95%CI: 0.61-3.55,  $P = 0.56$ ,  $I^2 = 0\%$ ) were reported in three studies<sup>[11-13]</sup>



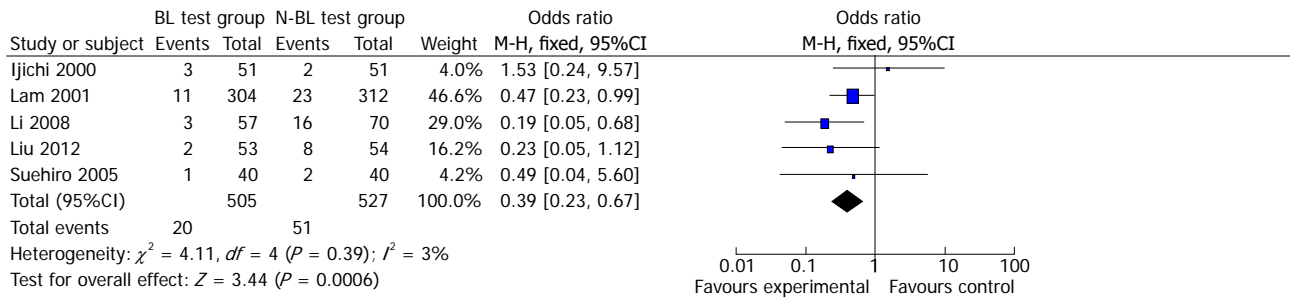


Figure 3 Bile Leakage between bile leakage test group vs non-bile leakage test group.

Table 1 Characteristics of clinical studies of bile leakage test

Study	Design	Comparison	Material	Sample (trial:control)	Inclusion criteria	Liver disease	Country
Ijichi <i>et al</i> <sup>[11]</sup> , (2000)	RCT	BL Test <i>vs</i> N-BL Test	Saline solution	51:51	Liver resection	Tumor	Japan
Liu <i>et al</i> <sup>[13]</sup> , (2012)	RCT	BL Test <i>vs</i> N-BL Test	Fat emulsion	53:54	Liver resection	Tumor or hepatolithiasis	China
Li <i>et al</i> <sup>[12]</sup> , (2009)	Controlled clinical trial	BL Test <i>vs</i> N-BL Test	Fat emulsion	57:70	Major liver resection	Tumor	Germany
Suehiro <i>et al</i> <sup>[24]</sup> , (2005)	Controlled clinical trial	BL Test <i>vs</i> N-BL Test	ICG	40:40	Liver resection	Liver donor	Japan
Lam <i>et al</i> <sup>[21]</sup> , (2001)	Controlled clinical trial	BL Test <i>vs</i> N-BL Test	Methylene blue	304:312	Liver resection	Tumor	Hong Kong
Leelawat <i>et al</i> <sup>[26]</sup> , (2012)	Controlled clinical trial	White Test <i>vs</i> Saline Solution Test	Fat emulsion	30:30	Liver resection	Tumor	Thailand
Sakaguchi <i>et al</i> <sup>[27]</sup> , (2010)	Controlled clinical trial	ICGF Test <i>vs</i> N-ICGF Test	ICG	27:32	Liver resection	Tumor	Japan
Kaibori <i>et al</i> <sup>[25]</sup> , (2011)	RCT	ICGF Test <i>vs</i> N-ICGF Test	ICG	52:50	Liver resection	Tumor	Japan

ICGF: Indocyanine green solution with fluorescent imaging; RCT: Randomized controlled trial; ICG: Indocyanine green; BL: Bile leakage.

Table 2 Quality assessment of the included randomized controlled trials based on the Jadad scoring system

Study	Randomized	Appropriate randomization	Appropriately double blinded	Description of withdrawals	Jadad score	Study quality
Ijichi <i>et al</i> <sup>[11]</sup> , (2000)	Yes	Yes	No	Yes	3	High
Liu <i>et al</i> <sup>[13]</sup> , (2012)	Yes	Yes	No	Yes	3	High
Kaibori <i>et al</i> <sup>[25]</sup> , (2011)	Yes	No	No	Yes	2	Low

Table 3 Quality assessment of the included non-randomized controlled trials based on the Newcastle-Ottawa scale

Study	Selection star	Comparability star	Outcome star	Total star	Study quality
Li <i>et al</i> <sup>[12]</sup> , (2009)	4	2	3	9	High
Suehiro <i>et al</i> <sup>[24]</sup> , (2005)	3	2	3	8	High
Lam <i>et al</i> <sup>[21]</sup> , (2001)	3	1	3	7	High
Leelawat <i>et al</i> <sup>[26]</sup> , (2012)	4	2	3	9	High
Sakaguchi <i>et al</i> <sup>[27]</sup> , (2010)	3	1	3	7	High

and no significant difference was observed between the bile leakage test group and non-bile leakage test group. Two studies<sup>[11,12]</sup> reported ileus (RR = 1.86, 95%CI: 0.24-14.24,  $P = 0.68$ ,  $I^2 = 0\%$ ) and liver insufficiency (RR = 1.18, 95%CI: 0.17-8.29,  $P = 0.21$ ,  $I^2 = 37\%$ ), and no significant difference was observed between the bile leakage test group and non-bile leakage test group. Intraperitoneal hemorrhage was reported in two

studies<sup>[11,13]</sup> and there was no significant difference between the bile leakage test group and non-bile leakage test group (RR = 1.01, 95%CI: 0.18-5.71,  $P = 0.37$ ,  $I^2 = 0\%$ ). Two trials<sup>[12,13]</sup> reported liver failure, and meta-analysis showed that there was no significant difference between the bile leakage test group and non-bile leakage test group (RR = 1.47, 95%CI: 0.41-5.29,  $P = 0.76$ ,  $I^2 = 0\%$ ).

**Table 4** Intraoperative positive bile leakage and postoperative bile leakage treatment

Study	Intraoperative bile leakage	Postoperative bile leakage	Conservative treatment (n)	Puncture drainage (n)	ENBD (n)	Reoperation (n)
Ijichi <i>et al</i> <sup>[11]</sup> , 2000	41.2% (21/51)	5.9% (3/51)	5	0	0	0
Liu <i>et al</i> <sup>[13]</sup> , 2012	62.3% (33/53)	3.8% (2/53)	7	0	2	1
Li <i>et al</i> <sup>[12]</sup> , 2009	71.4% (45/63)	5.3% (3/57)	No description	No description	1	2
Suehiro <i>et al</i> <sup>[24]</sup> , 2005	No description	2.5% (1/40)	No description	No description	No description	No description
Lam <i>et al</i> <sup>[2]</sup> , 2001	19.7% (60/304)	3.6% (11/304)	7	11	6	10
Leelawat <i>et al</i> <sup>[26]</sup> , 2012	63.3% (19/30)	No description	2	0	0	0
Sakaguchi <i>et al</i> <sup>[27]</sup> , 2010	29.6% (8/27)	0% (0/27)	2	0	0	0
Kaibori <i>et al</i> <sup>[25]</sup> , 2011	80.8% (42/52)	0% (0/52)	2	1	2	0
Total	39.3% (228/580)	3.42% (20/584)	25 (41.0%)	12 (19.8%)	11 (18.0%)	13 (21.2%)

ENBD: Endoscopic nasobiliary drainage.

### ICGF test vs N-ICGF test

**Bile leakage:** Both of the studies<sup>[25,27]</sup> provided data on postoperative bile leakage. Meta-analysis of the two studies revealed that there was no significant difference between the ICGF test group and N-ICGF test group (RR = 0.13, 95%CI: 0.02-1.00,  $P = 0.64$ ,  $I^2 = 0\%$ ).

**Other complications:** There was no significant difference in total complications (RR = 0.48, 95%CI: 0.21-1.07;  $P = 0.21$ ,  $I^2 = 36\%$ ) between the ICGF test group and N-ICGF test group<sup>[25,27]</sup>, nor in pleural effusion (RR = 1.78, 95%CI: 0.49-6.44,  $P = 0.73$ ,  $I^2 = 0\%$ ) or wound infection (RR = 0.42, 95%CI: 0.09-2.09,  $P = 0.71$ ,  $I^2 = 0\%$ ).

### White test vs saline solution test

Only one self-controlled study<sup>[26]</sup> compared the white test with the saline solution test. Only data on intraoperative bile leakage were provided and meta-analysis revealed that the white test had a higher rate of bile leakage points (RR = 2.38, 95%CI: 1.24-4.56,  $P = 0.009$ ).

**Other outcomes:** These studies did not provide enough data on operation time, blood loss, postoperative hospital stay, or duration of drainage for analysis. The bile leakage test showed intraoperative bile leakage points on the hepatic resection plane in an average of 39.3% patients (range, 19.7%-80.8%). The postoperative bile leakage rate was 0%-5.9% after suturing. Among the bile leakage patients, 25 (41.0%) were treated conservatively; 12 (19.8%) underwent puncture drainage; 11 (18.0%) underwent endoscopic nasobiliary drainage; and 13 (21.2%) required reoperation (Table 4).

## DISCUSSION

Bile leakage is a common complication after hepatic resection and seriously affects postoperative quality of life, and also causes intra-abdominal infection and liver failure<sup>[13]</sup>. During the operation, it is difficult for surgeons to identify bile leakage by traditional methods such as the gauze test. Technical diligence in liver resection is required intraoperatively to minimize bile leakage. The bile leakage test is considered to be an effective method to prevent intraoperative bile leakage. The aim of the

bile leakage test is to detect insufficiently closed stumps of bile ducts on the transected liver surface by elevating biliary pressure<sup>[24]</sup> and then the leakage site is sutured.

We conducted a systematic review and meta-analysis and eight trials including three aspects of the bile leakage test were included. In the bile leakage test group vs non-bile leakage test group, studies involving the bile leakage test drew different conclusions. Three studies<sup>[2,12,13]</sup> showed that the bile leakage test decreased postoperative bile leakage, whereas two studies<sup>[11,24]</sup> showed no significant difference. Our meta-analysis showed that the bile leakage test group had a lower incidence of bile leakage than the non-bile leakage test group. Moreover, there was no significant difference in complications between the two groups. This suggests an effective and safe method for prevention of postoperative bile leakage. In the ICGF test group, ICG was injected into the biliary duct and then bound with proteins in bile<sup>[28]</sup>. The ICG-bile mixture can evoke fluorescent images that are obtained using an infrared camera system. Bile leakage can be easily detected by the extra-biliary fluorescent signal<sup>[25,27]</sup>. Meta-analysis found no difference in bile leakage rate and postoperative complications. Only one self-controlled study<sup>[26]</sup> compared the white test with the saline solution test and only positive rate of the intraoperative bile leakage test could be used for analysis. Meta-analysis found that the white test was superior for the detection of bile leakage compared with the saline solution test. However, the limitations of the number of trials (only one) may have affected our results and interpretation. Therefore, our results showed that the bile leakage test reduced postoperative biliary leakage, and considering the solution used in the test, fat emulsion may be more effective than saline solution.

To perform the bile leakage test, incidental cholecystectomy and cystic duct exploration are always necessary. One meta-analysis<sup>[29]</sup> reported increased morbidity in patients undergoing incidental cholecystectomy. However, in our meta-analysis, total complications, wound infection, ileus, liver insufficiency, intraperitoneal hemorrhage, pulmonary disorder, and hepatic failure showed no significant difference. The detection of bile leakage depends on elevated pressure by injecting solution into the biliary tract, however, excessive bile duct pressure

could lead to regurgitation of bacteria and induce cholangitis and abdominal infection<sup>[15,16]</sup>. Although abdominal infection did occur in some trials, our meta-analysis showed that the bile leakage test did not increase the risk of abdominal infection. Therefore, the bile leakage test is a safe technique and does not cause additional complications.

In all the included studies, the bile leakage test detected leakage in an average of 39.3% patients (range, 19.7%-80.8%). After prophylactic closure of the leaking bile duct stump, bile leakage occurred in only an average of 3.42% of patients, which was significantly lower than before. This suggests that the bile leakage test can reduce bile leakage in liver resection, but it does not completely eliminate it. Several reasons could explain this. First, we need to consider the lack of refinement of surgical techniques. For example, the pressure on the bile duct was not high enough so that bile leakage was not observed during the operation. Inadequate suturing of the defect<sup>[2]</sup> also could lead to bile leakage. Second, minor bile ducts in the wound surface of the liver were obstructed by microliths, and the bile leakage was formed when the microliths dropped off after the operation<sup>[13]</sup>. Third, there were defects in the technique of the bile leakage test. Parts of the leaky bile ducts were not in communication with the biliary tree, therefore, the leakage sites could not be identified through the use of an intraoperative bile leakage test<sup>[13]</sup>. Moreover, solutions such as ICG and methylene blue have the drawback of staining surrounding tissues, and saline solution has poor sensitivity. These drawbacks make precise localization or identification of multiple sites of leakage difficult<sup>[12]</sup>. However, the white fat emulsion can be easily washed out from the bile ducts<sup>[26]</sup> and it does not contaminate the surface of the wound, and it can be used repeatedly in tests. This may be the reason why the white test can decrease bile leakage more than the saline solution test can.

However, some patients without the bile leakage test did not develop bile leakage, for the following reasons. First, partial minor leakage points can be closed spontaneously. Second, bile leakage from small biliary stumps with some communication to the main biliary tree would usually close spontaneously, with the restoration of peristalsis and papillary function<sup>[11]</sup>. Thus, the bile leakage test could not completely eliminate bile leakage, but just decrease it.

This review had some limitations. The first concerns the small number of RCTs included, and we also included non-randomized trials. Second, incomplete reporting of important methodological issues, such as randomization process and blinding assessment of trial quality, raises doubts about the adequate power of these studies. Third, the heterogeneity of the patients in the included trials may have influenced the conclusions because some trials included liver tumor patients and only one study included living donors. To overcome these limitations, more RCTs should be conducted with large numbers of patients to achieve a sufficient level of statistical power for accurate evaluation of the bile leakage test.

In conclusion, this review provides the best available evidence for the safety and efficacy of the bile leakage test. On the basis of this evidence, the bile leakage test appears to reduce the incidence of postoperative bile leakage and does not increase the incidence of other complications. In addition, fat emulsion may be the best choice of solution for use in the bile leakage test. Further trials are required to assess the role of the bile leakage test in liver resection patients.

## COMMENTS

### Background

Liver resection is an important treatment method for liver diseases, especially for liver cancer. Bile leakage is a common complication after hepatic resection and seriously affects patients' postoperative quality of life. Therefore, it is important to prevent bile leakage in liver resection. Many methods have been introduced to prevent bile leakage after liver transection and the bile leakage test is a common approach.

### Research frontiers

Several trials have evaluated the safety and efficacy of the bile leakage test; however, the results of this technique remain inconsistent. The authors conducted a systematic review and meta-analysis to evaluate the safety and efficacy of the bile leakage test in liver resection.

### Innovations and breakthroughs

The authors have provided the best available evidence for the safety and efficacy of the bile leakage test. This meta-analysis found that the bile leakage test lowered the incidence of postoperative bile leakage and could not increase the incidence of other complications. In addition, fat emulsion may be the best choice of solution for use in the bile leakage test.

### Applications

The study results suggest that the bile leakage test is an effective and safe method that could be used in preventing bile leakage after liver resection.

### Terminology

The bile leakage test is a common approach to reduce postoperative bile leakage. With this technique, after cholecystectomy and liver resection, a catheter is inserted through the cystic duct into the common bile duct and the distal common bile duct is occluded. Solution is slowly injected into the biliary tree and a clinical judgment is then made as to whether a bile leak is present on the transected surface of the liver. If so, the bile leak site will be closed steadily beforehand to avoid bile leakage.

### Peer review

The authors present a meta-analysis of the literature describing feasibility, safety and efficacy of a method to avoid complications after hepatic surgery, the so-called bile leakage test. The topic of the article is important for researchers and the literature analysis reported is interesting.

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## Magnetic resonance cholangiography in assessing biliary anatomy in living donors: A meta-analysis

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### Abstract

**AIM:** To establish the role of magnetic resonance cholangiography (MRC) in diagnosis of biliary anatomy in living-donor liver transplantation (LDLT) donors.

**METHODS:** A systematic review was performed by searching electronic bibliographic databases prior to March 2013. Studies with diagnostic results and fulfilled inclusion criteria were included. The methodological quality of the studies was assessed. Sensitivity, specificity and other measures of the accuracy of MRC for diagnosis of biliary anatomy in LDLT donors were summarized using a random-effects model or a fixed-effects model. Summary receiver operating characteristic (SROC) curves were used to summarize overall test performance. Publication bias was assessed using Deek's funnel plot asymmetry test. Sensitivity analysis was ad-

opted to explore the potential sources of heterogeneity.

**RESULTS:** Twelve studies involving 869 subjects were eligible to the analysis. The scores of Quality Assessment of Diagnostic Accuracy Studies for the included studies ranged from 11 to 14. The summary estimates of sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, diagnostic OR of MRC in diagnosis of biliary anatomy in LDLT donor were 0.88 (95%CI: 0.84-0.92), 0.95 (95%CI: 0.93-0.97), 15.33 (95%CI: 10.70-21.95), 0.15 (95%CI: 0.11-0.20) and 130.77 (95%CI: 75.91-225.27), respectively. No significant heterogeneity was detected in all the above four measures. Area under SROC curve was 0.971. Little publication bias was noted across the studies ( $P = 0.557$ ). Sensitivity analysis excluding a study with possible heterogeneity got a similar overall result, which suggested the little influence of this study on the overall results.

**CONCLUSION:** Our results suggest that MRC is a high specificity but moderate sensitivity technique in diagnosis of biliary anatomy in LDLT donors.

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**Key words:** Magnetic resonance imaging; Cholangiography; Biliary; Anatomy; Living donors

**Core tip:** The current findings on the value of magnetic resonance cholangiography (MRC) in diagnosis of biliary anatomy in living-donor liver transplantation (LDLT) donors are conflicting. This meta-analysis including 12 studies with 869 patients suggested that MRC has a high specificity in diagnosis of biliary anatomy in LDLT donors, but the sensitivity is moderate. This is the first meta-analysis to investigate the diagnostic accuracy of MRC in the detection of biliary anatomy in LDLT donors; and these results will provide valuable information to the doctors when they make a decision for the living liver donors.

Xu YB, Bai YL, Min ZG, Qin SY. Magnetic resonance cholangiography in assessing biliary anatomy in living donors: A meta-analysis. *World J Gastroenterol* 2013; 19(45): 8427-8434 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8427.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8427>

## INTRODUCTION

Adult living-donor liver transplantation (LDLT) is an alternative therapeutic option for patients with end-stage liver disease. Some transplantation centers have reported high rates of biliary complications following LDLT<sup>[1-3]</sup>. Biliary complications after LDLT are closely related to the complex anatomy of the donor's biliary tree. Therefore, preoperative knowledge of the donor's aberrant biliary anatomy can minimize postoperative morbidity in the recipient and maximize safety for the donor. Several techniques are currently being used in this setting; however, they all have some limitations. For example, endoscopic retrograde cholangiography (ERC) and intraoperative cholangiography (IOC) are both invasive techniques that can result in serious complications<sup>[4,5]</sup>, while the non-invasive multidetector computed tomography (MDCT) technique exposes the potential donor to ionizing radiation and the risks associated with nephrotoxic contrast agents<sup>[6]</sup>.

Magnetic resonance cholangiography (MRC) is a non-invasive imaging technique and has shown promise in the preoperative evaluation of the biliary anatomy of LDLT donors. Previously, two studies<sup>[7,8]</sup> reported meta-analyses on the value of MRC in the diagnosis of biliary complications after liver transplantation. However, neither evaluated the role of MRC in evaluating the biliary anatomy of LDLT donors. In addition, although some studies<sup>[9-12]</sup> reported a high diagnostic accuracy for MRC in the diagnosis of biliary anatomy, the sample sizes were small; thus, the results remained inconclusive. Given the importance of a preoperative evaluation of biliary anatomy and the uncertainty regarding the diagnostic accuracy of MRC, we performed a meta-analysis to determine the overall diagnostic accuracy of MRC in the evaluation of the biliary anatomy of LDLT donors.

## MATERIALS AND METHODS

### Search strategy and study selection

We systematically searched the Cochrane clinical trials database, MEDLINE/PubMed, and Embase to identify suitable studies prior to March 1, 2013. No starting date limit was applied. Articles were also identified using the related articles function in PubMed. References within the identified articles were also searched manually. The search terms included "magnetic resonance cholangiography" or "MRC," "biliary anatomy," and "donors."

The searches were limited to human studies. Potentially relevant articles were then screened by at least two independent reviewers. Disagreements were resolved by discussion or upon consensus from a third reviewer.

### Inclusion and exclusion criteria

A study was included in the meta-analysis when it provided data on both the sensitivity and specificity of MRC for the diagnosis of the biliary anatomy of living donors, or when it provided values in a scatterplot form, allowing test results for individual study subjects to be extracted. Studies were excluded if they were review articles, case reports, or animal studies. In order to obtain a more reliable estimation of the accuracy of MRC, we only included studies that fulfilled at least nine items of the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) criteria. Two reviewers independently judged the eligibility of the studies. Disagreements between reviewers were resolved by consensus. The authors of some publications were contacted for clarifications and additional information.

### Data extraction and quality assessment

The final set of articles was assessed independently by two reviewers. The data retrieved included the authors, publication year, the country where the study was conducted, the number of patients and their mean age, the reference standard (gold standard), true-positive, false-negative, false-positive, and true-negative values, and the quality of the methodology. The methodological quality of the studies was assessed using QUADAS, an evidence-based quality assessment tool developed for use in systematic reviews of studies of diagnostic accuracy and fully described by Whiting *et al.*<sup>[13]</sup>, with a maximum score of 14.

### Statistical analysis

Standard methods recommended for the meta-analysis of diagnostic test evaluations were used<sup>[14]</sup>. The following measurements of test accuracy were computed for each study: sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR). The analysis was based on a summary receiver operating characteristic curve (SROC); the results are described as the area under the curve (AUC) of the SROC, with its Q-point representing the maximal joint sensitivity and specificity<sup>[15,16]</sup>. The summary sensitivity, specificity, and other measures across MRC studies were calculated using a random-effects model and a fixed-effects model, respectively. A  $\chi^2$  test and an inconsistency index ( $I^2$ ) were used to detect statistically significant heterogeneity across studies. Publication bias was assessed using Deeks' Funnel Plot Asymmetry Test<sup>[17]</sup>. Analyses were performed using Meta-DiSc version 1.4 (Unit of Clinical Biostatistics, the Ramón y Cajal Hospital, Madrid, Spain) and Stata 11.2 (Stata Corporation, College Station, TX, United States) software.

**Table 1** Characteristics of included studies

Study	Country/years	No. of patients	Mean age	Study design	MRCP technique	Reference standard	QUADAS	TP	FP	FN	TN
Ayuso <i>et al</i> <sup>[9]</sup>	Spain/2004	25	NS	Prospective	MnDPDP-enhanced, MIP	IOC	12	15	0	1	9
Limanond <i>et al</i> <sup>[11]</sup>	United States/2004	26	37	Retrospective	T2 single-shot fast spin-echo, T2 HASTE	IOC	13	5	2	2	17
Kim <i>et al</i> <sup>[23]</sup>	Canada/2005	30	36	Prospective	T2 weighted SSFSE, Mn-DPDP	IOC	12	12	0	1	17
An <i>et al</i> <sup>[12]</sup>	South Korea/2006	24	29	Prospective	Gadobenate dimeglumine-enhanced T1-and T2-weighted, MIP	IOC	14	13	1	1	9
Sirvanci <i>et al</i> <sup>[25]</sup>	Turkey/2007	62	42	Retrospective	RARE, HASTE, 3D TSE	IOC	13	16	0	3	43
Song <i>et al</i> <sup>[26]</sup>	South Korea/2007	111	29	Prospective	Single-slab RARE or multislice HASTE	IOC	12	42	3	2	64
Basaran <i>et al</i> <sup>[19]</sup>	Turkey/2008	40	35	Prospective	T2-weighted PACE turbo spin-echo	IOC	12	13	3	0	24
Kashyap <i>et al</i> <sup>[22]</sup>	United States/2008	36	38	Retrospective	Thick and thin slab heavily T2 weighted	IOC	14	16	0	3	17
Artioli <i>et al</i> <sup>[27]</sup>	Italy/2010	32	38	Prospective	T2-weighted	Surgery	11	15	1	2	14
Kim <i>et al</i> <sup>[24]</sup>	South Korea/2010	52	33	Prospective	RARE, 3D SE T2-weighted sequences.	IOC	14	15	3	3	31
Hsu <i>et al</i> <sup>[21]</sup>	Taiwan/2011	203	32	Retrospective	RARE thin-slab	IOC	13	45	6	8	144
Chiang <i>et al</i> <sup>[20]</sup>	Taiwan/2012	228	30	Retrospective	T2-weighted GD-DTPA	IOC	13	55	7	9	157

SSFSE: Single shot fast spin echo; RARE: Rapid acquisition with relaxation enhancement; SE: Spin-echo; Mn-DPDP: Mangafodipir trisodium; GD-DTPA: Diethylenetriaminepentaacetic acid; MIP: Maximum intensity projection; PACE: Prospective acquisition correction; SSD: Shaded surface display; HASTE: Half-Fourier Acquisition Single-Shot Turbo Spin-Echo; QUADAS: Quality Assessment of Diagnostic Accuracy Studies.

## RESULTS

### Study selection

The primary literature search retrieved 25 studies that were considered eligible for the analysis. After a detailed evaluation, 13 studies were excluded, as six were laboratory studies, three were reviews, three provided insufficient data for calculations<sup>[1,10,18]</sup>, and one was irrelevant to the current analysis. Consequently, 12 studies<sup>[9,11,12,19-27]</sup> involving 869 subjects were finally included in the present meta-analysis.

### Characteristics of the studies and quality assessment

All included studies adequately described the MRC techniques used and the types of conventional and aberrant biliary anatomy; however, the screening techniques were different across the studies. All but one study were using IOC as the reference standard. Additional patient demographics from each of the included studies are listed in Table 1. The QUADAS scores for the included studies ranged from 11 to 14 (Table 2).

### Meta-analysis

The overall analysis of the 12 studies showed that the summary sensitivity, specificity, PLR, NLR, and DOR were 0.88 (95%CI: 0.84-0.92), 0.95 (95%CI: 0.93-0.97), 15.33 (95%CI: 10.70-21.95), 0.15 (95%CI: 0.11-0.20), and 130.77 (95%CI: 75.91-225.27), respectively; no significant heterogeneity was detected in any of the above four measures (all  $P > 0.05$ ). The AUC of the SROC was 0.971, suggesting a high diagnostic accuracy (Figures 1 and 2). The moderate sensitivity indicates that 12% of the cases with aberrant biliary anatomy could be missed, and the high specificity indicates a small probability of the pres-

ence of aberrant biliary anatomy when the MRC diagnosis is normal. A PLR of 15.33 suggests that patients with aberrant biliary anatomy have about a 15-fold higher chance of a positive test than those without. An NLR of 0.15 suggests that if the MRC result is negative, the probability of the patient having aberrant biliary anatomy is 15%.

### Sensitivity analysis

Because one of the studies<sup>[27]</sup> used surgery as the reference, we excluded it from the sensitivity analysis. The results were similar to the overall results; the summary sensitivity, specificity, PLR, NLR, DOR, and AUC of the SROC were 0.88 (95%CI: 0.84-0.92), 0.96 (95%CI: 0.93-0.97), 15.41 (95%CI: 10.69-22.22), 0.148 (95%CI: 0.11-0.20), 132.19 (95%CI: 75.72-230.76), and 0.972, respectively, which suggested that the excluded study had little influence on the overall results.

### Publication bias

Deeks' Funnel Plot Asymmetry Test for the overall analysis showed that no significant publication bias was found ( $P = 0.557$ ; Figure 3).

## DISCUSSION

Generally, ERC, IOC, MRC, and MDCT cholangiography have been used to evaluate the biliary anatomy of liver donors. However, these techniques all have their inherent strengths and weaknesses. Although ERC is an accurate method to identify biliary anatomy, the high incidence of serious complications caused by the invasiveness of the procedure makes it excessively risky to perform on healthy donors. IOC is considered the gold standard for

Table 2 Quality assessment tool for diagnostic accuracy systematic review of quality criteria of included studies

	Ayuso <i>et al</i> <sup>[9]</sup>	Limanond <i>et al</i> <sup>[11]</sup>	Kim <i>et al</i> <sup>[23]</sup>	An <i>et al</i> <sup>[12]</sup>	Sirvanci <i>et al</i> <sup>[25]</sup>	Song <i>et al</i> <sup>[24]</sup>	Basaran <i>et al</i> <sup>[19]</sup>	Kashyap <i>et al</i> <sup>[22]</sup>	Artioli <i>et al</i> <sup>[27]</sup>	Kim <i>et al</i> <sup>[24]</sup>	Hsu <i>et al</i> <sup>[21]</sup>	Chiang <i>et al</i> <sup>[20]</sup>
Patient spectrum representative	Yes	NA	Yes	Yes	NA	Yes	Yes	Yes	NA	Yes	NA	NA
Selection criteria described	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Reference standard appropriate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Time between tests appropriate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Uniform verification by reference standard	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Same reference test used	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Reference standard independent	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Reference standard described adequately	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Blinding to reference standard results	NA	Yes	NA	Yes	Yes	NA	NA	Yes	NA	Yes	Yes	Yes
Blinding to index test results	NA	Yes	NA	Yes	Yes	NA	NA	Yes	NA	Yes	Yes	Yes
Appropriate clinical data available	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Uninterpretable data reported	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Withdrawals explained	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
No. of criteria met out of 14	12	13	13	14	13	12	12	14	11	14	13	13

NA: Not available.

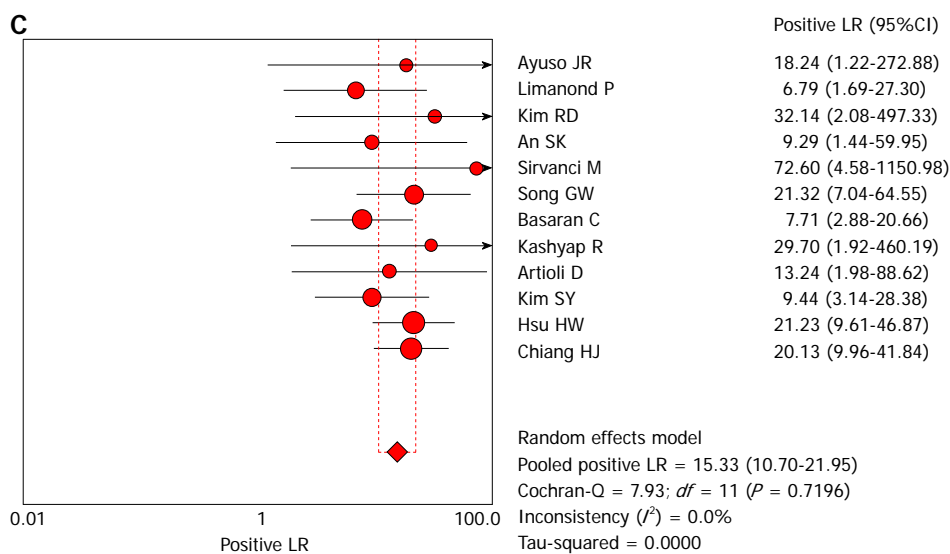
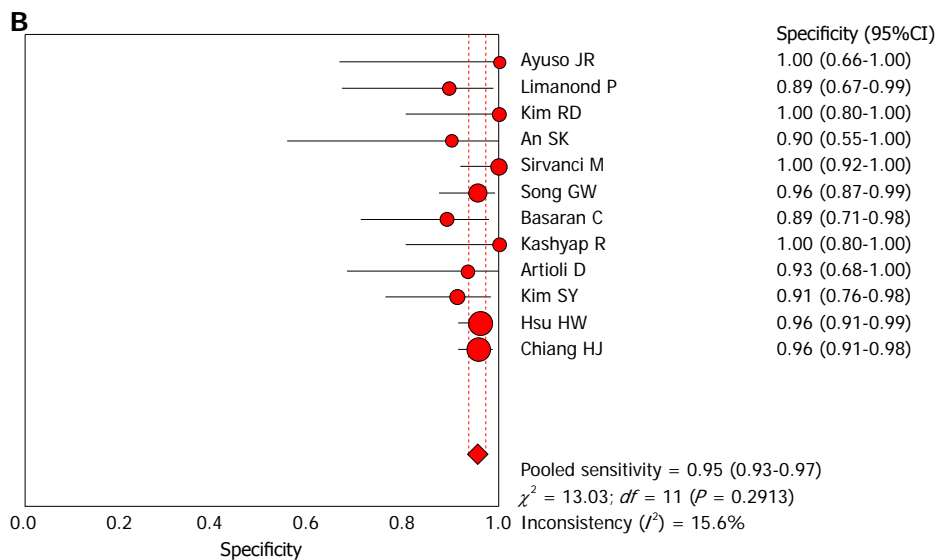
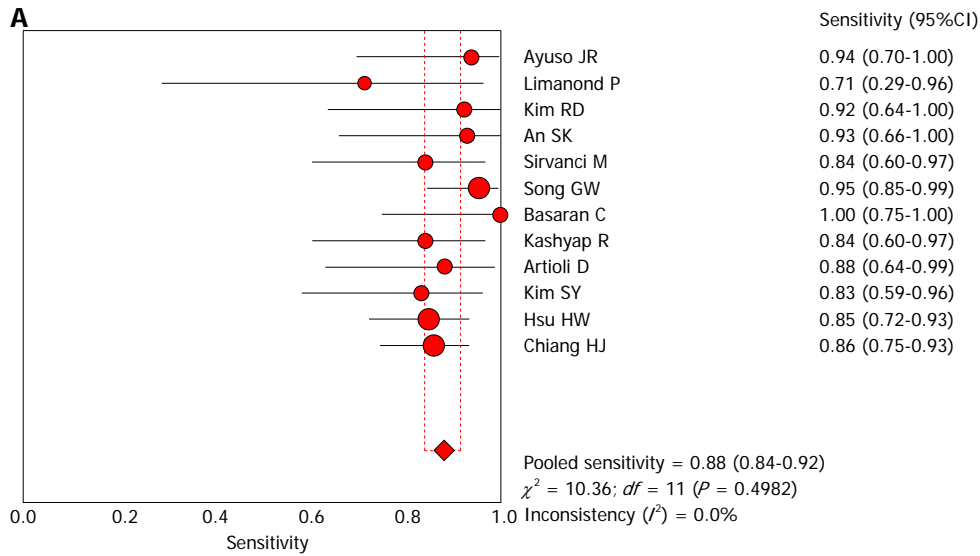
the imaging of the biliary anatomy; however, because it does not allow a three-dimensional (3D) view, this technique may not be helpful in obtaining a single bile duct orifice in some cases with anatomical variations. MDCT or MDCT cholangiography are both non-invasive techniques, and they have shown promise in delineating biliary anatomy in potential right lobe living donors<sup>[26,29]</sup>. However, compared with MRC, the obvious disadvantages of CT are the exposure to ionizing radiation and nephrotoxic contrast agents; especially, the intravenous contrast agent iodipamide meglumine is associated with poor patient tolerance and adverse reactions<sup>[6]</sup>. Several studies showed that MRC has a strong correlation with IOC in delineating the intrahepatic biliary anatomy. In addition to biliary anatomy imaging, MRC is also able to assess other aspects of a potential donor's liver anatomy. It can perform an assessment of the vascular and biliary anatomy and provide an assessment of iron and fat depositions in the liver in a single examination<sup>[23]</sup>.

One of the obvious advantages of the MRC technique is the elimination of the need for an invasive procedure, thus considerably reducing the cost and associated complications. However, this technique also has limitations. For example, MRC is more limited in temporal and spatial resolution than direct digital subtraction angiography and IOC. In addition, the conventional MRC technique (half-Fourier acquisition) provides a depiction of the biliary anatomy with limited detail, especially of non-dilated ducts. However, recent progress in MRC techniques seems to have overcome some of these limitations. Mangafodipir trisodium-enhanced 3D T1-weighted MRC is reported to provide an increased signal-to-noise ratio and a greater visualization rate of the ductal anatomy compared with T2-weighted MRC or two-dimensional (2D) gradient-echo images<sup>[30,31]</sup>. Gadobenate dimeglumine-enhanced T1-weighted MRC can provide information about the differentiation of cystic structures near bile ducts and the bile duct lumen<sup>[12]</sup>. In addition, gadobenate dimeglumine is easier to use and less expensive than mangafodipir trisodium.

The present meta-analysis suggests that MRC has a high diagnostic specificity and moderate sensitivity for biliary anatomy in LDLT donors. Likelihood ratios  $> 10$  or  $< 0.1$  generate large and often conclusive shifts from the pre-test to post-test probability<sup>[32,33]</sup>. DOR is a single indicator of test accuracy that combines the sensitivity and specificity data into a single number<sup>[34]</sup>. The SROC curve presents a global summary of test performance and shows the tradeoff between sensitivity and specificity<sup>[35]</sup>. The summary DOR and the AUC of the SROC were 130.77 and 0.971, respectively, indicating that the overall accuracy was as high as expected, and that MRC is helpful in the diagnosis of aberrant biliary anatomy.

To our knowledge, this is the first meta-analysis exploring the value of MRC in the detection of biliary anatomy of LDLT donors. Our study included more published articles with more subjects, which can provide a more reliable statistical power. In addition, the quality of the selected studies was higher, as all had QUADAS scores over 10. Furthermore, the negligible heterogeneity and publication bias suggest the robustness of the results. In the present study, although several MRC techniques were used and the diagnostic accuracy varied across the studies (the sensitivity ranged from 0.71 to 1.00), the screening techniques were not a significant factor contributing to differences in diag-





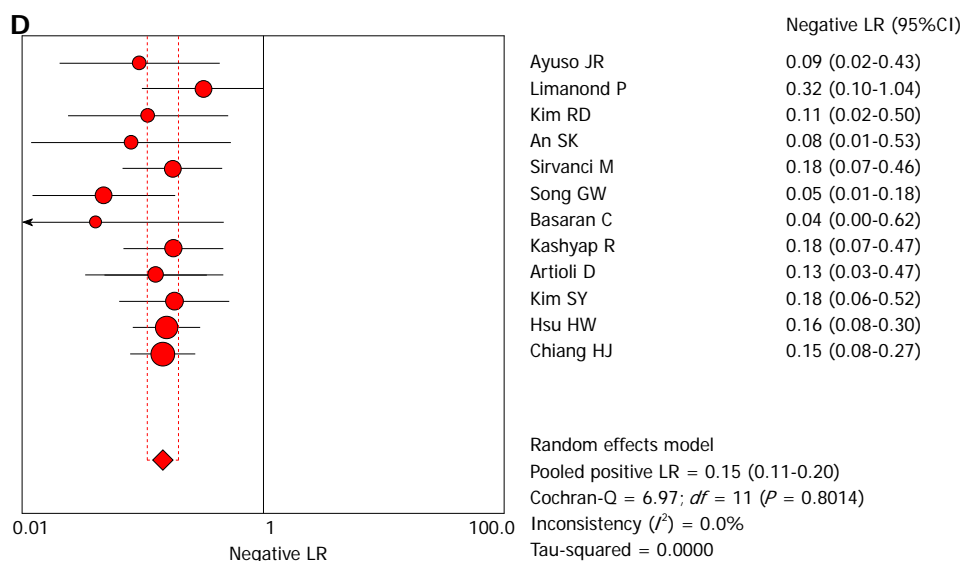


Figure 1 Forest plot of summary results of magnetic resonance cholangiography in the diagnosis of biliary anatomy in living-donor liver transplantation. A: Sensitivity; B: Specificity; C: Positive likelihood ratio; D: Negative likelihood ratio.

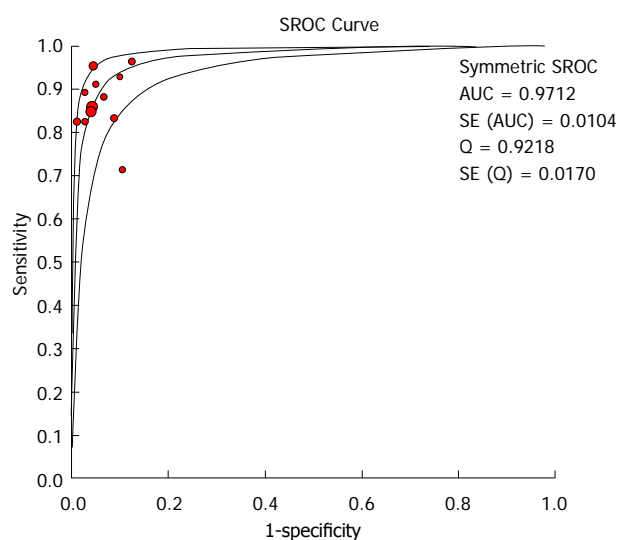


Figure 2 Summary receiver-operating characteristic curve of biliary anatomy in living-donor liver transplantation. SROC: Summary receiver-operating characteristic curve; AUC: Area under the curve.

nosis. Even though both were using a conventional MRC technique, Limanond *et al*<sup>[11]</sup> reported the lowest sensitivity of 0.71, while Song *et al*<sup>[26]</sup> reported a higher sensitivity of 0.95. Likewise, Kim *et al*<sup>[23]</sup> and Chiang *et al*<sup>[20]</sup> both used GD-DTPA MRC techniques, and their diagnostic sensitivities were not similar, reporting 0.92 and 0.86, respectively. These differences suggest that there were other factors, such as sample size, imaging treatment technique (2D, 3D, or maximum intensity projection), or the variability among different imaging readers. These factors may have led to the differing levels of diagnostic accuracy.

There were several limitations to the present meta-analysis. First, the MRC screening techniques were heterogeneous across the studies, although the techniques

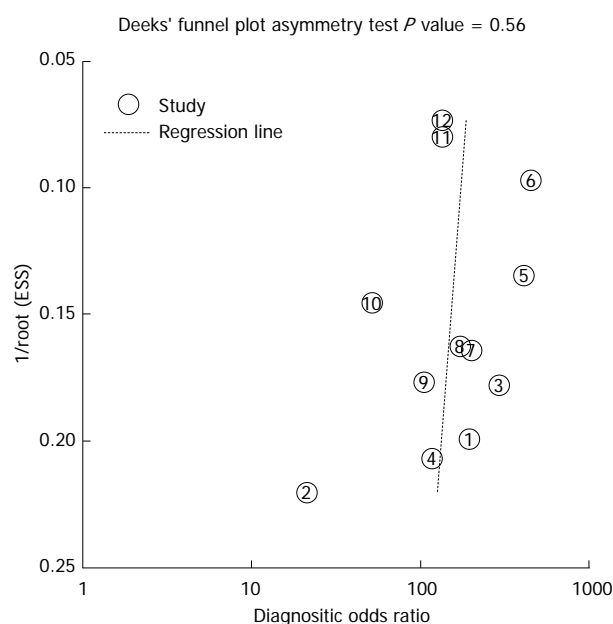


Figure 3 Deek's funnel plot asymmetry test for identifying publication bias in the diagnosis of biliary anatomy in living-donor liver transplantation.

are all considered good enough to make a correct diagnosis by our contributing radiologists (Min ZG and Zeng ZY). However, the heterogeneity of the MRC techniques across the studies may have led to a differential verification bias and could have falsely elevated the reported sensitivities. Second, five of the included studies<sup>[9,19,23,26,27]</sup> did not specifically state that the readers were blinded to the results of the MRC, thus, it might raise the possibility of a review bias. Third, five of the included studies<sup>[11,20-22,25]</sup> were retrospective in design; thus, the biases of retrospective design studies, such as selection bias and recall bias<sup>[36]</sup>, should not be neglected. Fourth, although there were 12 studies included, the number of subjects was rel-

atively small; a larger sample size of subjects is warranted to obtain more reliable results. Fifth, the eligible studies for the present study were all English publications, which may have led to the observed publication bias. Sixth, although all the included studies stated that the results of the MRC were evaluated by experienced radiologists, a reviewer bias caused by inter-observer variability among different readers should not be neglected.

In summary, this meta-analysis demonstrates that MRC is a diagnostic technique with high specificity, but moderate sensitivity, in the diagnosis of biliary anatomy in LDLT donors. Therefore, other techniques such as MDCT may be complementary methods to enhance the sensitivity of the evaluation.

## COMMENTS

### Background

Magnetic resonance cholangiography (MRC) is a non-invasive procedure in diagnosis of the biliary anatomy. The current findings on the value of MRC in diagnosis of biliary anatomy in living-donor liver transplantation (LDLT) donors are conflicting.

### Research frontiers

This study suggests that MRC has a high specificity in diagnosis of biliary anatomy in LDLT donors, but the sensitivity is moderate.

### Innovations and breakthroughs

This is the first meta-analysis to investigate the diagnostic accuracy of MRC in the detection of biliary anatomy in LDLT donors.

### Applications

The results will provide valuable information to the doctors when they make a decision for the living liver donors.

### Peer review

In LDLT, preoperative assessment of biliary duct is so important, especially in a case of posterior graft. Authors reported MRC is a high specificity but moderate sensitivity technique in diagnosis of biliary anatomy in LDLT donors. Other inspection of biliary duct is maybe invasive, and therefore MRC is a good tool for the first survey of the biliary tree. This report is informative for transplant surgeons, even though the sensitivity is moderate.

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## Endovascular pseudoaneurysm repair after distal pancreatectomy with celiac axis resection

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### Abstract

Erosive hemorrhage due to pseudoaneurysm is one of the most life-threatening complications after pancreatectomy. Here, we report an extremely rare case of rupture of a pseudoaneurysm of the common hepatic artery (CHA) stump that developed after distal pancreatectomy with en block celiac axis resection (DP-CAR), and was successfully treated through covered stent placement. The patient is a 66-year-old woman who underwent DP-CAR after adjuvant chemoradiotherapy for locally advanced pancreatic body cancer. She developed an intra-abdominal abscess around the remnant pancreas head 31 d after the surgery, and computed tomography (CT) showed an occluded portal vein due to the spreading inflammation around the abscess. Her general condition improved after CT-guided drain-

age of the abscess. However, 19 d later, she presented with melena, and CT showed a pseudoaneurysm arising from the CHA stump. Because the CHA had been resected during the DP-CAR, this artery could not be used as the access route for endovascular treatment, and instead, we placed a covered stent *via* the inferior pancreaticoduodenal artery originating from the superior mesenteric artery. After stent placement, cessation of bleeding and antegrade hepatic artery flow were confirmed, and the patient recovered well without any further complications. CT angiography at the 6-mo follow-up indicated the patency of the covered stent with sustained hepatic artery flow. To our knowledge, this is the first reported case of endovascular repair of a pseudoaneurysm that developed after DP-CAR.

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**Key words:** Distal pancreatectomy; En block celiac axis resection; Pseudoaneurysm; Endovascular treatment; Covered stent

**Core tip:** Erosive hemorrhage due to pseudoaneurysm is one of the most life-threatening complications after pancreatectomy. Here, we report an extremely rare case of rupture of a pseudoaneurysm of the common hepatic artery stump that developed after distal pancreatectomy with en block celiac axis resection. The pseudoaneurysm was successfully treated through covered stent placement *via* the inferior pancreaticoduodenal artery.

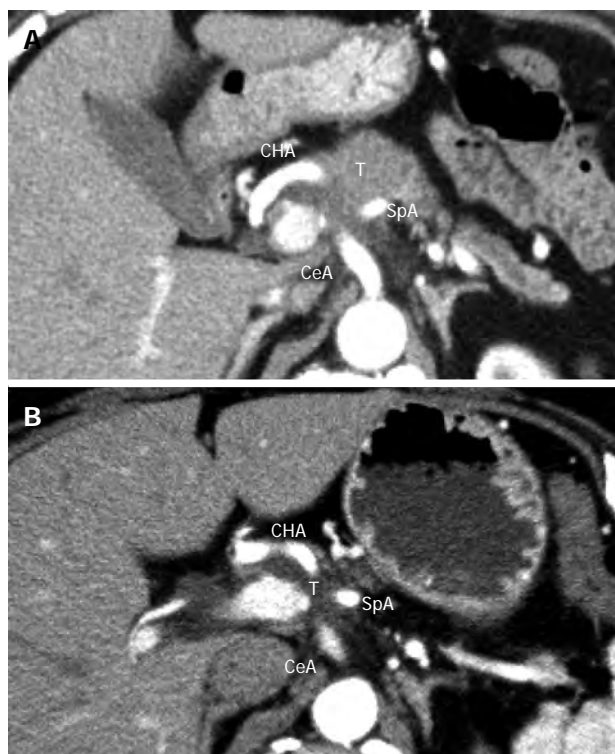
Sumiyoshi T, Shima Y, Noda Y, Hosoki S, Hata Y, Okabayashi T, Kozuki A, Nakamura T. Endovascular pseudoaneurysm repair after distal pancreatectomy with celiac axis resection. *World J Gastroenterol* 2013; 19(45): 8435-8439 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8435.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8435>

## INTRODUCTION

Erosive hemorrhage due to pseudoaneurysm, concomitant to a pancreatic fistula and intra-abdominal abscess, is one of the most serious complications after pancreatectomy<sup>[1]</sup>. Several recent reports have described the successful endovascular treatment of pseudoaneurysms that may develop after pancreaticoduodenectomy<sup>[1,2]</sup>. However, there has been no published report of endovascular repair of pseudoaneurysms that develop after distal pancreatectomy with en block celiac axis resection (DP-CAR) because of the difficulty of the endovascular approach associated with resection of the common hepatic artery (CHA) in this procedure. Here, we report an extremely rare case of a successfully treated pseudoaneurysm of the CHA stump that developed after DP-CAR by covered stent placement *via* the inferior pancreaticoduodenal artery (IPDA). To our knowledge, no similar case has been reported.

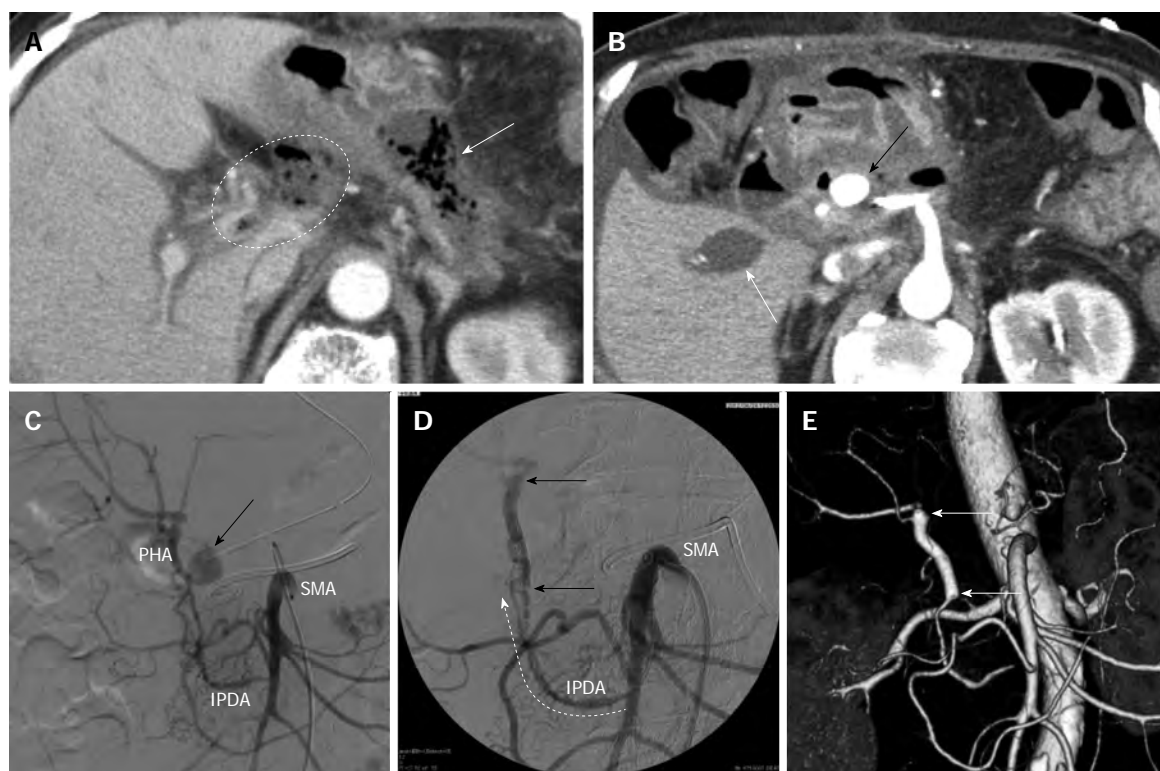
## CASE REPORT

A 66-year-old woman presented with intractable back pain. Laboratory test results showed a high level of carbohydrate antigen 19-9 (CA19-9; 936 U/mL) and contrast-enhanced computed tomography (CT) indicated the presence of a 4.3 cm low-density pancreatic body tumor that involved the CHA, splenic artery (SpA), celiac axis (CeA), and superior mesenteric artery (SMA) (Figure 1A). No distant metastasis was detected. We determined that the tumor was unresectable at that time, and initiated conversion chemoradiation therapy with gemcitabine (1000 mg/body per 2 weeks) and 50 Gy external beam radiation. After the first chemoradiation treatment, the patient received additional gemcitabine chemotherapy (1600 mg/body per 2 weeks) at the discretion of the medical oncologist. After 5 mo of this chemoradiation therapy, the CA 19-9 level had declined to the normal range (14.1 U/mL) and contrast-enhanced CT revealed that the maximum tumor diameter had decreased to 3.0 cm (Figure 1B). Furthermore, although the tumor still involved the CeA, SpA, and CHA, the invasion around the SMA was remarkably diminished. At this point, we determined that the tumor could be completely removed by DP-CAR, and the surgery was planned. At laparotomy, the SMA and the gastroduodenal artery (GDA) were free of tumor invasion. The resectability was confirmed, and the DP-CAR procedure with concomitant portal vein resection was performed. Histopathological examination showed a mucinous carcinoma with lymph node metastasis. Portal venous invasion and extrapancreatic perineural invasion were also confirmed; however, the resection margin of the specimen indicated a negative result for cancer (R0). According to the UICC-TNM classification system, the pancreatic tumor was classified as Stage III (T3N1M0). The patient's intractable back pain disappeared immediately after the surgery, and the postoperative course was uneventful. The patient was discharged on postoperative



**Figure 1** Computed tomography images before and after chemoradiation therapy. A: Computed tomography (CT) image showing a 4.3 cm low-density pancreatic body tumor (T) that involves the common hepatic artery (CHA), splenic artery (SpA), and celiac axis (CeA); B: CT image showing obvious tumor shrinkage after chemoradiation therapy, although the tumor still involves the CHA, SpA, and CeA.

day 13. One week after discharge, she was re-admitted because of appetite loss and oral feeding difficulty. Contrast-enhanced CT at 31 d after surgery showed formation of an intra-abdominal abscess around the remnant pancreas head as well as portal vein occlusion due to the spreading inflammation around the abscess (Figure 2A). CT-guided abscess drainage was performed, and the patient's general condition improved. However, 19 d after drainage of the abscess, the patient presented with melena, and contrast-enhanced CT showed a pseudoaneurysm arising from the CHA stump (Figure 2B). Emergent angiography also showed the pseudoaneurysm arising from the CHA stump (Figure 2C). Because the CHA had been resected during the distal pancreatectomy, the artery could not be used as the access route for endovascular treatment. Therefore, we performed covered stent placement *via* the inferior pancreaticoduodenal artery (IPDA) originating from the SMA (Figures 2D and 3) using a Jostent GraftMaster 3.0 mm × 16 mm stent (Abbott Vascular, Chicago, United States). After stent placement, cessation of bleeding and anterograde hepatic artery flow were confirmed. Although the portal vein remained occluded, the maximum values of GOT and GPT only reached 119 U/L and 70 U/L, respectively, on day 5 after stent placement, and the patient recovered well without any further complications. CT angiography at the 6-mo follow-up indicated the patency of the covered stent with



**Figure 2** Imaging findings before and after endovascular treatment for the pseudoaneurysm. A: Computed tomography (CT) image indicating formation of an intra-abdominal abscess around the remnant pancreas head (arrow) with an occluded portal vein due to the spreading inflammation around the abscess (dotted circle); B: CT image showing the pseudoaneurysm emerging from the common hepatic artery (CHA) stump (black arrow). The white arrow shows the occluded right portal vein; C: Angiography image confirming the pseudoaneurysm emerging from the CHA stump (arrow); D: Covered stent placement (arrows) performed via the IPDA (dotted arrow) originating from the superior mesenteric artery (SMA); E: CT angiography image at the 6-mo follow-up showing the patent covered stent (arrows) and sustained hepatic artery flow. IPDA: inferior pancreaticoduodenal artery; PHA: proper hepatic artery.

sustained hepatic artery flow (Figure 2E). Further, there was no sign of recurrence by 8 mo after DP-CAR.

## DISCUSSION

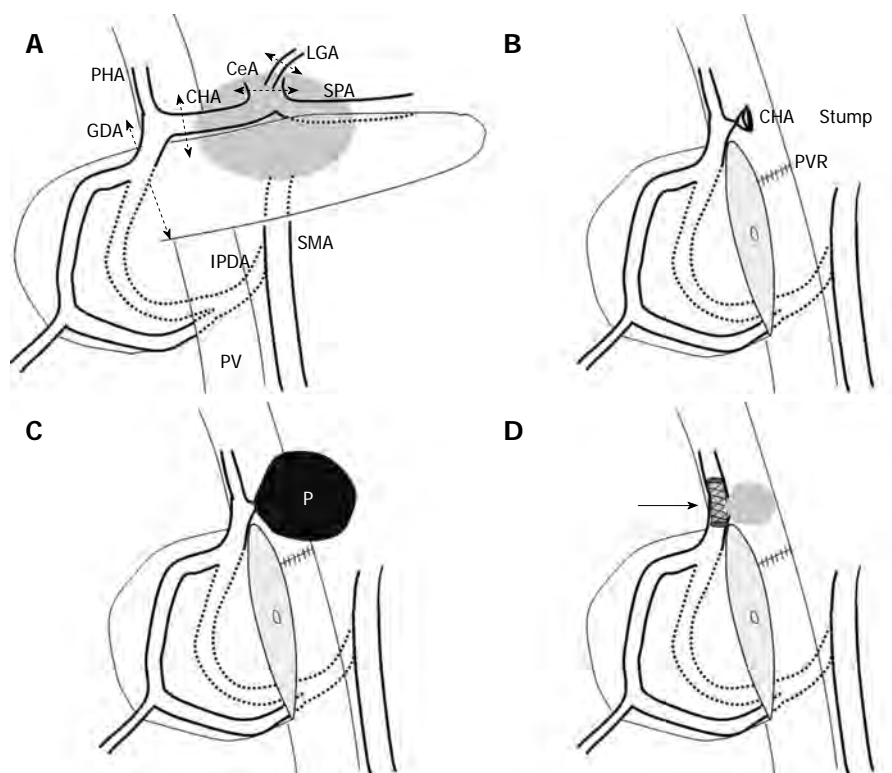
Perineural invasion of pancreatic body cancer can spread towards the celiac plexus and ganglia directly or *via* the nerve plexus surrounding the SpA and CHA<sup>[3]</sup>. Therefore, locally advanced pancreatic body cancer often involves the CHA and/or the celiac axis, and it was often regarded as an unresectable disease. DP-CAR is an operative method that can be adopted in such cases of locally advanced pancreatic body cancer<sup>[3,4]</sup>. The procedure includes distal pancreatectomy and en block resection of the CHA and the CeA with the surrounding neuroplexus. A drawback of this procedure is that, sufficient blood supply from the IPDA to the liver and stomach can not always be ensured. Angiography is routinely performed before DP-CAR in our institution in order to prevent ischemia-related complications in these organs after the surgery. After balloon occlusion of both the CHA and the left gastric artery (LGA), a superior mesenteric angiogram is obtained. If the angiogram indicates insufficient blood flow of the proper hepatic artery and the right gastroepiploic artery, subsequent coil embolization of both the CHA and the LGA is performed to increase the blood flow in the IPDA and prevent ischemia-related

complication after DP-CAR.

With DP-CAR, locally advanced pancreatic body cancers can be resected completely, and some authors have reported prolonged postoperative survival times<sup>[3,4]</sup>. Hirano *et al*<sup>[3]</sup> reported on 23 patients who underwent DP-CAR and described a high R0 resectability rate of 91% with a postoperative mortality rate of 0%. The estimated overall 1- and 5-year survival rates were 71% and 42%, respectively, and the median survival time was 21.0 mo. Baumgartner *et al*<sup>[4]</sup> reported on 11 patients who underwent DP-CAR after neoadjuvant therapy. These authors also described an R0 resectability rate of 91%, with a median overall survival of 26 mo.

Although DP-CAR can offer survival benefit and can potentially achieve complete local control in selected patients, the morbidity rate remains very high, ranging from 25% to 80%<sup>[5,6]</sup>. One of the most serious life-threatening morbidities after pancreatectomy is erosive hemorrhage due to pseudoaneurysm, concomitant to a pancreatic fistula and intra-abdominal abscess<sup>[1]</sup>. This requires emergent treatment, and the mortality rate of patients who develop intra-abdominal arterial hemorrhage after pancreaticoduodenectomy (PD) is reportedly 20%-50%<sup>[7-10]</sup>. Takahashi *et al*<sup>[11]</sup> successfully treated a case of pseudoaneurysm that developed after DP-CAR using relaparotomy. In their case, as in the current case, the pseudoaneurysm emerged on the stump of the CHA,





**Figure 3** Schema of the treatment course. A: The range of tumor invasion after the chemoradiation therapy (gray circle) and the resection line of the distal pancreatotomy with en-bloc celiac axis resection (DP-CAR) (double-headed arrow); B: After DP-CAR: Remnant pancreas head and the arcade of the pancreaticoduodenal artery; C: The pseudoaneurysm (P) at the CHA stump; D: Covered stent delivered *via* the IPDA (arrow). LGA: Left gastric artery; SpA: Splenic artery; CeA: Celiac axis; CHA: Common hepatic artery; SMA: Superior mesenteric artery; PV: Portal vein; PHA: Proper hepatic artery; GDA: Gastroduodenal artery; IPDA: Inferior pancreaticoduodenal artery; PVR: Portal vein resection.

and resection of the pseudoaneurysm with ligation of the GDA were performed.

In recent years, successful endovascular treatment for pseudoaneurysms that develop after PD has been described, and it is presently believed that endovascular treatment should be a first choice for hemorrhage resulting from the pseudoaneurysm<sup>[1,2]</sup>. Lee *et al*<sup>[2]</sup> reported on 27 patients who developed ruptured pseudoaneurysm after PD. Angiographic procedures for initial hemostasis were technically successful in 25 of the 26 patients who underwent angiography. The procedures for hemostasis included transarterial embolization (TAE) using a microcoil in 21 patients and stent graft in 4 patients. There was no recurrent bleeding in any patient in the group, and the selective microcoil embolization or stent graft procedures were effective for controlling the pseudoaneurysmal bleeding.

Among these endovascular treatments, TAE has been recommended as a first-line treatment, because it has been associated with 83%-100% success rates<sup>[2]</sup>. However, despite the dual blood supply from the hepatic artery and portal vein, there is a risk of the major complication of liver infarction or abscess after TAE for pseudoaneurysms arising from the hepatic arteries. In the present case, the portal vein was already occluded, and maintaining hepatic artery flow was indispensable in order to avoid hepatonecrosis and concomitant liver failure. Thus, we decided to use a covered stent to maintain hepatic ar-

tery flow, and the patient remained free of liver infarction and liver abscess after stent placement. We had to use the arcade of the pancreaticoduodenal artery as the access route for covered stent placement because the CHA had already been resected during the distal pancreatectomy. Although the diameter of the IPDA differs among patients and it is uncertain that the stent can always be successfully delivered *via* this route, we believe the method is worth attempting after DP-CAR in critical situations such as the one described herein.

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## Ileal polypoid lymphangiectasia bleeding diagnosed and treated by double balloon enteroscopy

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Author contributions: Park MS and Lee BJ designed the report; Park MS, Lee BJ, Park JJ, Joo MK, Gu DH, Kim KJ, Pyo JH and Lee YH reviewed the case and collected the data; Lee BJ organized the report; and Park MS wrote the paper.

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### Abstract

Intestinal lymphangiectasia is a rare disease characterized by focal or diffuse dilated enteric lymphatics with impaired lymph drainage. It causes protein-losing enteropathy and may lead to gastrointestinal bleeding. Commonly, lymphangiectasia presents as whitish spots or specks. To our knowledge, small bowel bleeding resulting from polypoid intestinal lymphangiectasia has not been reported. Here, we report a rare case of active bleeding from the small bowel caused by polypoid lymphangiectasia with a review of the relevant literature. An 80-year-old woman was hospitalized for melena. Esophagogastroduodenoscopy could not identify the source of bleeding. Subsequent colonoscopy showed fresh bloody material gushing from the small bowel. An abdominal-pelvic contrast-enhanced computed tomography scan did not reveal any abnormal findings. Video capsule endoscopy showed evidence of active and recent bleeding in the ileum. To localize the bleeding site, we performed double balloon enteros-

copy by the anal approach. A small, bleeding, polypoid lesion was found in the distal ileum and was successfully removed using endoscopic snare electrocautery.

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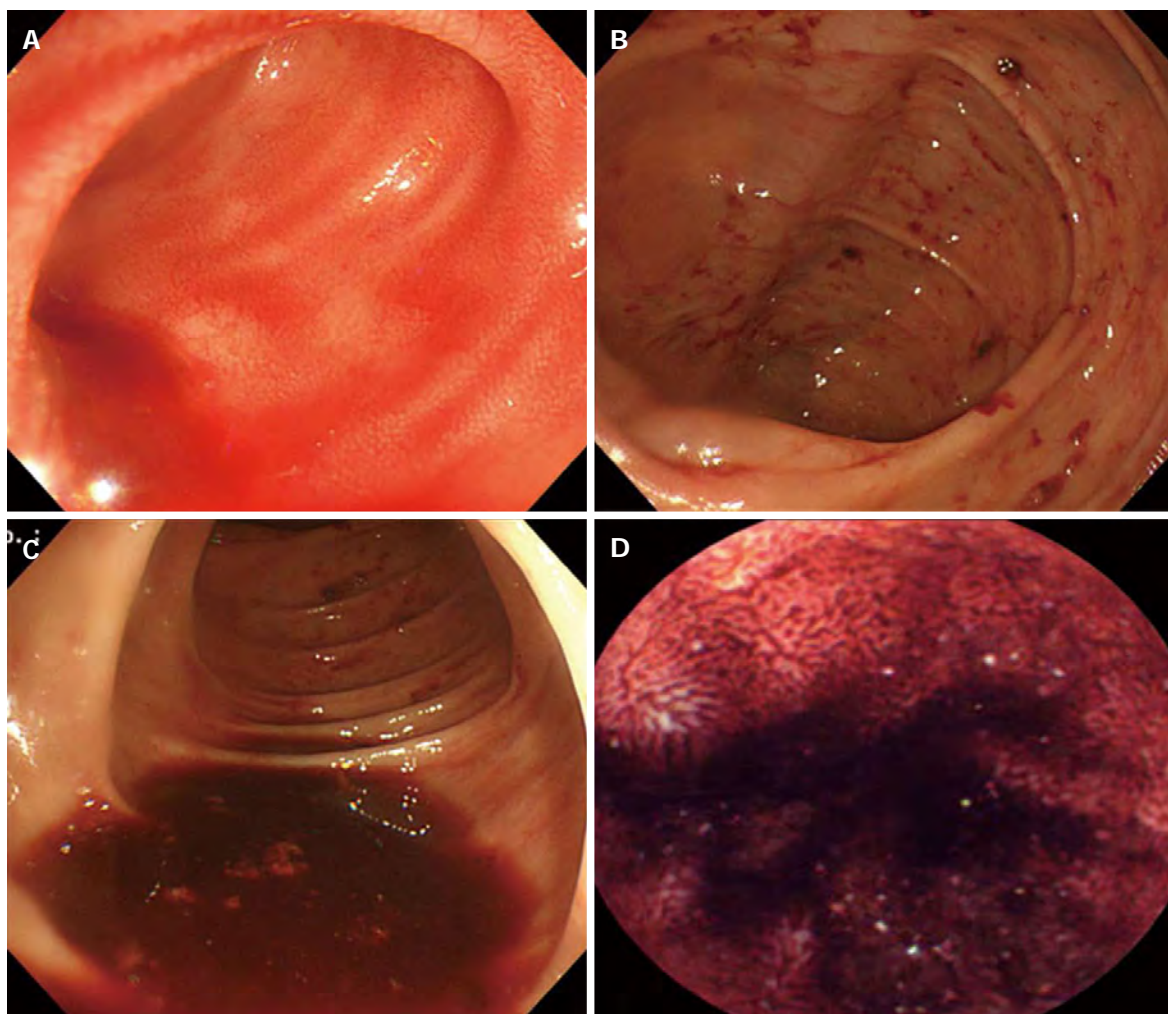
**Key words:** Intestinal lymphangiectasia; Small bowel bleeding; Double balloon endoscopy; Solitary ileal polypoid lesion; Endoscopic polypectomy

**Core tip:** Intestinal lymphangiectasia is a relatively rare disease. To date, only a few cases of small bowel bleeding resulting from intestinal lymphangiectasia have been reported. Herein, we report a case of active bleeding from the small bowel caused by polypoid lymphangiectasia with a review of the relevant literature.

Park MS, Lee BJ, Gu DH, Pyo JH, Kim KJ, Lee YH, Joo MK, Park JJ, Kim JS, Bak YT. Ileal polypoid lymphangiectasia bleeding diagnosed and treated by double balloon enteroscopy. *World J Gastroenterol* 2013; 19(45): 8440-8444 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8440.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8440>

### INTRODUCTION

Nonpathologic lymphangiectasias are commonly detected throughout the gastrointestinal (GI) tract<sup>[1]</sup>. Lymphangiectasias can be pathologic, thus leading to GI symptoms including abdominal pain, steatorrhea, ascites, and, rarely, mid-gastrointestinal bleeding<sup>[2-4]</sup>. Small bowel infections such as tuberculosis or parasitic infections that cause impaired lymph flow might lead to diffuse lymphangiectasia resulting in protein-losing enteropathy<sup>[5]</sup>. Other infectious diseases can also cause focal



**Figure 1** Colonoscopy and video capsule endoscopy findings. A-C: Colonoscopy shows fresh blood material that gushed from the small bowel; D: Subsequent video capsule endoscopy shows evidence of active and recent bleeding in the ileum.

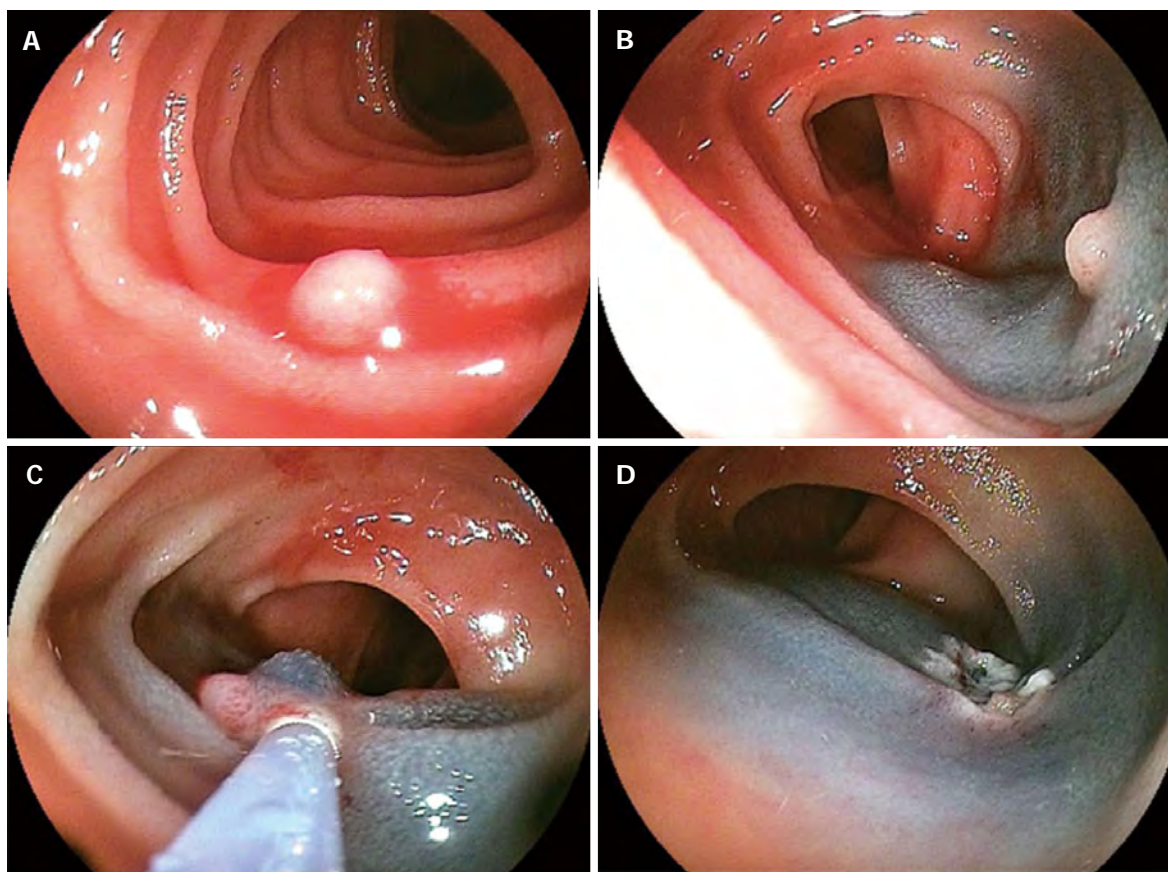
lymphangiectasia and obscure gastrointestinal bleeding. Lymphangiectasia presents either as whitish spots or specks, or yellowish, well-circumscribed, raised mucosal or submucosal lesions on endoscopy<sup>[6,7]</sup>. The polypoid form is extremely rare in patients with gastrointestinal vascular and lymphatic malformation. A few cases of small bowel bleeding resulting from lymphangiectasia have been reported. In this report, we present a case of ileal lymphangiectasia that was detected and treated by double balloon enteroscopy (DBE).

## CASE REPORT

An 80-year-old woman was referred to our department for investigation of gastrointestinal bleeding that she experienced for 7 d. She had chronic kidney disease and atrial fibrillation. She had been receiving hemodialysis twice a week for approximately 1 year prior to this episode, and had been taking warfarin for approximately 2 years. She had no history of habitual drinking or smoking and no specific family history of other diseases. On admission, her blood pressure was 120/80 mmHg, heart

rate was 66 bpm, body temperature was 36.1 °C, and respiratory rate was 22 breaths/min. On physical examination, the patient was alert and pale, and digital rectal examination revealed melena. Laboratory studies showed the following values: hemoglobin (Hb), 8.1 g/dL; hematocrit, 23.6%; white blood cells, 11300/ $\mu$ L (neutrophils, 79.1%; lymphocytes, 13.9%; eosinophils, 2.5%); platelets, 209/ $\mu$ L; prothrombin time, 27.3 s (International Normalized Ratio, 2.53); activated partial thromboplastin time; 28.8 s, protein, 8.2 g/dL; albumin, 4.5 g/dL; aspartate aminotransferase/alanine aminotransferase, 19/11 IU/L; alkaline phosphatase/gamma-glutamyl transpeptidase, 64/14 IU/L; total bilirubin, 0.43 mg/dL; and direct bilirubin, 0.15 mg/dL. We stopped warfarin on admission. We measured her Hb level every 4 h and administered packed red blood cell (RBC) transfusion when her Hb level was < 8.0 g/dL. Consequently, she received 4 pints of packed RBC by transfusion during hospitalization. An emergency esophagogastroduodenoscopy revealed atrophic mucosal changes and several raised erosions in the antrum; however, no active bleeding was found. A subsequent colonoscopy showed fresh





**Figure 2 Double balloon enteroscopy findings.** A: Double balloon enteroscopy shows a small, whitish polypoid lesion with active bleeding in the distal ileum; B, C: After submucosal injection of a saline-epinephrine mixture, polypectomy was performed; D: After the procedure, argon plasma coagulation was performed on the post-polypectomy ulcer to achieve hemostasis.

bloody material gushing from the small bowel (Figure 1A-C). An abdominal-pelvic contrast-enhanced computed tomography scan did not reveal any abnormal findings. Video capsule endoscopy (IntroMedic, Seoul, South Korea) showed evidence of active and recent bleeding in the ileum but could not localize the bleeding site (Figure 1D). To localize the bleeding site, we performed DBE. Retrograde DBE (EN450T5, Fujinon, Saitama, Japan) showed a small bleeding polyp in the distal ileum (Figure 2A). The ileal polyp was removed using an endoscopic snare (SD-9L-1; Olympus Optical Co., Ltd., Tokyo, Japan). Thereafter, electrocautery was performed after submucosal injection of a hypertonic saline-epinephrine solution (Figure 2B and C). After the procedure, argon plasma coagulation was performed for an ulcer caused by polypectomy to attain hemostasis (Figure 2D). The entire procedure lasted for approximately 150 min. The results of the histological examination were consistent with a diagnosis of lymphangiectasia characterized by dilated lymphatic channels in the lamina propria (Figure 3A and B). We administered warfarin 3 d after the procedure. After removal of the ileal polyp, the patient was discharged with no gastrointestinal bleeding. The patient has been followed up for 1 year and has shown no sign of recurrence.

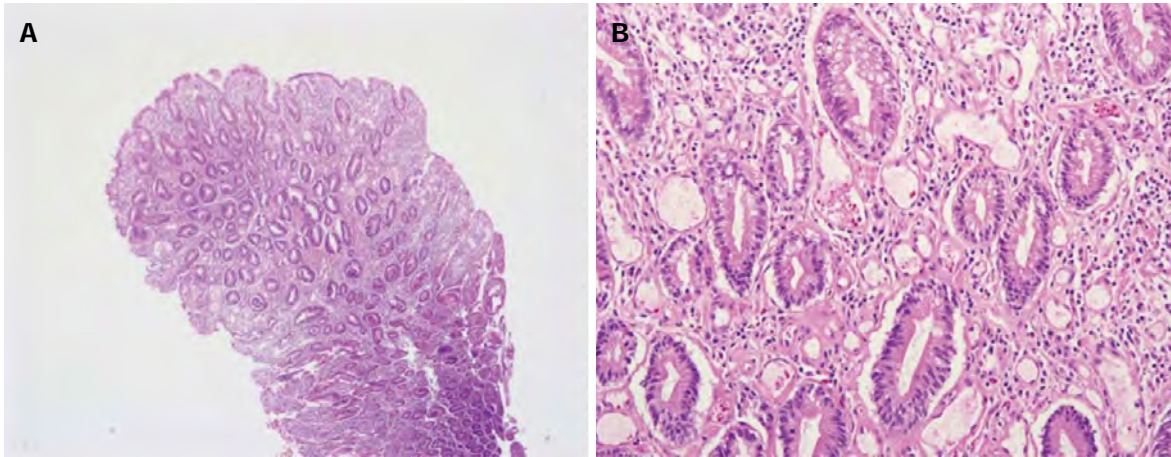
## DISCUSSION

Intestinal lymphangiectasia is a rare disease characterized by dilated intestinal lacteals causing loss of lymph into the lumen of the small intestine resulting in hypoproteinemia, hypogammaglobulinemia, hypoalbuminemia, and lymphopenia<sup>[8]</sup>. The most commonly affected site in the intestine is the duodenum<sup>[9,10]</sup>.

Gastrointestinal symptoms range from mild to severe presentations such as diarrhea, steatorrhea, abdominal mass, and mechanical ileus. Chronic occult blood loss may occur in some cases, and non-specific small bowel ulceration has been reported in others<sup>[9,11,12]</sup>. Rarely, massive bleeding has also been recorded<sup>[13,14]</sup>. The patient in our case had chronic kidney disease and was receiving treatment with warfarin for atrial fibrillation. Although we could not find any report on whether anticoagulation or hemodialysis increased the risk of bleeding in patients with lymphangiectasia, we consider that she had a bleeding diathesis and hence, had bleeding from the ileal polyp.

Several mechanisms have been suggested to interpret the pathophysiology of bleeding lymphangiectasias. Obstruction of the normal flow of chyle from the small intestine may increase intraluminal pressure sufficiently to open latent lymphatic-venous connections<sup>[15]</sup>. Conse-





**Figure 3** Histopathologic findings of intestinal lymphangiectasia. A, B: Microscopic examination shows dilated lymphatic channels in the lamina propria (hematoxylin and eosin,  $\times 40$ ). Protein-rich fluid can escape from these channels into the extracellular space of the lamina propria and ultimately into the gut lumen (hematoxylin and eosin,  $\times 200$ ).

quently, this pressure gradient opens latent lymphatic-arterial (rather than venous) connections<sup>[16]</sup>. Such openings into another closed system of higher pressure would allow the retrograde flow of blood into the lymphatics and as a result, bursting of blood-filled dilated lymphatics may lead to intestinal bleeding. However, we could find neither the pathologic lymphatic-blood vessel connection nor obstruction of lymphatic channel in the pathological sections of the biopsy specimen.

Intestinal lymphangiectasia was confirmed by the endoscopic findings and intestinal biopsy results. Marked dilation of the lymphatics was seen in the mucosa, sometimes extending into the submucosa. The overlying intestinal epithelium usually appears normal, but occasionally creamy yellow villi may be seen<sup>[17]</sup>. In recent years, the development of newer endoscopic methods, particularly video capsule endoscopy and DBE has simplified diagnosis and treatment of small bowel lesions. In our case, although small bowel bleeding was detected by video capsule endoscopy, the definite location and etiology of bleeding could not be confirmed. As the bleeding lesion was found to be limited to the ileum in video capsule endoscopy, DBE was performed by the anal approach. The oral approach was not used subsequently because there were no signs of bleeding after polypectomy and coagulation. We diagnosed and treated ileal polypoid intestinal lymphangiectasia using DBE.

To our knowledge, this is the first case to report small bowel bleeding from a solitary ileal polypoid intestinal lymphangiectasia. This case represents the successful detection and treatment of bleeding resulting from rare, solitary, ileal polypoid intestinal lymphangiectasia using DBE.

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**S- Editor:** Wen LL **L- Editor:** Cant MR **E- Editor:** Zhang DN



## Rare adult gastric duplication cyst mimicking a gastrointestinal stromal tumor

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Author contributions: Prachayakul V conceptualized the study; Aswakul P, Deesomsak M and Junyangdikul P acquired the data; Prachayakul V and Aswakul P wrote the manuscript.

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### Abstract

Gastric duplication cyst is a very rare gastrointestinal tract malformation that accounts for 2%-4% of alimentary tract duplications. Most cases are diagnosed within the first year of life, following presentation of abdominal pain, vomiting, and weight loss and clinical discovery of an abdominal palpable mass. This case report describes a very uncommon symptomatic gastric duplication cyst diagnosed for the first time in adulthood. Only a few other case reports of similar condition exist, and all were identified by endosonography. The current case involves a 52-year-old male who presented with a one-month history of progressive iron deficiency anemia without overt gastrointestinal bleeding. The patient underwent esophagogastroduodenoscopy, which revealed a 2.0 cm pinkish subepithelial lesion, suspected to be a gastrointestinal stromal tumor (GIST) and source of gastrointestinal bleeding. The endosonography showed

inhomogeneous hypoechoic lesions with focal anechoic areas arising from a second and third layer of the gastric wall. Differential diagnoses of GIST, neuroendocrine tumor, or pancreatic heterotopia were made. The lesion was removed using an endoscopic submucosal resection technique. Histopathology revealed an erosive gastric mass composed of a complex structure of dilated gastric glands surrounded by fibro-muscular tissue, fibroblasts, and smooth muscle bundles, which led to the diagnosis of gastric duplication.

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**Key words:** Gastric duplication cyst; Gastrointestinal bleeding; Endoscopy; Endoscopic ultrasonography; Endoscopic mucosal resection; Differential diagnosis; Adult

**Core tip:** Gastric duplication cyst is a very rare gastrointestinal malformation and most of the cases are diagnosed within the first year of life following clinical investigations of abdominal symptoms including pain, vomiting, and palpable mass. Gastrointestinal hemorrhage is a rare clinical manifestation. Here, we report a 52-year-old man who presented with a one-month history of progressive iron deficiency anemia without overt gastrointestinal bleeding. Esophagogastroduodenoscopy revealed general gastrointestinal bleeding and a 2.0 cm subepithelial tumor originating from the third layer of gastric wall. After complete removal by endoscopic mucosal resection, histopathological findings indicated a diagnosis of gastric duplication cyst with erosion.

Deesomsak M, Aswakul P, Junyangdikul P, Prachayakul V. Rare adult gastric duplication cyst mimicking a gastrointestinal stromal tumor. *World J Gastroenterol* 2013; 19(45): 8445-8448 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8445.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8445>

## INTRODUCTION

Gastric duplication cyst is a rare congenital malformation of the upper gastrointestinal tract, accounting for 2%-4% of all duplications of the alimentary tract<sup>[1]</sup>. Most cases are diagnosed in the first year of life, following presentation of abdominal pain, vomiting, and weight loss and clinical discovery of an abdominal palpable mass or upon clinical investigation of gastrointestinal obstruction and/or perforation<sup>[1-3]</sup>. Bleeding was a very rare clinical manifestation of this condition, and very few cases of gastric duplication cysts have been diagnosed in adulthood<sup>[2-6]</sup>. Some of the adult cases have been incidentally detected during upper endoscopy [endoscopic ultrasound (EUS) technology] or abdominal imaging studies for other reasons<sup>[3,5,6]</sup>. Yet, the reported radiographic and endosonographic findings from these limited cases have not revealed any characteristic profile useful for diagnosis.

Herein we describe an unusual case of gastric duplication cyst which was identified in an adult male following endosonographic investigations of subacute anemia and discovery of upper gastrointestinal bleeding.

## CASE REPORT

A 52-year-old man presented with a one-month history of progressive iron deficiency anemia without overt gastrointestinal bleeding. Laboratory tests indicated remarkably low hemoglobin level (9.0 mg/dL; reference range: 13.0-17.0 mg/dL) and slightly low ferritin level (18.0 ng/mL; reference range: 12-200 ng/mL). Esophagogastroduodenoscopy was performed and revealed a 2.0 cm pinkish umbilicated subepithelial lesion located at antrum and accompanied by evidence of ulceration and blood clotting (Figure 1A). Gastrointestinal stromal tumor (GIST) was suspected, and subsequent endosonographic evaluation was ordered.

In the endoscopic suite, the patient underwent total intravenous anesthesia using propofol with full anesthetic monitoring. A curvilinear echoscope (EG-3870UTK; Pentax, Miyaki, Japan) was used and revealed a 1.8 cm hypoechoic mass arising from the second and third layer of the gastric wall, with a centralized anechoic area lacking vascular flow (Figure 1B). The findings suggested small GIST with cystic change. An endoscopic submucosal resection (EMR) technique was applied with an 8.8 mm gastroscope (GIF-Q180; Olympus, Tokyo, Japan) and creation of a submucosal cushion by injecting a 4 mL mixture of adrenaline (1 mg), Indigo carmine (0.4% solution) and Gelofucine<sup>®</sup> (500 mL) using a 23 gauge needle [Heyinovo<sup>™</sup>; Wilson Instruments Co., Ltd., Shanghai, China]. The lesion was removed in an *en-bloc* fashion by an electrical snare (Figure 2).

Histopathological analysis of the resected tissue revealed an erosive gastric mass that was composed of a complex structure characterized by dilated gastric glands surrounded by fibromuscular tissue, fibroblasts, and smooth muscle bundles. Immunohistochemistry analysis

showed negativity for CD117, DOG-1, and S-100, but focal positivity for smooth muscle actin. Although these pathology findings suggested a differential diagnosis of pancreatic heterotopia, no evidence was found for this diagnosis (Figure 3) and the diagnosis of gastric duplication was made.

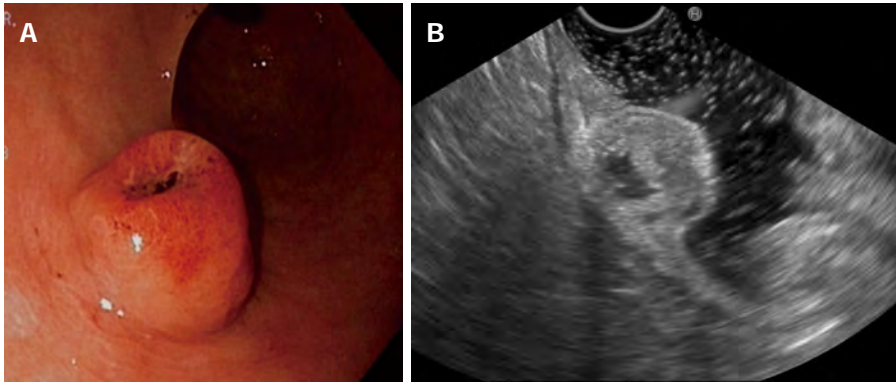
## DISCUSSION

Gastric duplication cysts are rare congenital malformations with frequent localization in the alimentary tract that follows the incidence hierarchy of ileum > esophagus > jejunum > colon > stomach > appendix<sup>[2-4]</sup>. Yet, the etiopathogenetic origin of gastric duplication cysts remains undefined. These duplications, however, are usually found to be distributed dorsally to the primitive gut during development, so that most gastric duplication cysts appear along the greater curvature of the stomach, adjacent to the gastric wall (only 5.5% are found in the lesser curvature<sup>[3]</sup>). The stomach is lined by typical gastric mucosa, which is often accompanied by patches of ectopic intestinal epithelium; in addition, a smooth muscle coat fuses with the muscularis propria of the stomach, making it contiguous with the wall of the stomach, and shares a common blood supply. The cyst in the current case was located at the greater curvature of the antral area.

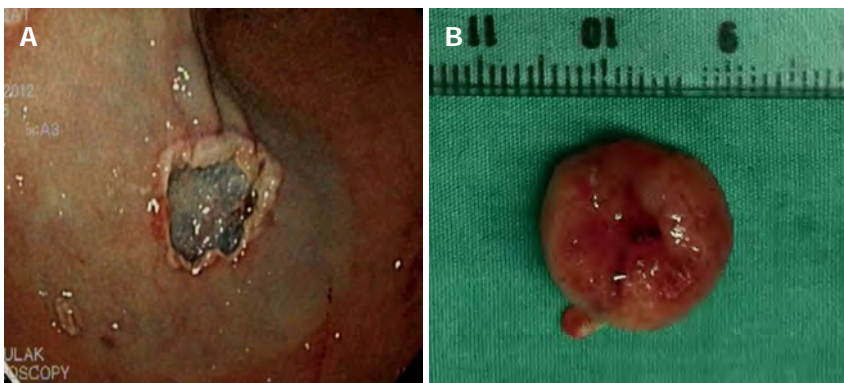
The rarity of the current case was furthered by the manifestation of symptoms and diagnosis having occurred in later adulthood. Furthermore, gastric duplication cysts are usually asymptomatic in adult patients, yet this case presented with anemia from upper gastrointestinal bleeding. Based upon the pathological findings, we speculate that acid erosion of the gastric lining near the cyst led to the hemorrhage, as well as the observed ulceration.

Throughout the case management course, various differential diagnoses were suggested and rejected. In particular, the endoscopic and EUS findings of small subepithelial lesions with umbilication suggested potential diagnoses of GIST, neuroendocrine tumor, or pancreatic heterotopia. The endosonographic characteristics of this patient are very distinctive from those reported for previous cases, even though all cases involved cystic lesions at greater curvature sites of the stomach. The cysts of previous cases have ranged in diameter from 5 to 7 cm, and were located adjacent to the fourth layer of the gastric wall. In addition, most of these cysts showed an inhomogeneous echoic pattern, which was suspected to reflect secretions from the gastric gland composed of both mucous and serous material<sup>[3,5,6]</sup>. We hypothesized the atypical endosonographic findings of the current case corresponded to the smaller cyst size and low fluid volume, which caused the lesion to appear more like a solid mass with cystic degeneration than a cystic lesion. Therefore, the final diagnosis was gastric duplication cyst. Finally, even though fine needle aspiration cytology may have been able to achieve the differential diagnosis<sup>[4]</sup>, we felt that complete removal of the lesion was justified by

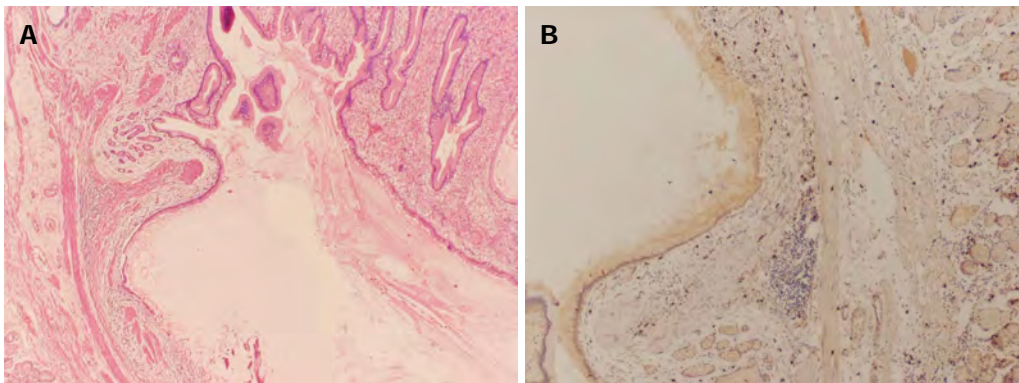




**Figure 1** Endoscopic view. A: Showing a subepithelial lesion with ulceration and blood clotting; B: Showing a hypoechoic lesion arising from the second and third layers of the gastric wall, with a central anechoic area.



**Figure 2** Lesion was removed in an en-bloc fashion by an electrical snare. A: Submucosal layer after completion of the endoscopic mucosal resection; B: En-bloc resected tissue.



**Figure 3** Histopathology and immunohistochemistry showing. A: The eroded gastric mass composed of dilated gastric glands surrounded by fibromuscular tissue, fibroblasts, and smooth muscle bundles; B: CD117-negative staining.

the patient's symptoms and unknown risk for malignant transformation<sup>[1]</sup>.

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## Ileo-ileal intussusception caused by diffuse large B-cell lymphoma of the ileum

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Author contributions: Xu XQ and Hong T designed the report; Xu XQ, Hong T, Li BL and Liu W were the patient's attending doctors; Xu X and Hong T performed the surgery; Xu XQ and Hong T organized the report; and Xu XQ wrote the paper; an author may have more than one contribution, and more than one author may have contributed to the same aspect.

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**Core tip:** Intussusception is a common cause of bowel obstruction in pediatric patients. Primary diffuse large B cell non-Hodgkin's lymphoma of the small intestine is rare. Intussusception due to primary diffuse large B cell non-Hodgkin's lymphoma in the small intestine is even rarer in adults and is often difficult to diagnose. This article describes the diagnosis and management of this rare disease.

Xu XQ, Hong T, Li BL, Liu W. Ileo-ileal intussusception caused by diffuse large B-cell lymphoma of the ileum. *World J Gastroenterol* 2013; 19(45): 8449-8452 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8449.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8449>

### Abstract

The occurrence of adult intussusception from small intestinal lymphoma is quite rare. We present an 82-year-old man with a two-month history of intermittent abdominal pain, nausea and fatigue. Clinical symptoms included moderate abdominal tenderness in the right lower abdomen. Computed tomography scan of the abdomen revealed a mass in the terminal ileum with the sign of "bowel within bowel" which was suspicious of ileo-ileum intussusception. The patient underwent laparoscopic segmental ileal resection. Pathologic evaluation revealed a diffuse large B cell non-Hodgkin's lymphoma of the ileum. The postoperative course was uneventful.

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**Key words:** Adult intussusception; Lymphoma; Small intestine; Ileum

### INTRODUCTION

Intussusception is most often seen in infants and children and only 5% of all cases occur in adults. It accounts for about 1% of all cases of adult bowel obstruction. Adult intussusception is rare and about 40% are related to malignant lesions<sup>[1]</sup>. In general, most lesions in the small intestine are benign. Malignant lesions account for up to 30% of all cases of intussusception in the small intestine. Intussusception occurring in the large bowel is more likely to be related to malignant lesions in 63%-68% of cases<sup>[2]</sup>. Primary malignant tumors of the small intestine are very rare, accounting for less than 2% of all gastrointestinal malignancies. Malignant lesions resulting in intussusception in the small intestine include primary adenocarcinoma, gastrointestinal stromal tumors (GISTs), lymphoma and carcinoid tumors<sup>[3]</sup>.

The gastrointestinal tract is the most common site of primary extranodal non-Hodgkin's lymphoma (NHL), accounting for 20%-40% of all extranodal disease<sup>[4]</sup>. The stomach (50%-60%) is the most frequently affected site, followed by the small bowel (20%-30%), whereas 85%

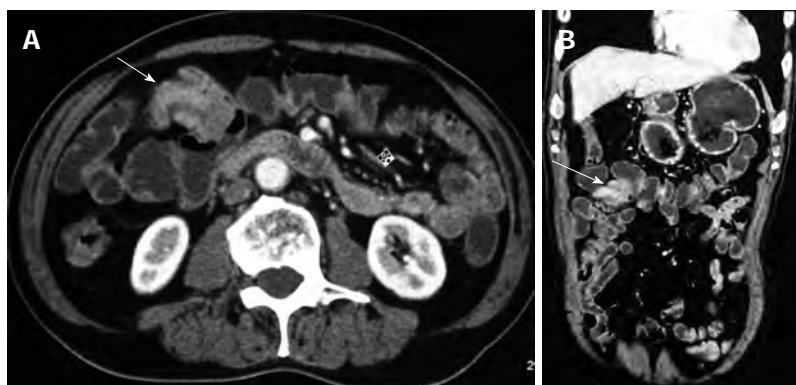


Figure 1 Contrast-enhanced computed tomography showing suspected right ileo-ileum intussusception with the sign of “bowel within bowel” in the ileum (A, arrow, axial view), (B, arrow, coronal view).



Figure 2 Positron emission tomography and computed tomography showing high metabolism in the right ileum (arrow) and multiple lymph nodes with high metabolism in the mesentery root of the small intestine, in which malignant lesions in the terminal ileum were suspected.

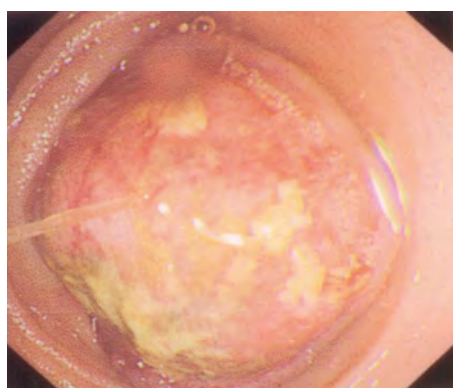


Figure 3 Endoscopic findings showing a mass approximately 50 cm away from the ileocecal valve, which almost filled the ileal cavity.

of primary gastrointestinal lymphomas and 60%-80% of intestinal lymphomas are B-cell type followed by T-cell NHL and Hodgkin's lymphoma<sup>[5]</sup>. The ileum is the most common site of small intestine lymphoma. Intussusception is very rarely seen in intestinal NHL and the most common type of lymphoma causing intussusception is diffuse B-cell NHL<sup>[5]</sup>.

We herein describe a case of adult ileal intussuscep-

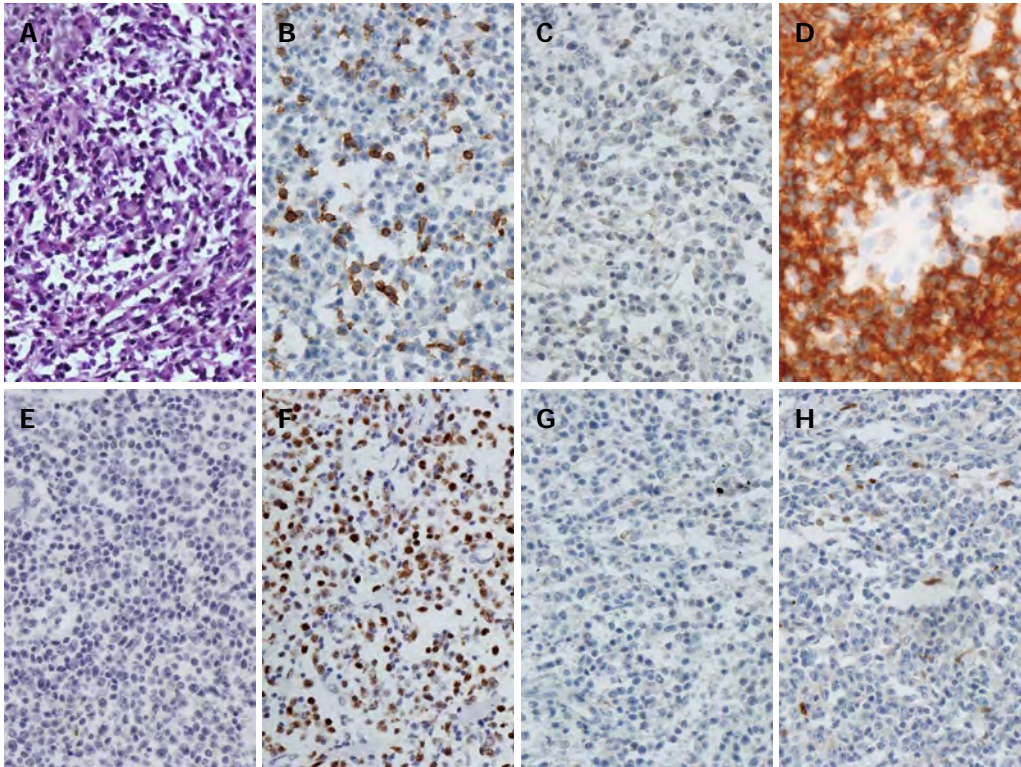
tion caused by diffuse large B-cell lymphoma of the small bowel in an 82-year-old male patient.

## CASE REPORT

An 82-year-old male was admitted with a two-month history of intermittent abdominal pain, nausea and fatigue. He also complained of marked weight loss (8 kg) during the last two months. He was diagnosed with incomplete intestinal obstruction and was supported with parenteral nutrition in the community hospital. His past medical history was hypertension for 20 years. Physical examination revealed moderate abdominal tenderness in the right lower abdomen.

Blood tests revealed a white blood cell count of  $4.1 \times 10^9/L$  with 63% neutrophils and a hemoglobin level of 96 g/L with a hematocrit of 29%. His liver and kidney function test results and tumor marker (CEA, CA19-9, CA242 and CA724) levels were all normal. The fecal occult blood test was positive. Plain abdominal radiography was unremarkable. Contrast-enhanced computed tomography (CT) showed multiple lymphadenoma in the mesentery root of the small intestine and posterior peritoneum, a mass in the terminal ileum with the sign of “bowel within bowel” was suspicious of ileo-ileum intussusception (Figure 1). Positron emission tomography and computed tomography (PET-CT) showed high metabolism in the terminal ileum and multiple lymph nodes with high metabolism in the mesentery root of the small intestine, in which malignant lesions in the terminal ileum were considered (Figure 2). Therefore, balloon-assisted enteroscopy was performed. A mass, approximately 50 cm away from the ileocecal valve, almost filled the ileal cavity (Figure 3) and did not allow the enteroscope to pass. A biopsy was taken from this lesion and the pathology result showed a diffuse large B cell NHL of the ileum (Figure 4). Laparoscopic exploration was performed due to low hemoglobin, weight loss and the mass with suspected intussusception on CT. A tumor mass of  $5.0 \text{ cm} \times 3.0 \text{ cm}$  was revealed with ileo-ileum intussusception which was 40 cm distal to the ileum with multiple lymphadenoma in the mesentery root





**Figure 4** Histological and immunohistological examination of the endoscopic and surgical specimens showing diffuse large B-cell non-Hodgkin's lymphoma. A:  $\times 400$ , HE staining; B:  $\times 400$ , CD5 (-); C:  $\times 400$ , CD10 (-); D:  $\times 400$ , CD20 (+); E:  $\times 400$ , CD23 (-); F:  $\times 400$ , MUM-1 (+); G:  $\times 400$ , Bcl-6 (-); H:  $\times 400$ , Cyclin D1 (-).

of the small intestine. A segmental ileal resection was performed laparoscopically. After opening the specimen, a mass of approximately 3 cm was found in the ileum wall almost filling the ileal cavity. The histopathological findings were suggestive of a diffuse large B cell NHL of the ileum (Figure 4), which was consistent with the preoperative pathological results of endoscopic biopsy. Tumor infiltration was detected in 2 of 16 lymph nodes. The postoperative course was uneventful. Bone marrow biopsies showed a normal marrow without infiltration. Cervical and chest PET-CT scans were unremarkable. Postoperative chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisolone combined with the monoclonal antibody, rituximab, was administered. At the 6-mo follow up, there was no evidence of tumor activity.

## DISCUSSION

Intussusception is rare in adults ( $< 5\%$ ), but it is the most common cause of intestinal obstruction in infants aged 6-18 mo<sup>[1]</sup>. The characteristic pediatric presentation triad of abdominal pain, palpable abdominal mass and bloody stool is rarely seen in adult cases. An abdominal mass is palpated in 12%-30% of cases, whereas 6%-25% of cases present with complications of the disease such as obstruction, hemorrhage, perforation, or intussusception<sup>[5]</sup>. Most patients present with subacute (24.4%) or chronic (51.2%) symptoms of abdominal pain, nausea, vomiting and constipation. The non-specific clinical

presentation makes the preoperative diagnosis difficult<sup>[6]</sup>. CT scan is one of the most useful preoperative diagnostic modalities for intussusception as it can show the thickened segment of bowel with an eccentrically placed crescent-like fatty area (bowel within bowel)<sup>[5]</sup>. If the patient does not have a complete intestinal obstruction, colonoscopy is helpful for the clinical and pathological diagnosis of intussusception. PET-CT is useful for detecting the primary benign or malignant lesion sites and the distal regions which may be involved in lymphoma, although it is not often routinely used<sup>[7]</sup>.

About 52%-55% of adult intussusceptions occur in the small intestine<sup>[8]</sup>. Sixty-three percent of adult small intestinal intussusception cases were associated with benign lesions, 23% cases were idiopathic, and 14% cases were associated with malignant lesions<sup>[9]</sup>. The incidence of primary lymphomas in the small intestine accounts for less than 2% of all gastrointestinal malignancies and 10%-20% of small intestine malignancies<sup>[1]</sup>.

The management of small intestine intussusception caused by NHL is mandatory surgical intervention for adult intussusception due to the high incidence of underlying malignancy in intussusceptions and the inability to differentiate non-operatively benign from malignant causes in enteric intussusceptions<sup>[5]</sup>. For adult small intestinal intussusception caused by NHL, primary surgical resection of the localized intestinal lesions with NHL is the treatment modality of choice, especially in patients who have complications of intussusception.

In conclusion, we describe the diagnosis of adult

ileal intussusception with detailed images of this specific case. Adult intussusception must be considered in the differential diagnosis of patients with abdominal pain and vomiting. The work-up must include X-ray, ultrasound and CT scan of the abdomen and PET-CT in special cases. Surgical intervention is required and warranted once the diagnosis of intussusception is made, due to the high risk of malignancy and bowel obstruction.

## COMMENTS

### Clinical diagnosis

Ileo-ileal intussusception caused by diffuse large B-cell lymphoma of the ileum.

### Differential diagnosis

Adult intussusception must be considered in the differential diagnosis of patients with abdominal pain and vomiting.

### Imaging diagnosis

Diagnostic imaging must include X-ray, ultrasound and computed tomography scan of the abdomen which can show the sign of "bowel within bowel".

### Treatment

Surgical intervention is required and warranted once the diagnosis of intussusception is made, due to the high risk of malignancy and bowel obstruction.

### Peer review

This is an interesting case report, which shows a rare case of ileo-ileal intussusception caused by diffuse large B-cell lymphoma of the ileum.

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## Successful interventional radiological management of postoperative complications of laparoscopic distal pancreatectomy

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### Abstract

During the past decade, laparoscopic distal pancreatectomy (LDP) has gained increasing acceptance in the surgical community as a viable treatment option for distal pancreatic lesions. However, the possible complication of post-LDP pancreatic leakage remains a challenge, because it may lead to a series of events resulting in intraperitoneal abscess formation, sepsis, pseudoaneurysm formation, and occasional fatal hemorrhage. Dealing with these complications is extremely difficult and not much experience has been reported to date. We report a case involving the aforementioned post-LDP complications successfully managed by interventional radiological techniques while avoiding reop-

eration. We conclude that these management options are attractive, safe and minimally invasive alternatives to standard protocols.

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**Key words:** Interventional radiology; Pancreatectomy; Laparoscopy; Postoperative complications

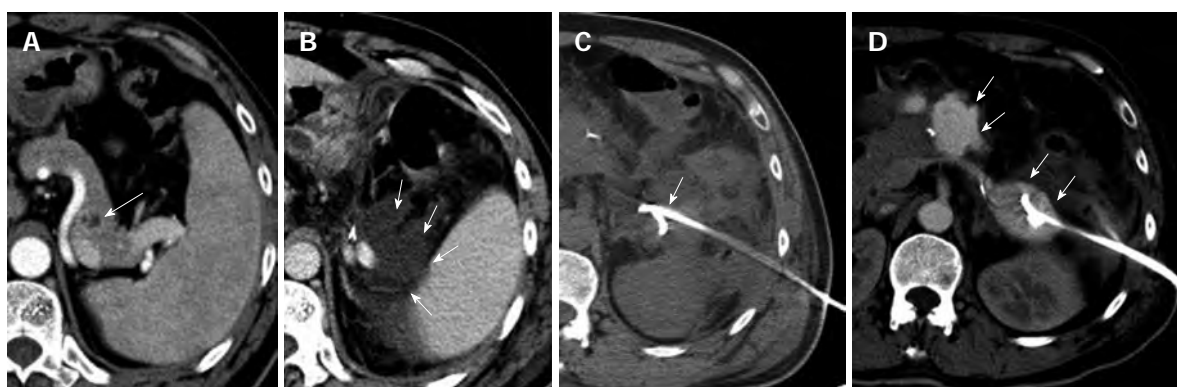
**Core tip:** A 48-year-old man who suffered from a series of complications of pancreatic leakage and resultant abscess formation, sepsis, and intra-abdominal hemorrhage after laparoscopic distal pancreatectomy (LDP), was treated successfully by interventional radiology, with avoidance of reoperation. These interventional radiological approaches seem to be attractive, safe and minimally invasive options for treatment of post-LDP complications.

Zhu YP, Ni JJ, Chen RB, Matro E, Xu XW, Li B, Hu HJ, Mou YP. Successful interventional radiological management of postoperative complications of laparoscopic distal pancreatectomy. *World J Gastroenterol* 2013; 19(45): 8453-8458 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i45/8453.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i45.8453>

### INTRODUCTION

Laparoscopic pancreatectomy was first reported in the early 1990s. Since then, a variety of procedures have been introduced, such as staging of pancreatic malignancy, debridement for acute necrotizing pancreatitis, cystoenterostomy for pancreatic pseudocysts, pancreatic tumor enucleation, and pancreaticoduodenectomy<sup>[1-4]</sup>. Laparoscopic distal pancreatectomy (LDP) is widely accepted by the surgical community due to its advantages





**Figure 1** Computed tomography. A: Distal pancreatic tumor; B: Hydrops adjacent to the pancreatic stump; C: A pigtail catheter (percutaneous catheter drainage tube 1) was placed into the left anterior pararenal space under computed tomography (CT) guidance; D: Abdominal contrast-enhanced CT revealed extravasation of contrast medium adjacent to the pancreatic stump.

of decreased postoperative pain, earlier normalization of bowel function, and shorter length of hospitalization compared to open surgery<sup>[5-8]</sup>. However, the complication of pancreatic leakage after LDP remains a challenge because it may lead to a series of events including intraperitoneal abscess, subsequent sepsis, pseudoaneurysm formation, and occasional fatal hemorrhage<sup>[9]</sup>. Dealing with these complications by reoperation is particularly difficult due to adhesion formation and the generally fragile postoperative condition of the patient.

## CASE REPORT

A 48-year-old man was admitted because of the incidental discovery of a pancreatic mass during a routine medical check-up. Abdominal computed tomography (CT) showed a low-density lesion of 1.5 cm × 1.3 cm with arterial enhancement in the pancreatic tail (Figure 1A). The patient underwent spleen-preserving LDP in April 2009. During the procedure, the splenic artery and vein were meticulously dissected from the pancreatic tissue and preserved. Branches from these two vessels to the pancreatic parenchyma were either severed by a harmonic scalpel or carefully ligated and separated. The pancreas was transected by an endoscopic linear stapler and the stump continuously sutured. A Jackson-Pratt drainage tube was placed in close proximity to the pancreatic stump. The procedure which lasted for 160 min was smooth. Postoperative pathological examination diagnosed a pancreatic insulinoma.

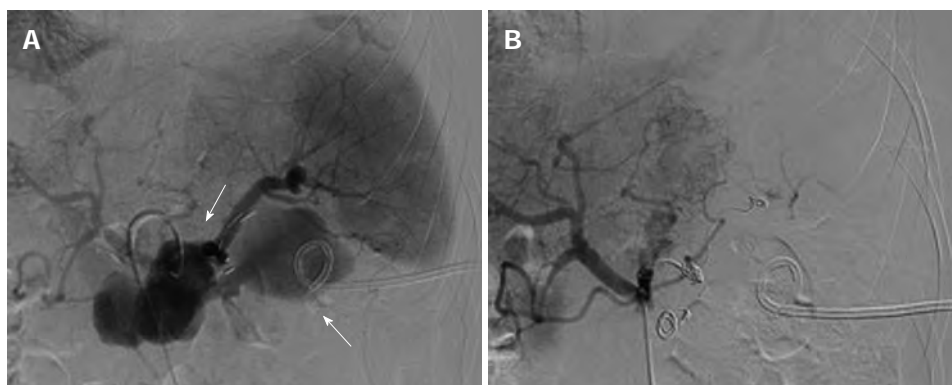
The patient complained of abdominal fullness at 4 d after the operation, with physical examination revealing fever (up to 38.8 °C), tachycardia, and left abdominal tenderness. Amylase-rich abdominal fluid (4389 IU/L) draining from the Jackson-Pratt tube was detected. An effusion was demonstrated on both abdominal ultrasonography and CT beside the pancreatic stump (Figure 1B). Pancreatic leakage from the stump was therefore diagnosed according to International Study Group for Pancreatic Fistula criteria<sup>[10]</sup>. It was also noted that the patient's sclera and skin were yellow in hue. Laboratory

findings showed obvious elevation of serum bilirubin (total/direct bilirubin: 6.7/3.4 mg/dL) without evidence of biliary obstruction on imaging studies.

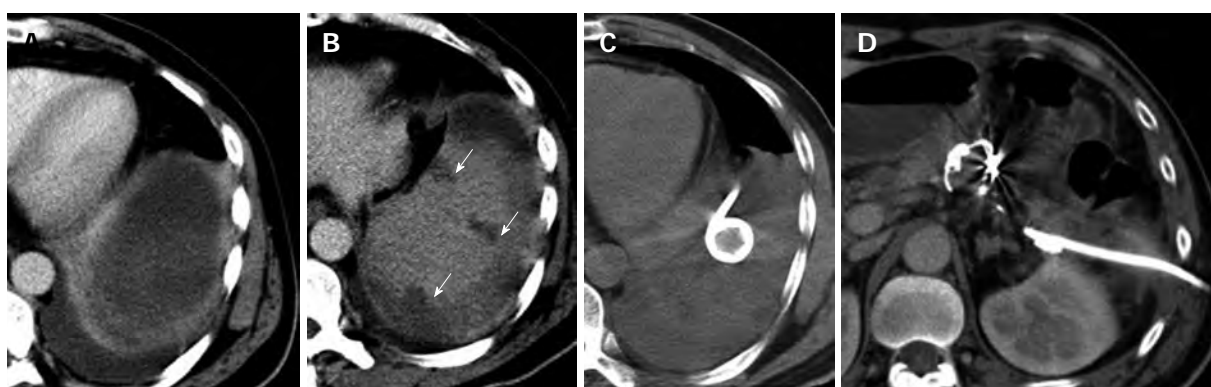
CT-guided percutaneous catheter drainage (PCD) was performed using Seldinger's method: A 12-Fr locking pigtail catheter (Angiotech, Stenlose, Denmark) was placed into the left anterior pararenal space (Figure 1C). Thick brown amylase-rich (19000 IU/L) fluid of about 50 mL in volume was drained out daily; drainage continued for 6 d after which the daily drainage decreased to < 10 mL. The patient's body temperature returned to normal 2 d after the PCD procedure. His white blood cell count and serum bilirubin level declined to the normal range in subsequent laboratory examinations. The patient was discharged with two drainage tubes in place on postoperative day 15.

Six days later, the patient was readmitted due to persistent epigastric pain. On readmission, his vital signs were stable. There was tenderness and rebound pain at the upper quadrant of his abdomen with moderate muscle guarding. Sentinel bleeding was suspected because bloody fluid was observed in the PCD tube. The patient underwent an emergency abdominal CT scan that showed extravasation of contrast medium beside the pancreatic stump (Figure 1D). Follow-up arteriography revealed a splenic arterial wall irregularity at the proximal portion 3 cm from the celiac trunk, suggesting a pseudoaneurysm (Figure 2A). Under interventional radiology, the pseudoaneurysm was occluded at both proximal and distal sites with microcoils (Figure 2B). There was no continuous intra-abdominal bleeding after embolization, however, leakage of pancreatic juice and bacterial infection did not cease despite administration of octreotide and antibiotics. On postoperative day 25, the patient complained of epigastric pain and fever again, whereas his abdominal drainage decreased to < 5 mL/d. Enhanced abdominal CT revealed a peripancreatic effusion of considerable size (Figure 3A) and multiple low-density splenic shadows (Figure 3B). A second PCD was subsequently performed for drainage of the peripancreatic fluid collection (Figure 3C). Dark brown turbid fluid of a total volume

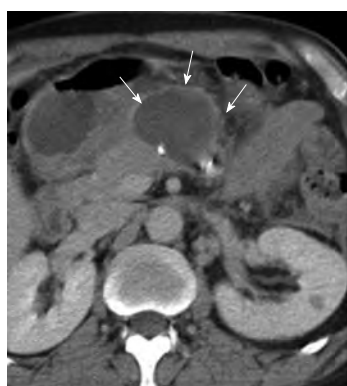




**Figure 2** Celiac trunk angiography. A: Celiac trunk angiography revealed pseudoaneurysm of the splenic artery; B: Celiac trunk angiography revealed no extravasation of contrast medium after embolization of the splenic artery with microcoils.



**Figure 3** Examination. A: Contrast-enhanced abdominal computed tomography revealed a peripancreatic effusion; B: Multiple focal ischemic lesions of the spleen; C: A pigtail catheter (percutaneous catheter drainage tube 2) was placed to drain the peripancreatic fluid collection; D: Absorption of the peripancreatic effusion.



**Figure 4** Encapsulated fluid collection anterior to the pancreatic stump.

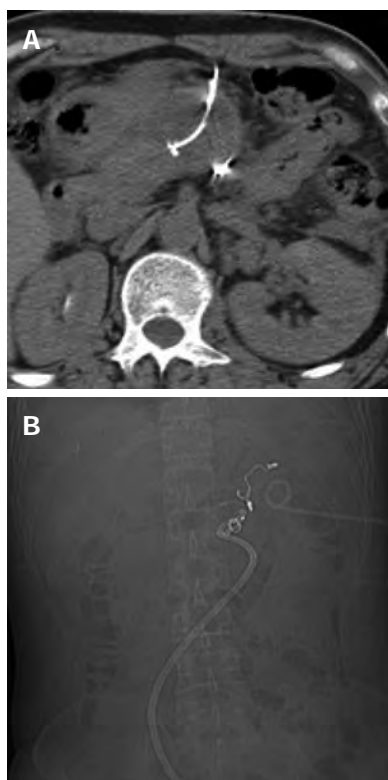
of 200 mL/d was drained out by the two PCD catheters (PCD tube 1: 20 mL/d, PCD tube 2: 180 mL/d) and the patient's symptoms were alleviated promptly. After 10 d, drainage from the two catheters began to decrease gradually and re-examination by CT showed apparent absorption of the peripancreatic effusion (Figure 3D). The second PCD tube was removed on postoperative day 40.

One week later, the patient suffered another episode of epigastric pain, fever, and leukocytosis despite cessation of drainage from PCD 1. Abdominal CT revealed

an encapsulated fluid collection in front of the pancreatic stump (Figure 4) and the third PCD was inserted under CT guidance (Figure 5). Continuous intra-abdominal lavage was established between the two drainage catheters (PCD tube 1 and PCD tube 3) afterwards. As a result, the encapsulated peripancreatic fluid collection was absorbed within 1 mo after the third PCD was inserted and the two catheters were eventually removed. The patient was discharged on postoperative day 80. The patient was well on subsequent follow-up and remained so until April 2013.

## DISCUSSION

LDP has been increasingly utilized as management for various lesions of the distal pancreas (*e.g.*, neuroendocrine tumors and cystic lesions) since development of the procedure towards the turn of the century. For benign lesions of the left pancreas, spleen-preserving LDP (SPLDP) has been proposed in order to reduce risks associated with splenectomy<sup>[11,12]</sup>. In SPLDP, the blood supply to the spleen can be maintained by either preserving the splenic artery and vein<sup>[13]</sup> or preserving collaterals of short gastric and left gastroepiploic arteries and veins while severing the splenic vessels (the Warshaw's method)<sup>[14]</sup>. The pancreatic lesion of the presented case, which was visualized as a hypodense mass on arterial enhance-



**Figure 5** A pigtail catheter (percutaneous catheter drainage tube 3) was placed anterior to the pancreatic stump to drain the effusion. A: Percutaneous catheter drainage (PCD) tube 3; B: PCD tubes 1 and 3.

ment of preoperative CT, was diagnosed as a neuroendocrine tumor. Tumor margins were clear with no apparent involvement of the splenic artery and vein, therefore, SPLDP was performed as surgical treatment.

The most common and clinically relevant complication of LDP is pancreatic leakage, which may lead to a series of sequelae including intra-abdominal abscess formation, sepsis, and even fatal bleeding. Although the incidence of pancreatic leakage after distal pancreatectomy has declined in the past decade, various high-volume centers have reported an occurrence rate of 5%-30% in recent studies<sup>[15-17]</sup>. A literature review revealed no significant variation in the incidence of pancreatic leakage after LDP and SPLDP<sup>[18-20]</sup>. Various techniques have been used to prevent pancreatic leakage, such as transecting the pancreatic parenchyma with endo-GI, or endo-GI closure with subsequent suturing, wound sealing with either fibrin-glue or an omental plug, and postoperative administration of octreotide. However, none of these aforementioned methods produced results of efficacious superiority.

On postoperative day 4, the patient complained of marked abdominal fullness. Physical examination revealed fever, tachycardia, and left abdominal tenderness. Amylase-rich fluid meeting the criteria for pancreatic leakage was detected from the Jackson-Pratt tube<sup>[10]</sup>. Fluid effusion was revealed adjacent to the pancreatic stump on ultrasonography and abdominal CT. The patient was noted to have jaundice on both physical and laboratory

examinations. Given that there was no evidence of biliary obstruction, sepsis associated with pancreatic leakage was determined to be a likely etiology.

Percutaneous catheter drainage has proven effective in evacuating intra-abdominal abscesses in the past decade, with a reported resolution rate of 50%-80%<sup>[21-24]</sup>. In the present case, the left anterior pararenal space was considered an ideal route for percutaneous drainage, and PCD was performed with resultant rapid mitigation of fever, leukocytosis, and jaundice.

Pseudoaneurysm formation and bleeding, as a fatal complication following pancreatic surgery, occurs in 2%-4% of patients with pancreatic leakage<sup>[25]</sup>. Such bleeding usually presents as a sudden or intermittent event in the late postoperative stage. According to the literature, most patients with pseudoaneurysm hemorrhage develop some form of sentinel bleeding prior to major onset<sup>[26]</sup>. Immediate diagnostic intervention of sentinel bleeding is essential to save patients from a foreseeable massive hemorrhage. Angiography has been established as the management of choice for sentinel bleeding, because it can simultaneously determine the origin of the hemorrhage and provide selective embolization<sup>[9]</sup>. In the present patient, the cause of pseudoaneurysm formation was considered to be exposure of underlying parenchymal vasculature to the leaked pancreatic juice, with subsequent regional infection. Furthermore, we speculate that there may have been several weak points on the trunk of the splenic artery postoperatively, particularly the origins of small branches from the splenic artery to the pancreatic parenchyma, which were severed via harmonic scalpel without ligation. These points might have been sensitive to erosion by pancreatic juice and thus were particularly prone to pseudoaneurysm formation. Therefore, we recommend that every visible branch between the splenic artery and pancreatic parenchyma should be ligated and severed. It is also questionable whether this patient's intra-abdominal bleeding was related to postoperative drainage catheter retention. However, iatrogenic etiology involving a pigtail catheter was deemed unlikely due to its coiled tip, which reduces friction between the catheter and surrounding tissue.

Based on the fact that radiological intervention has been widely recommended for pseudoaneurysm occlusion with a high success rate (67%-100%)<sup>[9,27-29]</sup>, transcatheter microcoil embolization of the splenic artery was performed in our patient. Both the distal and proximal portions of the pseudoaneurysm were blocked and hemorrhage was effectively controlled. Occlusion of the splenic artery, the main arterial blood supply of the spleen, might have led to splenic infarction and secondary infection. Post-embolization CT revealed multiple patchy ischemic changes in the spleen, without obvious progression of splenic infarction on subsequent CT. Delayed follow-up at 2 years postoperatively indicated that perfusion of the spleen recovered, apparently compensated for by adequate circulatory collateralization. It is therefore reasonable to assume that the short gastric and

gastroepiploic arteries are selectively preserved during SPLDP even when the splenic vessels are preserved.

The patient's hemorrhage ceased after splenic artery embolization. However, pancreatic leakage and sepsis continued and resulted in recurrence of abdominal abscess formation. According to Gervais *et al*<sup>[22]</sup>, repeated percutaneous drainage had a success rate of 91% in evacuating recurrent abdominal abscesses and was effective in obviating surgery in 56% of patients. In our case, two peripancreatic drainage catheters were inserted under interventional radiological guidance and peritoneal lavage was performed between the two tubes. Eventually, the pancreatic leakage ceased and the infection was controlled without further episodes of intra-abdominal bleeding.

In conclusion, apart from surgery, interventional radiological approaches are viable treatment options for post-LDP pancreatic leakage and ensuing secondary complications with safety and less invasiveness.

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## Liver physiology and liver diseases in the elderly

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### Abstract

The liver experiences various changes with aging that could affect clinical characteristics and outcomes in patients with liver diseases. Both liver volume and blood flow decrease significantly with age. These changes and decreased cytochrome P450 activity can affect drug metabolism, increasing susceptibility to drug-induced liver injury. Immune responses against pathogens or neoplastic cells are lower in the elderly, although these individuals may be predisposed to autoimmunity through impairment of dendritic cell maturation and reduction of regulatory T cells. These changes in immune functions could alter the pathogenesis of viral hepatitis and autoimmune liver diseases, as well as the development of hepatocellular carcinoma. Moreover, elderly patients have significantly decreased reserve functions of various organs, reducing their tolerability to treatments for liver diseases. Collectively, aged patients show various changes of the liver and other organs that could affect the clinical characteristics and management of liver diseases in these patients.

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**Key words:** Liver disease; Aging; Physiology; Immunology

**Core tip:** The morphology and physiology of the liver changes with aging and an understanding of those changes is important for the management of liver diseases. We first summarized the various changes in the liver with aging. We then reviewed the reported characteristics of liver diseases found in the elderly. This kind of information could be increasingly important in the near future, because the proportion of the world's population over 60 years old is increasing, especially in developed countries.

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### INTRODUCTION

The proportion of the world's population over 60 years old is increasing, especially in developed countries. Morphology and functions of the liver as well as other organs change with aging. Understanding these changes is important for the management of liver diseases in the elderly. In addition, the pathogenesis of many liver diseases is immune-mediated, and immune systems also change with aging, affecting the clinical picture of liver diseases.

### CHANGES IN LIVER MORPHOLOGY AND FUNCTIONS WITH AGING

#### *Morphology of the aged liver and microscopic or molecular characteristics of senescent hepatocytes*

In general, liver volume is reduced by 20%-40% in the elderly, with these reductions more marked in women (up to 44% decline) than in men<sup>[1]</sup>. Microscopically, elderly subjects have elevated numbers of hepatocytes with increased ploidy. Hepatocytes show decreased numbers of

mitochondria but increased volume of individual mitochondria, although functional impairment of mitochondria has not been demonstrated. Hepatocytes in elderly subjects contain denser body compartments, such as secondary lysosomes and lipofuscin, than do hepatocytes in younger subjects<sup>[2]</sup>. Lipofuscin accumulation has been associated with chronic oxidative stress and a failure to degrade damaged and denatured proteins<sup>[3]</sup>. Moreover, accumulating evidence suggests that lipofuscin interferes with cellular pathways due to its ability to trap metallic cations and facilitate further free radical formation<sup>[4]</sup>.

Vacuolation of hepatocyte nuclei has been associated with diabetes mellitus and non-alcoholic fatty liver disease. However, vacuolated hepatocyte nuclei were recently shown to be more abundant in senescent hepatocytes expressing p21 or  $\gamma$ H(2)AX<sup>[5]</sup>, suggesting they are a marker of hepatocyte senescence. Moreover, increased size of hepatocyte nuclei in nonalcoholic fatty liver disease (NAFLD) has been associated with telomere shortening and p21 upregulation<sup>[6]</sup>, suggesting that increased nuclear size is also a marker of hepatocyte senescence.

Cellular senescence is associated with aberrant activation of oncogenes, and senescent pre-malignant hepatocytes have been found to secrete cytokines and chemokines through interactions with their environment, resulting in immune-mediated clearance of these cells. Impairment of immune surveillance has been associated with the development of hepatocellular carcinoma (HCC)<sup>[7]</sup>. This scenario could account for the preferential development of HCC in aged patients with chronic liver diseases, irrespective of the etiology of these diseases<sup>[8]</sup>.

Recently, resistin, an adipokine that inhibits phosphorylation of AMP-activated protein kinase and modulates insulin resistance, has been shown to induce senescence-associated  $\beta$ -galactosidase in mouse hepatocytes<sup>[9]</sup>. Resistin has been shown to act by inhibiting the function of sirtuin 1, one of the 7 members of the sirtuin family of histone deacetylases shown to act as crucial negative regulators during the aging process<sup>[9]</sup>.

Molecular changes during hepatocyte senescence should be clarified in more detail in the near future. The identification of senescence-causing factors may be beneficial in preventing senescence-associated liver diseases.

### Blood flow

Liver blood flow is estimated to be decreased by 35%-50% in the elderly, and may be responsible for age-related reductions in liver volume<sup>[10]</sup>.

### Hepatic function

**Liver function tests:** Although interindividual differences have been observed, liver functions are relatively well preserved in elderly individuals. Hepatic enzymes and high-density lipoprotein cholesterol are well maintained, while bilirubin levels may decline with age due to reductions in muscle mass and hemoglobin concentrations<sup>[11]</sup>. Moreover, age was reported to be associated with modest decreases in albumin and  $\gamma$ -glutamyl transpeptidase con-

centrations, and increases in bilirubin concentration, after adjustments for sex, alcohol use, and components of the metabolic syndrome, suggesting that liver function may be decreased in these individuals<sup>[12]</sup>.

Alanine aminotransferase (ALT) concentrations have been reported to decrease with age in both men and women, independent of components of the metabolic syndrome. These findings suggest the need to identify an optimal cut-off point for normal ALT in elderly patients<sup>[12]</sup>.

### Drug metabolism

Phase I hepatic metabolism (first-pass hepatic uptake) of drugs has been reported to be decreased in the elderly, possibly due to reduced liver volume and hepatic blood flow, leading to a decline in hepatic drug metabolism. Metabolism of drugs with low phase I hepatic metabolism is likely to be impaired mainly by liver volume reduction.

A previous report suggested that drug metabolism is reduced by up to 30% after 70 years of age, and that a reduction in liver cytochrome P450 may also contribute to decreased drug metabolism. Cytochrome P450 activity was shown to be 32% lower in subjects > 70 years than in subjects aged 20-29 years<sup>[13]</sup>.

### Liver regeneration

Liver regeneration capacity has been reported to decline with age<sup>[14,15]</sup>. The mechanisms underlying the reductions in regeneration capability are complex. One of these mechanisms involves a decrease in the concentrations of circulating epidermal growth factor (EGF), with the response of hepatocytes to EGF also reduced due to age-associated loss of EGF receptors or deficits in signaling after EGF binds to its receptor<sup>[16]</sup>. Another mechanism underlying reduced hepatocyte proliferation capacity may be the inhibition of cyclin-dependent kinases by interaction with the chromatin remodeling protein Bim, which is expressed in aged hepatocytes<sup>[17]</sup>. Along with reductions in regenerative capacity, telomere length has been reported reduced in aged livers, especially in patients with liver diseases<sup>[18]</sup>.

### Immune system

Many liver diseases are mediated by the host immune response. Therefore, changes in immune functions may affect the clinical picture of various liver diseases. Several changes in the immune system have been observed in elderly individuals.

**Innate immunity:** Most of the immune cells involved in innate immunity, such as monocytes/macrophages and natural killer (NK) cells, show decreased function with aging<sup>[19]</sup>. Although the percentage and number of CD56<sup>bright</sup> NK cells gradually decline with age, the percentage and number of CD56<sup>dim</sup> NK cells progressively increase<sup>[20]</sup>.

In addition, dendritic cells (DCs), which are the most potent antigen-presenting cells, show significant function-



al changes with aging. DCs play pivotal roles in the onset and regulation of adaptive immune responses and control the state of tolerance to self-antigens<sup>[21]</sup>. Immature DCs promote tolerance through induction of regulatory T cells (Treg), whereas mature DCs stimulate effector T cells. DCs in the elderly show inappropriate maturation induced by infections or tissue injury, which may lead to alterations in the balance between the tolerogenic and immunogenic functions of DCs and instigate the development of autoimmune diseases<sup>[22]</sup>.

**Adoptive immunity:** T cell number and diversity of repertoire are decreased and T cell expansion, differentiation, and signaling intensity are impaired with aging. The numbers of CD4<sup>+</sup> T cells are decreased, while the numbers of CD8<sup>+</sup> T cells are increased. The expression of the costimulatory molecule CD28 is decreased on T cells, impairing their ability to proliferate and secrete interleukin-2<sup>[23]</sup>. Treg function is decreased after age 50 years, which may be associated with the increases in autoimmunity<sup>[24]</sup>.

The numbers of B cell precursors in the bone marrow (pre-B cells), as well as peripheral B cells, decrease with age<sup>[25]</sup>. In contrast, immunoglobulin concentrations may increase with age<sup>[26]</sup>, but the quantities of specific antibodies and the diversity of the B cell repertoire decrease<sup>[27]</sup>.

In summary, immune responses against foreign antigens and malignant cells seem to be impaired with age because of the reductions in number and functions of most immunocompetent cells. In contrast, the decrease in Tregs and the impairment of DC maturation may result in a predisposition to autoimmunity.

## LIVER DISEASES IN THE ELDERLY

The prevalence of some liver diseases increases with aging, and advanced liver disease is observed more often in older than in younger patients. Moreover, various physiological changes associated with aging may affect the pathogenesis of liver diseases. In addition, the decreased reserve capacity of most organs in elderly individuals may impair their ability to manage liver diseases.

### Viral hepatitis

**Hepatitis A:** Acute hepatitis A virus (HAV) infection is usually self-limiting. However, elderly patients with acute HAV infection experience hepatocellular dysfunction with frequent jaundice and coagulopathy, as well as an increased incidence of complications, such as prolonged cholestasis, pancreatitis, and ascites<sup>[28]</sup>. Higher hospitalization and mortality rates have been reported in elderly patients with HAV. For example, during an outbreak of HAV infection in the United States, 42% of patients aged 70 years or older required hospitalization compared with 3%-20% of adults aged 40-49 years<sup>[29]</sup>. Age-related differences in outcomes were also reported, with death rates of 0.004% in individuals aged 5-14 years and 2.7%

in those older than 49 years<sup>[30]</sup>. More recent data from the Centers for Disease Control and Prevention (CDC, 2009 Surveillance) also indicate that mortality due to HAV increases with age, with no fatalities reported in patients younger than 34 years of age. The mortality rates have been estimated to be 0.05 per 100000 patients aged 45-54 years, and 0.11 per 100000 patients older than 75 years. Vaccination for hepatitis A should, therefore, be considered for people, especially the elderly, who plan to travel to endemic areas<sup>[30]</sup>.

**Hepatitis B:** Acute hepatitis B virus (HBV) infection is uncommon in the elderly because the opportunities for HBV infection are estimated to be low in this population. However, hepatitis B and hepatitis C infections have been reported in aged residents of nursing homes<sup>[31,32]</sup>. Risk factors include sharing bath brushes, non-disposable syringes, and shaving blades, as well as sexual contact. Clinical manifestations of acute HBV infection are similar to those in younger adults. During an outbreak of acute HBV in elderly nursing home residents, most infected patients were asymptomatic, and no patient died or required hospitalization during the outbreak<sup>[31]</sup>. However, the rate of progression to chronic hepatitis B is higher in elderly than in younger patients. A report on an outbreak of acute HBV infection in a nursing home showed that 59% of patients older than 65 years of age developed chronic infection<sup>[31]</sup>. This may be due to a decreased immune response to the pathogens.

In chronic HBV infection, the prevalence rates of HBeAg and HBsAg are inversely related to patient age during the natural course of HBV infection. The prevalence rates of HBsAg in Taiwanese men and of HBeAg among HBsAg-positive men older than 60 years of age were reported to be 12.5% and 5.5%, respectively, while prevalence rates in patients aged 30-39 years were 23.8% and 23.3%, respectively<sup>[33]</sup>. Serum HBV DNA levels were found to vary by country and to be associated with HBeAg or HBV genotype<sup>[34]</sup>. Older age and male sex, in addition to serum HBV DNA levels, are regarded as risk factors not only for progression to cirrhosis<sup>[35]</sup>, but the development of HCC<sup>[36]</sup>.

Nucleos(t)ide analogs are effective in treating HBV infected patients, with similar efficacy in the elderly as in younger patients<sup>[37]</sup>. Although interferon-based therapy may also be effective for the treatment of chronic HBV infection, its therapeutic effects are inferior in elderly patients<sup>[38]</sup>.

**Hepatitis C:** The prevalence of hepatitis C virus (HCV) infection varies by age because HCV infection is transmitted by blood contact, such as blood transfusion (especially before 1992), military service, intravenous drug use, tattoos, hemodialysis, and health care work. In the United States, the prevalence of HCV infection is highest in patients aged 40-49 years (4.3%), whereas those aged 60-69 years and 70 years or older have lower prevalence rates of 0.9% and 1%, respectively<sup>[39,40]</sup>. A European

study showed that the prevalence of genotype 1 HCV increases with age, being 57% in patients aged < 65 years, 72% in those aged 65–80 years, and 84% in patients older than 80 years of age<sup>[41]</sup>. Older age at the time of infection, but not duration of infection, has been associated with fibrotic progression<sup>[41]</sup> and hepatocarcinogenesis<sup>[42]</sup>. Normal ALT levels are more likely observed in elderly than in younger HCV-infected patients (46% *vs* 10.6%, respectively)<sup>[43]</sup>. However, older patients often show more fibrosis regardless of serum ALT levels<sup>[41]</sup>. Moreover, the incidence of hepatocellular carcinoma increases with aging both in hepatitis C<sup>[42]</sup> and hepatitis B<sup>[44]</sup> infected patients. Therefore, progression of fibrosis and development of hepatocellular carcinoma should be considered, especially in elderly patients with chronic viral hepatitis.

Powerful regimens have been established for the treatment of chronic HCV infection, including pegylated interferon and ribavirin, have been established. However, adverse effects are observed more often in older patients<sup>[45]</sup>. The sustained viral response rate has been shown lower in elderly than in younger patients (46% *vs* 69.7%, respectively), perhaps due to the high proportion of elderly patients who stop antiviral therapy due to side effects<sup>[46]</sup>.

**Hepatitis E:** The prevalence of hepatitis E virus (HEV) infection differs markedly in endemic and non-endemic areas. However, recent reports suggested that exposure to HEV occurs frequently in Western countries. In the United States, 16% of blood donors younger than 60 years of age were positive for anti-HEV IgG, compared with 25.5% of those older than 60 years<sup>[47]</sup>. Furthermore, 3% of patients with acute liver injury suspected of being drug-induced liver injury were seropositive for anti-HEV IgM. Most patients with serology consistent with acute HEV infection were older than 60 years of age<sup>[48]</sup>.

### Autoimmune liver disease

The prevalence rates of autoimmune liver diseases, including autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC), are relatively high in older patients, whereas primary sclerosing cholangitis is more common in those in the third or fourth decade of life<sup>[49–51]</sup>. However, the results of laboratory tests associated with these autoimmune liver diseases are not associated with age, and treatment strategies are usually identical in older and younger patients.

**AIH:** Almost 20% of patients develop AIH after 60 years of age, and the disease is frequently progressive and unexpected because ascites and cirrhosis are common manifestations at presentation with few other symptoms<sup>[49,52,53]</sup>. Most elderly patients respond well to corticosteroid therapy<sup>[52]</sup>. Rates of treatment failure are lower in older than in younger patients (5% *vs* 24%), and elderly patients have lower rates of fatality from liver failure or need for liver transplantation (5% *vs* 21%)<sup>[52,53]</sup>. Notably,

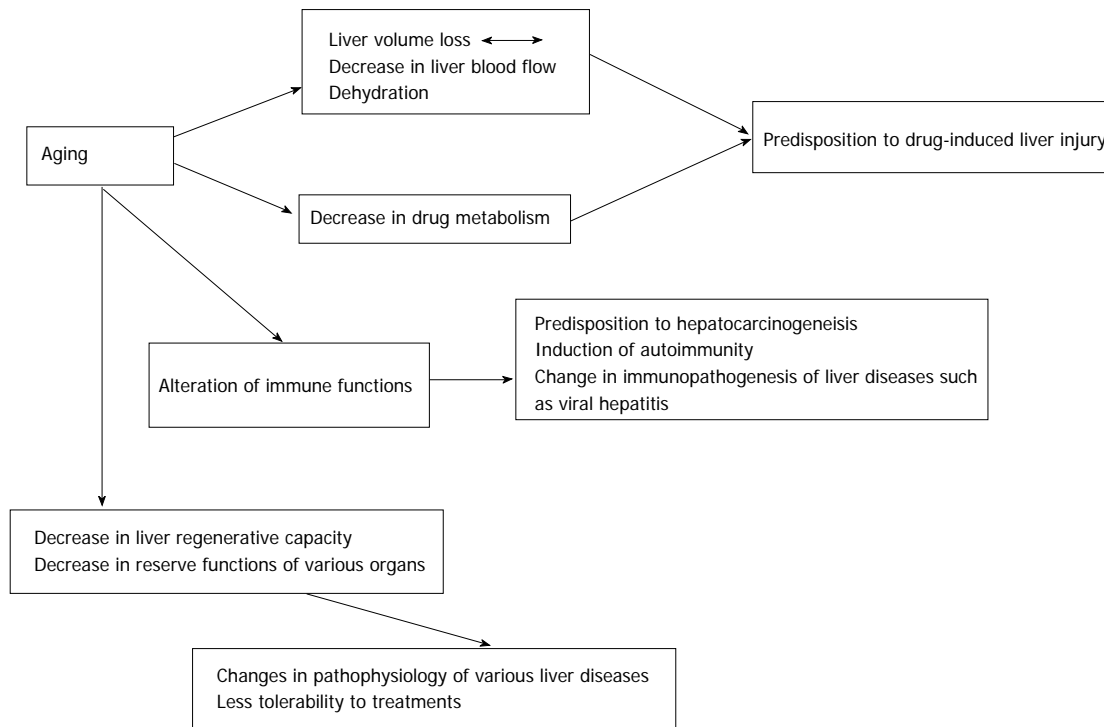
elderly patients are at risk of treatment-related complications, especially osteopenia and compression fracture<sup>[54]</sup>. Furthermore, they may have other comorbidities and medication requirements that complicate their management.

**PBC:** Advancing age has been associated with poor prognosis in patients with PBC, and elderly patients diagnosed with PBC at a young age are likely to show a poor prognosis<sup>[55]</sup>. In contrast, patients with PBC diagnosed after 65 years of age are less likely to have progressive or advanced disease<sup>[56]</sup>. Two types of phenotypic expression of PBC were recently reported: the classical asymptomatic onset in middle-late age with mild biochemical activity, and symptomatic onset at a younger age with high biochemical activity<sup>[57]</sup>. Administration of ursodeoxycholic acid, which is the only recommended therapy for PBC, appears to be safe and has few side effects. Osteoporosis should also be considered, especially in elderly patients.

**Alcoholic liver disease:** Alcohol consumption is common in the elderly. In a study of individuals in the United Kingdom, 62% of subjects aged 60–92 years were drinkers, with 13% of men and 2% of women being heavy drinkers<sup>[58]</sup>. Elderly people presenting with alcoholic liver disease (ALD) had more advanced disease than younger patients<sup>[59]</sup>. Half of the elderly patients who develop cirrhosis die within 1 year of diagnosis<sup>[60]</sup>. In patients with HCV infection, alcohol drinking was associated with accelerated disease progression<sup>[61]</sup>. Adverse effects of benzodiazepines as treatment for withdrawal symptoms, such as drowsiness, fatigue, confusion, ataxia, falls and incontinence, are more common with increasing age<sup>[62]</sup>.

**NAFLD:** NAFLD is a disease predominantly seen in middle-aged to older people. A significant proportion of cryptogenic cirrhosis may be due to the end-stage of NAFLD, and age has been reported to be a risk factor for liver fibrosis and higher mortality rate in patients with NAFLD<sup>[63]</sup>. Older patients have significantly more risk factors for NAFLD, including hypertension, obesity, diabetes, and hyperlipidemia<sup>[64]</sup>. A study of 351 consecutive patients in the United Kingdom found that albumin, alanine aminotransferase (ALT), ALT/aspartate aminotransferase ratio, and platelet counts were significantly reduced with advancing age<sup>[64]</sup>. Thus, aged patients with NAFLD are considered to have advanced liver disease.

Recently, sirtuin 1, a negative regulator of aging, was reported to play key roles in the regulation of lipid and glucose homeostasis<sup>[65]</sup>. This finding suggested that aging was associated with the development of NAFLD, and that activating sirtuin 1 may be a novel therapeutic strategy for patients with NAFLD. Several molecular characteristics of hepatocyte senescence have been observed in patients with NAFLD, with hepatocyte senescence being closely associated with advanced fibrosis stage and poor clinical outcome<sup>[66]</sup>. Thus, the development and patho-



**Figure 1** Physiological changes in elderly subjects associated with the development or pathophysiological modification of liver diseases. Aging is associated with decreases in liver volume, blood flow, drug metabolism and regenerative capacity, and alterations in immune functions. Changes due to decreased reserve functions of various organs could affect the clinical characteristics and management of liver diseases in the elderly.

genesis of NAFLD may be closely associated with the aging process.

**Drug-induced liver injury:** Old age is a risk factor of drug-induced liver injury (DILI) because the elderly are more susceptible to adverse drug reactions<sup>[67]</sup>. Moreover, patients over 75 years old required significantly longer hospitalization for DILI<sup>[68]</sup>. In contrast, a recent report suggested that older age is associated with a cholestatic type of liver injury<sup>[69]</sup>, and a study in Japan also showed rates of cholestatic liver injury was higher in patients > 65 than < 65 years of age (46% *vs* 31.6%).

Elderly patients may receive many types of drugs for treatment of comorbid conditions. For example, a Japanese study of patients with DILI showed that elderly patients > 75 years of age were taking significantly more concomitant drugs at the time of liver injury<sup>[68]</sup>. Other reports from Western countries also suggested greater drug usage among elderly patients. For example, a study of 466 patients in Germany > 70 years of age found that these patients were receiving an average of 3.7 prescribed medicines in addition to 1.4 over-the-counter medications daily<sup>[70]</sup>. In a prospective study in the Netherlands, 94.2% of elderly patients, mean age 82.3 years, were taking more than one drug, and 73.3% were prescribed four or more drugs<sup>[71]</sup>. Several pharmacokinetic and pharmacodynamic mechanisms that may predispose a patient on multiple medications to an increased risk of DILI have been proposed<sup>[72]</sup>. Adverse effects of both the individual drugs and their synergistic interactions must be taken into

consideration in elderly patients.

**Liver tumor:** HCC is more common in elderly patients with liver cirrhosis<sup>[73]</sup>. Elderly patients were reported to develop HCC even without fibrosis<sup>[74]</sup>, suggesting that aging itself may be a predisposing factor for hepatocarcinogenesis. The impact of viral eradication on HCC prevention was found to be less significant in older than in younger patients chronically infected with HCV, especially in patients at an advanced stage of liver disease<sup>[75]</sup>. These observations indicate the need for long-term follow-up of elderly patients with chronic HCV infection, even after viral eradication and especially in male patients with liver cirrhosis.

Hepatic resection for HCC can be performed safely and effectively in elderly patients<sup>[76]</sup>. Regional therapies, such as radiofrequency ablation and transarterial chemoembolization, may also be considered for elderly patients with HCC, if liver function and tumor stage are acceptable<sup>[77]</sup>.

**Liver transplantation:** The proportion of adult liver transplantation recipients in the United States older than 60 years of age increased from 10% in 1990 to more than 20% by 1999<sup>[78]</sup>. Some problems remain to be addressed regarding liver transplantation in elderly patients. Increased age has been associated with a poorer survival rate<sup>[79,80]</sup>, although other studies suggested that advanced age alone should not be a contraindication for liver transplantation<sup>[81,82]</sup>. Among 2141 patients who underwent

**Table 1 Clinical characteristics of liver diseases in patients**

Liver diseases	Characteristics
Viral hepatitis	
Hepatitis A	Higher hospitalization and mortality rates
Hepatitis B	More likely to progress to chronic hepatitis or cirrhosis
Hepatitis C	More likely to progress fibrosis Higher rates of hepatocellular carcinoma development Decreased tolerability to treatment
Autoimmune diseases	
Autoimmune hepatitis	Sometimes progressive
Primary biliary cirrhosis	Higher rates of treatment-related complications Sometimes progressive More likely to have osteoporosis
Alcoholic liver disease	Progressive
Nonalcoholic fatty liver disease	Higher prevalence Progressive
Hepatocellular carcinoma	Higher rates of development

retransplantation, more than 10% were over 60 years of age<sup>[82]</sup>. Being over 60 years of age was not independently associated with an increase in mortality when adjusted for factors that were found to influence survival<sup>[82]</sup>. Elderly patients may have multiple risk factors, including coronary artery disease or malignancy, and face age-associated quality of life impairments, such as instability, incontinence, immobility, dementia, and polypharmacy<sup>[83]</sup>. Moreover, aged recipients have a significantly lower quality of life, as assessed by physical functioning, bodily pain, general health, vitality, social functioning, role emotional, and physical component score<sup>[84]</sup>. Therefore, careful consideration is required in choosing liver transplantation for elderly patients.

## CONCLUSION

Aged patients show various changes in the liver, which could affect the clinical characteristics of liver diseases in these patients (Table 1). Decreases in functioning of the liver and other organs as well as alterations in immune functions should be taken into consideration in the management of the liver diseases (Figure 1).

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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

### Early stage colon cancer

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#### Abstract

Evidence has now accumulated that colonoscopy and removal of polyps, especially during screening and surveillance programs, is effective in overall risk reduction for colon cancer. After resection of malignant pedunculated colon polyps or early stage colon cancers, long-term repeated surveillance programs can also lead to detection and removal of asymptomatic high risk advanced adenomas and new early stage metachronous cancers. Early stage colon cancer can be defined as disease that appears to have been completely resected with no subsequent evidence of involvement of adjacent organs, lymph nodes or distant sites. This differs from the clinical setting of an apparent "curative" resection later pathologically upstaged following detection of malignant cells extending into adjacent organs, peritoneum, lymph nodes or other distant sites, including liver. This highly selected early stage colon cancer group remains at high risk for subsequent colon polyps and metachronous colon cancer. Precise staging is important, not only for assessing the need for adjuvant chemotherapy, but also for patient selection for continued surveillance. With advanced stages of colon cancer and a more guarded outlook, repeated surveillance should be limited. In future, novel imaging technologies (*e.g.*, confocal endomicroscopy), coupled with increased pathological recognition of high risk markers for lymph node involvement (*e.g.*, "tumor budding")

should lead to improved staging and clinical care.

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**Key words:** Colon cancer; Node-negative colon cancer; Staging of colon cancer; Nodal micrometastases; Follow-up and surveillance of early colon cancer

**Core tip:** Evidence has now accumulated that colonoscopy and removal of polyps, especially during screening and surveillance programs, is effective in overall risk reduction for colon cancer. After resection of malignant pedunculated colon polyps or early stage colon cancers, long-term repeated surveillance programs can also lead to detection and removal of asymptomatic high risk advanced adenomas and new early stage metachronous cancers. In future, novel imaging technologies (*e.g.*, confocal endomicroscopy), coupled with increased pathological recognition of high risk markers for lymph node involvement (*e.g.*, "tumor budding") should lead to improved staging and clinical care.

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#### INTRODUCTION

Adenocarcinoma of the colon, including rectum, is a major cause of morbidity and mortality among all internal malignant diseases in men and women. When the disease is at an advanced stage with documented metastatic involvement of lymph nodes or other organs, the prognosis is especially dismal. A number of different staging criteria have been used to estimate the depth of cancer penetration in the colon as well as the extent of extra-colonic disease involvement. Currently, a commonly used



**Table 1** Colon cancer staging

AJCC stage	TNM stage	TNM criteria
Stage 0	Tis N0 M0	Tumor confined to mucosa
Stage I	T1 N0 M0	Tumor invades submucosa
Stage II	T2 N0 M0	Tumor invades muscularis propria
Stage II A	T3 N0 M0	Tumor invades subserosa
Stage II B	T4 N0 M0	Tumor invades adjacent organs
Stage III A	T1-2 N1 M0	Tumor metastases to 1-3 nodes
Stage III B	T3-4 N1 M0	Tumor metastases to 1-3 nodes
Stage III C	Any T, N2, M0	Tumor metastases to 4 or more nodes
Stage IV	Any T or N, M1	Metastases to distant sites

AJCC: American Joint Committee on Cancer; TNM: Tumor/Nodes/Metastases. Other classification methods include: Dukes System: A, tumor confined to intestinal wall; B, tumor invading through the intestinal wall; C, tumor with lymph node involvement; D, tumor with distant metastases; and Astler-Coller System: A, Tumor limited to mucosa; B1, Tumor through muscularis mucosa but not muscularis propria; B2, Tumor beyond muscularis propria; C1, B1 with lymph node metastases; C2, B2 with lymph node metastases; D, Distant metastases. Other criteria include: venous and lymphatic invasion and differentiation.

staging method for colon cancer is based on the TNM (tumor/node/metastases) system as delineated by the American Joint Committee on Cancer (AJCC), now with a staging manual and atlas in its 7<sup>th</sup> edition<sup>[1]</sup>. These different AJCC stages are summarized in Table 1.

## EARLY STAGE COLON CANCER

Early stage colon cancer can be defined as disease that appears to have been completely resected with no subsequent evidence of involvement of adjacent organs, lymph nodes or distant sites. This definition differs from the clinical setting of an apparent “curative” resection later pathologically upstaged following detection of malignant cells extending into adjacent organs, peritoneum, lymph nodes, or other distant sites, including the liver.

This highly-selected group with disease localized in the colon still remains at especially high risk for subsequent development of colon polyps and metachronous colon cancer. Conceptually, this definition of early stage disease reflects increasing use of colonoscopic surveillance as an important tool in an emerging management approach. Precise staging, however, is critical, not only in assessing the need for adjuvant chemotherapy, but also for the selection of patients for continued surveillance. In patients with advanced stages of colon cancer and a more guarded outlook, repeated surveillance should be limited.

## IMAGING METHODS

Although imaging methods are important in defining suspected areas of involvement, complete staging currently requires pathological assessment of resected tissue, particularly to define early stage disease. Usually staging has been estimated after surgical removal of the colon cancer, however, experience has shown that complete staging is also possible after endoscopic resection

of a malignant pedunculated polyp that has minimal invasion. For these malignant polyps, however, deep histopathological assessment is not possible and lymph nodes are not removed. Further upstaging of colon cancer may result from employment of ultrasound, computed tomography (CT), magnetic resonance imaging or position emission tomography with pathological confirmation. In contrast, studies have already confirmed that methods such as fecal immunochemical testing (FIT) or CT have limited value in the detection of early stage colon cancer. For example, a high rate of false-negative results with FIT for early stage cancers was recently recorded<sup>[2]</sup> and CT was shown to have a low sensitivity for diagnosis of early T1 or T2 cancers<sup>[3]</sup>.

Studies to explore staging using evolving endoscopic methods have also appeared. For example, a recent report<sup>[4]</sup> compared new techniques for assessment of the actual depth of colon cancer invasion. Magnification chromoendoscopy and endoscopic ultrasound were found to have similar accuracy in estimating the depth of invasion, but neither procedure was believed to currently have sufficient diagnostic accuracy for use as a reliable or recommended standard<sup>[4]</sup>. Further investigative efforts are needed to explore novel and emerging imaging developments, particularly endoscope-based or probe-based confocal endomicroscopic methods. These offer the possibility for more rapid (and possibly for economical) differentiation of neoplastic from non-neoplastic colonic disease, earlier diagnosis of colorectal cancer, further evaluation of degree of differentiation and estimation of invasion depth for early colorectal cancer<sup>[5-8]</sup>.

## OUTCOME OF STAGING

Evidence has accumulated to show that a more advanced cancer stage is correlated with a worse clinical outcome. In patients with localized and limited disease confined to the submucosa or muscularis propria, the overall 5 year survival is about 70%. With more advanced disease extending beyond the subserosa into adjacent structures, peritoneum, lymph nodes or distant sites, the overall 5 year survival is about 30%. Even in early stage colorectal cancer, bowel perforation from the tumor itself or anastomotic leakage following surgery is associated with increased recurrence rates and an impaired disease-free survival<sup>[9]</sup>.

Early detection of colon cancer has been an important goal for physicians evaluating patients at increased risk for colon cancer. Colonoscopic regimens of surveillance have emerged based on good evidence that morbidity and mortality can be improved<sup>[10,11]</sup>. A number of guidelines have been developed for endoscopic surveillance of high risk groups to detect colon cancer. Some high risk categories have included a documented personal and family history of colon adenomas and colon cancer as well as inflammatory bowel disease. Among these high risk groups, a prior history of a completely resected colon cancer is a special group that should be considered for regular surveillance, particularly for those with early

stage disease<sup>[12]</sup>. Most important, recent publications have provided good evidence that colonoscopy is associated with reduced colorectal cancer mortality<sup>[13,14]</sup>. In addition, persistent and sustained reduction in colorectal cancer mortality has been attributed, in large part, to the effect of polypectomy<sup>[14]</sup>. For malignant colorectal polyps with localized submucosal invasion, similar long-term results have been recorded, although a risk for new colon polyps, including advanced adenomas, and metachronous colon cancer persists<sup>[15]</sup>.

## SURVEILLANCE AFTER COLON CANCER RESECTION

Earlier randomized clinical trials compared intense with less intense surveillance after a “curative” resection<sup>[16-20]</sup>. Unfortunately, a number of methodological flaws in these studies were noted<sup>[21]</sup>, particularly the inclusion of both early- (*i.e.*, node-negative) and late- (*i.e.*, node-positive) stage disease together in the comparison groups, regardless of the intensity of later surveillance. Perhaps, in these earlier studies, evaluation of more homogeneous populations, particularly with early-stage colon cancer, would have shown a positive effect of surveillance because prognosis for patients with nodal involvement, invasion of other structures and distant metastases would be expected to be much more limited<sup>[21]</sup>. Moreover, a more recent Cochrane evaluation has suggested a survival benefit for selected patients with more intense follow-up<sup>[22]</sup>. Finally, long-term studies of symptomatic early stage colon cancer patients followed over more than 10 years<sup>[23]</sup> demonstrated no locally recurrent disease. However, in the same study<sup>[23]</sup>, there was still an ongoing risk for new and asymptomatic neoplasms, including advanced adenomas and early-stage metachronous colon cancers.

## RISK OF LYMPH NODE METASTASES

A number of factors critical to accurate clinical and pathological staging have been explored in recent years, especially definition of high risk factors for lymph node involvement, if only early stage colon cancer with submucosal invasion (or T1) disease appears to be present. These factors include lymphatic invasion, venous invasion, tumor budding, poor tumor differentiation, extent (especially width) of submucosal invasion, complete disruption of muscularis mucosa. Indeed, some studies have suggested that up to 16% with localized submucosal invasive disease may already have lymph node metastases<sup>[24-30]</sup>.

For malignant pedunculated colon polyps, Haggitt *et al*<sup>[24]</sup> initially proposed a 4-level classification defined by increasing depths of cancer invasion into the submucosa, particularly if deeper than the polyp stalk. Level 4 invasion into the submucosa was thought to represent the highest risk for lymph node metastases. Some have used alternative measures of depth of invasion to ensure

complete electrocautery removal of malignant pedunculated polyps. For example, a distance from the leading invasive margin of the cancer to the cautery line of more than 2 mm has been empirically used as a guideline of an adequate resection of a pedunculated lesion with a stalk. If the cautery line is involved with malignant cells after removal of a malignant polyp, colectomy should be done.

For non-polypoid malignant lesions with submucosal invasion, assessment is more difficult. In these, level 4 invasion was traditionally defined<sup>[24]</sup>. Others have suggested a different classification schema, especially for surgically-resected specimens, defined by submucosal depth of invasion (*i.e.*, specifically, sm1, sm2, sm3) with greatest depth of invasion having greatest risk for lymph node involvement<sup>[27,31]</sup>. For endoscopic resection, complete removal of the submucosa may be more difficult pathologically to define, although a retrospective evaluation of colorectal cancer initially treated with endoscopic resection suggested that a positive vertical (rather than lateral) resection margin and inadequate lifting sign were positively correlated with risk of residual tumor and lymph node metastases<sup>[32]</sup>. Other pathological risk factors for node metastases have also been emphasized include venous or lymphatic invasion, moderately or poorly differentiated tumor grade, tumor “budding” at the submucosal invasive front of the cancer, or a completely cancer-disrupted muscularis mucosa<sup>[33]</sup>. A high CEA value may also be predictive of metastatic disease<sup>[34,35]</sup>. Because of this increased risk for node involvement after endoscopic resection with these high risk factors, colectomy may be recommended to ensure complete cancer removal and permit more detailed node sampling for metastatic disease.

## TUMOR BUDDING AND OTHER RISK FACTORS

“Tumor budding” is an independent prognostic indicator of risk for lymph node involvement, especially in early TNM stage colorectal cancer, as recently emphasized by expert pathologists<sup>[36]</sup>. This description of “tumor budding” was attributed to Imai who first postulated that this particular pathological feature of an invasive colon cancer represented a sudden or rapid growth of the leading or invasive edge of a carcinoma, in part, related to an interaction between epithelial and mesenchymal elements at the tumor margin<sup>[36]</sup>. Evidence has accumulated that tumor budding as well as high tumor grade or lymphovascular invasion are independent risk factors for lymph node metastases in patients with submucosally invasive colon cancer<sup>[37,38]</sup>. Patients with none of these high risk pathological features had only rare lymph node metastases (less than 1%) whereas the risk increased substantially with one (*i.e.*, about 20%) or multiple (*i.e.*, almost 40%) risk factors. In addition, this study showed that absence of extensive, particularly lateral, submucosal invasion (specifically, < 4 mm in width and < 2 mm in depth), had no apparent risk of metastases to lymph nodes (using an-

ti-cytokeratin immunohistochemical staining method for detection of lymph node micrometastases) if other high risk markers were absent. Similar observations have been independently reported<sup>[39-42]</sup>, including a recent evaluation following endoscopic removal of submucosal invasive T1 colorectal cancers<sup>[43]</sup>.

In future, the clinical relevance of other clinical and pathological methods of evaluation for staging, including stage II colon cancer, will need additional evaluation. These include number of lymph nodes surgically harvested<sup>[44-47]</sup>, techniques used for lymph node evaluation (including detection of micrometastases with novel immunohistochemical stains and polymerase chain reaction methods)<sup>[48-51]</sup> as well as definition of the precise role of sentinel node mapping for node sampling<sup>[52-54]</sup> and final staging.

## CONCLUSION

Colonoscopy screening and surveillance have a documented benefit in reducing the risk of colon cancer. As a result, more early stage colon cancers will be detected in surveillance programs and treated with endoscopic methods. Emerging imaging technologies, such as confocal endomicroscopic methods, may lead to further refinements in definition of patients with early stage disease as well their management. Pathological staging to define early stage disease also continues to evolve, particularly with the increased recognition of risk factors for lymph node disease in early stage colon cancers and immunohistochemical methods for lymph node evaluation, especially detection of lymph node micrometastases.

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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

# Evidence-based appraisal of the upfront treatment for unresectable metastatic colorectal cancer patients

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## Abstract

Colorectal cancer (CRC) is a significant health problem, with around 1 million new cases and 500000 deaths every year worldwide. Over the last two decades, the use of novel therapies and more complex treatment strategies have contributed to progressively increase the median survival of patients with unresectable advanced CRC up to approximately 30 mo. The availability of additional therapeutic options, however, has created new challenges and generated more complicated treatment algorithms. Moreover, several clinically important points are still in debate in first-line, such as the optimal treatment intensity, the most appropriate maintenance strategy, the preferred biologic to be used upfront in patients with KRAS wild-type CRC, and the need for more detailed information on tumor biology. In this moving landscape, this review analyses why the first-line treatment decision is crucial and how the choice

may impact on further treatment lines. In addition, it focuses on results of major phase III randomized trials.

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**Key words:** Colorectal cancer; Chemotherapy; Angiogenic inhibitors; Epidermal growth factor receptor inhibitors; Maintenance; First-line

**Core tip:** The choice of the first-line therapy is crucial for patients with advanced, unresectable colorectal cancer. The aim of this review is to critically focus on updated scientific data that medical oncologists need to interpret to make the most appropriate evidence-based choice among many possible treatment options.

Aprile G, Lutrino SE, Ferrari L, Casagrande M, Bonotto M, Ongaro E, Puglisi F. Evidence-based appraisal of the upfront treatment for unresectable metastatic colorectal cancer patients. *World J Gastroenterol* 2013; 19(46): 8474-8488 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8474.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8474>

## WHICH REASONING DOES LIE BENEATH THE CHOICE OF A FIRST-LINE TREATMENT?

Colorectal cancer (CRC) is currently the second most common cancer in Europe, with nearly 450000 new cases and approximately 215000 deaths occurred in 2012<sup>[1]</sup>. Half of those patients are either initially diagnosed at an advanced or metastatic stage or later develop distant metastases, and have a 5-year survival rate of 5%-10%<sup>[2]</sup>. While chemotherapy following resection of liver or lung

metastases has been reported to increase the chance of cure in selected patients, palliative systemic treatments may at least produce survival benefits for those presenting with diffuse unresectable disease. Over the last two decades, the median survival of patients with metastatic CRC has progressively improved, approaching 30 mo in recent reports. Notably, not only the widespread use of all available active agents (including 4 different chemotherapy drugs and 5 biologics) has shaped this clinical success, but also more patients have profited enhanced quality of life while receiving modified or less intensive maintenance treatments or while enjoying chemotherapy-free intervals. In fact, a smoother, more plastic concept embracing a “comprehensive treatment strategy” has substituted the rigid classical sequence of following structured treatment lines in the continuum of care. Notwithstanding those significant advances, the treatment landscape for unresectable advanced CRC has become increasingly complex. For all those incurable patients, mainstay of the treatment is to maximise survival while minimizing toxicities and maintaining optimal quality of life. The availability of more therapeutic options, however, has generated intricate algorithms of treatment decision-making and medical oncologists are often overwhelmed by a large number of trials providing unclear or conflicting results.

Unquestionably, when deciding the delivery of an optimally personalized treatment sequence, the ultimate treatment goal, outcome data from randomized clinical trials, different regimen-related toxicity profiles, molecular status of the disease, and patients' willingness should all be considered. However, while recent guidelines suggest to combine chemotherapy with targeted agents for the vast majority of those aged less than 75 years<sup>[3]</sup>, it is much less clear which patients deserve a higher treatment intensity and which is the best biologic to use upfront for CRC patients with KRAS wild-type disease<sup>[4]</sup>. Moreover, it should be acknowledged that the proportion of patients receiving therapy diminishes with subsequent lines and that efficacy results are the greatest in untreated patients and usually reduce along with treatment course because of a growing degree of chemoresistance. The foundation of the upfront treatment is, therefore, crucial: in first-line setting the highest number of patients may benefit therapies with the highest response rates and the longest median progression-free survival (PFS). Moreover, there is still a chance for unexpected resection and even cure, and for all those who will not be cured, first-line therapy may impact on overall survival (OS).

Actually, whenever discussing with a previously untreated patient the different first-line treatment options, some clinical considerations should be made: (1) How long will the patient survive and how long will the patient benefit from first-line treatment? (2) Does the patient need (and agree on) an aggressive strategy? (3) Will a deeper knowledge of tumor molecular biology aid in the decision-making process? (4) May the patient benefit from maintaining an antiangiogenic strategy across treat-

ment lines? and (5) Has the first-line choice potential impact on further treatment lines?

In addition, if the patients has previously received adjuvant chemotherapy (indeed, approximately 30% of metastatic CRC patients had), other questions arise: (1) How long have the patient lived without evidence of disease? (in other words, how long did the disease-free interval last?) and (2) May previous adjuvant treatments condition the first-line treatment choice?

Reporting as a springboard for discussion results from key randomized clinical trials (Table 1), aim of this viewpoint is to help clinicians making an evidence-based decision when choosing among possible first-line treatments for their medically-fit advanced unresectable CRC patients.

## WHEN TO TREAT PATIENTS WITH HIGHER INTENSITY? SEARCHING FOR THE OPTIMAL FINE-TUNING

The idea of combining all available drugs upfront with the aim to hit and immediately kill as many cancer cells as possible is certainly not new. In CRC, the combination of 5-fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI) was initially compared to 5-fluorouracil and irinotecan (FOLFIRI) in two independent studies<sup>[5,6]</sup>. Results from the phase III randomized Italian trial showed significant advantage for the triplet in terms of RR (66% *vs* 41%,  $P = 0.0002$ ), PFS (9.8 mo *vs* 6.9 mo, HR = 0.63), OS (22.6 mo *vs* 16.7 mo, HR = 0.70), and secondary resections for those with liver-limited disease (36% *vs* 12%,  $P = 0.01$ ), thus presenting such an intensive upfront regimen among the potential choices to be used when a significant tumor shrinkage is needed. Oppositely, although based on an encouraging preclinical<sup>[7]</sup> and clinical<sup>[8]</sup> background, final results of combining doublet chemotherapy with both bevacizumab and Epidermal Growth Factor Receptor (EGFR)-inhibitors were vastly disappointing<sup>[9,10]</sup>. Overall, both the randomized phase III CAIRO2 and PACCE studies showed significantly reduced PFS outcome results and increased toxicity profiles for the 4-drugs combination when compared to chemotherapy plus bevacizumab alone. The reasons for the unforeseen antagonism between the two biologic agents when combined with chemotherapy are still uncertain<sup>[11]</sup>. The issue regarding how much intense the chemotherapy backbone should be remains critical also in the era of targeted agents. Two randomized trials, phase III TRIBE<sup>[12]</sup> and phase II OLIVIA<sup>[13]</sup>, investigated the combination of the FOLFOX-IRI based-regimen with the antiangiogenic bevacizumab. In the first trial, 508 advanced CRC patients received upfront FOLFIRI or FOLFOXIRI plus bevacizumab. Patients in the experimental arm achieved a significantly longer PFS (12.1 mo *vs* 9.7 mo; HR = 0.77, 95%CI: 0.64-0.93,  $P = 0.006$ ). The triplet also provided a significant increase in RR (65% *vs* 53%,  $P = 0.006$ ), but not in radical resection rate (15% *vs* 12%,  $P = 0.327$ ). Neverthe-

**Table 1 Outcome results of major randomized phase III trials in the first-line setting in metastatic colorectal cancer patients**

Ref.	Regimen	n	Previous adjuvant treatment	ORR	Median PFS (mo)	Median OS (mo)	Post-study therapy
Hurwitz <i>et al</i> <sup>[80]</sup>	IFL	411	28%	34.8%	6.2	15.6	50%
Tebbutt <i>et al</i> <sup>[119]</sup>							
Hurwitz <i>et al</i> <sup>[80]</sup>	IFL+bevacizumab	402	24%	44.8%	10.6	20.3	50%
Cunningham <i>et al</i> <sup>[118]</sup>	Capecitabine	140	18.6%	10%	5.1	16.8	37%
	Capecitabine + bevacizumab	140	32.1%	19%	9.1	20.7	37%
Saltz <i>et al</i> <sup>[81]</sup>	XELOX/FOLFOX	701	25% <sup>1</sup>	38%	8	19.9	53%
	XELOX/FOLFOX + bevacizumab	699	24% <sup>1</sup>	38%	9.4	21.3	46%
Heinemann <i>et al</i> <sup>[81]</sup>	FOLFIRI + cetuximab	297	22.1%	62%	10	28.7	65.7%
	FOLFIRI + bevacizumab	295	18.9%	58%	10.3	25	61.7%
	Capecitabine	156	22%	30.3%	5.7	18.9	68%
Tebbutt <i>et al</i> <sup>[119]</sup>	Capecitabine + bevacizumab	157	28%	38.1%	8.5	18.9	62%
	Capecitabine + bevacizumab + MMC	158	16%	45.9%	8.4	16.4	61%
Falcone <i>et al</i> <sup>[112]</sup>	FOLFOXIRI + bevacizumab	252	12%	65%	12	31	NA <sup>3</sup>
	FOLFIRI + bevacizumab	256	12%	53%	9.7	25.8	NA <sup>3</sup>
Van Cutsem <i>et al</i> <sup>[82]</sup>	FOLFIRI	599	18.9%	39.7%	8.4	20	71.7%
	FOLFIRI + cetuximab	599	17.4%	57.3%	9.9	23.5	66%
Maughan <i>et al</i> <sup>[191]</sup>	XELOX/FOLFOX <sup>2</sup>	815	25% <sup>1</sup>	57%	8.6	17	62%
	XELOX/FOLFOX + cetuximab	815	25% <sup>1</sup>	64%	8.6	17.9	56%
Tveit <i>et al</i> <sup>[120]</sup>	FLOX	185	8% <sup>1</sup>	41%	7.9	20.4	73.5%
	FLOX + cetuximab	194	9% <sup>1</sup>	49%	8.3	19.7	75.8%
	FLOX intermittently + cetuximab	187	10% <sup>1</sup>	47%	7.3	20.3	64.2%
Douillard <i>et al</i> <sup>[90]</sup>	FOLFOX4	590	15% <sup>1</sup>	48%	8	19.7	63%
	FOLFOX4 + panitumumab	593	16.1% <sup>1</sup>	55%	9.6	23.9	53%
Schmoll <i>et al</i> <sup>[89]</sup>	FOLFOX + bevacizumab	713	19%	47.3%	10.3	21.3	23.8%
	FOLFOX + cediranib	709	17%	46.3%	9.9	22.8	28.2%
Díaz-Rubio <i>et al</i> <sup>[60]</sup>	XELOX + bevacizumab	239	13% <sup>1</sup>	47%	10.4	23.2	72%
	XELOX + bevacizumab→bevacizumab	241	17% <sup>1</sup>	49%	9.7	20	74%

<sup>1</sup>No previous oxaliplatin-based treatment allowed; <sup>2</sup>Both Arm A (continuously) and Arm B (intermittently) have been considered; <sup>3</sup>Data will be available in 2014. ORR: Overall response rate; PFS: Progression-free survival; OS: Overall survival; IFL: Irinotecan, fluorouracil, leucovorin therapy; FOLFIRI: 5-fluorouracil and irinotecan; XELOX: Capecitabine/oxaliplatin.

less, the study population was unselected for conversion to surgical resectability, since only 20% of randomized patients had liver-limited disease. Preliminary data showed a trend toward improved OS in the FOLFOX-IRI plus bevacizumab arm (31.0 mo *vs* 25.8 mo; HR = 0.83, 95%CI: 0.66-1.05). Phase II OLIVIA trial allocated 80 advanced CRC patients with liver-only unresectable metastases to receive 5-fluorouracil and oxaliplatin (FOLFOX) or FOLFOXIRI plus bevacizumab. Overall resection rate, the primary endpoint, was numerically higher in the FOLFOXIRI plus bevacizumab arm (61.0% *vs* 48.7%,  $P = 0.27$ ). The more intensive regimen provided both a higher RR (80.5% *vs* 61.5%,  $P = 0.061$ ) and radical (R0) resection rate (48.8% *vs* 23.1%,  $P = 0.017$ ), with longer PFS (18.8 mo *vs* 12.0 mo,  $P = 0.0002$ ). Moreover, retrospective data suggest that the addition of bevacizumab to the FOLFOXIRI regimen does not impact on liver toxicity while enhancing the rate of pathologic response and tumor necrosis<sup>[14]</sup>.

The combination of FOLFOXIRI with EGFR-inhibitors showed also interesting results in a phase II trial, but a formal phase III comparison of the added benefit of cetuximab or panitumumab to the triplet regimen is currently lacking. In the TRIP study, 37 highly molecularly selected patients (concomitant wild-type status for KRAS, BRAF, NRAS, and HRAS) received FOLFOXIRI plus panitumumab with a reported RR of 89%. Forty-three percent of them underwent secondary surgery of

metastases, and R0 resection was achieved in 13 cases (35%). After a median follow-up of 17.7 mo, median PFS was 11.3 mo<sup>[15]</sup>. Another phase II study enrolled 43 CRC patients with unresectable liver metastases to receive cetuximab plus chronomodulated irinotecan, 5-fluorouracil, leucovorin and oxaliplatin as neoadjuvant chemotherapy<sup>[16]</sup>. After a median number of 6 cycles, RR was noted in 79% of patients, and median OS was of 37 mo.

Based on available results, when should we opt for a very intensive treatment? The use of triplet plus bevacizumab could be considered a possible treatment option for those who parallel the trial's inclusion criteria (*i.e.*, unresectable, metastatic disease, age < 75 years; optimal ECOG PS, no major comorbidities), but this appears to be a much more intriguing and logical option for patients with symptomatic, bulky or aggressive disease or when conversion from unresectable to resectable status is deemed possible (liver-limited unresectable metastases). In the first circumstance, patients may benefit from a fast disease shrinkage that while reducing the tumor burden may better control cancer-related symptoms or avoid their occurrence. In the second condition, the advantage of using this highly active combination is that it may exert its effect in few cycles, avoiding a sustained exposure to chemotherapy that might potentially increase liver toxicity just before hepatic surgery. Although phase II studies results are promising, the use of a triplet regimen combined with EGFR-inhibitors outside of a clinical trial



should be currently discouraged, even in patients with optimal molecular selection. In order to ameliorate the tolerability, the intensification of the upfront therapy in never resectable patients usually requires to plan a short initial treatment period (induction phase) followed by a less intensive treatment (maintenance phase). To avoid excessive toxicity in a palliative setting, the strength of such an induction treatment should last no longer than 8 cycles. After that, patients are usually switched to an appropriate, more tolerable, maintenance regimen that may be continued for a long period. Ongoing studies are clarifying the role of the maintenance therapy and expounding which are the optimal agents to be used. Potential drawbacks of an intensive treatment include higher toxicity and more limited rescue options once the tumor has become resistant.

## WHICH BIOLOGIC SHOULD BE PREFERRED IN THE UPFRONT TREATMENT OF KRAS WILD-TYPE CRC PATIENTS?

Although the predictive role of G13D mutation still remains a matter of discussion<sup>[17-19]</sup>, having a KRAS mutation in codon 12 or 13 is a universally accepted marker for EGFR-inhibitor inefficacy<sup>[20,21]</sup>. Other germline mutations in *RAS* or *BRAF* genes also seem to predict unfavourable results<sup>[22,23]</sup>, and acquired secondary mutations may cause resistance to EGFR-inhibitors<sup>[24-26]</sup>. Moreover, retrospective data confirmed that using a more adequate technique RAS or BRAF mutations were found in approximately 20% of cancers initially classified as wild-type<sup>[20]</sup>, and this might help in refining the target population<sup>[27,28]</sup>. Current molecular selection has a negative predictive value, but it does not help in the clinical-decision process for patients with wild-type CRC. Actually, which targeted agent should be combined to first-line chemotherapy in KRAS wild-type patients is one of the hot-topics in colorectal oncology. Up today, the choice was essentially based on cross-trial comparisons and on meta-analyses estimating the magnitude of benefit provided by each targeted agent<sup>[29,30]</sup>. While EGFR-inhibitors were considered powerful shrinking agents, bevacizumab was preferred for its ability to delay tumor progression. FIRE-3, the first phase III randomized trial to provide results on the head-to-head comparison, randomized 592 KRAS wild-type CRC patients to upfront FOLFIRI plus either cetuximab or bevacizumab, with the aim to detect a difference of 12% in RR induced by FOLFIRI plus cetuximab (62%) compared to FOLFIRI plus bevacizumab (50%)<sup>[31]</sup>. Though unusual for a randomized phase III trial, RR was chosen as the primary endpoint of the study. Because of a higher than expected treatment activity reported for patients exposed to bevacizumab, RR resulted similar between treatment arms (62% in the FOLFIRI plus cetuximab arm *vs* 58% in the FOLFIRI plus bevacizumab arm, OR = 1.18, *P* = 0.18) and no differences

in PFS were documented (HR = 1.06; 95%CI: 0.88-1.26, *P* = 0.54). Of note, in the cohort of patients assessable for response (*n* = 526, 89%), encompassing all those who had received a minimum of 3 cycles and had performed at least a CT-scan evaluation following baseline, RR was significantly higher in favour of cetuximab-containing arm (72.2% *vs* 63.1%, OR = 1.52, *P* = 0.017). Although, no significant differences in median PFS were reported (10 mo *vs* 10.3 mo, HR = 1.03; 95%CI: 0.88-1.26), a clinically meaningful 3.7-month median advantage in OS was evidenced in favour of the cetuximab arm (28.7 mo *vs* 25 mo, HR = 0.77; 95%CI: 0.62-0.96), confirmed in all exploratory subgroups analysed. Disparities in subsequent treatment lines may hardly explain this unforeseen survival difference, being the proportion of patients who crossed over or received treatment beyond progression similar between treatment arms (65.7% in the cetuximab arm *vs* 61.7% in the bevacizumab arm, *P* = 0.34). Oppositely, the association of both early tumor shrinkage (at least 20% decrease in the sum of the longest diameter compared with baseline at week 8) and the deepness of response (percentage of tumor shrinkage observed at the smallest tumor size compared to baseline) to EGFR-inhibitors with the post-progression survival were advocated as possible reasons for success<sup>[32]</sup>. According to this theoretical model, the higher tumour shrinkage may result in a lower tumour load, as per RECIST, at the time of disease progression so that the benefit achieved in terms of deepness of response may influence the following history of patients' disease. Likewise, a significant correlation of the early objective tumor response (EOTR) with survival was demonstrated by an individual patient data meta-analysis of 15 randomized first-line trials enrolling approximately 12000 patients from the ARCAD database<sup>[33]</sup>. In the analysis, median PFS and median OS were consistently longer in patients with an EOTR at 6, 8 or 12 wk compared to those without. Overall, these results support the hypothesis that the advantage in terms of activity of an intensive upfront regimen may translate into a significant survival gain regardless the opportunity to achieve secondary resections. While a confirmatory correlation analysis is being conducted in FIRE-3 trial, outcome results from a larger intergroup phase III trial (CALGB 80405, NCT00265850) that aims to compare upfront chemotherapy with bevacizumab or cetuximab in over 1200 metastatic CRC patients are awaited. Differently from FIRE-3, OS is the primary endpoint of the CALGB and SWOG cooperative groups trial.

To simultaneously explore the head-to-head comparison and the treatment strategy, the GERCOR is sponsoring the phase III STRATEGIC-1 trial<sup>[34]</sup> that is designed to provide information on the optimal treatment sequence, with two different strategies each including all the currently available agents (oxaliplatin, irinotecan, fluoropyrimidines, bevacizumab, and EGFR-inhibitors), but in a different order. With disease control rate of the full strategy as the primary endpoint, nearly 500 patients with unresectable wild-type KRAS metastatic CRC will be

randomized to FOLFIRI-cetuximab, followed by an oxaliplatin-based chemotherapy with bevacizumab (Strategy A) or OPTIMOX-bevacizumab, followed by irinotecan-based chemotherapy with bevacizumab, followed by an EGFR-inhibitor with or without irinotecan (Strategy B). The study is starting soon the target recruitment.

## TOWARD A BETTER MOLECULAR SELECTION? BROADENING CRC BIOLOGIC KNOWLEDGE BEYOND KRAS

Since the acknowledgment that CRC is a highly heterogeneous disease with regards to clinical evolution and response to treatments and the fact that it may change over time or evolve under treatment pressure<sup>[35]</sup>, a more profound molecular knowledge of this cancer has been promoted<sup>[36]</sup>. Actually, a deeper understanding of the disease pathobiology and its molecular underpinnings allow clinicians to take advantage of a more detailed disease classification<sup>[37]</sup> and more robust information on predictive and prognostic biomarkers as well as resistance bioindicators for both antiangiogenic<sup>[38]</sup> and EGFR-inhibitors<sup>[39]</sup>. Whether serial tumor biopsies and repeated mutation testing may be useful to better capture the CRC heterogeneity and to systemically track its genomic evolution is a matter of debate<sup>[40,41]</sup>, but the application of innovative, low-invasive techniques may find acceptance from both scientific and ethical standpoints<sup>[42,43]</sup>. Specifically focusing on the treatment tailoring, the landscape has rapidly evolved beyond KRAS codon 12 and 13 mutational status<sup>[44]</sup>. For example, rare mutation occurring in other KRAS codons, such as mutation in codons 61 or 146, may result in reduced EGFR-inhibitor efficacy<sup>[22]</sup>. As well, V600E BRAF mutations occurring in approximately 10% of all KRAS wild-type CRC tumors<sup>[45]</sup> or more rare KRAS amplifications<sup>[46]</sup> seem to limit the benefit from EGFR-inhibitors<sup>[47-49]</sup>. However, while there is total agreement on its negative prognostic value, the negative predictive role of BRAF mutations with regards to EGFR-inhibitor therapy is not universally accepted<sup>[50-52]</sup> and loss of PTEN expression or activity<sup>[53,54]</sup> have also been associated to inferior benefit from EGFR-inhibitors, but the small sample size of the cohort analysed linked to the relatively rare events prevent to draw strong definitive conclusions.

Importantly, the use of EGFR-inhibitors in the clinical practice should be based on a deep molecular analysis with further refinement of tumor-specific genetic markers in order to simultaneously allow: (1) identification of a wider patient population that does not benefit from the target treatment or may have detrimental effect; and (2) selection of patients who may achieve a maximized survival improvement. A prospective-retrospective analyses of phase III PRIME trial<sup>[55]</sup> that randomized 1083 patients to upfront FOLFOX plus or minus panitumumab and a preplanned analysis of phase II PEAK study that assigned in first-line 285 patients to FOLFOX

plus either bevacizumab or panitumumab<sup>[56]</sup> consistently show that patients harbouring rare KRAS mutations in exon 3 (codons 59/61) and 4 (codons 117/146), or NRAS mutations in exon 2 (codons 12/13), 3 (codons 59/61), and 4 (codons 117/146) may not benefit from the EGFR-inhibitor. In the first analysis, patients without RAS mutations had a 2.2 mo median advantage in median PFS (10.1 mo *vs* 7.9 mo, HR = 0.72, 95%CI: 0.58-0.9, *P* = 0.004), and a 5.8 median advantage in OS (26 mo *vs* 20.2 mo, HR = 0.78, 95%CI: 0.62-0.99, *P* = 0.04). Impressively, patients with no RAS or BRAF mutations (*n* = 446) derived a 7.6 median survival benefit (28.3 mo *vs* 20.9 mo, HR = 0.74, 95%CI: 0.57-0.96, *P* = 0.02) if exposed to FOLFOX and panitumumab in first-line. An exploratory biomarker tumor analysis<sup>[57]</sup> of patients enrolled in the panitumumab *vs* BSC randomized phase III study<sup>[58]</sup> reported similar results. Importantly, the addition of panitumumab to first-line FOLFOX might be even detrimental in patients with less common RAS mutations and should be cautiously avoided. On the basis of these data, marketing authorization for panitumumab has been amended, including the analysis of NRAS status before prescription, and restraining its use to RAS wild-type CRC patients. Since it has been highlighted how a more detailed molecular profile may impact on the evidence-based decision making process, a more accurate selection of candidates to upfront EGFR-inhibitors is warranted. Results of a similar deeper molecular analysis in patients exposed to upfront cetuximab or bevacizumab combined with FOLFIRI in the FIRE-3 trial will be soon presented.

## ANGIOGENIC INHIBITORS UPFRONT AND IN THE FOLLOWING TREATMENT LINES? THE ISSUE OF MAINTENANCE AND TREATMENT BEYOND PROGRESSION

The choice of an upfront bevacizumab-based combination is considered a widely accepted standard treatment option for the majority of advanced CRC patients. Although supported by limited evidence, to continue the angiogenic inhibitor until disease progression is not uncommon in the clinical practice, especially for those patients who partially or entirely withhold the associated chemotherapy because of toxicity or towering cumulative doses of oxaliplatin<sup>[59]</sup>. Actually, results of randomized trials such as MACRO<sup>[60]</sup>, DREAM<sup>[61]</sup>, and COIN-B<sup>[62]</sup> suggest to continue bevacizumab as maintenance therapy until disease progression. In the MACRO trial, 480 CRC patients were randomly assigned to receive six cycles of bevacizumab, capecitabine, and oxaliplatin followed by bevacizumab either alone or combined with the same chemotherapy regimen until progression. A slightly longer median PFS was reported in the combination arm (10.4 mo *vs* 9.7 mo, HR = 1.1, *P* = 0.38), although burdened by a higher rate of severe sensory neuropathy (26% *vs* 8%, *P* = 0.0001) and HFS (13% *vs* 7%, *P* = 0.03). The primary analysis of DREAM demonstrated that a maintenance

therapy with bevacizumab and erlotinib may significantly prolong median PFS (10.2 mo *vs* 9.3 mo, HR = 0.76; 95%CI: 0.61-0.94,  $P = 0.009$ ) but not median OS (28.5 mo *vs* 27.0 mo, HR = 0.89; 95%CI: 0.7-1.12,  $P = 0.31$ ) after a first-line bevacizumab-based induction therapy<sup>[63]</sup>. The additive value of erlotinib to bevacizumab in this setting is however unconfirmed<sup>[64]</sup>. Yet, the issue regarding the role of bevacizumab in the maintenance phase was not formally addressed until recently. SAKK 41/06<sup>[65]</sup> and CAIRO-3<sup>[66]</sup> phase III trials compared observation to a maintenance strategy following an induction phase of chemotherapy plus bevacizumab. In the non-inferiority Swiss study, 262 CRC patients without disease progression at 4-6 mo since treatment start were randomized to continue on single-agent bevacizumab until disease progression or observation. Even though median PFS (+1.2 mo) and OS (+3.3 mo) were both longer for patients who continued on bevacizumab, the trial formally failed to meet its primary endpoint, since the median time to progression did not differ sufficiently between treatment arms (17.9 wk *vs* 12.6 wk; HR = 0.74; 95%CI: 0.57-0.94,  $P = 0.47$ ; with a non-inferiority limit for HR = 0.727). In CAIRO-3 trial, patients without disease progression after 6 cycles of capecitabine, oxaliplatin (CAPOX regimen) and bevacizumab were randomized to observation or continuing with capecitabine and bevacizumab. Upon the first disease progression, CAPOX plus bevacizumab was reintroduced and maintained until the second evidence of progression. The primary endpoint was the PFS2, defined as the time from randomization to progression upon treatment re-introduction. Patients in the maintenance arm achieved a significantly longer PFS2 (11.8 mo *vs* 10.5 mo, HR = 0.81; 95%CI: 0.67-0.98,  $P = 0.028$ ), PFS (8.5 mo *vs* 4.1 mo, HR = 0.44; 95%CI: 0.36-0.53,  $P < 0.00001$ ) and a non-significant advantage in OS (21.7 mo *vs* 18.2 mo, HR = 0.87; 95%CI: 0.71-1.06,  $P = 0.156$ ), that became significant in the adjusted analysis (HR = 0.80). AIO KRK0207, a phase III randomized trial comparing observation to maintenance with either bevacizumab alone or bevacizumab plus capecitabine, will clarify if a maintenance treatment, instead of a full holiday period, is actually needed for all patients. In conclusion, while reasonable, safe, and clinically feasible, whether a maintenance therapy is needed for all patients is still an open question.

The role of cetuximab in the maintenance therapy is also being investigated. The two-arm phase II COIN-B study randomized 169 patients with unresectable KRAS wild-type CRC to intermittent chemotherapy plus continuous or intermittent cetuximab as first-line treatment. Continuous cetuximab was associated with a longer failure free survival (FFS), chemotherapy-free interval (3.7 mo *vs* 5.1 mo) and time to progression (20.1 mo *vs* 18.4 mo). Median FFS was 12.0 and 13.7 mo, respectively<sup>[62]</sup>. The phase III Macbeth trial (EUDRACT 2011-000840-70) is an ongoing multicenter, randomized, open-label study designed to evaluate the efficacy and safety of eight cycles of FOLFOXIRI plus cetuximab

followed by maintenance with cetuximab or bevacizumab as first-line treatment for unresectable KRAS wild-type metastatic CRC patients.

Another point of discussion is the use of antiangiogenics beyond disease progression. Data from retrospective registries such as BRITe<sup>[67]</sup> or ARIES<sup>[68]</sup> suggested a survival benefit with the use of bevacizumab beyond disease progression. More recently, the randomized phase III ML18147 trial prospectively tested the efficacy of maintaining bevacizumab beyond disease progression<sup>[69]</sup>. After the failure of a bevacizumab-containing first-line treatment, 820 patients were randomized to receive a different second-line chemotherapy with or without bevacizumab. Those that continued on the antiangiogenic agent reported significantly longer OS (11.2 mo *vs* 9.8 mo; HR = 0.81; 95%CI: 0.69-0.94,  $P = 0.0062$ ) and PFS (5.7 mo *vs* 4.1 mo, HR = 0.68; 95%CI: 0.59-0.78,  $P < 0.0001$ ). Toxicity profiles were similar between the two arms, although more bleedings (2% *vs* 1%), venous thromboembolic events (5% *vs* 3%), and gastrointestinal perforations (2% *vs* < 1%) were noted among those receiving bevacizumab. In the phase III BEBYP trial<sup>[70]</sup>, 184 patients who had failed a bevacizumab-based first-line treatment were randomized to receive second-line chemotherapy with or without bevacizumab. The trial was stopped early, as soon as the positive results of the ML18147 were diffused. Performance status (ECOG PS 0 *vs* 1-2), length of the chemotherapy-free interval (< or > 3 mo), and type of second-line chemotherapy were considered as stratification factors. Two thirds of the patients received oxaliplatin-based combinations in both treatment arms. After a median follow-up of 22 mo, the results confirmed the benefit in PFS (6.8 mo *vs* 5 mo, HR = 0.72; 95%CI: 0.54-0.97,  $P = 0.029$ ) for those maintained on bevacizumab, while OS data are still immature to be analyzed.

Indirect evidence supports how CRC patients may benefit from further angiogenic treatments after disease progression while on bevacizumab. The phase III VELOUR trial showed the efficacy of aflibercept (a fusion protein with high affinity to all VEGF-A isoforms, VEGF-B, PlGF-1, and PlGF-2) in combination with FOLFIRI in 1,266 CRC patients who had failed a first-line oxaliplatin-based therapy<sup>[71]</sup>. Both median OS (13.5 mo *vs* 12.06 mo, HR = 0.817; 95%CI: 0.71-0.94,  $P = 0.0032$ ) and PFS (6.9 mo *vs* 4.67 mo, HR = 0.76) were significantly longer in those who received FOLFIRI and aflibercept. Importantly, prior exposition to antiangiogenics did not reduced the outcome effect. Actually, a similar benefit in PFS (6.7 mo *vs* 3.9 mo, HR = 0.66; 95%CI: 0.51-0.85) and OS (12.5 mo *vs* 11.7 mo, HR = 0.86; 95%CI: 0.67-1.10) was reported for the use of aflibercept in those who had received bevacizumab as part of their upfront treatment (approximately 28% in both treatment arms). Regorafenib is another agent with broad antiangiogenic properties<sup>[72]</sup>. In the CORRECT trial, 760 chemorefractory CRC patients were randomized 2:1 to regorafenib (160 mg daily in a 3-wk-on, 1-week-off schedule) or placebo<sup>[73]</sup>. All patients had previously re-



ceived bevacizumab. Median OS was 6.4 mo in the regorafenib group *vs* 5.0 mo in the placebo group (HR = 0.77; 95%CI: 0.64-0.94).

Large, international efforts have tried to define who are the patients more likely to benefit from the antiangiogenic strategy. Unfortunately, given the complexity of cancer-related angiogenesis, conflicting results have been reported both at molecular<sup>[74]</sup> or clinical levels<sup>[75,76]</sup>. The prospective validation of other single predictive biomarkers such as baseline LDH value<sup>[75]</sup>, number of circulating endothelial cells<sup>[77]</sup>, or level of miRNA<sup>[78]</sup> are still pending, but will unlikely succeed.

## WILL THE FIRST-LINE CHOICE IMPACT ON FOLLOWING TREATMENT LINES?

If and how the first-line therapy may influence further treatment is a matter of debate at many levels (molecular, clinical, regulatory). Nevertheless, how oncologists decide the sequence of treatment to use should be always based on a solid mainstay. The following reasoning is founded on a critical analysis of major phase III randomized studies.

Accordingly to the results of a pivotal phase III trial that compared FOLFOX6 followed by FOLFIRI to FOLFIRI followed by FOLFOX6 and showed similar outcomes regardless of the treatment sequence<sup>[79]</sup>, the backbone treatment used after first disease progression of disease is currently based on a crossover from an irinotecan- to an oxaliplatin-based regimen or vice-versa. In that trial, 220 patients were randomized to receive initially either FOLFIRI or FOLFOX6 and to switch to the other regimen at disease progression. Neither first-line RR (56% *vs* 54%), nor first-line median PFS (8.5 mo *vs* 8 mo, *P* = 0.26), nor median OS (21.5 mo *vs* 20.6 mo, *P* = 0.99) were statistically different between treatment arms.

Ten years after the widespread use of biologics has begun in the clinical practice, the scenario has become much more complicated, particularly in patients with KRAS wild-type tumors that may benefit from a scope of different treatments. The initial choice of the upfront chemotherapy regimen, however, retains its value.

When opting for an irinotecan-based first-line regimen, either bevacizumab<sup>[80]</sup> or cetuximab<sup>[81,82]</sup> could be used as optimal biologic partners. Either way the patient is started, survival results of the ECOG E3200 phase III trial<sup>[83]</sup> would suggest to use FOLFOX plus bevacizumab as second-line treatment after an irinotecan-based first-line failure. Later on, following on the treatment route, the choice of third-line may become critical. In this setting, while strong data support the use of EGFR-inhibitors either alone<sup>[84,58]</sup> or combined to irinotecan<sup>[85]</sup>, evidence suggesting potential benefit from retreating patients with EGFR-inhibitors is more shaggy<sup>[86,87]</sup> or under investigation<sup>[88]</sup>. Regorafenib, indeed, would be an appropriate choice for all highly pretreated patients<sup>[73]</sup>. Consequently, the treatment algorithm would offer 4 potential lines of treatment if the patient receive

upfront an irinotecan-based chemotherapy plus bevacizumab, but one treatment line would be lost if the patient starts with an irinotecan-based therapy plus cetuximab. This hypothetical reasoning may be revised (and even reversed) if the outcome results of CALGB 80405 trial will confirm the unexpected 3.7-mo median survival advantage reported in FIRE-3 for KRAS wild-type CRC patients receiving FOLFIRI and cetuximab in first-line.

When opting for a first-line treatment including oxaliplatin, antiangiogenic drugs<sup>[60,89]</sup> or EGFR-inhibitor<sup>[90,91]</sup> may be used in combination, although the upfront use of bevacizumab seems to be preferable because it may better fit in the maintenance strategy<sup>[92,93]</sup> for its convenience and safety when combined to capecitabine<sup>[94]</sup>. Moreover, the upfront combination of oxaliplatin with an EGFR-inhibitor requires more detailed molecular biology data (see paragraph 4) and increased watchfulness if using an oral fluoropyrimidine<sup>[91]</sup>. At disease progression, many reasons strongly support the choice of switching to an irinotecan-based regimen, including the potential cumulative neurotoxicity of prolonged oxaliplatin use. Since in second-line setting many alternative options exist, to establish which is the optimal biologic to be delivered is challenging and depends on the previous use of targeted agents. A number of second-line randomized trials have investigated the role of biological agents in the treatment of CRC patients not previously exposed to EGFR-inhibitors. Tested agents included bevacizumab<sup>[69]</sup>, aflibercept<sup>[71]</sup>, cetuximab<sup>[95]</sup>, or panitumumab<sup>[96,97]</sup>. Of note, in all those trials patients may have been upfront treated with bevacizumab, but the proportion of those who did receive the angiogenic inhibitors in first-line vastly varied, ranging from 2%<sup>[97]</sup> to 100%<sup>[69]</sup>. Results of ML18147 and VELOUR have been already discussed (see before). In the phase III EPIC study<sup>[92]</sup>, 1298 patients who had prior failed a first-line oxaliplatin-based regimen, were randomized to receive irinotecan plus cetuximab or irinotecan alone. The addition of cetuximab to irinotecan resulted in a significant improvement of PFS (4.0 mo *vs* 2.6 mo, HR = 0.69; 95%CI: 0.617-0.776, *P* < 0.0001), but no OS advantage was reported (10.7 mo *vs* 10.0 mo, HR = 0.97). Panitumumab was tested in another randomized phase III trial, comparing in 1,186 pretreated metastatic CRC patients, the addition of panitumumab itself to FOLFIRI, to placebo. A significant improvement in PFS was observed (5.9 mo *vs* 3.9 mo, HR = 0.73; 95%CI: 0.59-0.90, *P* = 0.004), with a trend for longer OS (14.5 mo *vs* 12.5 mo, HR = 0.85; 95%CI: 0.70-1.04, *P* = 0.12). Similarly, the PICCOLO study<sup>[97]</sup> reported higher RR (34% *vs* 12%, *P* < 0.001), longer PFS (HR = 0.78; 95%CI: 0.64-0.95, *P* = 0.015), but no survival advantage (10.9 mo *vs* 10.4 mo; HR = 1.01; 95%CI: 0.83-1.23, *P* = 0.91) for the use of panitumumab and irinotecan-based chemotherapy compared to irinotecan alone. If the upfront biologic was the EGFR-inhibitor, less options are permitted (see point A). Again, regorafenib may be considered as salvage treatment for all pretreated patients. As discussed before, if the patient is started with a EGFR-inhibitor, the number



of therapeutic options seems narrowed.

## CHOOSING A FIRST-LINE TREATMENT FOR CRC PATIENTS WHO HAVE FAILED ADJUVANT OXALIPLATIN - IS THERE ANY DIFFERENCE?

Since approximately 50% of stage III and 20% of stage II CRC patients do eventually recur, one third of patients present with metachronous metastatic disease, which is currently defined as more than 1 year between the occurrence of the primitive tumor and metastasis. Not surprisingly, a significant proportion of those patients may have already received an oxaliplatin-based chemotherapy, a universally confirmed standard regimen in the adjuvant setting<sup>[98-100]</sup>. Indeed, patients enrolled in first-line phase III randomized trials which had already been exposed to adjuvant chemotherapy ranged from 8% to 32% (Table 1). However, having received a previous treatment with oxaliplatin was sometimes included among the exclusion criteria, and even when it was permitted, how many of those pretreated patients had actually received an oxaliplatin-based regimen was rarely specified in the publication.

To fully understand the importance of this point, some data should be further discussed. The analysis of over 20000 CRC patients included in the ACCENT database showed that the risk of recurrence peaks between 18 and 24 mo after radical surgery, and then decreases over time<sup>[101]</sup>. Most patients who recur, therefore, develop metastatic disease within 18 mo since the end of postoperative chemotherapy.

The use of oxaliplatin is burdened by the frequent occurrence of chronic peripheral sensory neuropathy<sup>[102,103]</sup>, a dose-dependent disturbing toxicity characterized by dysesthesia and distal paresthesia, that often negatively impacts on patients' quality of life<sup>[104]</sup>. In addition, acute neuropathy (oral-facial and peripheral), which in some cases is induced or exacerbated by exposure to cold, was also reported. This neurological side-effect, quite unusual in the initial chemotherapy cycles, frequently appears during the treatment course as long as the cumulative dose of oxaliplatin increases.

The vast majority of the patients enrolled in randomized clinical trials that tested oxaliplatin in the adjuvant setting developed peripheral sensory neuropathy. In MOSAIC trial, any grade peripheral neurotoxicity was observed in 92% of patients, while grade 2 (moderate) or grade 3 (severe) was reported in 44%. Often, however, the symptoms ameliorated or resolved over time: one and four years after treatment, 30% and 15% of patients had minimal residual toxicity, respectively. In NSABP C-07 trial, grade 3-4 peripheral neuropathy was reported in 8.4% of patients. At 1 year from random assignment, the rate of severe neurotoxicity was 0.6%. The inferior rate of neurotoxicity may be due to the lower cumulative dose of oxaliplatin in NSABP C-07 (9 planned doses of 85 mg/m<sup>2</sup>) compared to MOSAIC (12 planned doses of

85 mg/m<sup>2</sup>).

In NO16968 study, any grade peripheral neuropathy occurred in 78% of patients exposed to oxaliplatin, and grade 3-4 in 11%. At the end of adjuvant treatment, residual neurotoxicity was still present in 68% of patients.

Toxicity data were confirmed in another randomized trial that tested the efficacy of bevacizumab combined to oxaliplatin-based chemotherapy in the adjuvant setting<sup>[105]</sup>. Grade 2 or grade 3 sensory neuropathy was reported in 43.7% of patients treated with FOLFOX6 and in 48.9 % of those treated with FOLFOX6 + bevacizumab, with the delivery of similar median doses of oxaliplatin. Notably, about 10%-20% of patients developed severe neurotoxicity after cumulative oxaliplatin dose of 750-850 mg/m<sup>2</sup><sup>[106]</sup>.

Recently, a number of studies reported on a long-lasting oxaliplatin-induced peripheral neurotoxicity<sup>[107,108]</sup>. Those studies showed that a not-negligible proportion of patients (5%-15%) still suffer from chronic neurotoxicity many years after treatment end, and refer troublesome numbness or tingling of hands and feet. Then, it is conceivable that a proportion of oxaliplatin-exposed patients may still have neurological symptoms at the time of recurrence. In order to prevent or reduce the incidence and intensity of this toxicity in the adjuvant setting, several strategies are being studied, including a reduced exposition to oxaliplatin<sup>[109]</sup> or the potential use of neuroprotectants such as glutathione<sup>[110]</sup>, oxcarbazepine<sup>[111]</sup>, or venlafaxine<sup>[112]</sup>, but no preventive treatment has been recognized as a standard. Moreover, retrospective studies suggested that the *iv* supplementation with calcium and magnesium may be useful<sup>[113]</sup>. However, a randomized phase III trial enrolling 362 radically resected CRC patients with no pre-existing peripheral neuropathy to compare calcium/magnesium supplementation *vs* placebo failed to show any significant difference among treatment arms in the rate of moderate or severe neuropathy<sup>[114]</sup>.

For all these reasons, whether the clinical outcome of an oxaliplatin-based first-line therapy is maintained in patients who had been already exposed to the drug in the adjuvant setting is unclear and few data are available on this regard. Recently, a retrospective study assessed the first-line RR to either FOLFIRI or FOLFOX in 32 patients with advanced CRC who had previously received adjuvant FOLFOX after radical surgery<sup>[115]</sup>. The median time between the beginning of adjuvant chemotherapy and disease recurrence was 1.7 years. The overall RR was 17% in the FOLFOX group *vs* 36% in the FOLFIRI group. Despite a trend in favor of FOLFIRI, the difference was not statistically significant ( $P = 0.22$ ).

For patients with residual neurotoxicity at the time of disease recurrence, the stop-and-go strategy may be an appropriate option to avoid the side-effect worsening while still using an active agent. Two different randomized trials showed a clinically significant reduction in the rate of severe neurotoxicity with the use of this strategy<sup>[116,117]</sup>. In conclusion, an oxaliplatin-based regimen could still be an option for patients without or with minimal residual neurotoxicity that become metastatic

after at least 12 mo since the end of an oxaliplatin-based adjuvant therapy. Oppositely, for those who relapse early (within 12 mo) or still have clinically significant neurotoxicity, it is reasonable to choose a regimen without oxaliplatin and delay as much as possible the reintroduction of the neurotoxic drug.

## CONCLUSION

The landscape of CRC treatment is changing very fast, and the availability of new therapeutic options has created new challenges and generated more complicated treatment algorithms. In conclusion, we would like to suggest the reader short possible answers to the initial questions. Undoubtedly, the optimal choice of the first-line treatment is still of great importance. When considering this choice, patients' performance status, comorbidities and desires should be considered as well as the ultimate goal of the treatment and the molecular features of the tumor. An highly intensive regimen is particularly indicated for younger patients without comorbid conditions or for those patients with aggressive colorectal carcinomas (symptomatic, bulky disease or BRAF mutant tumors). The application of a deeper molecular analysis not only helps identifying those patients who may benefit the most from EGFR-inhibitors but also has a prognostic value. In the majority of cases with RAS and BRAF wild-type status, a first-line combination with an EGFR-inhibitor seems to be the preferred treatment option, while the antiangiogenic strategy should be pursued in those with RAS mutated tumors or when a less aggressive treatment is favoured. The exposition to oxaliplatin in the adjuvant setting may somehow limit its use in the advanced phases of the disease due to possible cumulative neurotoxicity. Randomized trials, however, are verifying if a shorter oxaliplatin-based adjuvant treatment may be equally protecting and less toxic. Notably, many other new molecules are being studied in randomized trials and, hopefully, results of those studies will help clinicians further refining the current treatment paradigms.

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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

# Neo-adjuvant radiotherapy in rectal cancer

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## Abstract

In rectal cancer treatment, attention has focused on the local primary tumour and the regional tumour cell deposits to diminish the risk of a loco-regional recurrence. Several large randomized trials have also shown that combinations of surgery, radiotherapy and chemotherapy have markedly reduced the risk of a loco-regional recurrence, but this has not yet had any major influence on overall survival. The best results have been achieved when the radiotherapy has been given preoperatively. Preoperative radiotherapy improves loco-regional control even when surgery has been optimized to improve lateral clearance, *i.e.*, when a total mesorectal excision has been performed. The relative reduction is then 50%-70%. The value of radiotherapy has not been tested in combination with more extensive surgery including lateral lymph node clearance, as practised in some Asian countries. Many details about how the radiotherapy is performed are still open for discussion, and practice varies between countries. A highly fractionated radiation schedule (5 Gy × 5), proven efficacious in many trials, has gained much popularity in some countries, whereas a conventionally fractionated regimen (1.8-2.0 Gy × 25-28), often combined with chemotherapy, is used in other countries. The additional therapy adds morbidity to the morbidity that surgery causes, and should therefore be administered only

when the risk of loco-regional recurrence is sufficiently high. The best integration of the weakest modality, to date the drugs (conventional cytotoxics and biologicals) is not known. A new generation of trials exploring the best sequence of treatments is required. Furthermore, there is a great need to develop predictors of response, so that treatment can be further individualized and not solely based upon clinical factors and anatomic imaging.

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**Key words:** Chemotherapy; Chemoradiotherapy; Local control; Multidisciplinary; Organ preservation; Radiotherapy; Randomized trials; Rectal cancer

**Core tip:** Neo-adjuvant radiotherapy is beneficial to many rectal cancer patients since it reduces the risk of a local failure. Provided surgery is optimized, it does not substantially improve overall survival. This review describes the results of the randomized trials that form the basis for the present treatment recommendations. It also pinpoints reasons for differences in the care of rectal cancer patients seen worldwide. Finally, the concept of organ preservation is critically discussed.

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## INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and the second or third most common cause of cancer death. One third of the cancers arise in the rectum, the rest in the colon and most cases are adenocarcinomas. Survival has for decades been less favourable

in rectal than in colon cancer, but this is no longer the case<sup>[1-4]</sup>. Efforts to decrease rectal cancer loco-regional recurrence rates by better staging, improved surgery and incorporation of radiotherapy are the most likely reasons for the presently slightly better 5-year survival rates in rectal cancer. The local recurrence rates have also decreased from 30%-40% a few decades ago down to 5%-10% or even lower in some recent studies, and this has influenced survival in certain population-based studies. Survival still differs extensively between countries, and differences in therapy traditions are probably a major reason for this<sup>[5]</sup>.

Radical removal of the primary rectal cancer, together with all regional tumour cell deposits are prerequisites for cure, although occasional local recurrences can be salvaged by (chemo)radiotherapy [(C)RT] and secondary surgery. Avoidance of persistent or recurrent tumour in the pelvis is important, even if cure cannot be achieved since uncontrolled pelvic growth is usually associated with severe symptoms. Even if overall survival is not improved, improved local control is a legitimate outcome of different interventions in rectal cancer. The primary tumour in the bowel is usually not the major problem unless it grows extensively towards organs not readily removed. In these patients, preoperative therapy with the aim of sterilizing macroscopic tumour cells in the periphery of the tumour is required. The most prevalent clinical problem is rather to eradicate the microscopic tumour cell deposits, adjacent to the primary which the surgeon does not always manage to remove with a standard surgical approach, today usually encompassing a total (or partial) mesorectal excision (TME). In Japan and other Asian countries, more extensive surgery with lateral node excision is performed in patients with high risk tumours<sup>[6]</sup>, whereas in the western world, pre- or previously also postoperative (C)RT have been used to kill the subclinical tumour cells not removed by surgery. The (C)RT is then administered as adjuvant therapy after surgery, and as neo-adjuvant therapy before surgery.

An important aim is, thus, to treat so that the risk of residual disease in the pelvis is very low or preferably less than 5% in the population, in which curative treatment is intended. This should be possible in all but the few ( $\leq 10\%$ ) cases, who present with a fixed tumour growing into a non-readily resectable organ. At the same time, as little acute and late morbidity as possible should be aimed at. Surgery, particularly if extensive, may give rise to substantial morbidity and the additional treatments, whether given pre- or post-operatively, increase both acute and late morbidity. Thus, all additional treatments, as well as more extensive surgery, should be given only when the expected gains are sufficiently large to motivate the increased morbidity.

This review about the value of radiotherapy to improve loco-regional control and overall survival in rectal cancer is based upon a systematic approach to the scientific literature. The available literature has been identified in several systematic overviews and meta-analyses<sup>[7-11]</sup>. It

gives in addition some personal comments on observed developments during the past decades about sphincter- or organ preservation, where we lack good evidence of beneficial effects from controlled clinical trials.

### Diagnosis and staging of rectal cancers

Appropriate diagnosis and staging are fundamental as regards choice of therapy. Tumours with distal extension to 15 cm or less from the anal margin (as measured by rigid sigmoidoscopy) are classified as rectal, and more proximal tumours as colonic. Others, *e.g.*, in Japan<sup>[12]</sup>, prefer to separate colon and rectal cancers at the peritoneal reflection, or about 9-12 cm from the anal verge. Since the localization of the tumour in relation to other organs and structures and thus, the distance from the anal verge, is important for outcome and treatment, cancers between 10 and 15 cm are, in this author's opinion, best discussed as rectal cancers since radiotherapy (RT) is an important component of therapy, even if this is less common than for lower rectal cancers (0-10 cm)<sup>[13]</sup>. Lateral lymph node involvement is, however, rare in tumours above the peritoneal reflection<sup>[14]</sup>.

Rectal MRI is recommended for staging in order to select preoperative treatment and extent of surgery, although endoscopic ultrasonography can be used for the earliest tumours<sup>[15,16]</sup>. If MRI and ultrasound are combined, a study claimed that accuracy was improved<sup>[17]</sup>. The TNM staging system should be used. At present, the latest version 7 from 2010 is preferred by most, even if it shows marked interobserver variations in defining stages II and III<sup>[18]</sup>. There is a need for further subclassification of clinical stage T3 (cT3) (Table 1) in order to individualize therapy, *i.e.*, to decide whether surgery alone is appropriate or whether preoperative RT alone or with chemotherapy (CRT) should be recommended.

### Subdivision of rectal cancer with different therapeutic strategies

In order to define the extent of surgery and whether neo-adjuvant (or preoperative) (C)RT is required, rectal cancers can be divided into four groups, very early (some cT1), early (cT1-2, some cT3), intermediate (most cT3, some cT4) and locally advanced (some cT3, most cT4). Other factors than clinical T-stage, such as tumour height, closeness to the mesorectal fascia (mrf), potentially the circumferential margin (crm) (preoperatively, the term mrf is better than crm, since the crm cannot be defined until after surgery<sup>[19]</sup>), nodal (cN)-stage and vascular and nerve invasion are also relevant. It is not possible to precisely define which T and N sub-stages that belong to these groups. The terms "very favourable", "favourable or early or good", "intermediate or bad", and "locally advanced or ugly" can be used for categorizing the rectal cancers into these clinical subgroups. This subdivision (Table 2) is clinically relevant since primary treatment differs.

In many recent studies, the term "locally advanced" has been used for the "intermediate/bad" group, but is

**Table 1 Tumor node metastasis-7 classification (2010) with subclassification of stage T3**

TNM	Extension to
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Submucosa
T2	Muscularis propria
T3	Subserosa/perirectal tissue
T3a <sup>1</sup>	Less than 1 mm
T3b	1-5 mm
T3c	5-15 mm
T3d	15+ mm
T4	Perforation into visceral peritoneum (a) or invasion to other organs (b)
N1	1-3 regional nodes involved
N1a	1 lymph node
N1b	2-3 lymph nodes
N1c	Small deposits in the fat
N2	4 or more regional nodes involved
N2a	4-6 lymph nodes
N2b	7 or more lymph nodes
M1	Distant metastases
M1a	1 distant organ or set of lymph nodes
M1b	More than 1 organ or to the peritoneum

<sup>1</sup>This subclassification is based upon an evaluation using magnetic resonance imaging prior to treatment decision is clinically valuable, and recommended in this review. It can be used also in the histopathological classification but is not validated and not incorporated in TNM version 7. TNM: Tumor node metastasis.

best reserved for the truly “locally advanced/ugly” tumours<sup>[9,13,20]</sup>. Even if there is variability in what is called locally advanced there is consensus about the need to subgroup along these lines<sup>[13,21,22]</sup>. Subgrouping is an important step towards individualized therapy. Major discrepancies do, however, exist as regards which treatment is selected for these subgroups (Table 2).

### Different treatment principles in the world

There is marked difference in how the subclinical tumour deposits often seen in tumours below the peritoneal reflection are managed in Asia and in the rest of the world. Surgical removal of the lateral nodes on one or both sides has been the preferred option in Asia<sup>[6,23]</sup>, whereas the rest of the world has explored the value of radiation, in addition to surgery for the primary tumour in the bowel, to kill the tumour deposits. Since radiation does not selectively irradiate the lateral nodes, but also includes the primary tumour and the mesorectal nodes, the need for a meticulous surgical dissection technique has not been the same in the Western world as in Asia. Both extensive surgery and additional radiotherapy increase morbidity. It is not known which of the two alternatives is most efficient in eradicating all tumour cells, *i.e.*, preventing a local failure and which alternative results in the least morbidity since no randomized studies have compared the two strategies. Inter-trial comparisons have reported that the results are similar at specialized centres<sup>[24]</sup>. It is, however, probably more efficient to remove all subclinical cancer deposits using radiation rather than surgery, unless one can dissect in a surgical plane. The morbidity caused by

**Table 2 Subgrouping of localized rectal cancer assessed by magnetic resonance imaging<sup>1</sup> and the recommended primary treatment**

Favourable “good” group	Intermediate “bad” group	Advanced “ugly” group
Mid/upper rectum T1-3b Low rectum T1-2, T3a N0 mrf clear	Mid/upper rectum T3c/d low rectum also includes T3b T4 with peritoneal or vaginal involvement only N1/N2 mrf clear	T3 mrf positive T4 with overgrowth to prostate, seminal vesicles, base of urinary bladder, pelvic side walls or floor, sacrum positive lateral lymph nodes
5 yr LFR <sup>2</sup> < 10% 5 yr DFR <sup>3</sup> < 15% Primary surgery (TME) <sup>4</sup>	5 yr LFR <sup>2</sup> 10%-20% 5 yr DFR <sup>3</sup> 15%-60% Preop 5 × 5 Gy with immediate surgery <sup>5</sup>	5 yr LFR <sup>2</sup> 20%-100% 5 yr DFR <sup>3</sup> 30%-80% Preop CRT or 5 × 5 Gy with delayed surgery <sup>6</sup>

<sup>1</sup>The algorithm (modified from<sup>[102]</sup> with permission from the publisher Informa) does not primarily address the risk of systemic disease, although this risk also increases with the presence of many of “the risk factors”; however, not necessarily parallel to the local failure rate (LFR). The algorithm is also “too simplified”, in that a other factors like size of the mesorectum, anterior or posterior location, extramural vascular invasion (EMVI+) are also relevant. <sup>2</sup>Calculated in the group of patients planned for surgery, *i.e.*, irrespective of the surgical outcome. The table are valid if the surgeon is an experienced rectal cancer surgeon and no pre-treatment is given. <sup>3</sup>The 5-year risk of distant failure (DFR) is also given, although this risk is not well established. Risk factors detectable on magnetic resonance imaging for distant failure are N2 (versus N0 and N1), EMVI+, mrf+ and all T4 (a and b, see Table 1). These are also the risk factors used in the on-going trial<sup>[88]</sup>, where patients at high risk failing systemically are included. <sup>4</sup>A local procedure is possible in a few patients [chiefly pT1, sm1 (+2), N0]. This group is in the text referred to as “very favourable”. <sup>5</sup>Preoperative chemotherapy (CRT) is also a valid option according to international clinical guidelines<sup>[21]</sup>. <sup>6</sup>CRT means chemoradiotherapy to 50.4 Gy in 1.8 Gy fractions with 5-fluorouracil (capecitabine). 5 × 5 Gy with delayed surgery should be used only in patients not fit for CRT.

extensive surgery is very different from that caused by external RT and less extensive surgery, although the relevance of this on patient well-being differs between cultures.

In the Western world, preoperative RT was mainly explored in Europe whereas postoperative RT was explored in the US. A few small studies showed that postoperative CRT was better than postoperative RT in preventing local recurrence and that combined treatment was more effective than surgery alone. A NIH Consensus Conference and a subsequent NCI report in the early 1990s stated that postoperative CRT should be standard treatment in rectal cancer stages II and III<sup>[25,26]</sup>.

In Europe, several randomized trials compared surgery alone versus preoperative RT and surgery. These studies showed a relative reduction in local failure rates of 50%-60% if the radiation dose was moderately high (Table 3). If the radiation dose was lower, corresponding to a biologically effective dose (BED) below 30 Gy<sup>[7]</sup>, no or a more limited effect was shown. As a consequence, preoperative RT was recommended as routine therapy in many European countries<sup>[13]</sup> (Table 3).

Table 3 Major randomized radiotherapy trials in primary rectal cancer<sup>1</sup>

Study	Induction time	No of patients	Treatments		Radiation technique <sup>2</sup>	Increased postop death	Local recurrence			Increased survival	Comments
			Surgery alone	Preop (C)RT Postop (C)RT			Surgery alone	Preop RT + surgery	Postop RT		
Pre-TME era	1975-78	824	Yes	5 Gy × 1 2 Gy × 10	AP-PA	No	43%	45% 47%		No	Very low radiation dose, no benefit
			Yes	2.3 Gy × 15	AP-PA	No	28%	14% <sup>1</sup>		No	Decreased local recurrence risk
			Yes	1.75 Gy × 18	AP-PA	No	24%	17%		No	Marginally decreased local recurrence risk, comparably low dose
			Yes	5 Gy × 5	AP-PA	Yes	28%	14% <sup>2</sup>	-	No	Increased postop death (8% vs 2%), large target, suboptimal technique, decreased local recurrence risk.
Uppsala <sup>[95]</sup>	1980-85	471	-	5.1 Gy × 5	3D-C on RT	No	-	13% <sup>1</sup>	22%	No	Increased risk late complications
St Marks <sup>[96]</sup>	1980-84	395	Yes	5 Gy × 3	AP-PA	Yes	24%	17%		No	Preop 5 Gy × 5 is better than postop RT (60 Gy). Increased risk of late complications after postop RT
	1981-89	279	Yes	2 Gy × 20	AP-PA	No	46%	36% <sup>1</sup>		No	Increased postop death (9% vs 4%)
North-West <sup>[98]</sup>	1982-86	284	Yes	5 Gy × 4	3D-C on RT	No	41%	18% <sup>3</sup>		No	Slightly reduced risk of local failure, tendency to improved survival (HR = 0.79, 95%CI: 0.6-1.04)
	1987-90	1110	Yes	5 Gy × 5	3D-C on RT	No	27%	12% <sup>3</sup>	-	Yes	Decreased local recurrence risk, 10 × 10 cm beams
			Yes	5 Gy × 5	3D-RT	Yes	25%	12% <sup>3</sup>	-	Yes	Decreased local recurrence risk, no increased acute toxicity, some late toxicity after 10-15 yr
Stockholm II <sup>[99]</sup>	1987-93	557	Yes	5 Gy × 5	3D-RT	Yes					Overlaps to a large part SRCT, simplified radiation technique, tendency to increased postop mortality (4% vs 1%). Lower local recurrence risk, increased survival as in SRCT. Increased risk of late complications
Post-TME era	1993-03	1011	-	RT CRT <sup>3</sup>	3D-C on RT	No		17% 9% <sup>2</sup>		No	2 × 2 design, chemotherapy in addition to RT gives fewer local recurrences as first event than RT alone irrespective of whether concomitant (9%) or postoperative (10%), or both (8%), increased toxicity, no increased survival
			-	RT CRT	3D-C on RT	No		17% 8% <sup>1</sup>		No	Preop CRT results in fewer local recurrences than preop RT, increased toxicity, no survival difference
FFCD 9203 <sup>[97]</sup>	1993-03	742	-	RT CRT	3D-C on RT	No		6% <sup>2</sup>	13%	No	Preop CRT is less toxic and gives fewer local recurrences than postop CRT, no difference in survival
AIO-94 <sup>[90,100]</sup>	1995-02	823	-	CRT	3D-C on RT	No		5% <sup>3</sup>		No	No increased postop mortality. Decreased local recurrence risk even with TME, no improved survival, some risk of increased late complications after 5-10 yr
TME <sup>[54,101]</sup>	1996-99	1861	Yes	5 Gy × 5	3D-C on RT	No	11%			Yes	The only study in “ugly” rectal cancers, preop CRT gives better local control and better disease and cancer specific survival, tendency towards better survival (66% vs 53% after 5 yr). Increased acute and possibly late toxicity from CRT
LARCS <sup>[99]</sup>	1998-03	207	-	RT CRT	3D-C on RT	No		33% 18% <sup>1</sup>			Preop 5 Gy × 5 better than postop CRT if CRM+, marginally increased survival. No increase in late complications (3-5 yr)
MRC-CR07 <sup>[91]</sup>	1998-05	1350	-	5 Gy × 5 CRT if CRM+	3D-C on RT	No		5% <sup>2</sup>	11%	Yes	



Polish <sup>[33]</sup>	1999-02	312	-	5 Gy × 5 CRT	3D-C on RT	No	11% 16%	No	First study that shows less risk of acute toxicity from 5 × 5 compared with preop CRT, no difference in local recurrence and survival or late complications (3-5 yr)
TROG <sup>[34]</sup>	2001-06	326	-	5 Gy × 5 CRT	3D-C on RT	No	7% 4%	No	Same design as the Polish study, same results

<sup>1</sup>Only large studies of relevance for present treatment recommendations are included. Patients with tumours considered to be resectable were included in all studies but one (LARCS). Staging has varied considerably over the years, but most included patients belonged to the intermediate group (bad) except in the LARCS study where most tumours were locally advanced (ugly). <sup>2</sup>AP-PA, anterior posterior beams with no blocking, meaning high radiation doses to large normal tissue volumes. 3D-CRT, 3D-conformed radiotherapy. 3 or 4 beams with blocking of normal tissues that did not contain tumour cells. 3D-RT (in the Stockholm II study) means 4 beams but no blocking. <sup>3</sup>CRT means chemoradiotherapy with 1.8-2 Gy daily to 45-50.4 Gy. RT means the same radiotherapy as in the CRT arm without chemotherapy. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.001$ . TME: Total (or partial) mesorectal excision; CRT: Chemotherapy.

### How is the radiotherapy best given?

For about two decades, four questions have been particularly discussed, *viz* (1) should the RT be given before or after surgery; (2) should it be long-course or short-course; (3) should the long-course RT be given alone or with chemotherapy? In Europe researchers were not universally convinced of the advantages of adding concomitant chemotherapy considering the increased toxicity<sup>[27]</sup>, as stated in the US documents. Furthermore; (4) could sphincter-saving surgery be increased after preoperative CRT? More recently, a fifth question; and (5) has attracted much interest, *viz* if it is possible to avoid major surgery, *i.e.*, to preserve the organ, in patients who respond well to the preoperative CRT.

### Pre- or postoperative radiotherapy?

A randomized trial showed at an early stage that preoperative short-course RT (5 fractions of 5 Gy in one week) was more effective and less toxic than postoperative long-course RT (Table 3). In the trial<sup>[28,29]</sup>, significantly fewer local recurrences (13% *vs* 22%,  $P < 0.05$ ) was seen in the group of patients randomized to the brief preoperative schedule than to an "optimized" postoperative schedule (high total radiation dose, 60 Gy in 7-8 wk, only given to high risk groups, stages II + III). Subsequently, several trials comparing preoperative CRT with postoperative CRT were initiated. The only completed trial<sup>[30]</sup> again showed that preoperative therapy was more efficient and less toxic than postoperative. In the trial, fewer local recurrences (6% *vs* 13%,  $P < 0.01$ ) were seen in the group receiving preoperative CRT (Table 3). The preoperative treatment was also less toxic. No difference in survival was detected. Superiority of preoperative short-course RT over postoperative CRT was also shown in the MRC-CR07-trial; local recurrences were less commonly seen in the preoperatively irradiated group (5% *vs* 11%,  $P < 0.01$ )<sup>[31]</sup>. Most of the world has now accepted that additional (C)RT in rectal cancer should be given before, *i.e.*, neo-adjuvant, rather than after surgery. An analysis of data from the randomized studies also indicated that preoperative RT is more dose-efficient than postoperative RT<sup>[32]</sup>.

### Short- or long-course radiotherapy?

The question of whether the preoperative RT is best given as a short-course (5 Gy × 5) schedule or as long-course conventionally fractionated RT (1.8-2.0 Gy × 25-28) has been ongoing since the first results of the Uppsala trial were published in 1985<sup>[28]</sup>, and the matter has not yet been settled. The potential advantages and disadvantages of the two fractionation schedules are presented in Table 4. The most recent RT trial in rectal cancer in Sweden, the Stockholm III trial recently closed patient entry (Jan 2013). It has compared the different fractionation schedules in 845 patients randomized to either 5 Gy × 5 with immediate surgery, 5 Gy × 5 with delayed (4-8 wk) surgery and 2 Gy × 25, likewise with delayed surgery. Results of the primary outcome, local recurrences, will be available in 2015. Two other trials including 316 and 326 patients, respectively, could not find any differences in local recurrence rates, disease-free (DFS) and overall survival (OS) between the groups randomized to short-course RT alone or long-course CRT<sup>[33,34]</sup>. A German trial<sup>[35]</sup> with a similar design was initiated in 2004. No data has yet been released.

The short-course schedule has gained much popularity in Northern European countries where the health care system is rarely dependent upon private initiatives, whereas the long-course schedule is preferred in countries where physician and hospital budgets are influenced by the number of treatments given. Reimbursement has thus influenced routines, although this is seldom officially admitted. Many concerns have been expressed about the long-term consequences of hypofractionated RT. There is considerable evidence that the short-course schedule results in long-term morbidity, and the scale of that morbidity is well known<sup>[36]</sup>. The long-term morbidity of CRT, whether given pre- or

**Table 4** Main differences between and potential advantages of short-course and long-course preoperative radiotherapy in intermediate (bad) rectal cancers<sup>1</sup>

	Short-course	Long-course
Total (physical) radiation dose	25 Gy	45-50.4 Gy
Fraction size/number of fractions	5 Gy/5	1.8-2 Gy/23-28
Radiation duration	1 wk	4.5-5.5 wk
BED <sup>2</sup> , acute effects	37.5 Gy	37.5-44.4 Gy
BED <sup>2</sup> , late effects	66.7	72-84 Gy
Overall treatment time	About 10 d	10-14 wk
Demands of radiation resources	Planning + 5 fractions	Planning + 23-28 fractions
Concomitant chemotherapy <sup>3</sup>	No	Yes
Acute toxicity	Minimal	More
Late toxicity	Present, considered limited in the "bad" group	Present, but not extensively studied. Anticipated to be higher than after short-course
Down-sizing/ down-staging	No <sup>4</sup>	Yes <sup>5</sup>

<sup>1</sup>In locally advanced (ugly) tumours, long-course CRT is the preferred option although short-course RT with a delay to surgery is an option if CRT is not tolerated because of high age or co-morbidity; <sup>2</sup>Biologically effective dose according to the time-corrected linear quadratic model. Major uncertainties exist in the relative biological efficacy of the fractionation schedules concerning the acute, antitumour effects. The parameters selected for the acute effects were those used in the meta-analyses from 2001<sup>[7]</sup>, even if they can be criticized and probably are incorrect. For late effects, an  $\alpha/\beta$  of 3 Gy with no time correction is used. The anticipated antitumour effects do not thus differ substantially and late toxicity is at least not higher with short-course RT; <sup>3</sup>Improved local control with long-course RT, increased acute toxicity and probably also late toxicity. Should not be given with short-course RT; <sup>4</sup>Seen after short-course RT with delayed surgery; <sup>5</sup>Not relevant in these intermediate tumours (unless organ-preservation is aimed at), however, relevant in locally advanced (ugly) tumours. BED: Biologically effective dose.

postoperatively has not been studied systematically with the result that the extent of late morbidity is not precisely known. Both options, short-course 5 Gy  $\times$  5 and long-course CRT are considered valid in the intermediate group of rectal cancers, according to recent clinical guidelines<sup>[13,21]</sup>. The demands of radiation resources and the acute toxicity are much higher using long-course CRT than using short-course 5 Gy  $\times$  5. It is possible to conclude from the randomized trials that they have similar efficacy and do not differ in the risk of late toxicity; therefore, it is surprising to this author that they are considered equally valid (Table 4).

### Radiotherapy alone or with chemotherapy?

Three randomized trials, two in the intermediate group<sup>[37,38]</sup> and one in the locally advanced, ugly group<sup>[39]</sup>, have provided an answer to the third question. Local control was better in the combined treatment arm in all three studies, whereas a significant survival gain was only seen in the trial including locally advanced cancers<sup>[9,39]</sup>. Whenever a patient with a locally advanced, ugly rectal cancer receives preoperative treatment, CRT should be used unless the patient cannot tolerate this treatment. It

should, however, be recognized that the gains from the chemotherapy addition are rather limited and come with a rather high price with significantly increased acute toxicity<sup>[11]</sup>, and in all probability also increased late toxicity (see below).

The drug most extensively used to sensitize the RT has been 5-fluorouracil (5-FU), although oral capecitabine gives the same potentiation of the effects, and is more convenient<sup>[40]</sup>. Other oral fluoropyrimidines such as UFT<sup>[41,42]</sup> have also been explored, but have not yet been the subject of randomized trials. Combinations of 5-FU and other cytotoxic drugs such as oxaliplatin and irinotecan, and targeted drugs, have been extensively explored during the past decade. Multiple phase II studies in so-called "locally advanced rectal cancer" have claimed superior results [more down-sizing, higher pathological complete (pCR) rates]. It is likely that these apparently favourable results depend upon the inclusion of mainly early or intermediate cancers. Five large randomized trials have failed to show any superior results from the addition of oxaliplatin<sup>[43-47]</sup>. When cetuximab was added to CRT with capecitabine and neo-adjuvant chemotherapy with capecitabine-oxaliplatin in a randomized phase II study, the primary endpoint, pCR rate, was not increased, but more radiological responses (89% *vs* 72%,  $P = 0.002$ ) and improved OS (96% *vs* 81% at 3 years,  $P = 0.04$ ) were seen in the KRAS wild-type population ( $n = 90$ )<sup>[48]</sup>. These results need confirmation.

### Sphincter preservation, organ preservation

Trials, again chiefly run in Europe, have explored whether long-course (C)RT with a delay before surgery could increase sphincter preservation rates, whereas others took it for granted that this was the case. The trials could not show that this occurred to any meaningful extent<sup>[49]</sup>. The hopes about improved chances of sphincter saving influenced routines in many countries, particularly in Southern Europe, Germany and the United States. At present, hopes about organ preservation (see below) influence treatment decisions at many centres.

## TREATMENT ACCORDING TO RISK GROUP

### Very favourable rectal cancer

In the earliest rectal cancers, chiefly the malignant polyps [Haggitt 1-3, T1 sm 1(-2?) N0], a local procedure, e.g., using the transanal endoscopic microsurgery (TEM) technique, is sufficient for cure<sup>[50,51]</sup>. If the resection is not radical (R0), there are signs of vessel invasion, poor differentiation or if the tumour infiltrates more deeply into the submucosa (Haggitt 4, T1) or is a T2 tumour, the risk of recurrence is too high ( $\geq 10\%$ ) and the patient should be recommended postoperative CRT or, more safely, major (TME) surgery. If the cancer diagnosis is biopsy-verified, presurgical CRT is preferred if the intent is to perform a local procedure<sup>[50]</sup>. As an alternative to local surgery, alone or with CRT, local RT (brachytherapy

or contact therapy using the Papillon technique) can be used. Experience of these treatments is limited outside specialized centres<sup>[52]</sup> and more prospective studies are required before they could be a part of clinical routines.

### **Favourable, "good" rectal cancers**

In these cases cT1-2, some early cT3, N0 [cT3a(-b) and clear mrf (mrf-) according to MRI], "good" group, surgery alone using the TME technique is appropriate, since the risk of local failure is low unless the tumour is at the level of the levators<sup>[13]</sup>. Although the large randomized trials have indicated that short-course RT even further reduces local recurrence rates<sup>[31,53,54]</sup>, surgery alone is recommended since the addition of preoperative RT results in overtreatment of too many individuals<sup>[13]</sup>.

### **Intermediate, "bad" rectal cancers**

In this group most cT3 [cT3(b)c+ without threatened or involved mrf (mrf-) according to MRI], some cT4 (*e.g.*, vaginal or peritoneal involvement only, N+), preoperative RT is recommended since the risk of local failure is not negligible (> 8%-10%), even if proper surgery is performed. Even in the absence of signs of extramural growth on ultrasound or MRI (cT2) in very low tumours (0-5 cm), preoperative RT may be indicated because the distance to the mrf or the levator muscles is very small. Surgery alone, often an abdomino-perineal excision, will then again result in unacceptably high local recurrence rates. Twenty-five Gy delivered during one week and followed by immediate surgery (< 10 d from the first radiation fraction) has in randomized trials reduced the risk of local failure by 50%-70% *vs* surgery alone<sup>[31,53-55]</sup>. The relative efficacy is likely to be the same irrespective of tumour height, although this was not seen in the TME trial<sup>[54]</sup>. CRT to 46-50.4 Gy, 1.8-2.0 Gy/fraction with 5-FU (bolus, continuous infusion or peroral) is an alternative, although it is more demanding and not proven to be more effective<sup>[33,34,37,38]</sup>. CRT is preferred in low rectal cancers even at centres that otherwise use 5 Gy  $\times$  5. It must be stressed that RT (or CRT) cannot compensate for poor surgery. Surgery should aim at clear resection margins (crm-); therefore, in low rectal cancers requiring an abdomino-perineal excision, it is important to do the dissection so that a "waist" is avoided. As described above, two European trials<sup>[37,38]</sup> showed that the addition of 5-FU improved local control with a reduced risk of local failure as first event. After 5 years these were 17% in the preoperative RT arms alone and 8%-9% in the CRT arms. In the EORTC trial, the same reduction was seen whether the chemotherapy was administered concomitantly with the RT, only postoperatively or both pre- and postoperatively. Two randomized trials (Polish, TROG 1.04) could not detect any statistically significant differences in local recurrence rates, DFS and OS after preoperative 5  $\times$  5 Gy or preoperative CRT (5-FU + 50.4 Gy)<sup>[33,34]</sup>. In the TROG study, numerically more recurrences were seen in the group randomized to 5 Gy  $\times$  5 (6/48 *vs* 1/31,  $P = 0.21$ )<sup>[34]</sup>. In the MRC-CR07-trial including 1350 patients,

preoperative 5  $\times$  5 Gy was randomly compared with postoperative CRT if the crm was positive. Local recurrence rates favoured the preoperative arm (5% *vs* 17%,  $P < 0.001$ )<sup>[31]</sup>. DFS was also superior in the preoperative arm (HR = 0.76,  $P = 0.01$ ) whereas OS did not differ significantly (HR = 0.91,  $P = 0.04$ ).

### **Locally advanced, "ugly" rectal cancers**

In the locally advanced, frequently non-resectable cases [cT3 mrf+, cT4 with overgrowth to other organs (cT4b)], preoperative CRT, 50.4 Gy, 1.8 Gy/fraction with concomitant 5-FU-based therapy should be used<sup>[9,13,39]</sup>, followed by radical surgery 6-8 wk later. In a Nordic randomized trial (cT4NXM0), local control was significantly better after 5 years in the CRT arm (5-FU + 50 Gy) than in the RT only arm (82% *vs* 67%,  $P = 0.03$ ). Also DFS and cancer-specific survival were significantly better in the combined modality arm, whereas OS did not differ significantly (66% *vs* 53%,  $P = 0.09$ )<sup>[39]</sup>.

In very old patients ( $\geq 80$ -85 years) and in patients not fit for CRT, 5  $\times$  5 Gy with a delay of approximately 8 wk before surgery is an alternative option, based upon three retrospectively analyzed patient series revealing favourable results<sup>[56-58]</sup>. A randomized trial will in all probability never be performed in this patient group, which is not considered to tolerate standard therapy.

### **Organ preservation?**

Apart from the earliest tumours that can be treated with a local procedure or local RT, as described above, it has become increasingly popular to give CRT, then wait and re-stage the tumour<sup>[59-62]</sup>. If no signs of remaining tumour/no viable tumour cells are found when biopsies are performed, major surgery is not performed and the patient is monitored closely for at least 5 years. The hypothesis is that potential lymph node metastases have been eradicated parallel with the response of the primary tumour. Although this occurs in some patients, this strategy has not been the subject of properly controlled prospective studies. This excellent response will not be frequent in the intermediate and locally advanced cases<sup>[63,64]</sup>, but only in early cases. The cell kill effect of available CRT schedules is too small.

No major surgery and no rectal excision in very low tumours can be clearly beneficial for individuals who run a high risk of surgical therapy or who cannot accept a stoma. However, the disadvantages for many patients are seldom discussed. In most patients with an early rectal cancer, a low anterior resection alone is the reference treatment. Cure rates are high and morbidity is only a result of the surgery. If these patients are treated with the aim of organ preservation, all will receive CRT with its acute morbidity. Patients who respond with a clinical complete remission (cCR), and are not operated are the ones potentially having a benefit of a wait-and-see approach, although they will all suffer from the long-term toxicity that can be seen after CRT. If the tumour is located in the lower rectum, at least part of the sphincters



must be included in the irradiated volume, and suboptimal anal function can be a result. Those who do not achieve a cCR or those who recur during follow-up will require major surgery. These patients will thus suffer the morbidity from both CRT and major surgery. It is presently not possible to know the proportion of patients who do not require major surgery. With the CRT schedules available today, the group of patients having a true advantage is most probably much smaller than the group of patients who suffer extra morbidity.

### **Radiation therapy volumes and doses**

In the “intermediate/bad” group, with the aim of lowering the risk of local failure, the primary tumour with the mesorectum and lymph nodes outside the mesorectum, at risk to contain tumour cells more than exceptionally should be irradiated<sup>[65,66]</sup>. In the “early/good” group before or after a local procedure, only mesorectal nodes are at sufficient risk to be involved. The appropriate dose to subclinical disease should with 5-FU chemotherapy be at least 45 Gy in 1.8-2.0 Gy fractions. The relative reduction in local failure rates is then in the order of 50%-60%, and subsequently there is room for improvement. A boost of about 4-6 Gy in 2-4 fractions to the primary tumour is sometimes given<sup>[67]</sup>. A brachytherapy boost has also been tried; however, without any apparent advantage<sup>[68]</sup>. The clinical problem is not the primary tumour in the bowel, unless you aim at organ preservation (see above).

In the “locally advanced/ugly” tumours, the target is basically the same as in the intermediate group, although the primary tumour extends more laterally and more lymph nodes can be at risk. In these patients, a lateral boost to areas where it can be difficult to surgically remove all cells can be indicated<sup>[69]</sup>. It is not primarily motivated to boost the centre of the tumour, *e.g.*, where the PET-uptake is the highest, if this can surgically be removed.

The entire mesorectum is in most cases at great risk of having tumour deposits and should be included in the clinical target volume (CTV). In high tumours it is sufficient to include the 4 cm distal to the tumour. Besides the mesorectal nodes, the presacral nodes up to the level of S1-2 should be included in CTV. If presacral nodes are radiologically involved, the upper border of CTV should be even higher. Local recurrences above S1-2 are infrequent<sup>[70-72]</sup>. The lateral nodes, including the internal iliac nodes up to the bifurcation of the common iliac arteries should be included in tumours below the peritoneal reflection, *i.e.*, in tumours up to about 9-12 cm from the anal verge<sup>[73]</sup>. The risk of lateral node involvement in the Western world is not precisely known, but studies from Asia show that these lymph nodes are rarely involved in low-mid rectal pT1-2 tumours and in high tumours irrespective of T-stage<sup>[14,74]</sup>. External iliac nodes should only be included if an anterior organ such as the urinary bladder, prostate or female sexual organs are involved. The medial inguinal nodes need only to be prophylactically included when the tumour grows below the dentate line<sup>[75]</sup>.

The ischiorectal fossae should be included only when the levator muscles and the internal and external sphincters are involved. The fascia inside the levators is considered to be a strong barrier to tumour cell penetration<sup>[76]</sup>. Other opinions have been expressed<sup>[65]</sup>.

### **Late toxicity from rectal cancer radiotherapy**

The prevention of a local failure with the severe morbidity this may have must be weighed against the morbidity from (C)RT that all treated patients can develop. From the Swedish and Dutch randomized trials, the morbidity after 5 × 5 Gy RT is well described and reviewed in<sup>[36]</sup>. Increased risks of poor anal and sexual function, small bowel toxicity with obstruction and secondary malignancies have been reported. Studies have tried to estimate what minimal absolute gain should be present for patients to prefer RT. These studies are difficult to interpret, although many patients accept an absolute 3% difference in local recurrence risk for the known morbidity risks of RT<sup>[77]</sup>.

After having treated rectal cancer patients for over 30 years, and thus, seeing many patients with a local recurrence during the first part of the period, and being actively involved in research aimed at estimating the extent of late toxicity up to 20 years after the RT, it is my opinion that an absolute risk reduction of approximately 5% motivates the recommendation to irradiate. The recommendations given above, as well as in recent consensus statements<sup>[13,21]</sup> reflect this opinion. Furthermore, and very importantly, the RT we give today, and the RT that routinely can be given in only a few years<sup>[66,78,79]</sup>, will mean even less late toxicity than that seen in the follow-up studies of the RT delivered during the 1980s-1990s. Better understanding of internal movements will also allow more precise delivery of the radiation dose<sup>[80]</sup> and of dose-response relationships for *e.g.*, faecal incontinence<sup>[81]</sup>.

An important question is the late toxicity from 5 × 5 Gy compared with the late toxicity seen after 46-50 Gy in 25-28 fractions, usually administered with 5-FU. The long-term morbidity from 5 Gy × 5 up to at least 10 years follow-up (with yesterday's techniques) is known from studies including thousands of patients. This knowledge is not as solid from CRT. The Polish<sup>[33]</sup> and the MRC-CR07 trials<sup>[31]</sup> could not detect any differences between 5 × 5 Gy and CRT to 46-50 Gy after 4 years of follow-up. The short-course schedule uses a high fraction size of 5 Gy, compared with 1.8-2.0 Gy, whereas the total dose is less (25 Gy compared to 46-50 Gy). Both the fraction size and the total dose are relevant. The relationship between total dose, fraction size and late toxicity is, however, complex.

Another question is whether the addition of 5-FU increases late toxicity. In one of the two larger randomized trials in the intermediate risk group<sup>[37,38]</sup>, the addition of 5-FU negatively affected global QoL, social functioning and diarrhoea. Almost 60% of the patients suffered from faecal incontinence, impairing their social life<sup>[82]</sup>. In the trial in locally advanced/ugly cancers, more patients



had a stoma or a poor anal function in the CRT group than in the RT group (89% *vs* 70%,  $P = 0.046$ )<sup>[83]</sup>. If this means that the addition of chemotherapy results in more late toxicity or if this difference reflects the survival of patients with more advanced tumours in the CRT group cannot be deduced. No differences in QoL were seen after 4-8 years<sup>[84]</sup>.

## CONCLUSION

During the past three decades, a severely disabling condition for many rectal cancer patients, viz a local failure with uncontrolled growth of the cancer in the perineum and pelvis has disappeared, although, unfortunately, not yet at all centres. Multiple trials have confirmed the superiority of what can presently be considered as recommended care and treatment (Table 2). A multidisciplinary approach has been a must in this development, at present formalized as (weekly) multidisciplinary team (MDT) meetings, during which all patients are discussed before the first treatment decision, postoperatively, and at critical time points during the course of the disease. Many countries have successfully launched quality assurance and quality control programmes in rectal cancer surgery<sup>[85,86]</sup>. It is important that, besides surgical details, RT and CRT details are also fully integrated in the programmes.

Practically all details in the care of the patients have been the subject of prospective, frequently randomized trials. It should, however, also be recognized that many uncertainties about what is the best treatment still exist. Furthermore, alternative approaches to attain low local failure rates and improved survival together with as little negative consequences from the disease and its treatment as possible, also exist.

The trials have repeatedly shown that RT, whether alone or with chemotherapy, should be given before surgery to have the best efficacy and least toxicity. This was shown as early as 1985, but is only recently unanimously agreed upon. It is also a belief that systemic treatment, being the weakest part of the therapy, should be given before and not after the surgery in order to have greatest efficacy. Progression of the local primary should then not occur during the systemic treatment, presently requiring a duration of 5-6 mo. The discovery that the short-course schedule results in substantial down-staging, is tolerable and permits full chemotherapy starting soon after the RT<sup>[56,87]</sup>, has led to the next generation of studies, such as the multicentre "RAPIDO" trial<sup>[88]</sup>. Patients with ugly rectal cancers at high risk to recur are randomized to the present standard, CRT, surgery and adjuvant chemotherapy (even if not all consider this standard<sup>[89]</sup>) and an experimental arm with 5 × 5 Gy, neo-adjuvant chemotherapy and surgery at the end. A Polish study, likewise in locally advanced, unresectable rectal cancer, with a similar design is also ongoing<sup>[90]</sup>. In an interim analysis after 97 randomized patients, no major differences in acute toxicity and local efficacy were seen between the control group receiving CRT (50.4 Gy with 5-FU/FA/oxaliplatin) and

the experimental group (5 × 5 Gy followed by 3 FOLFOX-4 cycles preoperatively). No postoperative therapy is scheduled.

During the past 30 years, a better understanding of the molecular mechanisms involved in tumour development and progression has placed great expectations on improved diagnosis, staging, prognostic evaluation and selection of the individually best therapy. Much new and valuable information has been created, but no new clinically valuable markers have been identified. The number of mm's from the most peripheral part of the rectal tumour to the mrf (or crm postoperatively) is most informative. No predictor of which pre- (or post-)operative treatment to choose is available. The efforts to translate basic knowledge into clinically useful information must be intensified or explored along other paths. Sampling of representative and sufficient tumour material for diagnosis and research prior to, during and after therapy may help. Functional imaging showing where to sample, may be helpful. We need predictors and must find better ways of identifying them than has been possible in the past.

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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

# Colorectal cancer: Current imaging methods and future perspectives for the diagnosis, staging and therapeutic response evaluation

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## Abstract

In the last 10 years the mortality rate of colorectal cancer (CRC) has decreased by more than 20% due to the rising developments in diagnostic techniques and optimization of surgical, neoadjuvant and palliative therapies. Diagnostic methods currently used in the evaluation of CRC are heterogeneous and can vary within the countries and the institutions. This article aims to discuss in depth currently applied imaging modalities such as virtual computed tomography colonoscopy, endorectal ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) in the diagnosis of CRC. Special focus is put on the potential of recent diagnostic developments as diffusion weighted imaging MRI, MRI biomarkers (dynamic enhanced MRI), positron emission tomography with 2-(fluorine-18)-fluoro-2-deoxy-D-glucose (FDG-PET) combined with computed tomography (PET/CT) and new hepatobiliary MRI contrast agents. The precise role, advantage and disadvantages of these modalities are evaluated

controversially in local staging, metastatic spread and treatment monitoring of CRC. Finally, the authors will touch upon the future perspectives in functional imaging evaluating the role of integrated FDG-PET/CT with perfusion CT, MRI spectroscopy of primary CRC and hepatic transit time analysis using contrast enhanced ultrasound and MRI in the detection of liver metastases. Validation of these newer imaging techniques may lead to significant improvements in the management of patients with colorectal cancer.

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**Key words:** Colorectal cancer; Imaging; Staging; Computed tomography; Magnetic resonance imaging; Diffusion weighted imaging; Contrast enhanced ultrasound

**Core tip:** This state-of-the-art review article covers current and future contribution of various imaging modalities in the diagnosis of colorectal cancer. Primary local staging, metastatic spread, restaging and posttreatment response evaluation are discussed in depth using emerging techniques such as virtual computed tomography (CT) colonoscopy, endorectal ultrasound and positron emission tomography/CT. The role and indications of more recently developed techniques as magnetic resonance imaging (MRI) with diffusion weighted images and hepatobiliary contrast materials are evaluated. The challenges and evolving role of functional imaging with MRI spectroscopy and hepatic transit time analysis using MRI and contrast enhanced ultrasound in the detection of liver metastases are also covered.

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## INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in the western world, with a high lifetime incidence of 6%. The prognosis of CRC is like other tumors staging dependent and the 5 years survival lies in the range of 40%-60%. Due to optimization of surgical techniques, introduction of neoadjuvant therapies and recent developments in diagnostic imaging modalities, the mortality rate has decreased significantly by 20% in the last years.

Utilization of different imaging modalities in diagnosing of CRC vary between countries and institutions. While computed tomography virtual colonoscopy (CTC) is a validated tool in the primary diagnosis of CRC in the United States<sup>[1]</sup>, this method is used with caution in many European countries due to radiation exposure and is thus not included as a screening modality in asymptomatic patients<sup>[2]</sup>. The pros and cons of this rapidly evolving diagnostic modality compared to endoscopy are discussed controversially.

Imaging for surgical planning depicts the relationship of the tumor to surgical key landmarks and shows the presence of metastatic disease. Imaging features enable preoperative evaluation of prognostic features, which may guide patient selection for specific (*e.g.*, neoadjuvant) therapy<sup>[3]</sup>. Recent developments in imaging technologies and validation of newer imaging techniques may lead to significant improvements in the management of patients with CRC. Diagnostic techniques such as diffusion weighted imaging (DWI), Fluorodeoxyglucose positron emission tomography (FDG-PET) and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) are increasingly used and have shown to be clinically useful in tumor characterization<sup>[4-6]</sup>. Newly developed techniques such as perfusion computed tomography (CT) and MRI spectroscopy allowing insights in tumor biology have shown promising results, however they are not yet validated for clinical practice<sup>[7,8]</sup>.

This review discusses the current and future contribution of various imaging modalities to already established and recently developed techniques to improve the diagnosis for both-tumor detection and tumor characterization of CRC. In addition, the evolving role of newly developed methods for functional evaluation of otherwise “occult” hepatic metastases such as Doppler perfusion index (DPI)<sup>[9]</sup> and hepatic transit time (HTT) analysis using contrast enhanced MRI<sup>[10]</sup> will also be covered.

## PRIMARY DIAGNOSIS OF COLORECTAL CANCER

Considering the high diagnostic performance, optical colonoscopy (OC) remains the gold-standard investigation in the early detection of CRC. Colonoscopy allows

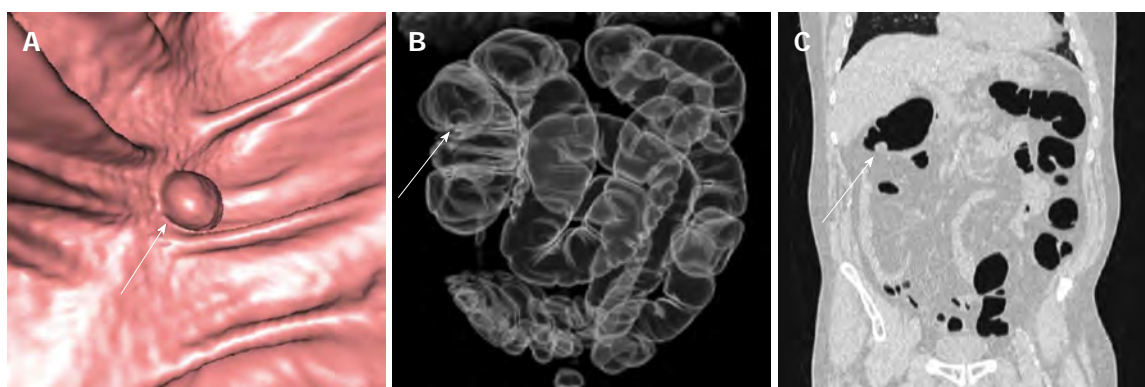
biopsy samples to be taken for definitive diagnosis with a simultaneous opportunity for a therapeutic polypectomy, therefore improving a long-term prevention of CRC deaths<sup>[11]</sup>. However, patients with tumor related stenosis, older patients and those with comorbidities are more likely to have an incomplete or difficult OC<sup>[12,13]</sup>.

## VIRTUAL CT COLONOSCOPY

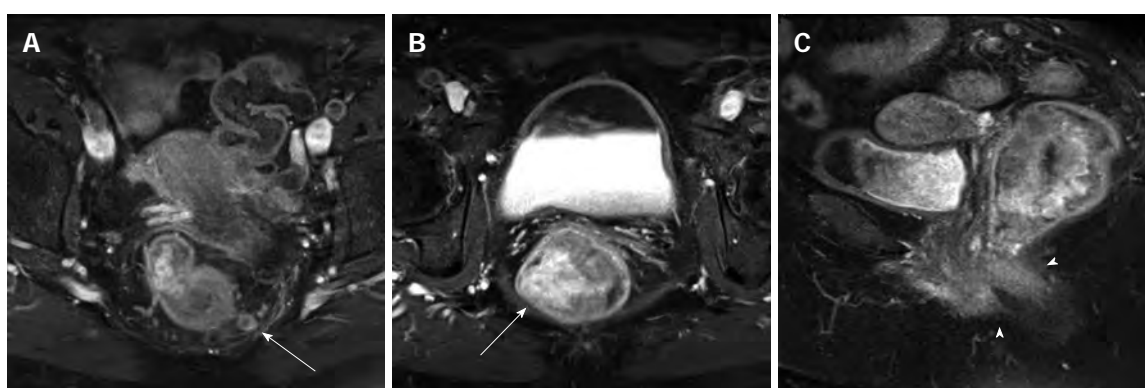
In recent years the role of CTC as a potential alternative to endoscopy has been widely studied<sup>[14-16]</sup>. CTC image formation is based on the X-ray attenuation of low-density; high-intrinsic-contrast objects such as the air contained in the colonic lumen versus the large bowel walls, acting as an interface between intra luminal air and the extra luminal compartment. Low X-ray energy is sufficient to achieve diagnostic CTC images, resulting in a low radiation dose. If CTC is aimed at the sole examination of the colon (*e.g.*, for CRC screening purposes), the use of low radiation dose CT acquisition protocols is warranted. Conversely, regular dose CT protocols can be used if CTC is part of a CT examination in which all abdominal organs have to be investigated. This method is applied in patients with known CRC and incomplete OC, in whom CT plays a role for both complete assessment of the colonic lumen and for oncological staging (Figure 1). For adequate colonic distention to be achieved, air or carbon dioxide is usually delivered into the patients colon with a thin rectal catheter prior to CTC. Air has the advantage of no cost and the ease of administration, but is less tolerated as it is not absorbed by the colonic mucosa. Conversely, carbon dioxide is more comfortable as it is gradually absorbed by the colonic walls, although larger volumes must be supplied compared with air. In practical terms, administration of 1.0-1.5 liter of air or 3-4 liter of carbon dioxide is usually sufficient<sup>[17]</sup>. The CT acquisition is usually performed twice: in supine and prone position (or vice versa). This is to optimize the distention of the various colonic segments depending on gravitational compression by the surrounding abdominal structures, as well as to distinguish polyps which may be fixed to the bowel walls from fluid and/or fecal residues. Colonic distention is also favored by parenteral administration of spasmolytic agents, such as glucagon or hyoscine-N-butyl bromide, which inhibit peristalsis and reduce the tone of the parietal musculature. By orally administering positive contrast material (barium or iodine), fecal and fluid tagging can be performed, helping to distinguish fecal/fluid residues from parietal polyps. Tagged residual fluid can then be electronically removed from CTC images by means of a dedicated software. 3D reconstructions enable accurate quantification of polyp volume, which can be helpful in a follow-up to assess growth of the polyps. Research is in progress on subtracting solid tagged stool in patients who do not undergo cathartic cleansing.

Pickhardt *et al*<sup>[14]</sup> found CTC comparable to colonoscopy in detection of bigger colorectal polyps. Two meta-analysis studies showed a high sensitivity (100%) of CTC in the detection of colon cancer and 87.9% for adenomas less than 10 mm<sup>[18,19]</sup>. Despite such promising data, there





**Figure 1** A 64-year-old male patient who underwent routine screening colonoscopy terminated due to severe discomfort. A: Virtual computed tomography (CT) colonography detected a 1 cm polyp (arrow) in right colonic flexure, biopsy proved as adenocarcinoma; Fly-through with a 3D view of the polyp; B: The virtual X-ray reconstruction; C: Coronal reconstruction using the lung window shows the tumor clearly.



**Figure 2** Abdominal magnetic resonance imaging for local staging of rectal adenocarcinoma in a 58-year-old female. A, B: Post-contrast fat-suppressed axial images show 7 cm long contrast enhancing neoplastic mass with lymph node metastases within the mesorectal fascia (arrow); C: Peripheral desmoplastic reaction (arrowheads) on T1 sagittal images.

is currently no transcontinental consensus on whether CTC should be used as a screening method in asymptomatic patients. Since 2008 CTC is recommended as a validated diagnostic tool by the American Cancer Society and is included among the screening tests of CRC<sup>[1]</sup>. This recommendation was revalidated in a recent large patient sample (1610 patients) multicenter randomized trial by Atkin *et al*<sup>[16]</sup>, concluding that CTC is a similarly sensitive, less invasive alternative to colonoscopy. However, in many European countries the use of CTC as a screening method in asymptomatic populations is prohibited due to radiation related consequences and only advised in cases of incomplete preoperative colonoscopy<sup>[2]</sup>.

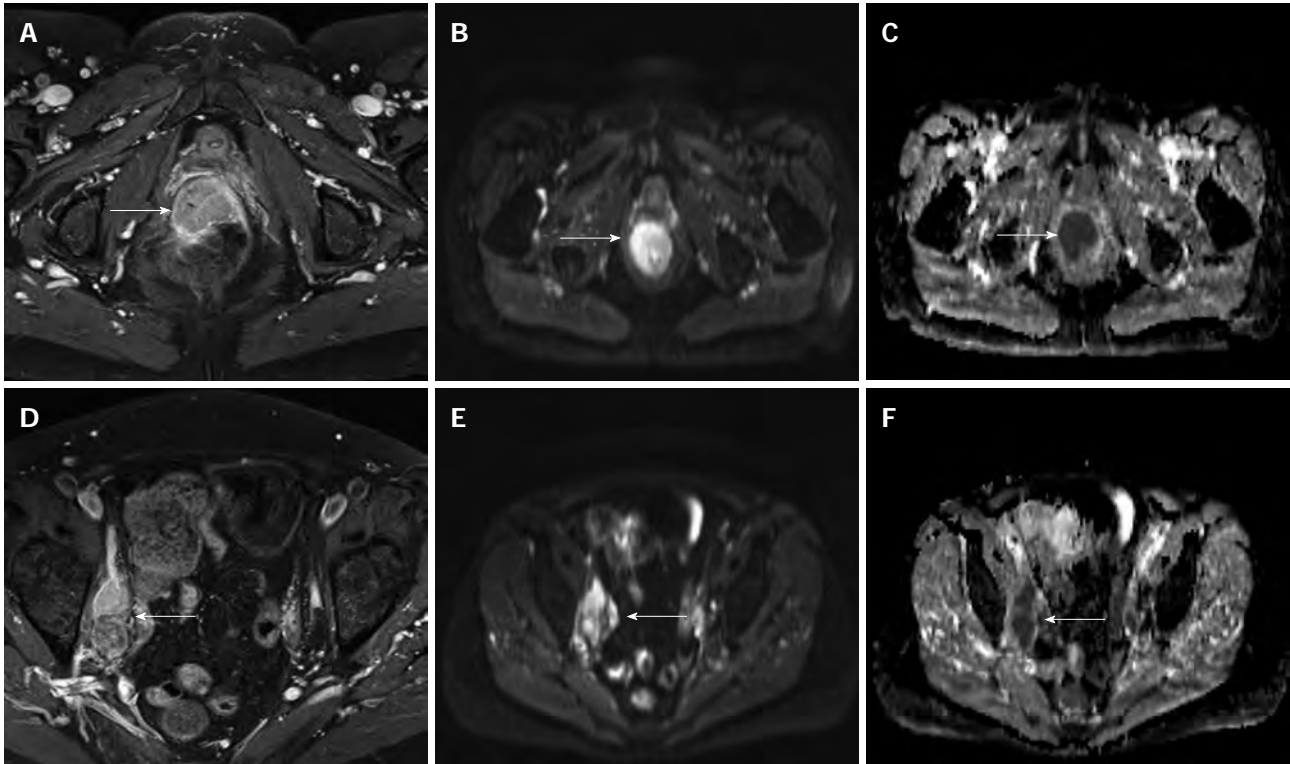
An alternative method to CTC could be MRI colonoscopy which is not radiation exposure related<sup>[20]</sup>. However, currently there are insufficient study results available to recommend this method as a screening modality.

## LOCAL STAGING OF CRC: MRI AND ENDORECTAL ULTRASOUND

The tumor node metastasis classification of the American Joint Committee on Cancer is the internationally accepted standard for the staging of CRC<sup>[21]</sup>. The accurate diagno-

sis of local tumour extension, location, T stage, potential circumferential resection margins, mesorectal fascial involvement and extramural or venous invasion is essential for defining the treatment strategy. For this reason, MRI is the recommended modality for initial staging, due to its high accuracy for the definition of localization, determining the total extension and the relationship of the tumor to the peritoneal reflection<sup>[22]</sup>. Furthermore, MRI is accurate in measuring the distance between the anorectal junction and the distal part of the tumor. It is also accurate for determining the length of the tumor. Although it has been the standard in the past, it is inappropriate to use the term circumferential resection margins (CRM) for initial clinical staging before surgery, since CRM can be defined only postoperatively by the surgical plane. The tumor growth on primary staging MRI should be best described in relation to an anatomical structure, like the mesorectal fascia<sup>[23]</sup>. Most staging failures with MRI occur in the differentiation of T2 stage and borderline T3 stage with overstaging as the main cause of errors<sup>[24]</sup>. Overstaging is often caused by desmoplastic reactions<sup>[5]</sup> and it is difficult to distinguish on MRI between spiculation in the perirectal fat caused by fibrosis alone (stage pT2) and spiculation caused by fibrosis that contains tumor cells in stage pT3 (Figure 2).





**Figure 3** A 58-year-old female with biopsy-proven adenocarcinoma of the rectum. A: Post-contrast fat-suppressed axial T1 images show a contrast-enhancing mass (arrow), extending from rectum into the anal canal and invading the posterior aspect of the vagina; B, E: Both, the primary tumor and the lymph node metastases, show an hyperintense signal on diffusion weighted imaging; C, F: An reduced apparent diffusion coefficient reflecting the tight tumor cellularity; D: Enlarged, contrast-enhancing lymph nodes along the right iliac axis (arrow).

Although previous studies have not shown much advantage of dedicated phased-array coils<sup>[25]</sup>, our clinical experience is positive and at our institution we use phased-array coils as a standard in the primary diagnosis of colorectal cancer. The advantage of high spatial resolution with a large field of view is making phased-array MRI suitable for staging of both superficial and advanced rectal tumors. A standard phased-array MRI protocol for rectal cancer consists of T2-weighted turbo spin-echo (TSE) MR sequences with high spatial resolution. The strength of T2-weighted turbo spin-echo MRI of rectal cancer is that fat tissue remains high in signal intensity. In this way, the tumor contrasts well with the surrounding fat tissue, and even very thin hypointense structures such as the mesorectal fascia can always be identified independent of the body habitus of the patient, owing to the high contrast between the hypointense fascia and the hyperintense fat tissue in and outside the mesorectum<sup>[5]</sup>.

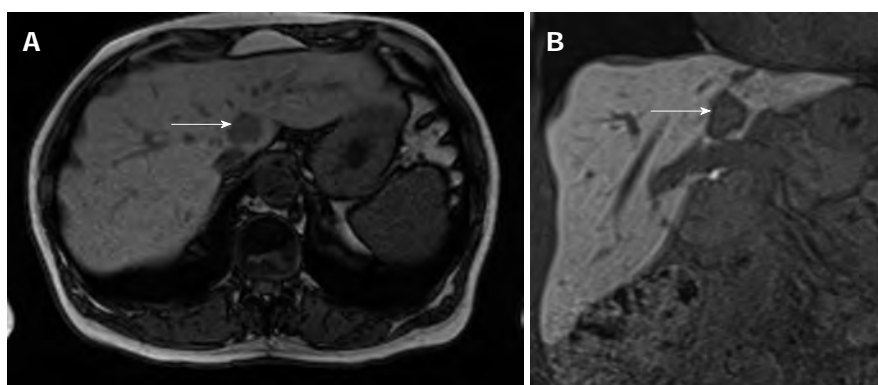
At our institution, phased-array MRI for primary rectal cancer staging is performed at 1.5 Tesla (Siemens Avanto and Espree, all Siemens Healthcare, Erlangen, Germany) and 3.0 Tesla (Siemens Prisma, Skyra and Verio). The protocol consists of a T2 SPACE 1.0 mm isovoxel sequence, a standard echo planar imaging sequence for diffusion (b-values: 0, 40, 400 and 800 s/mm<sup>2</sup>) including an apparent diffusion coefficient (ADC) map and a T1 TSE Dixon sequence with fat saturation (FS) and calculation of in-/opposed-/fat- and water maps before con-

trast administration (Figure 3). Post contrast sequences are just a standard transversal T1 TSE FS (SL 5 mm) and a T1 VIBE FS 1.2 mm isovoxel. The pre- and post contrast isovoxel sequences can be reconstructed in line with and perpendicular to the individual tumor.

Endorectal Ultrasound (ERUS) is now an established modality for evaluation of the integrity of the rectal wall layers. With accuracies for T staging varying between 69% and 97%, endorectal ultrasonography (US) is currently the most accurate imaging modality for the assessment of T1 tumors<sup>[2]</sup>. ERUS and endorectal MRI have similar accuracy in the differentiation between superficial (T1 and T2) and T3 tumors<sup>[26]</sup>. However, endorectal MRI is related to high costs, limited availability and is less patient friendly. Consequently, endorectal MRI is not recommended by the European Society for Medical Oncology Guidelines as a preferred imaging modality for clinical T stage in colorectal cancer<sup>[22]</sup>.

## METASTATIC SPREADING OF CRC

In 25% of patients with colonic cancer and in 18% of patients with rectal cancer, metastases are present at the time of the first diagnosis. The most frequently used imaging modalities for the detection of CRC metastases are US, CT, MRI and PET/CT<sup>[27]</sup>. Current National Comprehensive Cancer Network guidelines for initial staging of CRC suggest the use of chest/abdomen/pelvis CT or



**Figure 4** A 63-year-old female with colorectal cancer and suspected liver metastasis. A: Primovist images acquired 10 min *p.i.*, during the hepatobiliary phase using a T1 VIBE isovoxel sequence with coronal orientation; B: Due to the high resolution axial reconstructions are also done routinely. The lesion in segment I (arrow) is clearly demarcated as a contrast defect because of the missing hepatocytes in the metastasis while the other parts of the liver show a bright contrast enhancement.

MRI, while FDG-PET/CT is reserved for surveillance or problem solving.

### N staging

ERUS, CT and MRI use the size as the main criterion in the assessment of nodal involvement, although the lymph node size is not an ideal indicator of metastasis and lacks sufficient accuracy for clinical decision-making<sup>[28]</sup>. FDG-PET gives better insight in tumor biology, however, due to limited spatial resolution it does not allow for reliable detection of small lymph node metastases. FDG-PET/CT may provide additional information and could increase the accuracy of lymph node involvement significantly with a sensitivity and specificity of 51% and 85% for local lymph nodes and 62% and 92%, for distant lymph nodes<sup>[29]</sup>.

### M staging

Correct detection of hepatic and pulmonary metastases can be challenging considering the possible difficulties in differentiation with benign lesions in these organs. CT has a better diagnostic performance (sensitivity 74%-84%, specificity 95%-96%) compared to US in detection of CRC liver metastases<sup>[30]</sup>. A meta analysis of prospective studies comparing FDG-PET, MRI, and CT demonstrated a superior performance of MRI over the other two modalities on a lesion-by-lesion basis of the liver and in particular in evaluating lesions less than 1 cm in size (sensitivity 80%-88% and specificity 93%-97%)<sup>[6]</sup>.

Recently, DWI and hepatobiliary phase MRI with new hepatobiliary contrast agents have been integrated for the detection of liver metastases demonstrating improved sensitivity over routine MRI alone<sup>[31]</sup>. The newest hepatobiliary contrast agent available is Gd-EOB Primovist® in Europe and Eovist® in United States and Canada (Bayer Healthcare, Leverkusen, Germany). Uptake of contrast within the hepatocytes results in peak parenchymal enhancement approximately 10-20 min *p.i.*, referred to as the hepatobiliary phase. As expected, lesions like metastases without containing hepatocytes are strongly hypointense compared to the surrounding enhanced parenchyma in this phase (Figure 4).

For the detection of pulmonary metastases imaging can be limited to chest X-ray. Although CT detects more

lesions compared to chest X-ray (CXR), a large number of these lesions (4%-42%) does not allow for a definitive diagnosis. Only one quarter of unspecified pulmonary lesions found on CT are demonstrated to be metastases, therefore the high sensitivity of CT cannot guarantee important benefit for the patients<sup>[32]</sup>. This concept is supported by a recent study showing that preoperative staging chest CT is not beneficial for CRC patients without liver and lymph node metastasis on abdominal and pelvic CT who had a negative initial CXR finding<sup>[33]</sup>.

## RESTAGING: THERAPEUTIC RESPONSE EVALUATION

### General considerations

Patients after primary tumor resection and those treated with chemoradiation therapy (CRT) for locally advanced CRC require a regular post treatment evaluation. Within the first 5 years after curative therapy there is an increased chance for a locoregional relapse (3%-24%), occurrence of distant metastases (25%) and for developing metachronous secondary tumors (1.5%-10%). The introduction of preoperative adjuvant CRT has led to a reduction in local recurrency rates and has become standard of care for patients with locally advanced rectal cancer.

Several studies investigating the role of imaging for restaging after CRT suggest that neither MRI nor ERUS or FDG-PET are sufficiently accurate for identifying the true complete responders with positive predictive values ranging from 17%-50%<sup>[34-36]</sup>. T2 weighted MRI has been standardly used for local restaging (Figure 5). Many recent reports have shown that DWI MRI may be useful for the response evaluation after CRT<sup>[37,38]</sup>. DWI has shown to be feasible as an early marker of treatment response because cell death and vascular alterations typically occur before size changes. It also has been proved that DWI in addition to standard MRI significantly improves the performance of radiologists to select complete therapy responders compared to standard MRI only<sup>[39,40]</sup>. In a recent systematic review and meta analysis study including 1556 patients from thirty-three studies MRI has shown to be useful for tumor-free CRM restaging, however nodal staging remained challenging<sup>[41]</sup>. High b-value DWI is sensitive for detecting the location of lymph nodes, but

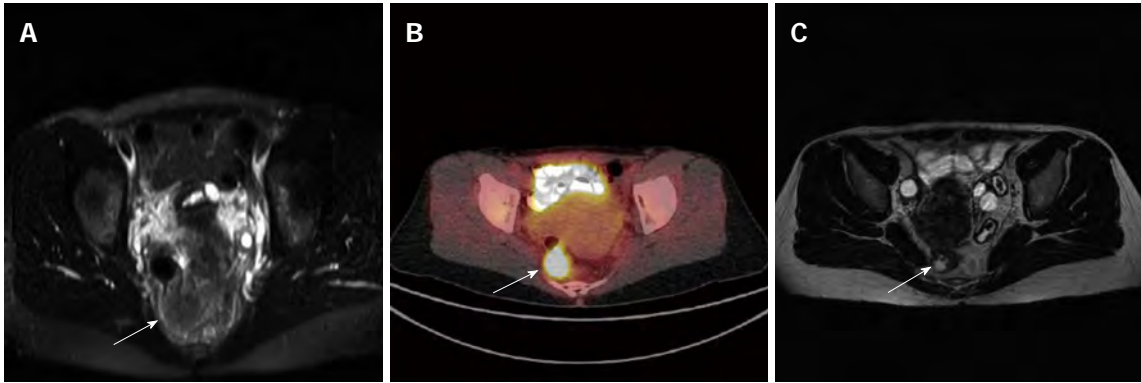


Figure 5 Initial rectal cancer staging of a 48 year old female. A: Occlusion of the rectum by solid tumor on magnetic resonance imaging (MRI) (arrow); B: Corresponding high Fluorodeoxyglucose metabolism in the Fluorodeoxyglucose positron emission tomography/computed tomography; C: Post treatment (chemoradiation therapy) magnetic resonance imaging shows a clear tumor size reduction (arrow) with a continuing lumen after chemoradiation therapy.

characterization of neoplastic nodes yields false-negative results and reactive hyperplastic nodes false-positive results.

It has been reported that transient decrease in the ADC may occur early in treatment related to cellular swelling, reduction in the blood flow or extravascular-extracellular space<sup>[42]</sup>. However, early decreases in ADC values are not consistently seen and it has recently been reported that increases in ADC value with therapy response occur within 3-7 d in responding CRC patients treated with chemotherapy<sup>[43]</sup>. Therefore the utilization of ADC values in the CRC evaluation needs further standardization and validation.

The role of FDG-PET in evaluating of recurrent colon cancer is controversial. Some of the previously published studies showed high specificity of this modality (up to 98%) based on evident FDG reduction after adjuvant CRT<sup>[44]</sup>. Metabolic changes in response to treatment occur before any structurally detectable change (*e.g.*, tumor shrinkage). In the neoadjuvant setting, serial FDG-PET examinations may aid treatment planning to decide the appropriate length of neoadjuvant chemotherapy to maximize tumor response before surgical resection. In this setting, FDG-PET could lead to changes in therapies for those patients with tumors that show no metabolic change<sup>[45]</sup>. On the contrary, other studies suggest that when radiation therapy is applied, FDG-PET cannot reliably identify pathologic complete response to CRT due to radiation related increased FDG uptake by rectal mucosa resulting in high false-positive data<sup>[46,47]</sup>. For this reason, when using FDG-PET to monitor tumor response, it is not advocated within the first 4 wk after completion of CRT. FDG-PET/CT is a unique combination of the cross-sectional anatomic information provided by CT and the quantitative metabolic information provided by FDG-PET. In the past years, FDG-PET/CT has taken an important place in treatment response assessment<sup>[48]</sup>. Limitations of FDG-PET/CT are that the technique is cost- and time-consuming (utilizing about 1.5 h per patient) and is not widely available.

Considering a very limited benefit of CRC follow-up in stage I tumors, described as only 1% increase in patient survival<sup>[49]</sup>, a regular follow-up in these patient group

is not indicated. In patients with advanced primary CRC (stage II and III), US is advised for the follow-up of liver metastases. US has a slightly lower sensitivity compared to CT in the detection of liver metastases, however the performed studies did not show a convincing advantage of CT over US in evaluation of asymptomatic patients<sup>[50]</sup>. Therefore, abdominal US can be indicated as a cost-effective, widely available and relatively simple diagnostic modality in the follow-up of CRC liver metastases.

Up to 7% of all curatively treated patients with CRC develop distant pulmonary metastases which in 3.4%-30.0% are detected with chest X-Ray<sup>[51]</sup>. Therefore CRX evaluation can be sufficient in follow-up of asymptomatic patients.

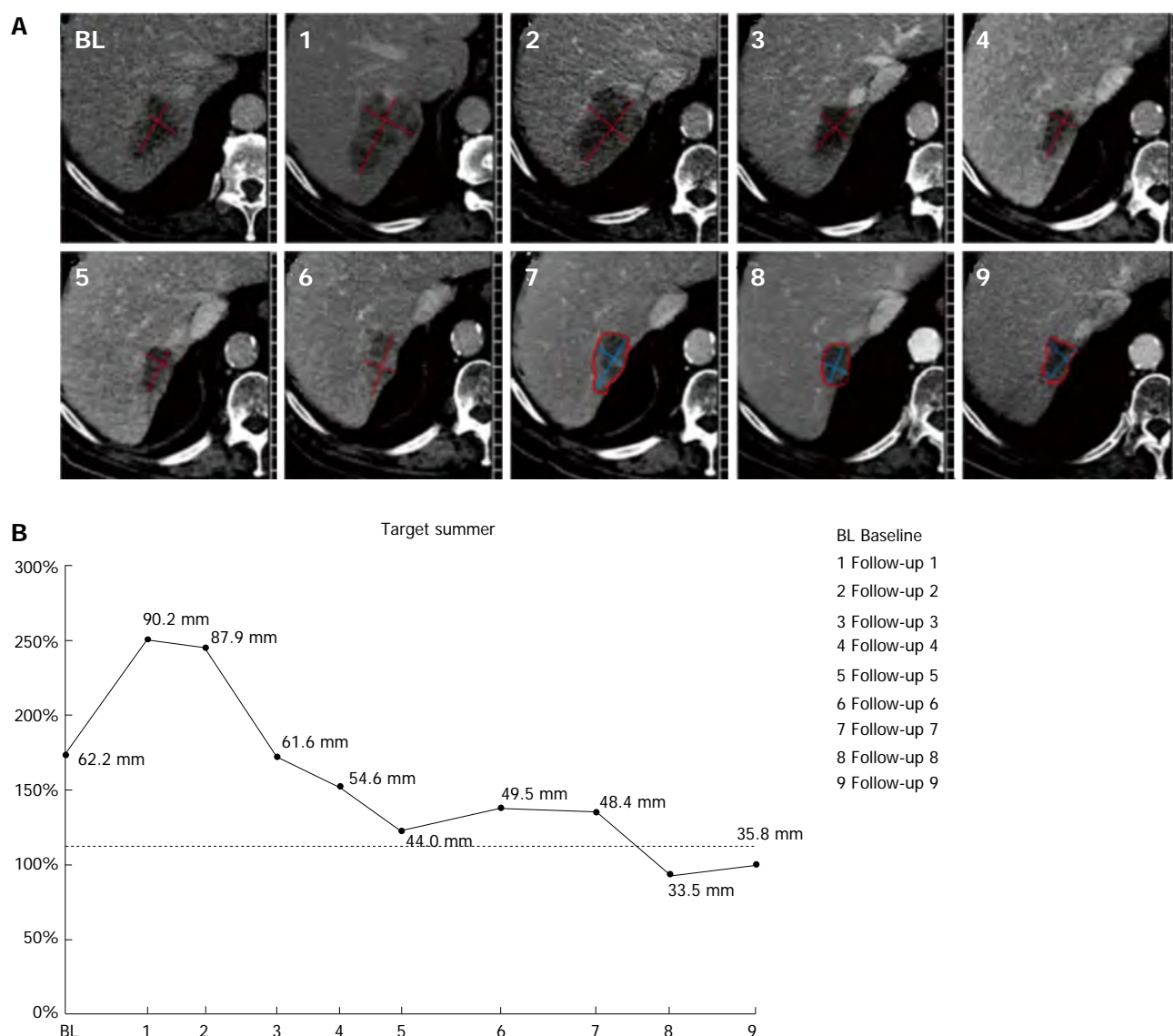
For anatomic objective response evaluation criteria based on assessment of the size of the tumor or metastases, Response Evaluation Criteria in Solid Tumors (RECIST) have been developed<sup>[52]</sup>. RECIST uses unidimensional measurements of the sum of the longest lesion diameters of target lesions. At our institution we use a commercially available software for RECIST analysis (mint Lesion®, Mint Medical GmbH, Heidelberg, Germany), which will be discussed below.

#### Software based follow-up

Proper response assessment and reporting of metastatic lesions are crucial. A major pitfall in tumor response monitoring is the increasing incidence of mixed response to chemotherapy and subjective measurements of the lesions, *e.g.*, liver and lung metastases, also lesion measurements are time-consuming and can be investigator dependent. Computerized tools able to optimize the radiologist's workflow of the image reading process are spreading as the need for a systematic, standardized follow-up procedure grows. For example, the syngo® CT Oncology software (Siemens Healthcare, Erlangen, Germany) is able to perform automated measurement of neoplastic lesions helping to solve the long-standing issue of interobserver variability.

Another automated tool is mint Lesion® (Mint Medical GmbH, Heidelberg, Germany), developed at the German Cancer Research Center (Heidelberg, Germany)





**Figure 6** An automated software (mint Lesion<sup>®</sup>, Mint Medical GmbH, Heidelberg, Germany) used at our Institution for tumor treatment response evaluation. A: Computed tomography images show liver metastasis in a patient with colorectal cancer on nine follow-up examinations; B: Graphical evaluation depicts the measurement of the lesion standardized throughout the whole staging period consisting of baseline (BL) and nine follow up assessments (1-9). Tumor response is evaluated according to Response Evaluation Criteria in Solid Tumors.

which is currently routinely used at our institution for oncological assessment. Connected with our Picture Archiving Computer System (PACS) the mint Lesion software is able to continually synchronize and upgrade its worklist retrieving and matching precedent patients' related studies allowing a workflow optimization. It covers management of patient cohorts in terms of disease and treatment, assessment of lesions with respect to the overall patient treatment course, statistical response evaluation in line with response criteria, and consistent and comprehensive automated reporting.

In the initial baseline assessment target and non-target lesions are defined. In subsequent follow-up exams the software is able to correlate and match images of the previous studies allowing a faster recognition of previously described lesions; by showing exactly how quantitative measurements (*i.e.*, volumetry, density and intensity) were performed in previous studies, interobserver variability

is thus reduced. Apart from the reproducible measurements, assessment notes, treatment outcome statistics for patient cohorts and individual patients, mint Lesion<sup>®</sup> provides an automatically generated, consolidated visual and textual overview of a single treatment course (Figure 6). Graphical charts help to identify the dimensions of tumor load change with respect to baseline, nadir and previous exams. Therapy course overview is clearly depicted in the results and can be sent as digital imaging and communications in medicine to the PACS as well as actively included in the report. By such means, the standardization of the read workflow contributes to the assessment quality of longitudinal follow-up sequences providing comprehensible information for an interdisciplinary assessment of the therapy response by a tumor board.

#### **Beyond resolution: Functional imaging**

Functional imaging now has a growing role in colorec-



tal cancer assessment. Recent developments in imaging technologies and validation of these newer imaging techniques may lead to significant improvements in the management of patients with colorectal cancer.

To date, FDG-PET does not have an established role in primary diagnosis of colon cancer reflecting limited availability of resources and lack of convincing cost-benefit data<sup>[53]</sup>. This technique has low sensitivity revealing mucinous adenocarcinomas in which metabolic activity is low. Partial volume averaging and necrotic lesions may cause false-negative results, and incidental physiologic bowel FDG uptake or inflammation will produce increased tracer uptake, giving rise to false-positive findings that can mimic a tumor. The controversial role of FDG-PET in the posttreatment setting has been already discussed above. Prediction of the nodal status by CRC remains problematic. A novel nanoparticle MRI lymphographic agent - ultrasmall superparamagnetic iron oxide particles showed an overall sensitivity and specificity of 88% and 96% in the detection of lymph node metastases of CRC<sup>[54]</sup>. Regretfully these MRI contrast agents are not yet available for clinical practice.

Dynamic contrast-enhanced (DCE) CT and MRI have been described as potential prognostic biomarkers in CRC. The results of the studies evaluating DCE-CT as a biomarker for chemoradiation are controversial: while baseline low perfusion values were described to be associated with a poorer response in the study by Bellomi *et al.*<sup>[55]</sup>, another group reported the contrary<sup>[56]</sup>. DCE-MRI data uses two compartments for contrast agent accumulation: blood plasma and extravascular-extracellular space.  $K^{trans}$  (volume transfer constant between the blood plasma and the extravascular-extracellular space, the washout rate, measured in minutes<sup>-1</sup>) and  $K_{ep}$  (rate constant between the extravascular-extracellular space back to the blood plasma, the washout rate, measured in minutes<sup>-1</sup>) determine the transport between these two compartments. Rectal tumors with higher  $K^{trans}$  values at presentation appear to respond better to CRT than those with lower values. After CRT, usually  $K^{trans}$  values are reduced, while persistent raised values indicate residual active disease<sup>[57]</sup>.

### Experimental techniques in primary colorectal cancer diagnosis

In a study by Ng *et al.*<sup>[58]</sup>, CT texture features of primary colorectal cancer were studied in relation to 5-year overall survival rate. The authors studied the tumor heterogeneity using a range of parameters, including entropy, uniformity, kurtosis, skewness, and standard deviation of the pixel distribution histogram. According to this study tumors demonstrating less heterogeneity at fine filter levels were associated with poorer survival, concluding that the addition of texture analysis to staging contrast-enhanced CT may improve prognostication in patients with primary colorectal cancer. Goh *et al.*<sup>[8]</sup> assessed an interobserver agreement in a prospective study with integrated FDG-PET/CT and perfusion CT to evaluate the relationship between tumor glucose metabolism and vascularization.

FDG-PET/CT was used to localize the colorectal tumor, and CT coordinates were used to plan the subsequent perfusion. The study showed good intra- and interobserver agreement for the metabolic-flow differences, suggesting this approach as a robust parameter for clinical practice.

The role of MR Spectroscopy (MRS) has been of great interest in the recent years to improve the primary diagnosis of various cancer groups. In a small sample ex vivo prospective study on 24 subjects with colorectal cancer without neoadjuvant treatment, MRS was able to discriminate healthy from neoplastic tissue and to distinguish patients with different prognoses<sup>[7]</sup>.

### Functional imaging in liver metastases of colorectal cancer

The liver is the first organ most likely to develop distant metastases from CRC. Knowledge of hepatic metastatic involvement during identification of the primary tumor is therefore crucial. The idea is not new and we can follow several attempts to get access to that information back to the nineteen-eighties. The approach is to detect the arterialization of the liver blood supply during the onset and development of liver metastases. In a normal healthy individual approximately two thirds of the blood supply of the liver arrives *via* the portal vein and one third *via* the hepatic artery. During the development of liver metastases, this relation changes: the above mentioned arterialization occurs, which means the arterial portion of the liver blood supply increases while the portal vein portion decreases<sup>[59]</sup>. This has been shown first with technetium colloid scintigraphy to estimate the so called hepatic perfusion index (HPI) in overt liver metastases<sup>[60-62]</sup>. Meanwhile it has been shown that the hemodynamic changes occur already at an early microscopic stage of metastasis formation<sup>[63,64]</sup>.

Leen *et al.*<sup>[65]</sup> developed a Doppler ultrasound method to get a parameter similar to the HPI, the DPI, which gives the hepatic arterial blood flow relative to the portal venous flow. This ratio was raised in patients with liver metastases. The method demonstrated not only the possibility to detect overt liver metastases but also the arterialization due to occult metastases for the standard morphology based imaging methods. This study showed that patients with colorectal cancer, without liver metastases on first imaging and a raised DPI, had a much higher risk of developing liver metastases in the following five years than those with normal DPI. The DPI method thus seems to detect the presence of metastases which were occult to all other imaging modalities<sup>[9]</sup>. Unfortunately, DPI measurements are strongly operator dependent and other groups could not reproduce Leen's results<sup>[66,67]</sup>. HTT analysis of a microbubble ultrasound contrast agent has then been proposed as an alternative technique for detecting hepatic arterialization<sup>[68]</sup>. It was initially used to show arterialization in patients with hepatic cirrhosis<sup>[69,70]</sup>. Meanwhile several studies have shown that the method is able to detect hemodynamic changes in liver metastases but depends on the used contrast agent<sup>[10,71-73]</sup> (Table 1).

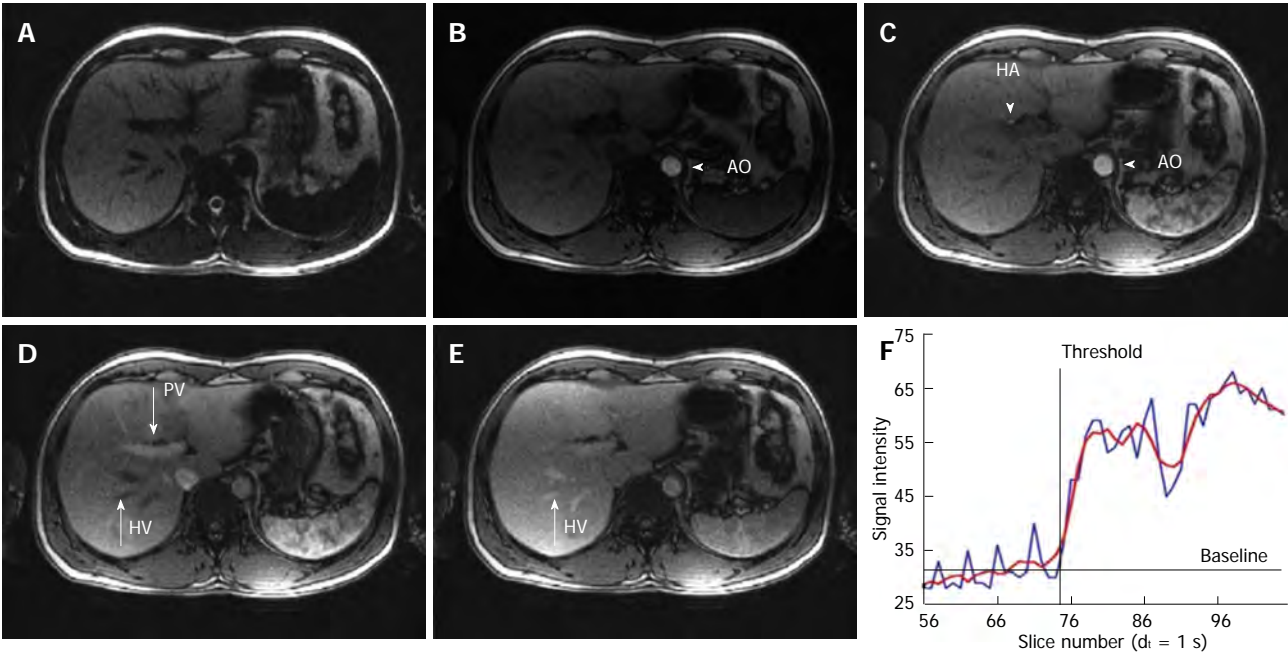


Figure 7 Magnetic resonance imaging images of the T1-weighted sequence for hepatic transit time analysis at different time points. A: Baseline image without contrast; B: Arterial phase with opacification of the aorta (AO); C: Arterial phase with opacification of the AO and the hepatic artery (HA); D: Portal venous phase with additional enhancement of the portal vein (PV); Note that the hepatic veins (HV) are still not enhanced; E: Venous phase with complete opacification of all vessels including the HV; F: Example of a typical time intensity curve acquired from a ROI placed at the position of the HA; The raw data in the graph (blue line) has a modulation due to patient breathing. Therefore the curve has to be fitted and smoothed (red line). The calculated baseline as well as threshold point, demonstrating the arrival time of the contrast agent is drawn.

Study	Number of patients	Hepatic transit time (arterial to venous) (s)		contrast agent	modality
		Liver metastases	No liver metastases		
Bernatik <i>et al</i> <sup>[73]</sup>	28/36	6.7	15.4	Optison <sup>1</sup>	CEUS
Hohmann <i>et al</i> <sup>[10]</sup>	22/22	7.4	11.1	SonoVue <sup>2</sup>	CEUS
Zhang <i>et al</i> <sup>[71]</sup>	5/3	6.2	11.3	SonoVue <sup>2</sup>	CEUS
Haendl <i>et al</i> <sup>[72]</sup>	20/15	6.3	9.3	SonoVue <sup>2</sup>	CEUS
Haendl <i>et al</i> <sup>[72]</sup>	12/14	9.9	14.8	Levovist <sup>3</sup>	CEUS
Haendl <i>et al</i> <sup>[72]</sup>	20/15	6.3	9.2	Luminity <sup>4</sup>	CEUS
Hohmann <i>et al</i> <sup>[78]</sup>	20/21	7.1	13.5	MultiHance <sup>2</sup>	MRI

<sup>1</sup>Amersham, Little Chalfont, United Kingdom; <sup>2</sup>Bracco, Milano, Italy; <sup>3</sup>Bayer, Berlin, Germany; <sup>4</sup>Lantheus, N. Billerica, MA, United States. These are mainly studies using CEUS but also one study which used MRI. All other MRI studies used slightly different approaches and did not measure directly comparable values. CEUS: Contrast enhanced ultrasound; MRI: Magnetic resonance imaging.

There have also been other attempts to measure hepatic blood supply changes with CT and MRI. With CT this is usually a perfusion measurement with the calculation of different perfusion parameters such as hepatic artery and portal vein perfusion and the HPI<sup>[74,75]</sup>. The major drawback of CT measurements is radiation exposure and, therefore, most of the studies are animal studies. Even though the results are promising, there are probably no realistic possibilities for CT perfusion measurements in humans.

With MRI the approaches are different which are

summarized under the term diffusion/perfusion measurements (Figure 7). Especially with focal or global perfusion methods, MRI seems to have great potential to detect hemodynamic changes due to focal liver lesions<sup>[76]</sup>. While the first studies just measured perfusion parameters in one single plane<sup>[77,78]</sup>, this changed to measurements of the whole liver with 3D Datasets but with limited time resolution<sup>[79,80]</sup>. It should currently be possible to increase this time resolution in further studies. All the previous mentioned methods required intravenous contrast material, which might have an influence on the results similar to the results on MRI as it was shown with CEUS. Therefore new methods without contrast material, like hemodynamic response imaging, which has proven to show therapy response in experimental settings, are very promising<sup>[81]</sup>.

Overall, for functional imaging in patients with colorectal cancer, MRI of the liver offers the widest variety of possibilities in the future. This might be essential for the detection of occult liver metastases at the time of first diagnosis of colorectal cancer and will then result in different therapeutic approaches due to the results of the measurement.

CONCLUSION

In recent years several attempts have been made to improve the diagnostic performance of imaging modalities for better characterization of CRC. To date, OC remains the most precise modality in the detection of primary CRC simultaneously allowing biopsy and therapeutic

polypectomy. Virtual CT colonoscopy is gaining importance as a potential alternative to OC, recently showing a similar diagnostic performance<sup>[16]</sup>. However, radiation exposure and the lack of instantaneous therapeutic possibilities remain a primary concern. To date, there are insufficient study results to recommend MR Colonoscopy as a screening modality.

MRI and ERUS at present show the best results in the local staging of rectal carcinoma<sup>[5,22,23]</sup>. MRI is the superior imaging modality for the evaluation of primary tumor location, extension and mesorectal fascia involvement. Overstaging remains problematic on MRI, related to difficulties in differentiating desmoplastic reaction caused by fibrosis alone (stage pT2) and by fibrosis that contains tumor cells (stage pT3). ERUS, with an accuracy of up to 97%, is currently the most accurate imaging modality in the assessment of T1 rectal tumor<sup>[2]</sup>. For the detection of CRC distant metastases, US and CT are the most advocated modalities. Although FDG-PET/CT shows an increased accuracy in metastatic lymph node assessment, utilization of this modality is limited and cannot be applied broadly. Recent studies support the concept that in preoperative staging chest CT is not beneficial and imaging of the patients without hepatic and lymphatic metastases can be limited to CXR<sup>[81]</sup>.

Newer techniques in functional imaging may lead to significant improvements in the management of CRC. The hepatobiliary MRI contrast agent (Gd-EOB Primovist/Eovist®, Bayer Healthcare) is available to improve the detection of liver metastases and could be problem-solving in difficult cases. In treatment response monitoring, DWI is gaining a promising role as a reliable marker to improve MRI performance, however, characterization of metastatic lymph nodes remains challenging. Other MRI biomarkers in the treatment response evaluation such as Dynamic contrast enhanced MRI and perfusion CT might improve the insights in tumor biology to better characterize residual tumor. Experimental studies on MRI spectroscopy of primary CRC, MRI diffusion/perfusion and hepatic transit time analysis using MRI in the detection of metastatic liver disease are promising. However, further research in larger series is needed to be applicable in clinical practice.

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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

# Lymph node staging in colorectal cancer: Old controversies and recent advances

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## Abstract

Outcome prediction based on tumor stage reflected by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) tumor node metastasis (TNM) system is currently regarded as the strongest prognostic parameter for patients with colorectal cancer. For affected patients, the indication for adjuvant therapy is mainly guided by the presence of regional lymph node metastasis. In addition to the extent of surgical lymph node removal and the thoroughness of the pathologist in dissecting the resection specimen, several parameters that are related to the pathological work-up of the dissected nodes may affect the clinical significance of lymph node staging. These include changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the AJCC/UICC TNM system as well as the minimum number of nodes to be dissected. Methods to increase the lymph node yield in the fatty tissue include methylene blue injection and acetone compression. Outcome prediction based on the lymph node ratio, defined as the number of positive lymph nodes divided by the total number of retrieved nodes, may be superior to the absolute numbers of involved nodes. Extracapsular invasion has been identified as additional prognostic factor. Adding step sectioning and immunohistochemistry to the pathological work-up may result in higher

accuracy of histological diagnosis. The clinical value of more recent technical advances, such as sentinel lymph node biopsy and molecular analysis of lymph nodes tissue still remains to be defined.

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**Key words:** Colon cancer; Rectum cancer; Tumor staging; Lymph node metastasis; Prognosis; Sentinel lymph node; Lymph node ratio; Extracapsular invasion; Immunohistochemistry; Molecular analysis

**Core tip:** For patients with colorectal cancer, the indication for adjuvant therapy is mainly guided by the presence of regional lymph node metastasis. This review provides an in depth analysis of parameters affecting the clinical significance of lymph node staging, focusing on changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the American Joint Committee on Cancer/Union for International Cancer Control tumor node metastasis staging system, the minimum number of lymph nodes that should be evaluated, lymph node ratio, extracapsular invasion, sentinel node biopsy, and the potential benefit of ancillary techniques, such as immunohistochemistry and molecular analysis.

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## INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide. In the United States, approximately 102480

new cases of colon cancer and 40340 new cases of rectal cancer have been estimated for 2013. For the same time period, 50830 deaths from colorectal cancer have been calculated, accounting for about 9% of all cancer deaths<sup>[1]</sup>.

Surgical resection is the treatment of choice for patients with locally confined disease. Outcome prediction based on tumor stage reflected by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) tumor node metastasis (TNM) system is currently regarded as the strongest prognostic parameter. Adjuvant chemotherapy, which is primarily based on 5-fluorouracil, has decreased tumor recurrence in AJCC/UICC stage III patients, while neoadjuvant chemotherapy and total mesorectal excision have improved local control in patients with rectal cancer. The indication for adjuvant therapy is mainly guided by the presence of regional lymph node metastasis<sup>[2-4]</sup>.

A plethora of controversies exists how the evaluation of resected lymph nodes should be performed, many of these affecting the clinical significance of lymph node staging in daily routine practice (Table 1). This already starts with varying definitions of lymph nodes as such, lymph nodes involved by metastatic tumor tissue, and their differentiation from tumor deposits, as is reflected by changing criteria in different editions of the AJCC/UICC TNM staging system<sup>[5]</sup>. The number of examined lymph nodes has been identified as an additional important issue. Some investigators claim the lymph node ratio, defined as the number of positive lymph nodes divided by the total number of retrieved nodes, to be more important than the absolute number of positive nodes<sup>[6-9]</sup>. Likewise, the identification of extracapsular invasion by cancer cells may help to improve the prognostic significance of lymph node staging<sup>[10-13]</sup>.

Manual dissection with subsequent histological assessment based on routinely hematoxylin and eosin (HE) stained slides is the standard approach in the examination of regional lymph nodes in cancer specimens (Figure 1)<sup>[14]</sup>. However, some studies have raised the suspicion that analysis based solely on HE stained slides is insufficient for a proper evaluation. This notion has led to the introduction of new techniques, such as sentinel node biopsy, immunohistochemical and molecular analyses in the work-up of cancer specimens<sup>[15]</sup>.

In this review, we will refer to the controversies mentioned above in detail, focusing on both clinical impact and technical issues. Data for this review were compiled using MEDLINE/PubMed and Thomson Reuters Web of Science®, assessing articles published before August 2013. The search terms included colorectal cancer, colon cancer, rectum cancer, TNM classification, lymph node metastasis, lymph node ratio, extracapsular invasion, sentinel lymph node, immunohistochemistry, and molecular analysis. Only articles published in English were considered.

## LYMPH NODE STAGING ACCORDING TO THE AJCC/UICC TNM SYSTEM

Quantitative lymph node evaluation has repeatedly been

**Table 1 Parameters affecting the clinical significance of lymph node staging in colorectal cancer**

Extent of surgical lymph node removal
Thoroughness of the pathologist in dissecting the resection specimen
Technical methods to increase lymph node yield
Methylene blue injection
Fat clearing
Acetone compression
Changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the AJCC/UICC TNM staging system
History of neoadjuvant treatment
Absolute number of retrieved lymph nodes
Absolute number of positive lymph nodes
Lymph node ratio
Presence of extracapsular invasion
Sentinel node biopsy
Number of histological sections
Use of immunohistochemistry to identify micrometastasis and/or isolated tumor cells
Use of molecular techniques to identify minimal tumor disease in lymph node tissue

AJCC: American Joint Committee on Cancer; UICC: Union for International Cancer Control; TNM: Tumor node metastasis.

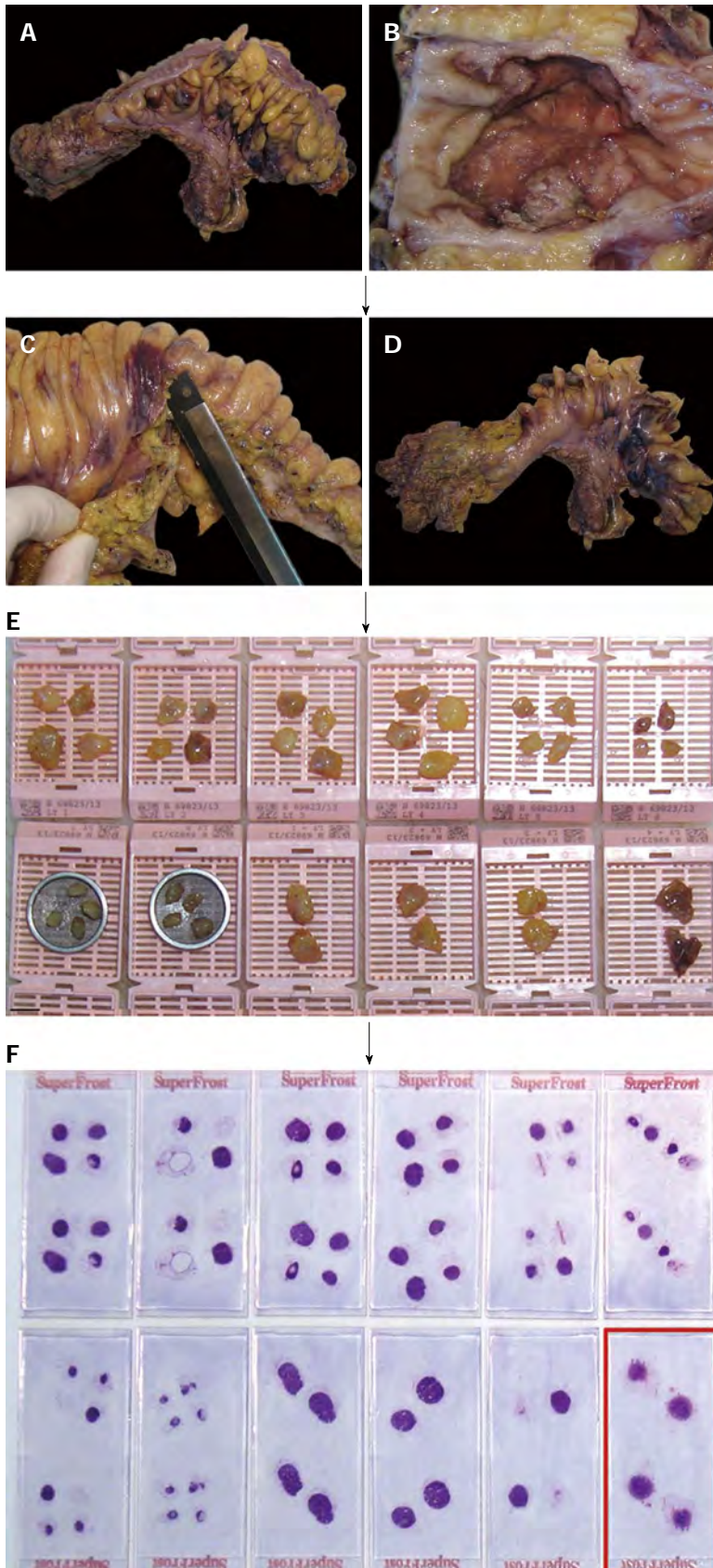
validated as a powerful prognostic tool in patients with colorectal cancer. In particular, the absolute number of positive nodes has been identified as a highly effective predictor of adverse outcome, as shown by worsening of prognosis with increasing number of lymph nodes involved by cancer<sup>[16,17]</sup>.

Hence, in the AJCC/UICC staging system the prognostic stratification of nodal disease is based on the absolute number of positive lymph nodes. Difficulties, however, arise with respect to changing definitions of lymph nodes as such, involved lymph nodes, and/or tumor deposits (satellites) in different editions<sup>[5]</sup>. Tumor deposits are macroscopic or microscopic nests or nodules of cancer found in the pericolic and/or perirectal adipose tissue's lymph drainage area of a primary carcinoma (away from the leading edge of the infiltrating tumor) without histological evidence of residual lymph node in the nodule. They are histologically heterogeneous and may be seen associated with distinct anatomic structures, such as veins<sup>[18]</sup>. These deposits may represent discontinuous primary tumor spread, venous invasion with extravascular spread, or a totally replaced lymph node (Figure 2A-C).

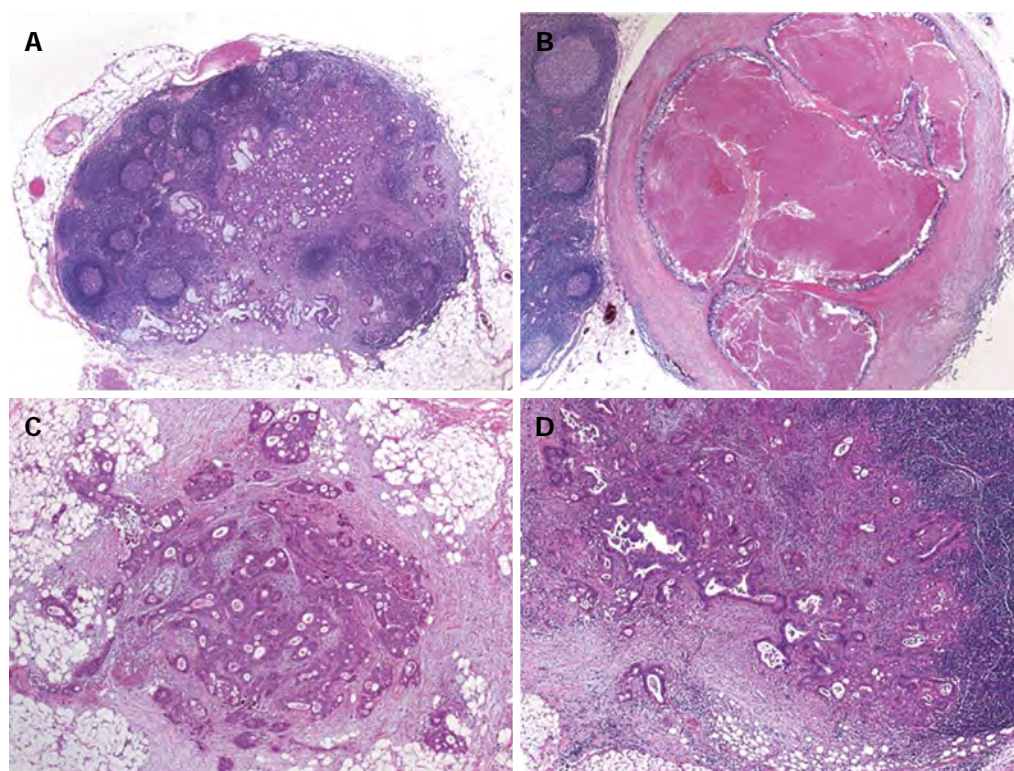
The main differences between the different editions of the AJCC/UICC TNM system regarding lymph node staging are as follows: The 5<sup>th</sup> edition of the TNM system (TNM-5) introduced the 3 mm rule for their classification, providing a tool based exclusively on the size of the lesions<sup>[19]</sup>. The 6<sup>th</sup> edition (TNM-6) discarded the size criterion and referred to the contour of the lesions<sup>[20]</sup>. The 7<sup>th</sup> edition (TNM-7) focused on the differentiation of lymph node metastases from tumor deposits, including the latter in the pN category (pN<sub>1c</sub>)<sup>[21]</sup>. Details are presented in Table 2.

Nagtegaal *et al*<sup>[5]</sup> have proven lymph node staging according to TNM-5 to be superior to TNM-6, as demon-





**Figure 1** Manual dissection with subsequent histological assessment based on routinely hematoxylin and eosin stained slides is the standard approach in the examination of regional lymph nodes in cancer specimens. A: Rectum cancer specimen of a 56-year-old female; B: Ulcerated primary tumor, measuring 5 cm in largest diameter; C: After preparation of the primary tumor (including the fatty tissue underneath the lesion and the circumferential margin) the remaining perirectal/mesocolic fatty tissue is carefully removed; D: Specimen for subsequent manual lymph node dissection; E: 36 presumed lymph nodes are isolated, of which the largest four are cut into halves and embedded on their own, respectively (lower right); F: 31 lymph nodes are confirmed on hematoxylin and eosin stained slides, one of which with metastatic cancer tissue (encircled).



**Figure 2** Lymph node metastases and tumor deposits in patients with colorectal cancer. A: Metastatic adenocarcinoma within a mesocolic lymph node [hematoxylin and eosin (HE) original magnification,  $\times 100$ ]; B: Mesocolic lymph node totally replaced by metastatic cancer tissue, note the smooth contour of the lesion (HE, original magnification,  $\times 150$ ); C: Tumor deposit (satellite) within the mesocolic fatty tissue, note the irregular contour of the lesion (HE, original magnification,  $\times 250$ ); D: Mesocolic lymph node metastasis with extracapsular extension of cancer tissue (original magnification,  $\times 250$ ).

strated in two independent populations. Therefore, several national guidelines in Europe still refer to TNM-5 for classification. It is simpler, more reproducible, allows for comparison with preoperative imaging, and is effective and accurate<sup>[5]</sup>. The potential value of TNM-7 remains to be evaluated in larger prospective studies. The fact that all patients with tumor deposits will now be classified in the node-positive group has raised major concerns. This holds true particular for the evaluation of tumor regression and residual tumor foci after neoadjuvant therapy. In the group of patients who did not receive preoperative treatment, however, staging according to TNM-7 appears to be highly prognostic and possibly superior to TNM-5 and TNM-6<sup>[5]</sup>. The reproducibility of the definitions given in the latest version may, however, be imperfect. In a recent interobserver variability study of lymph nodes and tumor deposits by Rock *et al.*<sup>[22]</sup>, seven gastrointestinal pathologists completely agreed on only 11 of 25 lesions ( $\kappa$ -value 0.48; 95%CI: 0.28-0.67). Top-ranked features for the differentiation of lymph node metastases from tumor deposits included round shape, peripheral lymphocyte rim, peripheral lymphoid follicles, subcapsular sinus, residual lymph node surrounding fibroadipose tissue, and thick capsule. As inconsistency remains even under expert pathologists, it is currently unclear whether the criteria that are available for the distinction of lymph node metastases from tumor deposits are feasible in everyday routine practice performed by general pathologists.

## MINIMUM NUMBER OF LYMPH NODES

Adequate assessment of nodal status depends on the total number of retrieved lymph nodes that are available for histological evaluation. A recommendation put forward by Fielding *et al.*<sup>[23]</sup> stated the ideal minimum to be 12 nodes since below this cut-off value there is a high risk of false-negative reporting of lymph node involvement due to inadequate sampling<sup>[16]</sup>. This recommendation was adopted by the AJCC/UICC TNM system and has been included in various clinical practice guidelines<sup>[2-4,24]</sup>. The minimum number of lymph nodes that should be assessed ensures adequate staging, prognostication, and accurate treatment, since affected lymph nodes are the primary determinant for the use of adjuvant chemotherapy.

The variability in the number of retrieved lymph nodes remains to be a major problem in patient management since often the recommended minimum number of 12 lymph nodes is not achieved. This may be due to differences in the extent of surgical lymph node removal, the thoroughness of the pathologist in dissecting the cancer specimen, and/or the actual number of regional lymph nodes that is related to tumor location<sup>[25,26]</sup>. In rectal cancer, the increasing use of neoadjuvant therapy represents another important factor affecting lymph node yield. Under combined chemo- and radiotherapy regional lymph nodes undergo a process of regression. Thus, the recommended number of 12 lymph nodes was



**Table 2 Changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the American Joint Committee on Cancer/Union for International Cancer Control tumor node metastasis staging system**

TNM-5	A tumor nodule greater than 3 mm in diameter in perirectal or pericolic adipose tissue without histological evidence of a residual lymph node in the nodule is classified as regional lymph node metastasis. However, a tumor nodule up to 3 mm in diameter is classified in the T category as discontinuous extension, <i>i.e.</i> , T3
TNM-6	A tumor nodule in the pericolic/perirectal adipose tissue without histological evidence of residual lymph node in the nodule is classified in the pN category as a regional lymph node metastasis if the nodule has the form and smooth contour of a lymph node. If the nodule has an irregular contour, it should be classified in the T category and also coded as V1 (microscopic venous invasion) or V2, if it was grossly evident, because there is a strong likelihood that it represents venous invasion.
TNM-7	Tumor deposits (satellites), <i>i.e.</i> , macroscopic or microscopic nests or nodules, in the pericorectal adipose tissue's lymph drainage area of a primary carcinoma without histological evidence of residual lymph node in the nodule, may represent discontinuous spread, venous invasion with extravascular spread (V1/2) or a totally replaced lymph node (N1/2). If such deposits are observed with lesions that would otherwise be classified as T1 or T2, then the T classification is not changed, but the nodule(s) is recorded N1c. If a nodule is considered by the pathologist to be a totally replaced lymph node (generally having a smooth contour), it should be recorded as a positive lymph node and not as a satellite, and each nodule should be counted separately as lymph node in the final pN determination.

TNM: Tumor node metastasis.

reached in only about 20% of cases in large international trials that investigated the benefit of neoadjuvant therapy in rectal cancer. This observation prompted the question whether the insufficient number of lymph nodes is due to the disappearance of the nodes, or just reflects progressive atrophy and fibrosis with subsequent reduction in lymph node size, rendering them undetectable during routine pathological work-up<sup>[27]</sup>.

Due to the fact that the recommended number of nodes is often not reached by traditional manual dissection new technical methods were introduced to facilitate lymph node harvest in the fatty tissue. These include fat clearing methods, methylene blue-assisted lymph node dissection, as well as acetone elution with subsequent compression of adipose tissue ("acetone compression"). The method of methylene blue-assisted lymph node dissection was introduced in 2007 as a cheap and simple tool<sup>[28]</sup>. The method is based on *ex vivo* intraarterial injection of 15-20 mL of methylene blue solution in the fresh or shortly formalin-fixed resection specimen. After fixing overnight lymph nodes are dissected manually. This technique results in dramatically increased lymph node counts compared to conventional dissection. The effect is particularly evident in rectal cancer patients after neoadjuvant therapy and helps to ensure a sufficient lymph node harvest in these patients. However, according to a

recently published study<sup>[29]</sup>, the application of this technique does not seem to be associated with an increased detection of lymph node metastases. In this study, comparing methylene blue assisted dissection with standard dissection, neither the rate of nodal positive cases, nor the rate of pN<sub>2</sub> cases differed between the two groups. The most probable explanation for this finding is the fact that mostly involved lymph nodes are enlarged and therefore easy to find<sup>[30]</sup>.

The acetone elution and compression method was introduced by Basten *et al.*<sup>[31]</sup>. After manual dissection for large palpable lymph nodes (usually > 1 cm in diameter) the mesorectal fat is perforated with a needle roller and transferred to acetone. After elution in acetone, tissue samples are mechanically compressed using a manual squeezing machine, as described in detail by Gehoff *et al.*<sup>[27]</sup>. By this method a reduction of about 90% of mesorectal fat volume is achieved. Specifically, acetone compression facilitates the detection of any tumor deposit in mesorectal and mesenteric fatty tissue and therefore provides a reliable survey of tumor cell deposits including perineural cancer infiltrates, particularly after neoadjuvant therapy<sup>[27]</sup>. As for methylene blue-assisted lymph node dissection, the total number of harvested lymph nodes markedly increased in that study, the number of positive lymph nodes, however, did not change. From a biological standpoint it is interesting to note that, basically, the number of lymph nodes is independent of pretreatment status<sup>[27]</sup>.

## LYMPH NODE RATIO OR ABSOLUTE NUMBER OF INVOLVED LYMPH NODES?

Several studies have demonstrated that simply the analysis of a larger number of lymph nodes results in a survival advantage for patients with stage II and III disease, while the situation for stage I disease is less clear<sup>[32-36]</sup>. A study by Lykke *et al.*<sup>[36]</sup> demonstrated that in patients with more than 12 nodes, there was a significantly higher proportion of stage III disease, indicating that stage migration takes place when high numbers of lymph nodes are harvested. To overcome the dependence on the number of harvested lymph nodes, a ratio-based node staging system has been proposed.

The lymph node ratio, defined as the number of positive lymph nodes divided by the total number of retrieved nodes, has gained increasing attention. A large number of studies showed that the prognostic significance of lymph node ratio is superior to that of the absolute number of involved lymph nodes<sup>[6,8,36-44]</sup>. Lymph node ratio was identified as an independent predictor of disease-free survival, overall survival, and cancer-specific survival in stage III disease. Notably, lymph node ratio remains to be an independent prognosticator even after neoadjuvant therapy, despite reduction of the absolute number of retrieved nodes<sup>[45]</sup>. The lymph node ratio may thus improve TNM-based prognostic stratification and may help to identify patients at high risk of disease recurrence and/or

progression.

Problems, however, remain, particularly as different cut-off values were applied in the studies that identified lymph node ratio as promising tool. Currently, we do not know which cut-off value is ideal and whether this value may be the best for both colon and rectum cancer. Future prospective studies, applying a data-driven approach are urgently needed to accurately define these cut-off values, as obviously “not one size fits all”<sup>[40]</sup>.

Although the concept of lymph node ratio was developed to generate a prognostic marker that is independent from the number of examined nodes data are still conflicting in this regard. According to Chen *et al.*<sup>[43]</sup>, lymph node ratio independently estimates survival, irrespective of the number of nodes examined. In the study by Berger *et al.*<sup>[38]</sup>, lymph node ratio was a significant parameter when 10 or more lymph nodes were removed, but not for patients with less than 10 lymph nodes.

## EXTRACAPSULAR LYMPH NODE INVASION

Extracapsular lymph node invasion refers to the extension of cancer cells through the nodal capsule into the perinodal fatty tissue (Figure 2D)<sup>[46]</sup>. This phenomenon has gained considerable attention as prognostic variable in several solid organ tumors, particularly in cancers originating from breast and head and neck region as well as in several gastrointestinal malignancies.

In colorectal cancer, extracapsular invasion has been observed in 18% to 68% of stage III tumors<sup>[10-13,46]</sup>. According to Komuta *et al.*<sup>[10]</sup>, extracapsular invasion occurs more likely in lymph nodes that are occupied for more than 50% by cancer cells, compared to lymph nodes with less than 50% occupation. Its occurrence has been related to high pT-classification, high number of involved nodes, and presence of positive distant lymph nodes, which allows the conclusion that extracapsular invasion is more likely to be found in advanced tumor stage<sup>[11-13]</sup>.

The ability of metastatic nodes to recruit degradation factors that permit cancer cells to break through the lymph node capsule reflects the invasiveness and aggressiveness of the primary tumor, even in an immunologically hostile environment<sup>[46]</sup>. Thus, patients with extracapsular invasion at metastatic sites are at particularly high risk to develop disease progression and distant cancer spread<sup>[12,13]</sup>. In particular, survival and recurrence rates of patients with extracapsular invasion are significantly worse than those of patients without, and extracapsular invasion has been identified as independent predictor of disease-free and overall survival in patients with node positive cancers<sup>[11,12,47,48]</sup>.

Overall, the detection and, possibly, quantification of extracapsular invasion may help to individualize post-operative treatment strategies by identification of a subgroup of patients with significantly poorer long-term survival and poorer local control who might benefit from intensified adjuvant therapy<sup>[46]</sup>.

## SENTINEL LYMPH NODE BIOPSY

The sentinel lymph node, defined as the first lymphatic station within a given lymph drainage area, is considered to be of eminent importance in oncologic practice. Sentinel node detection may be accomplished by injection of blue dye (*e.g.*, methylene blue) or radiotracers near to the tumor. Afterwards the surgeon detects the node by visual inspection or by use of gamma probe or Geiger counter. In clinical practice, sentinel lymph node biopsy has been found to be highly effective in correctly predicting the nodal status in malignant melanoma and breast cancer<sup>[49]</sup>. Commonly, a frozen section procedure is employed so if neoplasia is detected further lymph node dissection may be performed. If, however, the sentinel node is free of cancer the extent of operation may be kept to a minimum.

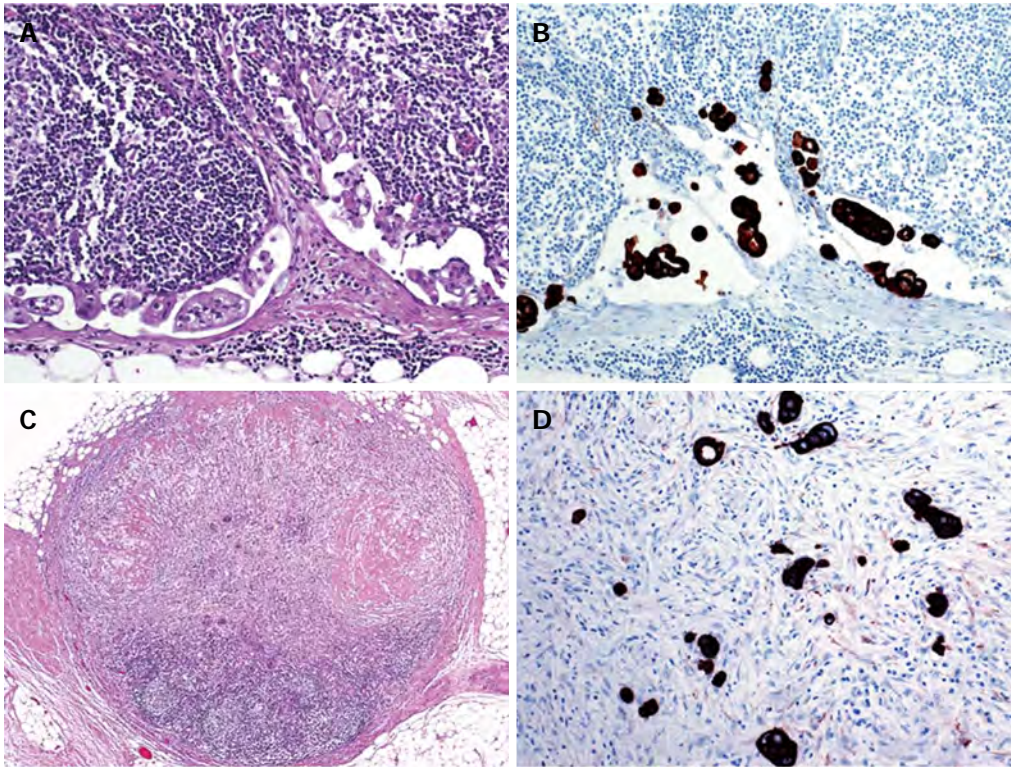
Within the last two decades, several investigators aimed to enlarge the field of application and have evaluated sentinel lymph node biopsy in various malignancies<sup>[50]</sup>. In colorectal cancer, the potential benefit of sentinel lymph node biopsy is different from that of malignant melanoma and breast cancer. Here, the method does not intend to reduce the extent of surgery but aims to identify conditions that might lead to more extensive surgical lymph node dissection. Another purpose is to establish more accurate lymph node staging in order to identify patients at risk for disease recurrence and/or progression<sup>[51]</sup>.

According to a recent meta-analysis<sup>[52]</sup>, the pooled sentinel node identification rate is approximately 90% in patients with colorectal cancer, with a significantly higher rate in studies including more than 100 patients or studies using an *ex vivo* approach. The pooled sensitivity of the procedure is approximately 70%. Subgroups with significantly higher sensitivity could be identified. These include individuals with  $\geq 4$  sentinel nodes identified (*vs* individuals  $< 4$  nodes), colonic location (*vs* rectal location), and early, *i.e.*, pT<sub>1/2</sub> carcinomas (*vs* advanced, *i.e.*, pT<sub>3/4</sub> carcinomas).

How sentinel lymph node biopsy may be successfully incorporated in routine practice has recently been illustrated in a study by Saha *et al.*<sup>[53]</sup>. The authors investigated 192 patients undergoing surgery for colon cancer and identified aberrant drainage, *i.e.*, drainage outside the standard resection margin requiring change of the extent of operation, in 22% of patients. Notably, nodal positivity was higher in patients undergoing change of operation (62%) compared to those undergoing only standard oncologic resection (43%).

Major drawbacks remain to be the still imperfect detection rate and the comparably low sensitivity for the identification of nodal disease. The detection rate is significantly influenced by several patient- and disease-specific factors, the most important of which being body mass index, center experience, and learning curve<sup>[49]</sup>. The considerably high false-negative rate to identify node-positive patients may be explained by aberrant drainage sites and the presence of skip lesions. It is known that skip lesions





**Figure 3** Value of immunohistochemistry in the evaluation of lymph nodes from patients with colorectal cancer. A: Micrometastasis in the subcapsular sinus of a mesocolic sentinel node evaluated by standard hematoxylin and eosin (HE) staining (original magnification,  $\times 400$ ); B: Micrometastasis in the subcapsular sinus of a mesocolic sentinel node evaluated by immunohistochemistry using an antibody preparation directed against pankeratin (serial section to A, original magnification,  $\times 400$ ); C: Atrophic perirectal lymph node with marked fibrosis after neoadjuvant treatment (original magnification,  $\times 100$ ); D: Identification of residual cancer cells by pankeratin immunostaining (original magnification,  $\times 400$ ).

occur when lymphatics are obstructed by tumor. Retter *et al*<sup>[54]</sup> showed that in 63% of their false negative tumors, lymphatic and venous invasion by cancer cells was present.

The extent of the pathological work-up is another major factor with significant impact on the performance and clinical significance of the sentinel node biopsy concept. According to the meta-analysis cited above, adding step sectioning and immunohistochemistry, *e.g.*, using antibodies directed against pankeratin (Figure 3), to the pathological work-up resulted in a mean upstaging in 18.9% (range 0%-50%). True upstaging defined as micrometastases [pN1(mi)] rather than isolated tumor cells [pN0(i+)] occurred in 7.7%<sup>[52]</sup>. The optimal technical method how sentinel lymph nodes should be evaluated still has to be defined. Several papers have addressed this topic, the three most relevant will be referred to in detail.

In the study by Bembenek *et al*<sup>[49]</sup>, a total of 141 of 186 patients classified as nodal negative by routine HE staining underwent step sectioning and immunohistochemical analysis for pankeratin (MNF116) of their sentinel lymph nodes. Thirty of these patients revealed micrometastases ( $n = 7$ ) or isolated tumor cells ( $n = 23$ ), resulting in an overall upstaging rate of 30 of 141 (21.3%). In the clinically important subgroup of stage II patients, upstaging occurred in 24.2% (21 of 91).

In the study by van der Zaag *et al*<sup>[51]</sup>, three serial sections (cut at 500  $\mu$ m intervals) of all 908 lymph nodes

from 58 patients with pN<sub>0</sub> carcinomas (according to standard evaluation on HE stained slides) were examined with three different antibodies [directed against pankeratin (Cam5.2), keratin 20, and Ber-EP4]. The examination revealed occult tumor cells in 33% (19 of 58) of histologically pN<sub>0</sub> patients (12% micrometastases and 21% isolated tumor cells). Occult tumor cells were predominantly found in sentinel nodes with an overall sensitivity of sentinel mapping for occult tumor cells of 88%.

In the study by Märkl *et al*<sup>[53]</sup>, applying methylene blue injection in an *ex vivo* approach lymph node metastases were found in 20 of 47 (43%) cases with skip metastases occurring in four of them. Performing three additional HE step sections and immunohistochemical staining for pankeratin (MNF116) in sentinel lymph nodes and all other lymph nodes, resulted in true upstaging (N0N1mi) in 1 of 23 cases (4%).

## MOLECULAR ANALYSIS OF LYMPH NODES - A FEASIBLE APPROACH?

The identification of lymph node involvement is the most important factor to predict outcome and qualify affected patients for adjuvant chemotherapy<sup>[55]</sup>. Manual dissection of fatty tissue and histopathology based on HE stained sections remain to be the standard approach in pathological lymph node evaluation.

**Table 3** Markers for molecular lymph node staging

Keratin 20
Keratin 19 (including one-step nucleic acid amplification technique)
Mucin apoprotein 2
Guanylyl cylase C
Carcinoembryonic antigen
CEACAM6
CEACAM1-S
CEACAM1-L
CEACAM7-1
CEACAM7-2
c-Met
K-ras mutation
Estrogen receptor promoter methylation

This may, however, lead to underestimation of disease and understaging of patients. About 30% of the patients with histopathology-negative lymph nodes (AJCC/UICC TNM stages I and II) develop recurrent and/or progressive disease, likely associated with undetected metastatic deposits<sup>[15,56-59]</sup>. As shown above, the use of additional step sections and immunohistochemistry may improve the identification of positive lymph nodes. Of note, many patients initially staged lymph node-negative, who experienced disease recurrence had isolated tumor cells and/or micrometastases after advanced evaluation<sup>[60]</sup>. A major limitation of the histological examination is the fact that only a small portion of the lymph node, usually the section(s) with the largest cut surface, is assessed leaving most parts of the nodes uninspected<sup>[61]</sup>.

As current techniques for nodal examination may be inadequate for the detection of micrometastases and/or isolated tumor cells, molecular analysis of lymph node tissue has been introduced as additional tool in the work-up of cancer patients. The identification of minimal disease in lymph nodes by molecular techniques may help to identify patients at high risk for recurrence and/or progression, who could benefit from adjuvant therapy<sup>[62]</sup>. The following features are relevant: (1) no expression of the respective marker in immune cells; (2) no or weak downregulation in tumors compared to normal tissue; and (3) relatively high and constant expression in tumor tissue irrespective of tumor stage<sup>[63]</sup>. Several molecular markers have been applied (Table 3). In the following we will refer to some of them in detail.

Keratin 20 (K20) is constitutively expressed in intestinal epithelia and is the most important keratin subtype expressed in colorectal cancer. It can be found in more than 90% of primary tumors. Immunoreactivity in metastatic tissues is known to match well with that of corresponding primary tumors, with high concordance for lymph node and distant metastases, respectively<sup>[64]</sup>. The significance of quantitative real-time polymerase chain reaction (RT-PCR) for the detection of K20 mRNA in regional lymph nodes of cancer patients has been investigated by several groups, mainly in sentinel node biopsies<sup>[15,57,65-71]</sup>. In general, these studies demonstrated a higher sensitivity of molecular analysis compared to standard evaluation based on HE stained slides and also

compared to advanced evaluation applying immunohistochemistry.

MUC2 apoprotein, which is secreted from non-neoplastic intestinal goblet cells and is expressed in the majority of colorectal cancers, has been introduced as another promising marker<sup>[15,63,72,73]</sup>. Some groups investigated carcinoembryonic antigen<sup>[63,68,69,72,74]</sup>, while others referred to guanylyl cylase C (GCC)<sup>[58,68,75]</sup>. GCC is a receptor for bacterial enterotoxins and the paracrine ligands guanylin and uroguanylin and is expressed selectively by intestinal epithelium. Comparable to mucin apoprotein 2 (MUC2), the expression of GCC is preserved throughout the transition from adenoma to carcinoma in colorectal tissues<sup>[56,58]</sup>. Most recently, so-called one-step nucleic acid amplification (OSNA) has been introduced to detect keratin 19 (K19) mRNA as a surrogate for lymph node metastasis. K19 is expressed in many types of cancer, albeit in varying frequencies. OSNA is based on reverse transcription-loop-mediated isothermal amplification to amplify K19 mRNA<sup>[59,61,76]</sup>.

All these techniques allow the examination of the entire lymph node, thereby overcoming the problem of sampling bias due to insufficient analysis of material in the standard histological approach. This may lead to improved staging and better selection of patients for adjuvant chemotherapy. More importantly the molecular detection of tumor cells in regional lymph nodes has been associated with disease recurrence and poor survival in node-negative colorectal cancer<sup>[14,77]</sup>.

Problems, however, remain. The value of quantitative RT-PCR assays for the detection of occult tumor cells in regional lymph nodes relies on the balance between sensitivity and specificity in order to minimize the occurrence of false-positive or false-negative results<sup>[78]</sup>. None of the markers are really specific. K19 has been used as a molecular marker in a variety of studies dealing with several types of cancer, including colorectal cancer. Doubt has arisen about the tissue specificity of K19 gene expression. Already in 1996, Gunn *et al.*<sup>[79]</sup> noted *K19* gene expression in 34 of 40 lymph nodes from patients who underwent bowel resection for benign disease. The reasons for the observed false-positivity rate are not entirely clear. In addition to simple contamination or dissemination of tumor cells and/or tumor cell fragments *via* the lymphatics during the procedure, amplification of *K19* pseudogenes may play a role<sup>[78]</sup>. Finally, Bustin *et al.*<sup>[68]</sup> detected K20, carcinoembryonic antigen, and GCC mRNA in 47%, 89% and 13% of 149 lymph nodes, respectively from patients with benign disease indicating that K19 is not the only marker for which specificity problems remain to be solved.

Nevertheless, the molecular approach has opened new options concerning the diagnosis of isolated tumor cells and micrometastases in patients with histopathology-negative lymph nodes<sup>[57]</sup>. Benefits of the molecular approach have to be weighed against potential drawbacks. A major reason for controversy is the lack of standardization of molecular analyses hampering comparison of different studies as well as inclusion of molecular techniques into



routine practice<sup>[57]</sup>. According to current practice guidelines, AJCC/UICC stage III patients receive adjuvant treatment. This strategy results in significantly improved outcome when nodal disease is proven histologically. However, it is currently not entirely clear whether the patients with nodal disease proven on a molecular level experience similar benefits if chemotherapy is given.

## CONCLUSION

Lymph node staging is a major prognostic factor in colorectal cancer and remains to be the most important criterion to select patients for adjuvant treatment. Changing definition of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the AJCC/UICC TNM system have influenced the significance of lymph node staging in the past. The standard approach for lymph node evaluation is based on manual dissection and histological evaluation of HE stained slides. Methylene blue injection and fat clearing methods increase lymph node harvest in cancer specimens. Adding step sectioning and immunohistochemistry to the pathological work-up may result in higher accuracy of histological diagnosis. The clinical value of more recent techniques, such as sentinel lymph node biopsy and molecular analysis of lymph nodes tissue still remains to be defined.

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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

# Different standards for healthy screenees than patients in routine clinics?

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## Abstract

Less than 5% of colorectal adenomas will become malignant, but we do not have sufficient knowledge about their natural course to target removal of these 5% only. Thus, 95% of polypectomies are a waste of time exposing patients to a small risk of complications. Recently, a new type of polyps, sessile serrated polyps, has attracted attention. Previously considered innocuous, they are now found to have molecular similarities to cancer and some guidelines recommend to have them removed. These lesions are often flat, covered by mucous, not easily seen and situated in the proximal colon where the bowel wall is thinner. Thus, polypectomy carries a higher risk of perforation than predominantly left-sided, stalked adenomas - and we do not know what is gained in terms of cancer prevention. Screening is a neat balance between harms and benefit for presumptively healthy participants not interested in risk exposure to obtain confirmation of being healthy. The situation is quite different for patient worried about symptom. Thus, the standards set for evidence-based practice may be higher for screening than for routine clinics - a mechanism which may benefit patients in the long run.

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**Key words:** Colonoscopy; Screening; Quality assurance; Standards

**Core tip:** There is a basic difference in incitements to attend for screening when you are healthy and for routine clinics when you are ill. This article points out logical mechanisms which may set standards for screening higher than for routine clinics, but this may prove to be of benefit for clinical services and patients in the long run. This is highlighted by sessile serrated polyps which were previously classified as innocuous hyperplastic polyps. Recent guidelines now recommend polypectomy of these lesions for cancer prevention, but we do not know the benefit gained - only the increased risk of perforation by polypectomy.

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Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8527.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8527>

## MECHANISMS FAVOURING DIFFERENT STANDARDS

We know that there are endoscopist-dependent variations in colonoscopy performance - whether this service is provided in routine clinics or screening<sup>[1-4]</sup>. Quality assurance (QA) initiatives driven by health care providers may be half-hearted - particularly when demands for colonoscopy outnumber available capacity and reducing unacceptable waiting lists is first priority. Within the European Union, however, it has been stated explicitly for screening that only organized screening that can be evaluated is to be

accepted, “performance indicators should be monitored regularly” and the population should be protected from “poor-quality screening”<sup>[5]</sup>. Independent of such policy statements for QA which may have its counterparts in clinical non-screening services in many countries, client or patient-driven QA may have a stronger impact in screening programmes than in routine clinics. The option of not attending if the quality is sub-standard is both more realistic and a dreadful threat to screening programmes compared to routine clinical services. Whichever colorectal cancer (CRC) screening method is used, high attendance rates are crucial for the success of any screening programme - “the best screening test is the one that gets done”<sup>[6]</sup>.

There is a basic difference between screening participants and patients. Screenshoters are presumptively healthy individuals who seek confirmation that they are just that - healthy. Patients have symptoms and disease for which they seek whatever help may be offered. This means that patients may be more willing to accept some risk of complications, harms and discomfort to be cured. It is reasonable that screenshoters are not willing to subject themselves to risks and discomfort to obtain confirmation of being healthy.

**Screening participants:** Presumptively healthy seeking confirmation of being healthy. Not willing to take risks to obtain this confirmation. They request documentation of benefits and harms - “what is in it for me?”

**Patients:** They have symptoms or known disease for which they seek whatever help they may be offered. It may be a matter of clinging to a hope of cure with great willingness to pay and few questions asked on documentation of effect - “please, just do something”

Since high attendance rates are crucial for screening programmes, it is important to understand the reasons for non-attendance. This is far from a primary issue in routine clinics serving patients. In focus groups addressing CRC screening, both representatives of target populations and family doctors have expressed scepticism to screening, questioning the evidence of its effectiveness<sup>[7]</sup>. To meet these critics, facts about risks and benefits and defining fields of uncertainty must be produced and made accessible in a trustworthy and understandable format to provide a basis for informed decision-making by members of the target population<sup>[8,9]</sup>. This is quite a different exercise from campaigning for screening by appealing to fear, guilt and personal responsibility - methods that may have been used too frequently in the short history of screening to improve attendance<sup>[10]</sup>. Such campaigning will only tear down any trustworthiness there may have been. There should be a strong incitement to provide high-degree level of evidence to support (or discard) screening - evidence that can withstand scepticism and critics generated by poor-level evidence and over-selling screening services<sup>[10]</sup>.

## SIZE OF THE PROBLEM AND THE HIGH INTENTIONS OF DOING GOOD

On a worldwide basis, there are more than 1.2 million new cases of CRC diagnosed annually with prospects of 5-year survival for 50%-60% of patients<sup>[11,12]</sup>. Symptoms often appear late and they are unspecific - mimicking common and more trivial conditions like haemorrhoids and irritable bowel. Although progress is being made on treatment of advanced CRC, new drugs are driving costs, but the best bet for cure remains early diagnosis and surgery. Both to get at the cancer at an early, asymptomatic stage to save lives and suffering - and to save costs for treatment of advanced disease<sup>[13]</sup>, CRC screening is recommended in several countries<sup>[14]</sup>. There are several screening methods, but only fecal occult blood tests (FOBT) and flexible sigmoidoscopy (FS) have been subjected to randomized trials (RCT) with long-term follow-up<sup>[15-18]</sup>. By intention-to-treat analyses, FOBT screening reduces CRC mortality by 15%-18% with no effect on CRC incidence. FS screening reduces mortality by 28% and incidence by 18%<sup>[18]</sup>. Intuitively, colonoscopy screening should be twice as good as FS (“half-way colonoscopy”) combining “gold standard” sensitivity for CRC and polyp detection with tissue sampling and removal of CRC precursor lesions (polyps). There are RCTs on colonoscopy underway, but results are not expected for many years<sup>[19,20]</sup>. Retrospective studies, however, have suggested that colonoscopy screening may not be as effective as expected in reducing right-sided CRC<sup>[21]</sup>. It has been suggested that right-sided (proximal) sessile serrated polyps, which are easily overlooked and share molecular similarities to CRC, may represent an additional polyp-carcinoma pathway similar to the traditional adenoma-carcinoma pathway<sup>[22]</sup>. This may explain poorer results than expected for colonoscopy in reducing the burden of right-sided CRC. When the trials on FOBT and FS screening were done, endoscopists and pathologists largely considered sessile serrated polyps to be hyperplastic and non-neoplastic with no intrinsic potential to develop into CRC. Changing to go aggressively for these right-sided sessile lesion has its implications (e.g., higher risk of perforation at polypectomy) and we really do not know what there is to be gained - *i.e.*, we cannot quantify expectations of a reduced risk of CRC.

## OVERTREATMENT WITH A FEAR OF NOT DOING ENOUGH

Screening is a neat balance between benefits and harms - benefit for the few (those few discovered to have asymptomatic CRC or advanced adenoma) *vs* inconvenience and potential risks for the many (all other participants). Providing data on CRC mortality and/or incidence reduction is a prerequisite before implementing screening



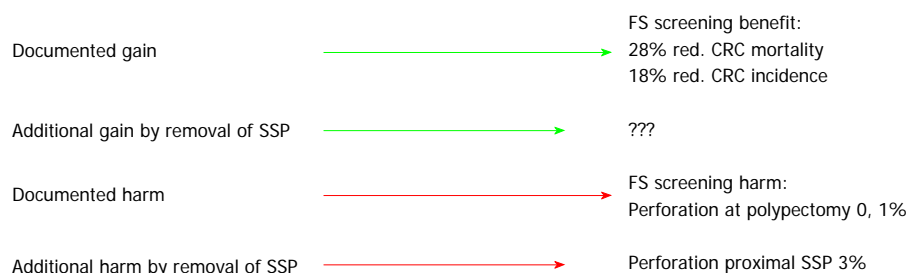


Figure 1 Overtreatment with a fear of not doing enough. Introducing uncertainty in the balance between benefits and harms of flexible sigmoidoscopy (FS) screening by adding systematic removal of proximal sessile serrated polyps (SSP) associated with 3% risk of perforation for these lesions<sup>[23]</sup> and unknown benefit compared to 0.1% risk of perforation<sup>[27]</sup> and known benefit. CRC: colorectal cancer.

programmes<sup>[15]</sup>, but the target population should also receive valid information on the downsides of screening, like the risk of perforation and bleeding when polypectomy is recommended. We now have long-term results from RCTs on FOBT and FS screening based on the standards used in the trials, including work-up colonoscopies and surgical treatment, and we can provide the target population with information of what is to be gained in terms of mortality and incidence reduction and the risks involved with endoscopy, polyp removal and surgery when required. This is very much a satisfactory level of practicing “evidence-based medicine”.

Our current practice of polyp treatment and surveillance is largely based on consensus guidelines. If we change our practice in screening programmes from the standards used in trials preceding the programmes, we do this because we believe such adjustments are for the good. The intentions may be the best, but is the evidence up to standards required for the target population to feel it worthwhile attending for screening? RCTs on FS screening give 18% reduced risk of CRC with a 0.04% risk of perforation and 0.1% risk of perforation at work-up colonoscopy<sup>[18]</sup>. But - more meticulous search and removal of proximal sessile serrated polyps may involve a risk of 3% for severe complications (perforation and bleeding) for these lesions<sup>[23]</sup> with no evidence of what to be gained (Figure 1). This is a level of uncertainty that may not be questioned by patients, but more likely tilt the decision of the potential screenee towards not attending.

Overdiagnosis and overtreatment of cancer is a recent issue that has emerged from screening activity - not from routine clinical work<sup>[24]</sup>. For CRC, we know that more than 95% of polypectomies are a waste of time involving unnecessary risks, but we do not know which 5% to go for. After more than 120 years of the adenoma-carcinoma sequence theory<sup>[25]</sup>, we do not know the natural history of adenomas. We can say very little about future risk of CRC in a polypectomized adenoma - had it not been removed. It is desirable with better definition and targeting of high-risk polyps to be removed and low-risk lesions to be ignored at colonoscopy. It is hard to see how this knowledge-gap can be filled without accepting prospective studies on in-situ polyps. With a low risk for complications at polypectomy, this may not be acceptable. Moving towards more aggressive interventions without knowing the mag-

nitude of expected benefit, we may eventually reach a line when screening either is to be stopped or modified due to complications. At that point in time the problems of overtreatment may become so pronounced that in-situ research with all possible security measures may be accepted. There may be more at stake for screening programme providers and participants (screenees) on this issue than for patients, and it may be that comparative effectiveness research (CER) within screening programmes<sup>[26]</sup> may provide possibilities to fill this and other knowledge-gaps - also for the benefit of clinical practice. Among 45 original publications on the main study and sub-studies published so far from the Norwegian Colorectal Cancer Prevention trial (NORCCAP) there were several findings of transfer value to routine clinical practice - particularly on endoscopy technique and technology (listed in [www.kreftregisteret.no/norccap](http://www.kreftregisteret.no/norccap)).

## CONCLUSION

There is a basic difference in incitements to attend for screening when you are healthy and for routine clinics when you are ill. This may be more clearly brought forward by an increasing demand for patients and clients to have a say in QA of health care provisions - both in screening and routine clinics. There are logical mechanisms which may set standards for screening higher than for routine clinics, but this may prove to be of benefit for clinical services and patients in the long run.

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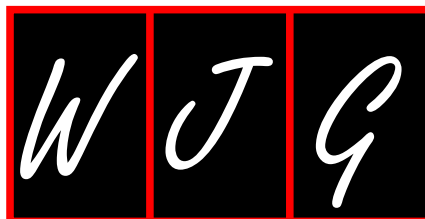
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## WJG 20<sup>th</sup> Anniversary Special Issues (5): Colorectal cancer

### Immunotherapy for colorectal cancer

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py and radiation therapy can improve survival rates, it is imperative to integrate alternative strategies such as immunotherapy to improve outcomes for patients with advanced CRC. In this review, we will discuss the effect of immunotherapy for inducing cytotoxic T lymphocytes and the major immunotherapeutic approaches for CRC that are currently in clinical trials, including peptide vaccines, dendritic cell-based cancer vaccines, whole tumor cell vaccines, viral vector-based cancer vaccines, adoptive cell transfer therapy, antibody-based cancer immunotherapy, and cytokine therapy. The possibility of combination therapies will also be discussed along with the challenges presented by tumor escape mechanisms.

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**Key words:** Colorectal cancer; Cytotoxic T lymphocyte; Dendritic cell; Immunotherapy; Vaccine

**Core tip:** The prognosis for patients with recurrent or metastatic colorectal cancer (CRC) is extremely poor. Immunotherapy may be effective for treating CRC patients and/or preventing relapse. The immunotherapeutic approaches for CRC, including peptide vaccines, dendritic cell-based cancer vaccines, whole tumor cell vaccines, viral vector-based cancer vaccines, adoptive cell transfer therapy, antibody-based cancer immunotherapy, and cytokine therapy have been demonstrated. The blockade of multiple immune regulatory checkpoints combined with immunotherapy and/or conventional chemotherapy may be effective in treating patients with advanced CRC.

### Abstract

The incidence of colorectal cancer (CRC) is on the rise, and the prognosis for patients with recurrent or metastatic disease is extremely poor. Although chemothera-

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## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men (accounting for 10.0% of all cancers) and the second most common cancer in women (accounting for 9.4% of all cancers) worldwide. Additionally, CRC is the fourth most common cause of cancer-related death<sup>[1]</sup>. Optimization of surgical resection for patients with localized disease has dramatically improved 5 year and 10 year survival rates. The prognosis for patients with resectable tumors depends on the disease stage. CRC patients with distant metastasis have a 12% survival rate<sup>[2]</sup>, and more than 20% of CRC patients have metastatic disease at the time of diagnosis<sup>[3,4]</sup>. Moreover, despite the fact that 80% of CRC patients with localized disease demonstrate complete macroscopic clearance of the tumor by surgery, 50% of CRC patients will relapse due to the presence of micro-metastasis at the time of surgery<sup>[5]</sup>. Chemotherapy is approved for the treatment of regionally metastatic CRC, but it shows only modest efficacy and is ineffective against distant metastases<sup>[6]</sup>. The prognosis for patients with advanced disease remains unfavorable due to the frequency of recurrence, distant metastasis, and resistance to chemotherapy. Thus, novel treatment modalities are needed. Interestingly, tumors that develop chemotherapy or radiation resistance are still suitable targets for immunotherapy<sup>[7-10]</sup>. Therefore, cancer immunotherapy may be effective for treating CRC patients and/or preventing relapse.

## ANTITUMOR IMMUNE RESPONSES

### T cells

Tumor cells degrade endogenous and exogenous tumor-associated antigens (TAAs) into short peptides (usually 8-10 amino acids) and present them on the cell surface in the context of major histocompatibility complex (MHC) class I molecules. T cell receptor (TCR) interaction with the complex of peptides and MHC class I molecules on tumor cells is a critical event in the T cell-mediated antitumor immune response. T cells that express the  $\alpha\beta$  TCR generally express CD4+ (helper T cells) or CD8+ (cytotoxic T cells) lineage markers<sup>[11]</sup>. In particular, CD8+ T cells recognize peptides (usually 8-10 amino acids) derived from TAAs bound by MHC class I molecules on tumor cells. Thus, immunotherapy may promote cancer cell killing by eliciting antitumor immune responses by recognizing specific TAAs on tumor cells.

To induce antigen-specific CD8+ cytotoxic T lymphocytes (CTLs), peptides derived from TAAs must be presented on the surface of antigen presenting cells (APCs) in the context of MHC class I molecules. In contrast, CD4+ T cells recognize peptides (usually 10-30

amino acids) in association with MHC class II molecules on APCs and enhance the persistence of antigen-specific CD8+ CTLs through secretion of interleukin (IL)-2 and interferon (IFN)- $\gamma$ <sup>[12]</sup>. Therefore, the interaction of the  $\alpha\beta$  TCR with complexes of peptides and MHC class I and class II molecules on APCs is a central event in cancer immunotherapy. The  $\alpha\beta$  TCR expressed by CD8+ CTLs recognizes MHC class I-peptide complexes on tumor cells and leads to tumor cell killing through effector molecules such as perforin and granzyme B<sup>[13]</sup>. Moreover, there is increasing evidence that CD4+ T cells play a more direct role in generating efficient antitumor immunity beyond simply assisting<sup>[14]</sup>. Therefore, effective antitumor responses depend on the presence and function of T cells that recognize and eliminate tumor cells<sup>[14,15]</sup>.

A unique subset of human T cells expresses the TCR- $\gamma\delta$ . Human  $\gamma\delta$ T cells include several subsets of cells defined by their TCR. The most common subset of TCR- $\gamma\delta$ T cells in circulating blood express the V $\gamma$ 9V $\delta$ 2 receptor<sup>[16,17]</sup>. Although cancer immunotherapy strategies primarily focus on activation of these MHC-restricted T cells,  $\gamma\delta$ T cells and  $\alpha\beta$ T cells share certain effector functions such as cytokine production and potent cytotoxic activity. However, they recognize different sets of antigens, usually in a non-MHC-restricted fashion<sup>[16,18]</sup>. Thus, T cells can attack tumors in their HLA-unrestricted cytotoxic capacity, as well as by secreting cytokines. Indeed, tumor-infiltrating  $\gamma\delta$ T cells have been detected in a broad range of cancers, including CRC<sup>[19]</sup>. Importantly, activated  $\gamma\delta$ T cells can kill cells from metastatic renal cell carcinomas, mammary carcinomas, prostate carcinomas and colorectal carcinomas, while sparing normal, untransformed cells<sup>[18,19]</sup>.

### Natural killer cells

Natural killer (NK) cells are component of innate immunity responses to tumor cells<sup>[20]</sup>. NK cells can rapidly kill certain target cells, including tumor cells with down-regulated MHC class I molecules<sup>[21]</sup>. Thus, NK cells play a critical role in antitumor immunity. NK cells recognize tumor cells *via* cellular stress or DNA damage signals<sup>[22]</sup>. Activated NK cells directly kill target tumor cells through several mechanisms, including<sup>[23]</sup>: (1) cytoplasmic granules such as perforin and granzyme B<sup>[24]</sup>; (2) tumor necrosis factor-related apoptosis-inducing ligand and Fas ligand (FasL)<sup>[25,26]</sup>; (3) effector molecules such as IFN- $\gamma$  and nitric oxide (NO)<sup>[24,27]</sup>; and (4) antibody-dependent cellular cytotoxicity (ADCC)<sup>[28]</sup>. NK cell activators (IL-2, IL-12, IL-15, and IL-18), have also been validated in preclinical cancer models<sup>[23]</sup>.

### Dendritic cells

Dendritic cells (DCs) are potent APCs that have been used in cancer vaccines due to their ability to initiate antitumor immune responses<sup>[12]</sup>. DCs are characterized by expression of MHC class I, class II, and costimulatory molecules (B7, ICAM-1, LFA-1, LFA-3, and CD40)<sup>[29-31]</sup>.



These molecules function in concert to generate a network of secondary signals essential for reinforcing the primary antigen-specific signal in T-cell activation<sup>[29,31]</sup>. DCs process endogenously synthesized antigens into antigenic peptides, which are presented on the cell surface in MHC class I-peptide and recognized by the  $\alpha\beta$  TCR on naïve CD8+ T cells<sup>[12]</sup>. DCs can also capture and process exogenous antigens, which are then presented by MHC class I molecules through an endogenous pathway in a process known as “cross-presentation”<sup>[32]</sup>. Moreover, exogenous antigens from the extracellular environment are also captured by DCs and delivered to the endosomal/lysosomal compartment, where they are degraded to antigenic peptides by proteases and peptidases. These antigens then complex with MHC class II for recognition by the  $\alpha\beta$  TCR of naïve CD4+ T cells<sup>[12]</sup>. Efficient antigen presentation by MHC class I and class II on DCs is essential for evoking tumor-specific immune responses<sup>[33]</sup>. Mature DCs are significantly better at processing and presenting MHC-peptide to the TCR and inducing CTLs due to higher expression of MHC class I and class II and costimulatory molecules<sup>[33]</sup>.

Immature DCs reside in peripheral tissues where they take up and process antigens that are degraded to peptides. These peptides are then bound to MHC class I molecules for presentation to CD8+ CTLs or bound to MHC class II molecules for presentation to CD4+ T helper (Th) cells. Differentiation of the immature DCs into mature DCs is triggered by molecular stimuli that are released in response to tissue disturbance and local inflammatory responses caused by pathogens<sup>[34]</sup>. After antigen uptake and stimulation by the inflammatory response, immature DCs in the peripheral tissues undergo a maturation process characterized by the up-regulation of MHC class I and class II and costimulatory molecules, the up-regulation of chemokine receptors such as CCR7, and the secretion of cytokines such as IL-12<sup>[34,35]</sup>. The mature DCs migrate to secondary lymphoid organs, where they present antigens to CD4+ and CD8+ T cells through the MHC class I and class II pathways, respectively<sup>[12,34]</sup>. Therefore, the aim of immunotherapy is to simultaneously activate CD8+ CTLs (which recognize TAA) and CD4+ Th cells.

### Immune suppressive cells

CD4+ Th cells are critical for inducing and regulating immune responses. Immune homeostasis is primarily controlled by two distinct helper T cell subsets, Th1 and Th2 cells<sup>[36]</sup>. Th1 cells secrete IFN- $\gamma$ , which can further sensitize tumor cells to CTLs by inducing the up-regulation of MHC class I molecule expression on tumor cells and antigen-processing machinery in DCs<sup>[12]</sup>. Th2 cells secrete type II cytokines such as IL-4 and IL-10 that enhance humoral immunity (the antibody-based antitumor response)<sup>[12]</sup>. Importantly, tumor cell-derived soluble factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-10 induce tolerance by promoting the expansion of the CD4+ $\alpha$ -2R (CD25)+ forkhead box P3 (Foxp3)+ natural

Treg subset<sup>[37]</sup>. Induced Tregs (CD4+CD25+Foxp3-) secrete TGF- $\beta$  and IL-10 and suppress Th1 and Th2 phenotypes<sup>[38,39]</sup>. Therefore, Tregs play a pivotal role in tumor progression and the suppression of antitumor immunity.

The cancer microenvironment consists not only of cancer cells but also stromal cells such as cancer-associated fibroblasts, tolerogenic DCs, myeloid-derived suppressor cells, immunosuppressive tumor-associated macrophages (TAMs), and Tregs. These immune suppressive cells secrete vascular endothelial growth factor (VEGF), IL-6, IL-10, TGF- $\beta$ , soluble FasL, and indolamine-2,3-dioxygenase (IDO)<sup>[40]</sup>, which inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and NO. Moreover, the tumor microenvironment promotes the accumulation of Tregs that suppress CD8+ CTL function due to the secretion of IL-10 or TGF- $\beta$  from Tregs and tumor cells<sup>[40]</sup> (Figure 1).

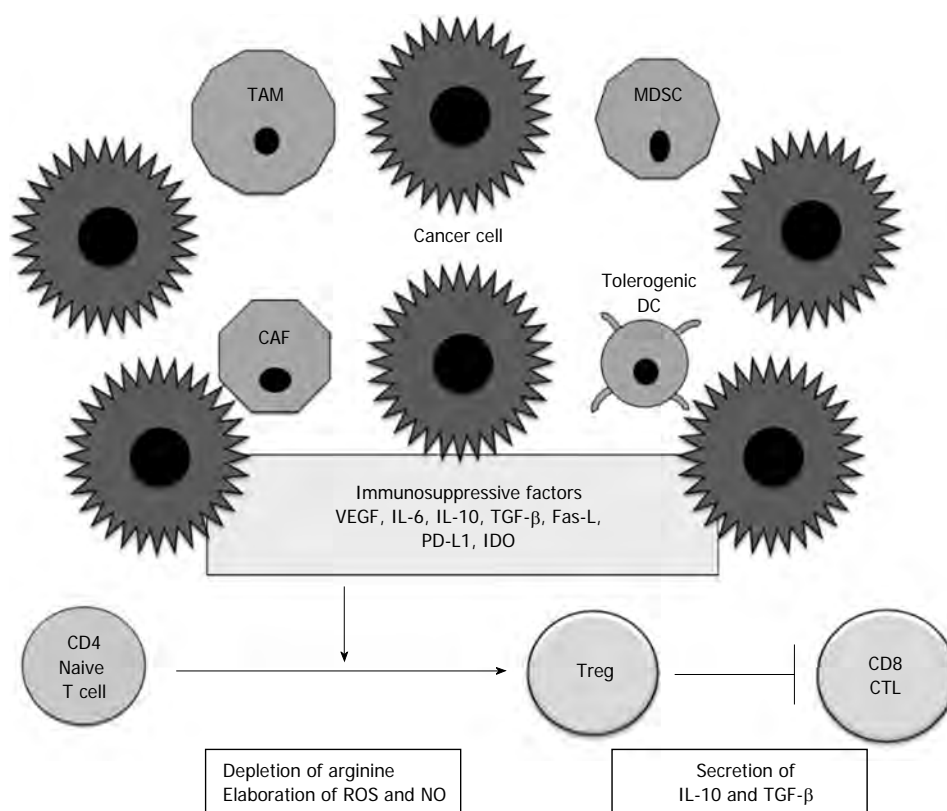
## IMMUNOTHERAPY

Immunotherapy is an active therapeutic approach designed to trigger the immune system to respond to tumor-specific antigens and attack tumor cells. Immunotherapy strategies include the use of peptides derived from TAAs, whole tumor cells, *in vitro*-generated DCs, or viral vector-based cancer vaccines (Table 1).

### Peptide vaccines

A peptide vaccine is based on the identification and synthesis of epitopes, which can induce TAA-specific antitumor immune responses. CRC cells express TAAs such as carcinoembryonic antigen (CEA)<sup>[41,42]</sup>, mucin 1<sup>[41-43]</sup>, epidermal growth factor receptor (EGFR)<sup>[44]</sup>, squamous cell carcinoma antigen recognized by T cells 3 (SART3)<sup>[45]</sup>,  $\beta$ -human chorionic gonadotropin ( $\beta$ -hCG)<sup>[46]</sup>, Wilms' Tumor antigen 1 (WT1)<sup>[47]</sup>, Survivin-2B<sup>[48]</sup>, MAGE3<sup>[49]</sup>, p53<sup>[50]</sup>, or mutated KRAS<sup>[51]</sup>, which are potential targets for immunotherapy. Peptide vaccines targeting these TAAs may be a useful approach for immunotherapy in CRC patients.

Peptide vaccines are simple, safe, stable, and economical. Multiple MHC class I-binding peptides have been identified and tested for immunogenicity. Several peptide vaccines for CRC have been tested in phase I clinical trials. Fifteen patients with advanced or recurrent CRC expressing survivin were vaccinated with a peptide derived from HLA-A\*2402-restricted epitopes<sup>[48]</sup>. In 6 patients, tumor marker levels (CEA and CA19-9) decreased transiently during the survivin-2B peptide vaccination. Moreover, in phase I trial of peptide-cocktail vaccines derived from ring finger protein 43 (RNF43) and translocase of the outer mitochondrial membrane 34 (TOMM34), 8 of 21 patients exhibited antigen-specific CTL responses to both RNF43 and TOMM34, and 12 patients exhibited CTL responses to one of the peptides only<sup>[52]</sup>. The patients who did not demonstrate any CTL responses had the lowest survival rates. By vaccination with a 13-mer



**Figure 1** Immunosuppression in the tumor microenvironment. Cancer cells secrete various factors such as vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-10, transforming growth factor- $\beta$  (TGF- $\beta$ ), Fas ligand (FasL), PD1 ligand 1 (PD-L1), and indolamine-2,3-dioxygenase (IDO), all of which promote the accumulation of heterogeneous populations of cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and tolerogenic dendritic cells (DCs). These immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitric oxide (NO). The tumor microenvironment promotes the accumulation of Tregs that suppress CD8+ cytotoxic T lymphocyte (CTL) function through secretion of IL-10 and TGF- $\beta$ .

mutant ras peptide, 2 of 7 CRC patients showed antitumor immune responses that were significantly associated with prolonged overall survival<sup>[53]</sup>. Moreover, in a phase II trial, vaccination with the  $\beta$ -hCG peptide induced anti- $\beta$ -hCG antibody production in 56 of 77 CRC patients<sup>[46]</sup>. Interestingly, anti- $\beta$ -hCG antibody induction was associated with longer overall survival<sup>[46]</sup>. However, some clinical trials report a discrepancy between clinical and immunological responses. In SART3 peptide vaccine therapy, IgE-type anti-peptide antibodies were detected after vaccination; however, immunological responses were not detected in the patients<sup>[45]</sup>. Peptide vaccines for CRC patients are generally well-tolerated, with no patients requiring cessation due to toxicity; however, a high frequency of reactions were observed at the injection site due to the use of adjuvants such as incomplete Freund's adjuvant, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and bacillus Calmette-Guerin (BCG). Importantly, peptide vaccines have shown only limited success in clinical trials. There are several drawbacks to the peptide vaccination strategy, including: (1) limitations due to the patient's HLA type<sup>[54]</sup>; (2) ineffectiveness of CD8+ CTLs due to the down-regulation of certain antigens and MHC class I molecules; (3) impaired DC function in patients with advanced cancer<sup>[55]</sup>; and (4) tumor microenvironments, where immune suppressive cells such as Tregs

exist<sup>[13]</sup>. Synthetic long peptides may be more attractive candidates for peptide-based vaccines. In a phase I/II trial, 10 CRC patients were vaccinated twice with a set of 10 overlapping p53 synthetic long peptides<sup>[50]</sup>. P53-specific CD4+ and CD8+ T-cell responses were observed in 9 of 10 CRC patients, and 6 of 9 tested patients maintained p53-specific T-cell reactivity for at least 6 mo. New trials may focus on improving the long peptide vaccine-induced antitumor immune responses.

### DC vaccines

Three signals were required for induction of efficient CTL responses: (1) simultaneous presentation of multiple TAAs by both MHC class I and class II molecules; (2) costimulation by membrane-bound receptor-ligand pairs; and (3) cytokines to direct polarization of the resultant antitumor immune responses. DCs can provide all three of these signals that are essential for the induction of antitumor immunity<sup>[33]</sup>. Therefore, many clinical trials of antigen-pulsed DCs have been conducted in patients with various types of tumors, including CRC.

To date, various strategies for delivering TAAs to DCs have been developed to generate potent CTL responses against tumor cells. DCs have been pulsed with synthetic peptides derived from the known TAAs<sup>[56]</sup>, tumor cell lysates<sup>[57]</sup>, apoptotic tumor cells<sup>[32]</sup>, and tumor RNA<sup>[58]</sup>, or

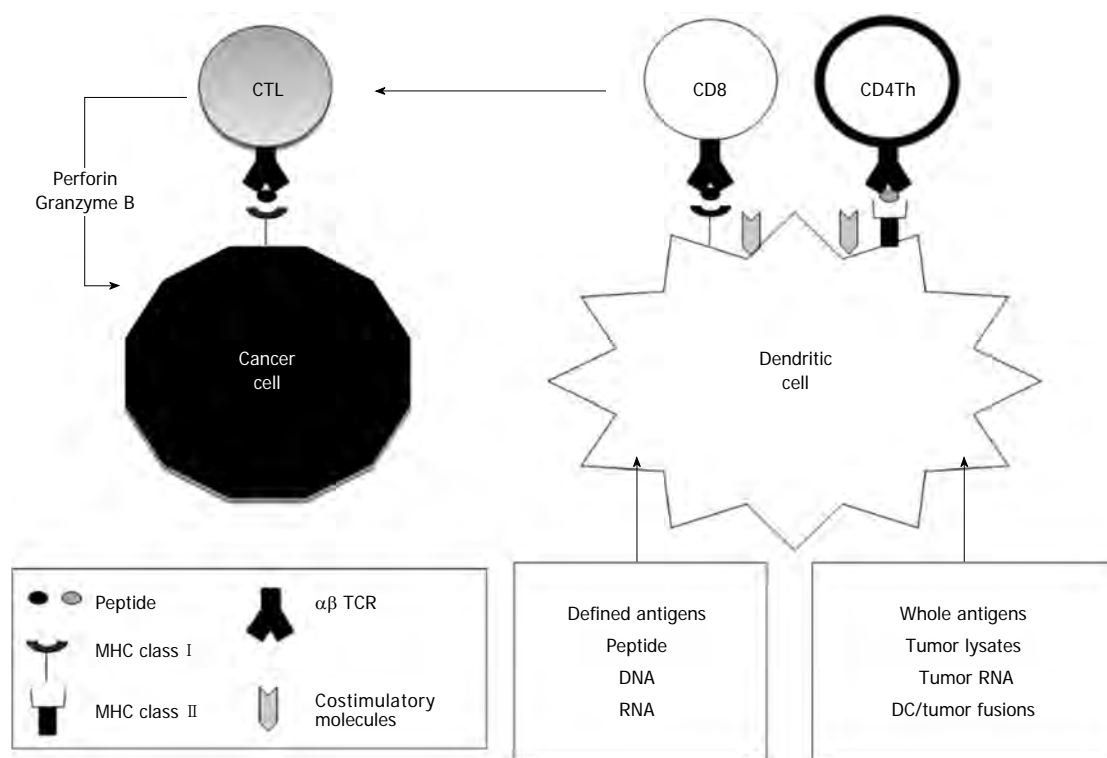
**Table 1** Immunotherapy strategies for colorectal cancer

Vaccine	Clinical response	Immunological response	Ref.
Peptide			
Survivin-2B	PR (1/15) SD (3/15) PD (11/15) Temporary decrease of CEA level 40% (6/15)	Increase of Survivin-2B-specific CTL frequency DTH 40% (6/15)	[48]
Combination chemotherapy with peptide vaccine against RNF43 and TOMM34	SD (16/19) PD (3/19)	8 of 21 patients exhibited antigen-specific CTL responses to both RNF43 and TOMM34, and 12 patients exhibited CTL responses to one of the peptides only	[52]
13-mer mutant ras	Of nine patients who completed all six vaccinations, seven patients showed no remaining evidence of disease	Two CRC patients showed immunological responses, and the antitumor immune response was significantly associated with prolonged overall survival	[53]
β-hCG	Prolongation of survival in patients with a high level of anti-peptide antibodies	Induction of serum anti-peptide antibody (56/77)	[46]
SART3	Diagnosis at 5 mo after first vaccination: SD (1/19) PD (10/19)	Increased CTL activity (2/11), induction of serum anti-peptide IgG (2/12), IgE (5/12), DTH (0/12)	[45]
A set of 10 overlapping p53 synthetic long peptides		Induction of p53-specific CD4+ and CD8+ T-cell responses (9/10), maintained p53-specific CTL reactivity for at least 6 mo (6/9)	[50]
DC			
DC pulsed with CEA peptide or CEA mRNA	Disease stabilization was observed in several patients	The majority of CRC patients demonstrated induction of CEA-specific T cell responses	[60-65]
DCs pulsed with CEA-derived altered peptides combined with the adjuvant Flt3 ligand	2 of 12 patients exhibited SD for 3 and 6 mo; 2 patients exhibited CR for more than 10 mo; 1 patient had a mixed response with some regression of liver metastases	Expansion of CD8+ T cells that recognize both the native and altered epitopes and possess an effector CTL phenotype	[64]
Whole tumor cell			
Autologous tumor cells combined with BCG	No significant clinical benefit was seen with whole tumor cell vaccines in surgically resected patients with stage II or III CRC	When treatment compliance was evaluated, the trend indicated benefits from vaccination in terms of disease-free survival ( $P = 0.078$ ) and overall survival ( $P = 0.12$ )	[68]
NDV-infected irradiated autologous tumor cells	A randomized phase III study of 50 patients with resectable CRC liver metastases demonstrated that vaccination with NDV-infected whole tumor cell did not significantly improve overall survival.	DTH (21/31)	[74,75]
Viral vector			
Replication-defective recombinant fowlpox and vaccinia viruses encoding the CEA antigen and TRICOM (B7.1, ICAM-1, and LFA-3)	SD (3/9)	Induction of CEA-specific CTL (3/9)	[79]
Combination chemotherapy and vaccination with a nonreplicating canarypox virus (ALVAC) expressing the CEA and T-cell costimulatory molecule B7.1 (ALVAC-CEA/B7.1)	Objective response (42/118)	Increases in CEA-specific T cells were detected in patients treated with chemotherapy and booster vaccination	[80]

Immunotherapy strategies including peptides derived from tumor-associated antigens, whole tumor cells, *in vitro*-generated dendritic cells (DCs), or viral vector-based cancer vaccine. PD: Programmed cell death protein; CTL: Cytotoxic T lymphocytes; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; RNF43: Ring finger protein 43; TOMM34: Translocase of outer mitochondrial membrane 34; β-hCG: β-human chorionic gonadotropin; SART3: Squamous cell carcinoma antigen recognized by T cells 3; NDV: Newcastle disease virus.

physically fused with whole tumor cells<sup>[59]</sup> to induce efficient antitumor immune responses (Figure 2). Because CEA is a tumor-associated antigen expressed by most CRCs, DCs have been pulsed with CEA peptides<sup>[60-64]</sup> or CEA mRNA<sup>[63,65]</sup>. In these phase I clinical trials, the majority of vaccinated CRC patients demonstrated the induction of CEA-specific T cell responses. Moreover, disease progression stabilized in several patients, and the vaccine was safe and well-tolerated. As CEA is a self-antigen and poorly immunogenic, Fong *et al.*<sup>[64]</sup> generated altered peptide ligands that were derived from native T

cell epitopes and contained amino acid substitutions that either increased the peptide affinity for the MHC peptide-binding groove or modified interactions with the T cell receptor. In this trial, 12 patients were immunized with DCs loaded with altered peptides derived from CEA and the Flt3 adjuvant ligand. Two of 12 patients showed disease stabilization for 3 mo and 6 mo, 2 patients showed complete responses for more than 10 mo, and one patient had a mixed response with some regression of liver metastases. To improve the clinical efficacy of DC-based cancer vaccines, it is crucial to design novel strategies that boost



**Figure 2** Dendritic cell vaccines. Dendritic cells (DCs) are loaded with antigens, which are taken up and degraded into peptide fragments by antigen-presenting cells such as immature DCs. DCs process tumor-derived peptides and major histocompatibility complex (MHC) class I peptides derived from DCs. They form MHC class I-peptide complexes in the endoplasmic reticulum, which are then transported to the surface of the DCs and presented to CD8+ T cells. DCs also synthesize MHC class II peptides in the endoplasmic reticulum, which are transported to the cytoplasm where MHC class II-peptide complexes are assembled with tumor-derived peptides and presented to CD4+ T cells. CD8+ T cells become cytotoxic T lymphocytes (CTLs), which destroy cancer cells through effector molecules such as perforin and granzyme B. TCR: T cell receptor.

adaptive antitumor immunity to overcome tolerance.

### Whole tumor cell vaccines

Because autologous tumor cells express a whole TAAs including those known and unidentified, using whole tumor cells could greatly diminish the chance of tumor escape compared to using a single epitope peptide<sup>[41,42]</sup>. A significant disadvantage to this approach is the difficulty in generating a “universal” vaccine that could be applicable to all patients with a given cancer. Autologous whole tumor cells have been used as cancer vaccines to induce polyclonal CTL induction in several cancer types<sup>[66,67]</sup>, including CRC<sup>[68]</sup>. A randomized phase III clinical trial of a combined autologous whole tumor cell plus BCG vaccine was conducted to determine whether surgical resection plus vaccination was more beneficial than resection alone in 412 stage II and III CRC patients. This study showed no significant clinical benefit from whole tumor cell vaccination in surgically resected patients with stage II or III CRC. However, effective immune responses were associated with the improved disease-free and overall survival. Only a small proportion of the proteins in an autologous whole tumor cell vaccine are specific to tumor cells, while a vast majority of antigens in the vaccine are shared with normal cells. Moreover, whole tumor cell vaccines are likely to be poorly immunogenic. Therefore, the immune response generated by whole tumor cell vaccines is gener-

ally insufficient to provide benefit to patients. To improve the immunogenicity of whole tumor cell vaccines, autologous tumor cells have been genetically modified to secrete GM-CSF and then re-administered to the patient<sup>[69]</sup>. The trials have shown promising results in patients with prostate carcinoma<sup>[70]</sup>, renal cell carcinoma<sup>[71]</sup>, metastatic non-small-cell lung carcinoma<sup>[72]</sup>, and melanoma<sup>[73]</sup>. This approach is based on the fact that GM-CSF is required at the site of the tumor to effectively prime TAA-specific immunity<sup>[69]</sup>. Another tumor cell vaccine approach utilizes Newcastle disease virus (NDV)-infected irradiated tumor cells as autologous CRC vaccines<sup>[74]</sup>. This approach resulted in a 97.9% two-year survival rate in patients with resected CRC, compared to 66.7% when treated with autologous tumor cells combined with BCG. However, a randomized phase III study of 50 patients with resectable CRC liver metastases demonstrated that vaccination with NDV-infected whole tumor cells did not significantly improve overall survival, disease-free survival or metastases-free survival, although subgroup analyses suggested that the vaccines were somewhat beneficial<sup>[75]</sup>. The immunogenicity of whole tumor cells needs to be improved for this vaccination strategy to be effective. However, it is unclear which specific agents (such as cytotoxic chemotherapeutics, ionizing irradiation, and chemical agents) are best suited for killing tumor cells to generate highly immunogenic whole tumor cell vaccines.



### Viral vector vaccines

Recombinant viral vectors are potentially useful vaccine vehicles for cancer therapy. Many types of recombinant viruses are naturally immunogenic, infect APCs (specifically DCs), and express TAAs<sup>[76]</sup>. The natural immunogenicity of viral vectors acts as an adjuvant to help boost TAA-specific immune responses. In one study, CRC patients were immunized with vaccinia virus or a replication-defective avian poxvirus encoding CEA. In this phase I study, viral-based vaccination with replication-defective recombinant fowlpox and vaccinia viruses encoding the CEA antigen and TRICOM (B7.1, ICAM-1, and LFA-3) induced CEA-specific T cell responses<sup>[77]</sup> and disease stabilization in 40% of patients with metastatic cancer (including CRC) for at least 4 mo<sup>[78]</sup>. A phase II clinical trial in patients with metastatic CRC examined the efficacy of chemotherapy combined with vaccination with a nonreplicating canarypox virus (ALVAC) expressing the CEA and T-cell costimulatory molecule, B7.1 (ALVAC-CEA/B7.1). Anti-CEA-specific T cell responses were successfully generated in 50% of patients undergoing chemotherapy and booster vaccination. Objective clinical responses were observed in 40% of the patients<sup>[79,80]</sup>. Interestingly, chemotherapy does not appear to inhibit vaccine-mediated immunity.

### ADOPTIVE CELL TRANSFER THERAPY

Cancer immunotherapy can be either active or passive. In passive cellular immunotherapy, specific effector cells are directly infused and are not induced or expanded within the cancer patient. Because most tumor cells express MHC class I-peptide, which can be recognized by antigen-specific CD8<sup>+</sup> CTLs. Therefore, adoptive transfer of activated CTLs successfully used in patients with advanced cancer<sup>[81]</sup>. In adoptive cell transfer therapy (ACT), autologous T cells are removed from patients, activated, expanded to large numbers *in vitro* and transferred back into the patients. ACT overcomes tolerogenic mechanisms by enabling the selection and activation of highly reactive T cell subpopulations and manipulating the host environment into which the T cells are introduced. Indeed, upon the successful induction of specific CTLs *in vitro*, a clinical response to adoptive immunotherapy can be expected even in patients with advanced CRC<sup>[82]</sup>. Moreover, injection of IFN promotes the MHC class I-peptide on the cell surface, thus antitumor immune responses are augmented. However, there are several drawbacks to ACT that should be considered, including a potential lack of immune memory, poor persistence of activated effector cells in patients, the prohibitive expense, and the time required to expand the cells.

A new approach using T cells genetically modified to express receptors that recognize TAAs with high specificity to tumor cells may provide significant clinical benefits, especially for large solid tumors<sup>[83]</sup>. Recently, several clinical trials have demonstrated the therapeutic potential of this approach, which lead to impressive tumor regression in cancer patients<sup>[84]</sup>. A phase I trial in CRC patients

examined human T cells engineered to express a high-avidity CEA-specific TCRs<sup>[85]</sup>. In this study, autologous T cells genetically engineered to express a murine TCR against human CEA were administered to three patients with metastatic colorectal cancer that was refractory to standard treatments. All patients experienced profound decreases in serum CEA levels (74%-99%), and one patient had an objective regression of cancer metastatic to the lung and liver. However, all three patients developed severe transient inflammatory colitis.

## ANTIBODY-BASED CANCER IMMUNOTHERAPY

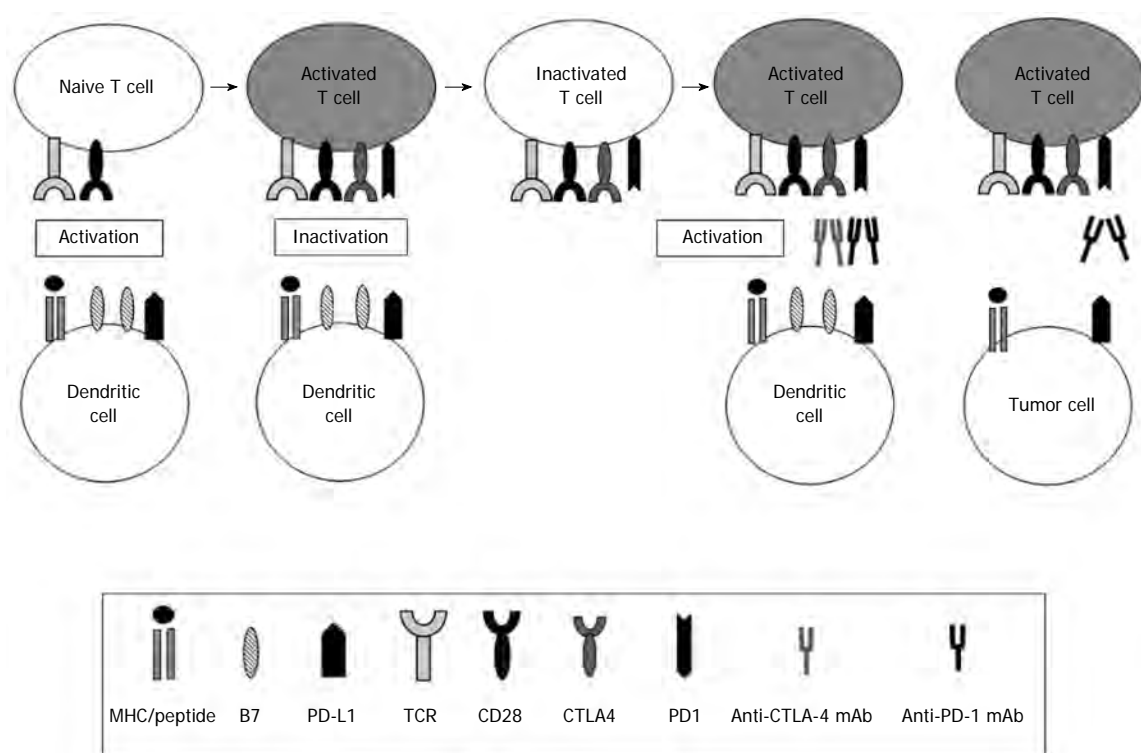
Monoclonal antibodies (mAbs) that target surface antigens expressed on tumor cells are clinically effective as cancer therapeutics<sup>[86]</sup>. Three mAbs (Cetuximab, Bevacizumab and Panitumumab) are approved for the treatment of CRC in the United States, and many other mAbs are being tested in clinical trials<sup>[87]</sup>. Treatment with mAbs can induce tumor cell death by several mechanisms, including interference with vital signaling pathways. Moreover, it is becoming apparent that innate immune effector mechanisms that engage the Fc portion of the antibody *via* Fc receptors are equally important<sup>[88]</sup>. The immune cytotoxicity includes ADCC, complement-mediated cytotoxicity, and antibody-dependent cellular phagocytosis. Bevacizumab, a recombinant humanized monoclonal antibody that selectively binds to human VEGF, is effective in KRAS wild-type CRC patients<sup>[89]</sup>. Recent evidence has also shown clinical benefits from treatment with anti-EGFR, Cetuximab and Panitumumab in KRAS wild-type CRC patients<sup>[90]</sup>.

### CYTOKINE THERAPY

Cytokines are substances proteins and glycoproteins that are secreted by immune cells. They have autocrine and paracrine functions and function locally or at a distance to enhance or suppress antitumor immunity. To date, IL-2 and IFN- $\alpha$  are two cytokines approved by the FDA for cancer therapy. Cytokines may be useful for treating hematologic malignancies (hairy cell leukemia and chronic myelogenous leukemia) or immunogenic tumors (melanoma and renal cell carcinoma). The major cytokines currently in use or under evaluation for cancer therapy are IFN- $\alpha$ , IL-2, GM-CSF, and IL-12.

### COMBINED IMMUNOTHERAPY

It is well known that even if CRC appears to have been eradicated by chemotherapy and radiation, a small cancer stem cell (CSC) fraction that can self-propagate and sustain tumor growth frequently persists, leading to relapse and therapeutic failure. Although CSC is often resistant to a variety of treatments, including chemotherapy and radiotherapy, immunotherapy may still be effective<sup>[8-10]</sup>. A combined approach that uses conventional chemotherapy



**Figure 3** Immune regulatory checkpoints in cancer immunotherapy. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1) are two well-described co-inhibitory molecules that are expressed on naïve or memory T cells and decrease antitumor immune responses. The CTLA-4-mediated immune checkpoint is induced in T cells at the initial response to antigen (early activation phase). After the T cell receptor (TCR) is triggered by antigen encounter, CTLA-4 is transported to the surface of naïve or memory T cells. In contrast, the major role of the PD1 pathway is not at the initial T cell activation stage but rather the regulation of inflammatory responses by effector T cells that recognize antigen in peripheral tissue cells. Thus, PD-1 is highly expressed by antigen-specific cytotoxic T lymphocytes (CTLs) in malignancies and is associated with impaired T-cell function. The best-characterized signal for PD1 ligand 1 (PD-L1) induction is interferon- $\gamma$  (IFN- $\gamma$ ), which is predominantly produced by Th1 cells. Although PD-L2 expression is limited to dendritic cells (DCs) and macrophages, PD-L1 is broadly expressed in tissues and is considered a molecular shield that protects cells from auto-reactive attack. In some tumors, PD-L1 is not constitutively expressed but is induced in response to inflammatory signals that are produced by an active antitumor immune response. Loading DCs with soluble PD1 decreases their function. Therefore, antibodies can be used to block inhibitory ligand:receptor interactions by acting on tumor cells, DCs (*e.g.*, anti-PD-L1) or T cells (*e.g.*, anti-CTLA-4 or anti-PD1). Combining the blockade of multiple inhibitory pathways synergistically decreases T cell anergy and improves T cell responsiveness against tumors.

or radiation to kill the bulk of cancer cells and immunotherapy to keep residual CSCs and differentiated cancer cells in check may abrogate the replenishing pool of CRC cells<sup>[91]</sup>. In addition, treatment with chemotherapy such as cyclophosphamide or gemcitabine can augment the antitumor effects of cancer immunotherapy by depleting Treg, potentially enhancing antitumor immune responses<sup>[92]</sup>. Therefore, chemotherapy can kill cancer cells and boost antitumor immune responses all at the same time<sup>[93,94]</sup>. A recent study reported that immune checkpoint blockade with monoclonal antibodies targeting the inhibitory immune receptors cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), PD-1, and PD-L1 can be used to successfully treat patients with advanced melanoma (Figure 3)<sup>[95-98]</sup>. Combined, these approaches have the potential to significantly improve patient outcomes compared to treatment with conventional cancer therapies such as chemotherapy, radiation, monoclonal antibodies, hormonal therapy, and photodynamic therapy.

## FUTURE PERSPECTIVE

Improved treatment options that selectively target cancer-dependent pathways with little or no toxicity to

normal tissues are urgently needed. Work in our laboratory focuses on these key aspects by combining the use of DCs pulsed with MHC class I and II peptides and conventional chemotherapy. Immunotherapy may be combined with conventional therapy to reduce Tregs and enhance CTL responses. Knockdown of PD-L1 and PD-L2 on monocyte-derived DCs and tumor cells may help decrease tolerance (Figure 3). DCs electroporated with PD-L small-interfering RNAs are highly effective in enhancing T cell proliferation and cytokine production and are therefore attractive candidates for improving the efficacy of DC vaccines in cancer patients<sup>[99]</sup>. Moreover, combined blockade of PD1 and CTLA-4, which play key roles in inhibiting T-cell activation, enhances antitumor immune responses compared to either agent alone (Figure 3)<sup>[100]</sup>. Combining immunotherapies, particularly agents that target different immune checkpoints, may be a promising approach. Preliminary clinical findings indicate that combined targeted therapies and simultaneous blockade of multiple immune checkpoints could promote therapeutic synergy and long-term antitumor immunity to improve clinical outcomes for melanoma patients<sup>[101]</sup>. In the metastatic CT26 CRC mouse model, simultaneous blockade of CTLA-4 and PD-L1 enhanced antitumor

activity in an interleukin-15-dependent manner<sup>[102]</sup>.

## CONCLUSION

The limitations of surgery and adjuvant chemo/radio/antibody therapies in treating CRC patients necessitate the development of novel approaches, including immunotherapy. Despite tremendous progress in basic immunological research, effective immunotherapies for most types of cancer, including CRC, are still lacking. Immunotherapy alone may be insufficient for treating advanced CRC patients. The most promising therapeutic approach for activating antitumor immunity in cancer patients may be blockade of inhibitory immune regulatory proteins such as immune checkpoint ligands and receptors. Therefore, it is important to develop cancer vaccines that do not express inhibitory molecules such as PD-L1, but do express high levels of molecules that enhance CTL priming, such as CD80 and 4-1BBL. The blockade of multiple immune regulatory checkpoints combined with immunotherapy and/or conventional therapy may be effective in treating patients with advanced CRC.

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# Early rehabilitation programs after laparoscopic colorectal surgery: Evidence and criticism

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## Abstract

During the past several decades, early rehabilitation programs for the care of patients with colorectal surgery have gained popularity. Several randomized controlled trials and meta-analyses have confirmed that the implementation of these evidence-based detailed perioperative care protocols is useful for early recovery of patients after colorectal resection. Patients cared for based on these protocols had a rapid recovery of bowel movement, shortened length of hospital stay, and fewer complications compared with traditional care programs. However, most of the previous evidence was obtained from studies of early rehabilitation programs adapted to open colonic resection. Currently, limited evidence exists on the effects of early rehabilitation after laparoscopic rectal resection, although this procedure seems to be associated with a higher morbidity than that reported with traditional care. In this article, we review previous studies and guidelines on early rehabilitation programs in patients undergoing rectal surgery. We investigated the status of early rehabilitation programs in rectal surgery and analyzed the limitations of these

studies. We also summarized indications and detailed protocol components of current early rehabilitation programs after rectal surgery, focusing on laparoscopic resection.

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**Key words:** Colorectal cancer; Enhanced recovery after surgery; Early rehabilitation; Fast-track; Laparoscopy; Rectal

**Core tip:** Several randomized trials and meta-analyses have confirmed that the implementation of early rehabilitation programs for perioperative care is useful for recovery of patients after colorectal resection. However, most of the previous evidence is obtained from studies of early rehabilitation programs adapted to open colonic resection. Currently, early rehabilitation combined with laparoscopic rectal surgery can be a feasible alternative in some selected patients, but indications are not established. Current evidence fails to support the safety of early rehabilitation combined with laparoscopic rectal surgery compared to that reported for laparoscopic colon surgery.

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## INTRODUCTION

Previously, patients undergoing colorectal surgery received traditional perioperative care, which comprised sufficient mechanical bowel preparation, insertion of a



nasogastric tube, preoperative fasting, postoperative fasting for up to 1 wk, and multiple intra-abdominal drains. Eventually, early rehabilitation programs were developed to decrease postoperative pain, perioperative physiological stress, and organ dysfunction, and to promote patient motivation, leading to enhanced recovery after surgery; decreased postoperative morbidity, length of hospital stay, and health care resources; and improved overall outcomes<sup>[1]</sup>. Since their first introduction in the mid-1990s, early rehabilitation programs, also known as fast-track pathways or enhanced recovery after surgery (ERAS), have become increasingly popular in the care for patients with colorectal surgery<sup>[2]</sup>.

During the past several decades, many studies have reported the results of early rehabilitation programs in colorectal surgery. Several randomized controlled trials and meta-analyses have indicated that the implementation of these evidence-based detailed perioperative care protocols is useful for early recovery of patients after colorectal resection<sup>[3-7]</sup>. Patients who underwent these programs showed rapid recovery of bowel movement, shortened length of hospital stay, and fewer complications compared with traditional care programs. However, most evidence from previous studies corresponded to patients undergoing colonic surgery for various diseases. Currently, the strongest evidence for early rehabilitation programs was adopted from open colonic resection<sup>[8]</sup>. At present, early rehabilitation programs in rectal surgery require standardization and can be adopted only after validation with high-level evidence from well-designed randomized controlled trials.

In this review, we summarized early rehabilitation programs reported in previous studies and guidelines including patients undergoing rectal surgery, and we analyzed the limitations of these studies. We also summarized indications and details of current early rehabilitation programs after rectal surgery, focusing on a laparoscopic resection perspective.

## EARLY REHABILITATION PROGRAMS AFTER RECTAL SURGERY: STATUS QUO

### *Early rehabilitation and laparoscopic colonic surgery*

Laparoscopic colorectal surgery has been established as a comparable alternative to open surgery with respect to its feasibility, safety and long-term outcomes. For malignant diseases, laparoscopic colonic resection performed by an experienced surgeon involves adequate lymph node harvest, sufficient surgical margins, and reduced operative time and intraoperative blood loss<sup>[8]</sup>. A previous study suggested that laparoscopic surgery could reduce the prevalence of postoperative immunosuppression<sup>[9]</sup>. Prospective randomized trials have shown that laparoscopic surgery for colon cancer can achieve earlier recovery in organ function and long-term oncological results equal to those for open colonic resection<sup>[10-12]</sup>. However, these trials did not apply early rehabilitation programs. Both laparoscopic surgery and early rehabilitation programs focus

on minimizing surgical pain and perioperative stress, and enhancing recovery after surgery. Many cohort series, meta-analyses, and several prospective randomized studies showed early rehabilitation programs and laparoscopic surgery can have a synergistic effect in enhancing recovery after laparoscopic surgery for colon disease<sup>[9,13,14]</sup>. Recently, the Laparoscopy and/or Fast-track Multimodal Management Versus Standard Care (LAFA) study, the largest multicenter randomized controlled trial thus far, reported comparative results between laparoscopic and open colectomy<sup>[9]</sup>. The total length of hospital stay was 2 d less than that after laparoscopic surgery. Furthermore, laparoscopic surgery was the only predictive factor associated with reduced hospital stay and morbidity. The results from the LAFA study also indicated that early oral intake, early mobilization, and laparoscopic surgery were independent determinants of early recovery<sup>[9,15]</sup>. In a previous study, we evaluated the efficacy of a rehabilitation program after laparoscopic colon surgery in the context of a randomized controlled trial. We found that the recovery time was shorter in the early rehabilitation program group than in the conventional care group, without differences in complication rates, quality of life, and pain<sup>[13]</sup>. Previous studies representative of laparoscopic colon surgery with early rehabilitation are summarized in Table 1. As early rehabilitation programs became more popular in the management of patients undergoing colon surgery, an international collaborative research group proposed a set of guidelines for perioperative care in elective colonic surgery, with the participation of the ERAS Society for Perioperative Care, The European Society for Clinical Nutrition and Metabolism, and The International Association for Surgical Metabolism and Nutrition<sup>[16]</sup>. These guidelines recommend detailed protocols for each component ranging from patient selection to hospital discharge, and provide additional consideration points in the setting of laparoscopic surgery.

### *Early rehabilitation and laparoscopic rectal surgery*

Laparoscopic rectal resection for various benign and malignant diseases, including total mesorectal excision, is considered technically challenging and has not gained popularity compared to laparoscopic colon resection. However, several studies have demonstrated that it is a feasible and safe alternative to open rectal surgery; some authors have reported that the short- and long-term oncological results were equal to those with open surgery<sup>[17-20]</sup>. We also reported the results of our multicenter study comparing open *vs* laparoscopic surgery for mid-rectal and low rectal cancer after neoadjuvant chemoradiotherapy (COREAN trial), which showed that laparoscopic surgery was safe and had short-term benefits, including earlier recovery of bowel function, shorter time to resume a normal diet, shorter time to first defecation, and less requirement for morphine, compared with open surgery<sup>[21]</sup>. Similarly, the quality of oncological resection was equivalent. Patients enrolled in the COREAN trial received postoperative management consisting of tradi-



Table 1 Previous representative studies of colonic surgery with early rehabilitation programs

Ref.	Country	Study design	Inclusion period	Patients (n)	Operations	Approach	LOS (d)		Readmissions		Morbidity		Mortality	
							ERP	CC	ERP	CC	ERP	CC	ERP	CC
Anderson <i>et al</i> <sup>[3]</sup> , 2003	United Kingdom	RCT	ND	25 (ERP: 14, CC: 11) Cancer: 18 (72) ERP: 11, CC: 7	RH: 14 (ERP: 9, CC: 5) LH: 11 (ERP: 5, CC: 6)	ND	3 (2-7)	7 (4-10) <sup>a</sup>	0 (0)	0 (0)	4 (29)	5 (45)	0 (0)	1 (9)
Gatt <i>et al</i> <sup>[4]</sup> , 2005	United Kingdom	RCT	ND	39 (ERP: 19, CC: 20) Cancer: 27 (69) ERP: 12, CC: 15	RH: 10 (ERP: 5, CC: 5) AR: 15 (ERP: 5, CC: 10) Others: 14 (ERP: 9, CC: 5)	ND	5 (4-9)	7.5 (6-10) <sup>a</sup>	1 (5)	4 (20)	9 (47)	15 (75)	1 (5)	0 (0)
Khoo <i>et al</i> <sup>[5]</sup> , 2007	United Kingdom	RCT	2003-2004	70 (ERP: 35, CC: 35) Cancer: 70 (100)	Colonic: 47 (ERP: 22, CC: 25) Rectal: 23 (ERP: 13, CC: 10)	Open	5 (3-37)	7 (4-63) <sup>a</sup>	3 (9)	1 (3)	9 (26)	16 (46)	0 (0)	2 (6)
Muller <i>et al</i> <sup>[6]</sup> , 2009	Switzerland	RCT	2004-2006	151 (ERP: 76, CC: 75) Cancer: 131 (87) ERP: 67, CC: 64	RH: 48 (ERP: 26, CC: 22) AR/LH: 101 (ERP: 30, CC: 51)	Open	5 (2-30)	9 (6-30) <sup>a</sup>	3 (4)	2 (3)	16 (21)	37 (49) <sup>a</sup>	0 (0)	0 (0)
Serclova <i>et al</i> <sup>[7]</sup> , 2009	Czech	RCT	2005-2007	103 (ERP: 51, CC: 52) Cancer: 7 (7) ERP: 3, CC: 4	Simple: (ERP: 47.1 %, CC: 61.5) Multiple: (ERP: 29.4 %, CC: 21.2)	Open	7 (5-11)	9 (7-22) <sup>a</sup>	0 (0)	0 (0)	11 (22)	25 (48) <sup>a</sup>	0 (0)	0 (0)
Lee <i>et al</i> <sup>[13]</sup> , 2011	South Korea	RCT	2007-2009	100 (ERP: 46, CC: 54) Cancer: 100 (100)	RH: 38 (ERP: 17, CC: 21) LH: 15 (ERP: 5, CC: 10) AR: 47 (ERP: 24, CC: 23)	Lap	7 (6-8)	8 (7-9)	0 (0)	0 (0)	6 (11)	14 (20)	0 (0)	0 (0)
Vlug <i>et al</i> <sup>[9]</sup> , 2011	Netherlands	RCT	200-2009	400 (ERP: 193, CC: 207) Cancer: 400 (100)	RH: 179 (ERP: 80, CC: 99) LH: 221 (ERP: 120, CC: 101)	Open/lap	Open: 7 (5-11) Lap: 5 (4-8)	Open: 7 (6-13) Lap: 6 (4.5-9.5) <sup>a</sup>	13 (7)	14 (7)	125 (65)	132 (64)	6 (3)	4 (2)
Wang <i>et al</i> <sup>[29]</sup> , 2012	China	RCT	2006-2009	78 (ERP: 40, CC: 38) Cancer: 78 (100)	RH: 13 (ERP: 7, CC: 6) Sig: 34 (ERP: 18, CC: 16) AR: 25 (ERP: 13, CC: 12)	Lap	5.5 (5-6)	7.0 (6-8) <sup>a</sup>	ND	ND	2 (5)	8 (21)	0 (0)	0 (0)

<sup>a</sup>*P* < 0.05 *vs* early rehabilitation program (ERP) group. LOS: Length of hospital stay; CC: Conventional care; RCT: Randomized controlled trial; RH: Right hemicolectomy; LH: Left hemicolectomy; SB: Small bowel; AR: Anterior resection; IBD: Inflammatory bowel disease; Lap: Laparoscopic; LAR: Low anterior resection; APR: Abdominoperineal resection; Sig: Sigmoidectomy; ND: Not documented. Continuous data are given as median (range) or mean  $\pm$  SD.

tional standard care instead of an early rehabilitation program. Only a few study results support the hypothesis that laparoscopic rectal surgery and a subsequent early rehabilitation program can act synergistically to enhance postoperative recovery and surgical outcomes.

During the past decade, some studies including prospective cohort studies and randomized controlled trials have shown that early rehabilitation programs enhance recovery after laparoscopic rectal resection and shorten the length of hospital stay<sup>[22-26]</sup>. However, these studies were heterogeneous: mixed open surgery or laparoscopy, colorectal disease or rectal disease, diverting stoma, and sphincter preservation, which makes it difficult to accept the validity of their results. Additionally, differences exist in the detailed components of individual early rehabilitation programs, which are classified into three categories of preoperative preparation, intraoperative intervention, and postoperative management, making it difficult to interpret a causal relationship between the components and positive/negative outcomes. To the best of our knowledge and based on the results of this literature review, only five studies have reported the results of implementation of early rehabilitation programs after laparoscopic rectal surgery: three prospective cohort studies<sup>[22,27,28]</sup>, one retrospective case-control study<sup>[29]</sup> and one randomized controlled trial<sup>[30]</sup>. The characteristics of these studies are summarized in Table 2.

A prospective cohort study by Lindsetmo *et al*<sup>[22]</sup> reported the results of 37 patients undergoing laparoscopic rectal resection. The mean hospital stay was 3.0 d (range, 1-8 d), in which 90% of patients were discharged < 5 d after surgery. No anastomotic leaks or mortality occurred, and the in-hospital complication rate was 8% (1 surgical-site infection

**Table 2 Summary of previous studies that evaluated early rehabilitation programs after laparoscopic rectal surgery**

Ref.	Country	Study design	Inclusion period	Patients (n)	Operations	Clinical effectiveness (LOS and complications)
Lindsetmo <i>et al</i> <sup>[22]</sup> , 2009	United States	Prospective cohort study	2005-2007	37 Cancer: 17 (46) Polyp: 4 (11) Others: 16 (43)	SPS: 37 (100) Diverting ileostomy: 7 (19)	Mean LOS: 3.0 d (range 1-8 d) Overall complications: 6 (16) UTI: 1; SSI: 2 Readmission < 30 d: 3 (8)
Chen <i>et al</i> <sup>[27]</sup> , 2011	Taiwan	Prospective cohort study	2007-2009	80 Cancer: 76 (95) Benign: 4 (5)	APR: 15 (19) SPS: 65 (81) Diverting ileostomy: 32 (49)	Mean LOS: 5.0d (range 3-22) Overall complications: 11 (14) AL: 1; pelvic abscess 2; ileus: 1 Readmission < 30 d: 7 (9)
Stottmeier <i>et al</i> <sup>[28]</sup> , 2012	Denmark	Prospective cohort study	2006-2009	102 Cancer: 102 (100)	APR: 19 (19) Hartmann: 6 (6) SPS: 77 (75) Diverting colostomy: 38 (37) Diverting ileostomy: 3 (3)	Median LOS: 5 d (range 2-42 d) Overall complications: 25 (25) AL: 3; intra-abdominal abscess: 3 Readmission < 30 d: 15 (15)
Huibers <i>et al</i> <sup>[29]</sup> , 2012	Netherlands	Retrospective case-control study	2004-2009	76 (ERP: 43, CC: 33) Cancer: 76 (100)	APR: 24 (32) ERP: 16 (37) CC: 8 (24) SPS: 52 (68) ERP: 27 (63) CC: 25 (76)	Median LOS: ( <i>P</i> = 0.042) ERP: 7 d (range 2-83 d) CC: 10 d (range 4-74 d) Overall complications: ERP: 17 (40) AL: 5; intra-abdominal abscess: 7 CC: 9 (27) AL: 4; intra-abdominal abscess: 3 Readmission < 30 d: ( <i>P</i> = 0.421) ERP: 5 (12) CC: 6 (18)
Lee <i>et al</i> <sup>[30]</sup> , 2013	South Korea	RCT	2007-2011	98 (ERP: 52, CC: 46) Cancer 98 (100)	SPS: 98 (100) Diverting ileostomy: 98 (100)	Median recovery time <sup>1</sup> : ( <i>P</i> = 0.47) ERP: 137 h (range 107-188 h) CC: 146.5 h (range 115-183 h) Overall complications: ( <i>P</i> = 0.054) ERP: 22 (42) AL: 1; POI: 15; acute voiding difficulty: 9 CC: 11 (24) AL: 1; POI: 6; acute voiding difficulty: 2 Readmission < 30 d: 0 (0)

<sup>1</sup>Defined by tolerable diet for 24 h, safe ambulation, analgesic-free and afebrile without complication. LOS: Length of hospital stay; SPS: Sphincter preserving surgery; UTI: Urinary tract infection; SSI: Surgical site infection; APR: Abdominoperineal resection; AL: anastomosis leakage; ERP: Early rehabilitation program; CC: Conventional care; RCT: Randomized controlled trial; POI: Postoperative ileus.

and 1 urinary tract infection). Readmission was required in three patients (8%) because of medical illness. The authors suggested that laparoscopy in conjunction with modern perioperative care allows rapid recovery with efficient use of hospital resources.

In contrast, two cohort studies by Stottmeier *et al*<sup>[28]</sup> and Chen *et al*<sup>[27]</sup> highlighted that postoperative morbidity remains substantial after laparoscopic rectal surgery combined with early rehabilitation program, even though performed by experienced surgeons. Stottmeier *et al*<sup>[28]</sup> reported a median hospital stay of 5 d and a postoperative complication rate of 25% among 102 consecutive patients who had undergone elective fast-track laparoscopic rectal cancer surgery. Although about 40% of the patients had a diverting colostomy or ileostomy, reoperation was needed in 15% owing to anastomotic leakage, colonic ischemia, intra-abdominal abscess, or mechanical obstruction. Postoperative mortality (< 30 d) occurred in 3% of the patients; one with postoperative septicemia and pneumonia, one with postoperative multiorgan failure, and one with intraoperative splenic bleeding. Chen *et al*<sup>[27]</sup> calculated the success rate of their enhanced recovery program and rein-

vestigated factors that may have affected the results of the enhanced recovery program combined with laparoscopic rectal surgery. As designated by their program, patients were scheduled to be discharged on postoperative day 5. The criteria of discharge included absence of fever or tachycardia, successful passage of flatus or stool, tolerance of three meals per day, pain relief with oral nonopioid analgesics, and independent ambulation. They reported a success rate of 52.5%, and this failure was related to low rectal lesion sites (< 7 cm from the anal verge) and surgery-related complications, with a rate of 13.8%. The authors concluded that the enhanced recovery program for laparoscopic rectal surgery is feasible but is not advised for all cases requiring laparoscopic rectal surgery.

Previously, we had designed a prospective, randomized, controlled parallel group trial to compare the outcomes of an early rehabilitation program *vs* conventional care after laparoscopic low anterior resection in patients with mid-rectal or low rectal cancer ( $\leq 10$  cm from the anal verge)<sup>[30]</sup>. The primary endpoint was recovery within 4 postoperative days and the criteria for recovery were as follows: tolerable diet for 24 h, safe ambulation, analgesic-

**Table 3** Protocols used in previous studies for evaluating early rehabilitation programs after laparoscopic rectal surgery

Protocols	Lindsetmo <i>et al.</i> <sup>[22]</sup> , 2009	Chen <i>et al.</i> <sup>[27]</sup> , 2011	Stottmeier <i>et al.</i> <sup>[28]</sup> , 2012	Huibers <i>et al.</i> <sup>[29]</sup> , 2012	Lee <i>et al.</i> <sup>[30]</sup> , 2013
Preoperative stage					
General considerations	Patient education	Patient education and ERP explanation	Thorough information Establishing a contract	ND	Operative risk assessment Counseling, informed consent
Oral bowel preparation	Yes	Yes	No (enema for left-sided tumors)	No (2 enemas)	Yes
NPO	ND	8 h before surgery	Fluid until 2 h before surgery	2 h before surgery	8 h before surgery
Oral carbohydrate solution	No	No	No	Yes	No
Epidural analgesia	No	No	Yes	Yes	No
Prophylactic antibiotics	ND	Single dose	Single dose (ampicillin + metronidazole + gentamicin)	Single dose (cefazoline + metronidazole)	ND
DVT prophylaxis	ND	ND	LMWH 2 h before surgery Compression stockings	LMWH until discharge	ND
Perioperative stage					
Operation approach	Laparoscopic	Laparoscopic	Laparoscopic	Laparoscopic	Laparoscopic
Anesthesia	ND	Short-acting anesthetics	Propofol, remifentanyl and muscle relaxant	ND	ND
Fluid	ND	Perioperative fluid restriction	Avoid both hypovolemia and fluid overload	ND	ND
Urinary drainage	Urethral catheter	Urethral catheter	Suprapubic or urethral catheter	Urethral catheter	Urethral catheter
Nasogastric tube	Yes (orogastric tube, removed before extubation)	No	No	No	No
Intra-abdominal drain	Rarely	Yes	No	Yes (one)	Yes (one)
Postoperative stage					
Pain control	IV PCA (12-18 h) Ketorolac Oral analgesia	Oral NSAIDs immediately after surgery Opioid for 1 d if needed	Epidural analgesia Paracetamol, ibuprofen Opioid if needed	Epidural analgesia Paracetamol, diclofenac Opioid avoided	IV PCA till POD 2
Sipping water	Immediately after surgery	Immediately after surgery	Immediately after surgery	Immediately after surgery	Immediately after surgery
Oral food intake	POD 1	POD 1	Evening of the day of surgery	Liquid diet in the evening POD 2	Semi-fluid diet, POD 1
Removal of urinary catheter	POD 1	POD 1	Immediately after surgery	POD 2	POD 3
Removal of intra-abdominal drain	No drain	POD 4	No drain	POD 2	ND
Mobilization	As soon as possible	Immediately after surgery	Two hours after surgery	POD 1	POD 1
Regular laxatives	ND	Sennoside	MgSO <sub>4</sub> 1 g two times daily	MgO	MgO
Routine discharge	ND	POD 5	POD 3	ND	ND
Discharge criteria	Tolerance of fluids and solid diet, adequate oral analgesia, passage of flatus or stool, adequate home support	No fever, no tachycardia, successful passage of flatus/stool, tolerance for 3 meals/d, comfort in taking oral non-opioid analgesics, independent ambulation, adequate self-care ability	Adequate bladder and bowel function, ability to drink, eat, walk without problems, manageable pain	No remaining lines or catheters, toleration of solid food, passage of stool, controllable pain, self-care ability	ND (Recovery: tolerance of diet for 24 h, analgesic-free, safe ambulation, afebrile status without major complications)

ERP: Early rehabilitation program; DVT: Deep vein thrombosis; LMWH: Low-molecular-weight heparin; NSAID: Non-steroidal anti-inflammatory drug; PCA: Patient-controlled analgesia; POD: Postoperative day; ND: Not described.

free, and afebrile status without major complications. The sample size was based on a superiority design. All patients were between 20 and 80 years of age and had undergone temporary loop ileostomy with laparoscopic low anterior resection. Protocols for perioperative care programs and

interventions were modified from previously described protocols for colonic surgery (Table 3). Ninety-eight patients were randomized on a 1:1 basis to an early rehabilitation or conventional care program. The recovery rates were no different in both groups; however, more com-

plications were observed in the rehabilitation program group (42.3% *vs* 24.0%,  $P = 0.054$ ), which were related to postoperative ileus (28.8% *vs* 13.0%,  $P = 0.057$ ), and acute voiding difficulty (19.6% *vs* 4.7%,  $P = 0.032$ ). Our randomized trial did not show that an early rehabilitation program was beneficial after laparoscopic low anterior resection. These results support those of previous studies in that postoperative morbidity might be a major obstacle to the ERAS in rectal cancer surgery.

## CURRENT EVIDENCE-BASED RECOMMENDATIONS FOR EARLY REHABILITATION AFTER RECTAL SURGERY

### *Consideration points for adopting early rehabilitation program in rectal surgery*

For the successful application of early rehabilitation programs to patients undergoing laparoscopic rectal resection, we need to recognize that colon surgery is entirely different from rectal surgery, which requires a deep pelvic dissection and is frequently accompanied by higher complication rates, longer hospital stay, and associated with unique complications such as sexual dysfunction, urinary retention, and pelvic organ injury (*e.g.*, hypogastric nerves and ureters) not seen in intra-abdominal colonic resection. Compared with colonic segmental resection, rectal surgery has higher technical complexity, longer operative times, and use of retraction known to increase perioperative morbidity<sup>[8]</sup>. Therefore, previous studies involving early rehabilitation programs excluded patients undergoing rectal resection<sup>[1,3,4,8]</sup>. In some studies, the results of rectal resection were mixed in the overall analysis of the application of early rehabilitation program protocols<sup>[23,24,26,31]</sup>.

The available guidelines for perioperative care in rectal surgery are currently limited<sup>[2,8]</sup>. Recently, guidelines for perioperative care in elective rectal surgery were published by the ERAS Society, which had also published colonic guidelines<sup>[8,16]</sup>. In these guidelines, the authors remarked that they specifically considered the application of ERAS principles to a special population of rectal resection patients, because of the differences between colonic and rectal surgery. Until now, ERAS Society recommendations seem to be the best evidence-based guidelines for each item of the perioperative treatment pathway. These recommendations were derived from extensive review of meta-analyses, randomized controlled trials, and large prospective cohorts. However, these guidelines are basically intended for open rectal surgery, and are not focused on laparoscopic surgery. ERAS Society recommendations assess the quality of evidence ("high", "moderate", "low", "very low"), and decide the strength of recommendations as follows: strong recommendations indicate that the panel is confident that the desirable effects of adherence to a recommendation outweigh the undesirable effects; and weak recommenda-

tions indicate that the desirable effects of adherence to a recommendation probably outweigh the undesirable effects, but the panel is less confident<sup>[8]</sup>. Many items in the recommendations are based on low or moderate level of evidence. Some items are recommended by a high level of evidence, such as prophylaxis against thromboembolism or preoperative bowel preparation; however, studies on these items are based on the results of patients undergoing open surgery or in a population undergoing both open and laparoscopic surgery. Specific validation for these items in patients undergoing laparoscopic rectal resection remains insufficient.

Currently, no early rehabilitation protocol perfectly fits all patients undergoing laparoscopic rectal surgery<sup>[2]</sup>. For each individual patient, these guidelines, which are suggestions on the basic concept for early rehabilitation, should be modified to optimize perioperative care, minimize postoperative morbidity, and improve overall patient outcomes.

### *Patient selection, counseling and risk assessment*

The first step is selecting patients. Extensive discussion with candidate patients on the entire surgical procedure followed by early rehabilitation program may be the most important step. This step can give patients the best insight into the benefits and risks and motivate them to make an effort to enhance their recovery after surgery because the success of early rehabilitation is affected by the active participation of the enrolled patient<sup>[2]</sup>. Previous studies and guidelines recommended direct interview, leaflets, or multimedia as information-providing methods<sup>[8]</sup>. Generally, patients who are bedridden, severely malnourished, and with an American Society of Anesthesia (ASA) score  $\geq 3$ , who are planning to receive emergency rectal surgery are excluded, and any healthy patients with ASA 1-2 are included<sup>[8,32]</sup>. It is also important to improve the patient's medical condition by correcting anemia, malnutrition, or hyperglycemia, and promoting cessation of smoking and alcohol consumption at least 4 wk before surgery<sup>[33]</sup>.

### *Bowel preparation*

Mechanical bowel preparation (MBP) is considered a necessary step before colorectal surgery, and it is believed to decrease the risk of infectious complications and anastomotic leakage. However, several studies, including large meta-analyses, showed no difference between the MBP and no MBP groups on infection rates or anastomotic leakage after colorectal surgery<sup>[8,34-36]</sup>. Some studies suggested that MBP increased dehydration and electrolyte imbalance<sup>[37]</sup>. On the contrary, a recent multicenter randomized trial showed that overall and infectious complications were higher in the no MBP group compared with the MBP group in patients undergoing low anterior resection. In this study, a non-significant trend to a two-fold higher risk of anastomotic leak (19% in no MBP *vs* 11% in MBP) was also observed<sup>[38]</sup>. Current guidelines support omitting MBP in colonic surgery but indicate insufficient evidence supporting this omission in rectal



surgery<sup>[8,39,40]</sup>. There has been no study on MBP efficacy in the context of early rehabilitation programs. The Society of American Gastrointestinal and Endoscopic Surgeons Guidelines comments that MBP may be helpful in laparoscopic colorectal surgery, because it can make laparoscopic colorectal manipulation easier<sup>[40]</sup>. Further studies comparing MBP with no MBP in patients undergoing laparoscopic rectal surgery are necessary.

### Postoperative pain

Postoperative analgesia is critical to enhance patient recovery because it directly affects early ambulation and patients comfort. Postoperative analgesia requires a multimodal approach consisting of the collaboration of the patient, surgeon, nurse, anesthesiologist and pain specialist<sup>[2]</sup>. Patient-controlled opioid analgesia (PCA) usually shows satisfactory result after rectal surgery<sup>[41]</sup>. However, PCA has some side effects influencing early recovery of patients, such as nausea, vomiting, and prolongation of postoperative ileus as well as sedation and respiratory suppression<sup>[2]</sup>.

Two recent guidelines recommended continuous epidural analgesia (CEA) for open rectal surgery during 48-72 h, with intravenous administration of lidocaine in view of the superior efficacy of pain relief compared with systemic opioids<sup>[2,8,42]</sup>. CEA has the benefit of delivering a combination of local and opioid analgesia directly to the dorsal horn of the spinal cord, thus providing pain relief without systemic opioid effects<sup>[43]</sup>. However, this method involves an invasive procedure for catheter insertion and has some side effects, including pruritus, urinary retention, and arterial hypotension<sup>[44]</sup>. Some authors have advocated CEA use in the context of early rehabilitation in patients without contraindications<sup>[45,46]</sup>. They have suggested that the superiority of CEA seems to be greatest in the first 2-3 d postoperatively, and thus, routine removal of CEA after 2 or 3 d postoperatively may be a useful strategy. Some studies have shown that, in laparoscopic approaches that use only several small incisions instead of a single, large vertical incision from the umbilicus down, continuous intravenous infusion of lidocaine or PCA, as alternatives for CEA, also provide good pain relief in the first 24 h with a similar time to return of bowel function or length of hospital stay<sup>[8,47]</sup>.

### Pelvic drainage

The use of pelvic drainage after low anterior resection has been a controversial issue in rectal surgery. Some surgeons still prefer insertion of a drain into the pelvic cavity to prevent bloody ascites and its adverse effect on anastomosis. Several randomized trials and meta-analyses have shown that the routine use of a pelvic drain does not affect the anastomotic leakage or overall complications<sup>[48-50]</sup>. However, the use of a drain should be considered in cases of clinical indications, such as high-risk individuals or suspicion of tenuous anastomosis<sup>[8]</sup>.

### Prevention of ileus

Prevention of postoperative ileus is a crucial element not

only for success of early rehabilitation, but also postoperative morbidity, readmission, and overall outcomes. To promote bowel motility after abdominal surgery, several methods have been evaluated, including gum chewing, oral magnesium oxide, and bisacodyl suppositories<sup>[51-54]</sup>. These methods have been reported to reduce time to bowel movement by 1-2 d, but there was no effect in the length of hospital stay or overall outcomes. However, the association of these medications with anastomotic dehiscence has not been addressed in a randomized trial of sufficient size. Furthermore, anastomotic leakage and temporary stoma should be considered in the use of stimulant laxatives after rectal surgery. Ileostomy has been reported as an independent risk factor for postoperative ileus, which developed in 22.8% of patients<sup>[55]</sup>. Our previous randomized controlled trial to evaluate the efficacy of an early rehabilitation program after laparoscopic rectal surgery also indicated a similar result, showing that a rehabilitation program introducing an early oral diet could increase postoperative ileus. Thus, further studies are necessary<sup>[30]</sup>.

## CONCLUSION

Early rehabilitation combined with laparoscopic rectal surgery is a feasible alternative in some selected patients, but indications have not been established. Current evidence fails to support the safety of early rehabilitation combined with laparoscopic rectal surgery compared to that reported for laparoscopic colonic surgery. Long-term outcomes, which might be affected by postoperative complications, in patients with malignant disease are unknown after laparoscopic rectal surgery followed by an early rehabilitation program. More data from well-designed clinical trials should be accumulated for widening the adoption of early rehabilitation programs to patients undergoing laparoscopic rectal surgery.

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## Pathophysiology of cystic fibrosis and drugs used in associated digestive tract diseases

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Author contributions: Haack A worked on the design and planning, in the acquisition, analysis and interpretation of data, in writing and critical review; Aragon GG contributed to the manuscript writing and interpreting data; Novaes MRCG worked on the design and planning, data interpretation, the writing and the final revision.

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### Abstract

Cystic fibrosis (CF) causes chronic infections in the respiratory tract and alters the digestive tract. This paper reviews the most important aspects of drug treatment and changes in the digestive tract of patients with CF. This is a review of the literature, emphasizing the discoveries made within the last 15 years by analyzing scientific papers published in journals indexed in the Scientific Electronic Library Online, Sciences Information, United States National Library of Medicine and Medical Literature Analysis and Retrieval System Online databases, both in English and Portuguese, using the key words: cystic fibrosis, medication, therapeutic, absorption, digestion. Randomized, observational, experimental, and epidemiological clinical studies were selected, among others, with statistical significance of 5%. This review evaluates the changes found in the digestive tract of CF patients including pancreatic insufficiency, constipation and liver diseases. Changes in nutritional status are also described. Clinical treatment, nutritional supplementation and drug management were classified in this review as essential to the quality of life of CF pa-

tients, and became available through public policies for monitoring and treating CF. The information gathered on CF and a multi professional approach to the disease is essential in the treatment of these patients.

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**Key words:** Cystic fibrosis; Medication; Therapeutic; Absorption; Digestion

**Core tip:** Cystic fibrosis (CF) has been studied in Brazil and in many other countries. Digestive manifestations may significantly compromise the nutritional status of CF patients, leading to numerous symptoms. Supplementation with enzymes, vitamins and nutrients is usually necessary. When infections are present, antibiotics are necessary, and these infections are often multisystemic, involving the digestive tract. The pharmaceutical assistance included in public policies, especially those which are financed, and the constant incentive to study the digestive manifestations in CF patients are essential, as without them, there would be infinite clinical changes which would compromise patient survival.

Haack A, Aragão GG, Novaes MRCG. Pathophysiology of cystic fibrosis and drugs used in associated digestive tract diseases. *World J Gastroenterol* 2013; 19(46): 8552-8561 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8552.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8552>

### INTRODUCTION

Cystic fibrosis (CF) is a chronic progressive disease, it exists in every ethnic group and it is equally common in both sexes. The CF gene has been isolated, cloned and sequenced, enabling the study of biochemical mechanisms responsible for the physiopathogenesis of the



disease. It also enables easier treatment of the patient's complications, such as the thick and viscous fluids which obstruct the lungs, the pancreas and the biliary duct<sup>[1,2]</sup>.

The prevalence of CF varies according to ethnicity, from 1/1800 to 1/5000 in Caucasians born alive in Europe, in the United States and in Canada, 1/14000 in Afro-Americans, and 1/40000 in Finland. It is considered a rare disease among Asians and Africans. In Brazil, local studies show variable statistical data which suggest an approximate incidence of 1/7000. The average lifetime of CF patients has increased in the last few years, which is the result of early diagnosis and specialized treatment in the early stages of the disease<sup>[1,3,4]</sup>.

The treatment of CF aims to clear the lungs using aerosols and respiratory physiotherapy, and to maintain nutritional status with nutrient supplementation and pancreatic enzymes. Recent medical advances have improved survival, but with increased costs, especially when the disease has progressed and when hospitalization is required. When infections are present, antibiotics are necessary, usually due to clinical complications which are often multisystemic, and involve the digestive tract<sup>[5,6]</sup>. Due to many involved systems and the variety and chronicity of the disease, a multitask approach is essential to help the patients and their families to comprehend the disease and undergo medical treatment<sup>[7]</sup>.

The current therapy for CF includes the maintenance of nutritional status, clearance of the pulmonary tract, utilization of antibiotics and other medication, treatment and monitoring of gastric, pancreatic and hepatobiliary changes, in addition to dietary supplementation with hypercaloric and hyperproteic foods, and the utilization of enzymes, minerals and vitamins<sup>[1,8,9]</sup>.

When chronic CF is diagnosed, with many clinical manifestations, the continuous use of medication (antibiotics, bronchodilators, mucolytics) and related procedures (respiratory physiotherapy, oxygen therapy, lung transplantation, digestive enzyme replacement and nutritional support) are required<sup>[8,10]</sup>. Due to the chronicity and the need for precautions in CF, the development of a Reference Center and the establishment of an organization that involves family members is crucial, together with an increase in cooperation between groups of CF patients and other organizations<sup>[4,11,12]</sup>.

CF requires the continuous use of medication which increases the average cost of treatment, and is too expensive for families. For that reason, CF patients and their families have the right to receive government help under the Unique Health System. The clinical record of the Health Ministry guarantees access to alpha dornase for pulmonary complications and pancreatic enzymes in patients with pancreatic insufficiency<sup>[3]</sup>. There are many deeds in every unit of the federation, including the Distrito Federal, to promote early diagnosis and even provide special formulas such as the alimentary supplements provided by Ordinance number 94/1809, published at the Distrito Federal in 2009<sup>[13]</sup>.

In Brazil, the dedication to diagnosing CF during

infancy is significant, with the use of programs for newborn screening or sweat testing. It is known that early treatment, including drug treatment, contributes to the prognosis and survival of CF patients<sup>[14-17]</sup>.

The objective of this study was to review the most important aspects of drug treatment and changes in the digestive tract of patients with CF. We also aimed to assess the pharmaceutical monitoring offered to CF patients undergoing treatment by public agents from the public health care system.

## REVIEW OF LITERATURE

This review focused on CF literature over the last 15 years, and included scientific papers indexed in the databases of Scientific Electronic Library Online, Sciences Information, United States National Library of Medicine and Medical Literature Analysis and Retrieval System Online, using the key words: cystic fibrosis, medication, therapeutic, absorption, digestion. Studies in English and Portuguese were selected.

The survey focused on the major advances in the understanding of CF during this period, both in understanding the disease and its treatment.

Articles that included at least one of the mentioned key words were selected. Controlled clinical studies were included, as well as observational epidemiological studies and meta-analyses, among others. Papers which did not include information on the diagnosis of CF or adherence to treatment were excluded as were experimental animal studies and gene therapy studies and those published in languages other than English and Portuguese.

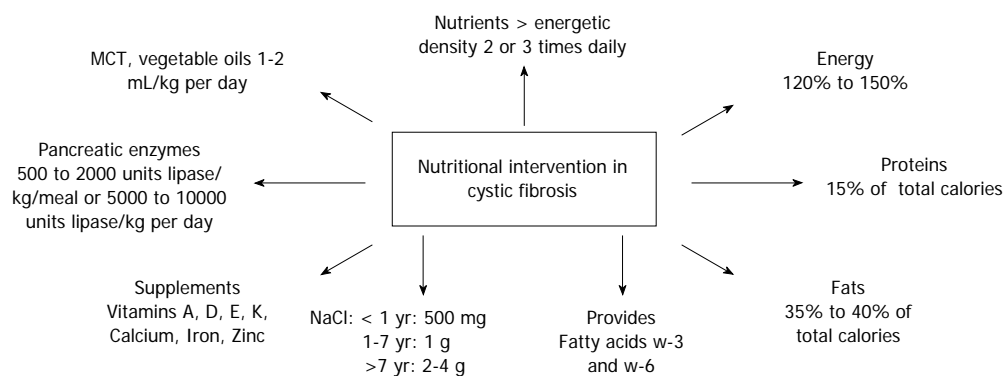
## RESULTS AND DISCUSSION

### *Physiopathology of the disease: overall symptoms*

The manifestation of CF is very changeable and may appear in the neonatal period or later in life. Some patients are completely asymptomatic for several years. The most common clinical signs of CF include a chronic cough, chronic diarrhea and malnutrition; however, the disease can appear in other ways, and can affect multiple systems and organs<sup>[18]</sup>.

Mutation of the CF gene causes absence or dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which works as a chloride canal in the apical membranes of epithelial cells. The CFTR also affects the production of mucus, secretory granules and intracellular organelles. This defect affects cells in many organs, not all organs have similar clinical responses, and different organs may be affected. Involvement of the respiratory tract is associated with a higher death rate and leads to death in 90% of patients<sup>[18-20]</sup>.

The most common and important symptom which affects the digestive tract is exocrine pancreatic insufficiency, characterized by chronic diarrhea with undigested food present. A decrease in the secretion of sodium bicarbonate reduces the efficacy of pancreatic enzymes



**Figure 1** Macronutrients and micronutrients essential for the recovery and maintenance of nutritional status in cystic fibrosis patients<sup>[7]</sup>. MCT: Medium chain triglycerides.

and the precipitation of bile salts, which results in a more acidic pH in the duodenum, contributing to malabsorption<sup>[18]</sup>.

The obstruction of pancreatic canaliculi by mucous plugs prevents the release of enzymes into the duodenum, which causes poor digestion of fat, proteins and carbohydrates. Malabsorption is caused by pre-epithelial dysfunction, which occurs after the rejection of non-hydrolysable nutrients in the lumen. Therefore, malnutrition occurs due to inadequate food digestion and increased energy needs (dietary recommendations) that are rarely achieved by CF patients due to anorexia and recurrent respiratory disease among other diseases<sup>[18,21-23]</sup>.

The endocrine pancreas also undergoes changes and the prevalence of CF related to glucose intolerance has increased proportionally with the rate of survival. The main cause of diabetes is damage caused to the pancreas, leading to a decrease in insulin secretion. Diabetes in CF patients results from microvascular and macrovascular complications associated with accelerated lung deterioration, consequently increasing the death rate. Since nutrition is critical in CF patients, blood glucose should be monitored and the insulin dose should be adapted, with a focus on adequate intake of nutrients<sup>[24]</sup>.

Symptomatic vitamin A and vitamin E deficiency has been reported in patients with CF presenting with deficit nutrient consumption and absorption<sup>[25,26]</sup>.

Many newly diagnosed infants have low levels of one or more fat-soluble vitamins<sup>[27,28]</sup> and due to the prevalence of fat-soluble vitamin deficiency, all infants with CF should receive standard, age-appropriate non-fat-soluble vitamins and vitamins A, D, E, and K as recommended in the CF Foundation Consensus Report on Nutrition for Pediatrics<sup>[29]</sup>.

Most patients who are vitamin deficient can be treated adequately with the doses of fat-soluble vitamins recommended in the CF Foundation Consensus Report on Nutrition for Pediatric Patients<sup>[30]</sup>.

Figure 1 shows relevant information on the nutritional care of CF patients<sup>[7]</sup>.

Among other events related to CF, meconium ileus, obstruction of the terminal ileum by thick meconium, is the first signal of pancreatic insufficiency, which affects

15% of babies. Therefore, treating patients with meconium ileus is very important until proved otherwise<sup>[31]</sup>.

Early diagnosis and the treatment of complications of the respiratory and gastrointestinal tract in CF can lead to an improvement in the survival rate of CF patients. Those who live beyond the fourth decade have a higher risk of developing additional diseases associated with chronic manifestations; hence, patients with a higher risk of chronic diseases should be monitored closely to improve the chances of early diagnosis<sup>[32]</sup>.

Figure 2 summarizes the majority of abnormalities observed in the digestive tract of patients diagnosed with CF from intrauterine life to adulthood.

### Gastrointestinal disease

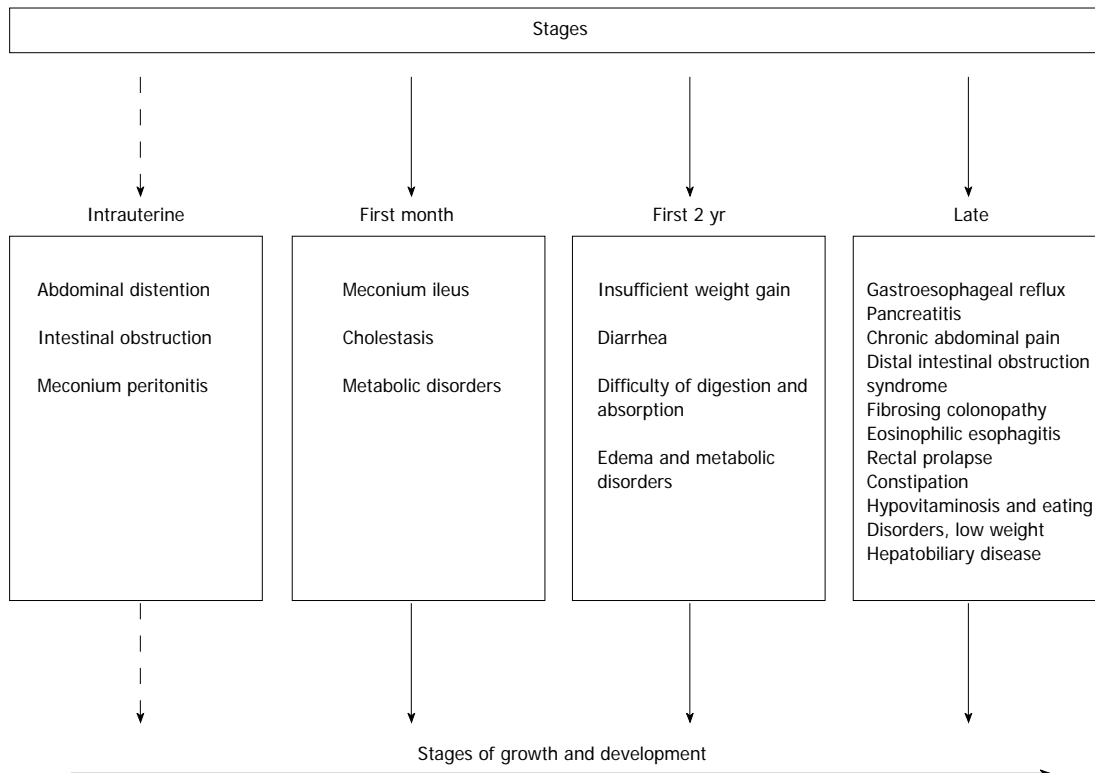
In CF patients gastrointestinal symptoms, such as nausea, vomiting, malnutrition and indigestion are frequent. In addition, gastroesophageal reflux disease, esophageal adenocarcinoma, distal intestinal syndrome and cholelithiasis are often seen in CF patients<sup>[33-35]</sup>.

There is increasing evidence to suggest that chronic inflammation is present in the gastrointestinal tract of CF patients. Some CF patients continue to have many severe gastrointestinal symptoms despite conventional CF treatment<sup>[36]</sup>.

A recent publication indicated the presence of eosinophilic esophagitis (EoE) in CF patients aged from 4, 12 and 15 years. Patients with CF may have clinically persistent emesis, food aversion and failure to thrive. It is possible that EoE has been underappreciated in CF due to symptom overlap with other common gastrointestinal disorders, including gastroesophageal reflux disease, infections, medication side effects or others conditions<sup>[37]</sup>.

Because the symptoms in EoE are non-specific and are also common in CF, when a patient with CF presents with food avoidance, regurgitation, heartburn or dysphagia, EoE should be considered, particularly if symptoms do not respond to empiric treatment and if endoscopic evaluation is contemplated<sup>[38-40]</sup>.

Secretory cells of CF patients show modification in their absorptive-digestive function in the gastrointestinal tract and the entire digestive process is altered, which results in malabsorption of nutrients, malnutrition and



**Figure 2** Summary of major abnormalities observed in the digestive tract of patients diagnosed with cystic fibrosis from intrauterine life to adulthood.

several gastrointestinal tract-related symptoms<sup>[34,41]</sup>.

Abdominal pain is a common complaint in CF patients, and distal bowel obstruction syndrome and fibrosing colonopathy are characteristics of gastrointestinal complications in CF patients. The main causes of epigastric pain in patients with CF are gastroesophageal reflux disease, biliary tract disease, pancreatitis and gastritis<sup>[42,43]</sup>.

Among the frequently observed gastrointestinal manifestations, gastroparesis has been diagnosed by a variety of methods and has been described by CF patients. Gastroparesis is a frequent complication of lung or heart-lung transplantation. It is predominantly found in children and individuals with severe deterioration of the pulmonary tract<sup>[43,44]</sup>.

After meconium ileus, the main area affected by distal bowel obstruction syndrome (DIOS) is the right colon. DIOS is more common in patients with pancreatic insufficiency. Several factors can trigger the syndrome, such as dehydration, the use of medicines which interfere with intestinal motility and pancreatic enzyme replacement. The most common signs and symptoms of DIOS are decreased defecation and colic pain in the right lower quadrant. During clinical examination, a reduction in intestinal peristalsis can be observed, with the possibility of cessation at some point. In some cases, a mass in the lower right quadrant can be palpated, which is related to distention of the cecum and right colon<sup>[45]</sup>.

Intestinal obstruction syndrome is similar to meconium ileus; however, one of the differences between these conditions is patient age. Intestinal obstruction syndrome is characterized by the impaction of fecal residues

in the terminal ileum and one of the precipitant factors for obstruction is dehydration. This obstruction can be total or partial, and may cause symptoms such as abdominal distention, constipation, anorexia, vomiting, and early satiety, which result in weight loss<sup>[45]</sup>.

Fibrosing colonopathy is another characteristic of CF, and includes a change in the colon submucosa, inflammation, and progressive fibrosis associated with managing the high doses of pancreatic enzymes. The clinical symptoms are pain and abdominal distention after ingesting food, anorexia, difficulty in gaining weight and digestive bleeding<sup>[34,46]</sup>.

### Pancreatic disease

The pancreas is one of the main organs affected by dysfunction of the CFTR. The exocrine pancreas is responsible for producing enzymes for food digestion in the intestinal lumen and exocrine pancreatic insufficiency is a well-known complication of CF and leads to fat loss in feces. Loss of function of the pancreas is associated with every genotype of CFTR mutation, leading to pancreatic insufficiency<sup>[47-49]</sup>.

Pancreatic exocrine insufficiency (PEI) is considered the main cause of intestinal malabsorption in CF, affecting 85% to 90% of patients<sup>[50]</sup>, and if inadequately treated high stool energy losses will occur, which is an important determinant of energy imbalance and malnutrition<sup>[51]</sup>.

Intestinal malabsorption is usually of early onset: signs and symptoms of maldigestion are often present at birth, and in the majority of patients, during the first years of life. At the time of diagnosis, at least 50% of in-

**Table 1** Fat-soluble vitamins used for supplementation in cystic fibrosis patients<sup>[1]</sup>

Vitamins	Dosage	Dosage
A	400-10000 UI (approximately 2240 µg)	Daily
D	400-1800 UI (approximately 18 µg)	Daily
E	50 mg (1 yr)	Daily
	100 mg (1- 10 yr)	
	180 mg (adolescents and adults)	
K	0.3-0.5 mg	Daily

fants identified by neonatal screening have PEI, and most of those carrying severe CFTR mutations on both alleles develop PEI during the first years of life<sup>[52-55]</sup>.

PEI is clinically characterized by weight loss or difficulty in gaining weight, diarrhea with a greasy appearance and malabsorption of fat-soluble vitamins A, D, E and K. Thus, the supplementation of these vitamins is routinely recommended, followed by blood examinations to manage the dose and the correct nutrients according to the patient's needs<sup>[27,56-58]</sup>.

Vitamin D is of great interest in CF due to its role in bone mineralization and its deficiency has been hypothesized to play a role in the development of depression. Hypovitaminosis is almost universal in patients with CF. Insufficient levels are widely reported and is associated with increasing age and obesity. Vitamin D screening and supplementation should be considered in all children with chronic illness, particularly those who are overweight<sup>[59-62]</sup>.

Table 1 shows treatments with fat-soluble vitamin supplementation in CF patients<sup>[1]</sup>.

### Hepatobiliary disease

The primary hepatic changes in CF involve a genetic defect in the CFTR protein, leading to the production of a thick biliary secretion, followed by biliary fibrosis<sup>[34]</sup>. Cirrhosis, ascites, portal hypertension, esophageal varices and bleeding are complications of hepatobiliary disease associated with CF, and frequently affect teenagers and adults<sup>[33]</sup>.

This dysfunction is predicted to result in defective (sluggish) bile flow, and is associated with a cholangio-cyte-induced inflammatory response with activation and proliferation of hepatic stellate cells, which results in cholangitis and fibrosis in focal portal tracts<sup>[63-66]</sup>.

Approximately 5%-10% of CF patients develop multilobular cirrhosis during their first decade of life. Subsequently, most tend to develop signs of hypertension with complications, especially variceal bleeding. Annual examinations are recommended to detect hepatic disease, and when presymptomatic signs are present therapy with ursodeoxycholic acid is recommended, which can prevent disease progression<sup>[67,68]</sup>.

Cystic fibrosis-related liver disease (CFLD) is defined if at least 2 of the following conditions are present on at least 2 consecutive examinations spanning a 1-year period: (1) Ultrasound confirmed hepatomegaly; (2) Elevated serum levels of alanine aminotransferase, aspartate

aminotransferase, alkaline phosphatase, gamma-glutamyl-transferase; and (3) Ultrasound abnormalities other than hepatomegaly (*i.e.*, increased, heterogeneous echogenicity, nodularity, irregular margins, splenomegaly). An ultrasonographic pattern of simple liver steatosis does not represent a diagnostic criterion. In the case of distinct ultrasonographic signs of liver cirrhosis (*i.e.*, coarse nodularity, presence of portal hypertension and rarefaction of peripheral portal veins) and clinical signs (*e.g.*, esophageal varices, splenomegaly) of liver cirrhosis, CFLD patients are classified as cirrhotics<sup>[63,69]</sup>.

Liver disease can only be taken into consideration if the physical examination is abnormal and abnormal hepatic function persists, and the latter has to be proved using ultrasound. If there are any doubts, a liver biopsy is suggested. All patients with liver disease require to be monitored annually to evaluate the progress of hypertension, portal cirrhosis or liver failure. Prophylactic measures for liver disease are nutrition monitoring, bleeding prevention and variceal decompression. In liver transplantation, deterioration of the organ has to be taken into consideration, especially in children with hepatic dysfunction or advanced hypertension<sup>[68]</sup>.

### Treatment

Treatment with pancreatic enzymes in patients with pancreatic insufficiency is associated with an increase in the coefficient of fat absorption, a decrease in bowel movement frequency, an improvement in the consistency of feces and weight gain. One of the aims of pancreatic enzyme replacement therapy is to abolish unpleasant gastrointestinal symptoms<sup>[45]</sup>.

The response to treatment is individually evaluated, and doses are adjusted according to nutritional status. The use of antacids is recommended in patients taking enzymes to increase bioavailability, although, there is insufficient evidence to indicate whether there is an improvement in quality of life or survival<sup>[1,70]</sup>.

In young children whose fat intake is known to vary with age, particular attention needs to be paid to fat malabsorption during pancreatic enzyme supplementation. More importantly, young children often have difficulty swallowing the available enzyme formulations, which may lead to suboptimal compliance and treatment effects<sup>[71]</sup>.

The initial dose of pancreatic enzymes can be calculated based on the weight of the patient taking into consideration the dietary fat intake. 500 to 1000 U of lipase/kg is administered per main meal, the dosage can be increased according to clinical signs, and the maximum daily dose should not exceed 2500 U/kg per meal or 10000 U/kg per day of lipase<sup>[3]</sup>.

Figure 1 summarizes pancreatic enzyme dosage<sup>[7]</sup>.

The guidelines recommend that if dose increases are required, they should be increased with careful monitoring of body weight and stool fat content. When controlled clinical trials are designed to assess the safety and efficacy of pancreatic enzyme replacement therapy, the dose in terms of lipase units is usually limited to a level



within the recommended range. However, in everyday clinical practice it is possible that maldigestion is not adequately controlled by the recommended doses in a proportion of CF patients: these patients may, therefore, require higher lipase doses<sup>[72-74]</sup>.

The United Kingdom Cystic Fibrosis database indicates that lipase dose often exceeds 10000 U/kg per day for extended periods in clinical practice, both with standard-dose and high-dose pancreatic enzyme preparations. These high-dose regimens appear to have good safety and tolerability profiles, and fibrosing colonopathy has not been reported in recent years. However, it is essential that the safety and efficacy of higher doses of pancreatic enzyme replacement therapy are fully explored, particularly in the long-term, clinical practice setting<sup>[74,75]</sup>.

There are several options for the treatment of EoE, including pharmaceutical agents and dietary elimination. Consensus recommendations advocate first-line treatment with oral corticosteroids (*e.g.*, fluticasone, budesonide) or dietary therapy depending on patient preference and illness severity<sup>[38]</sup>.

Dietary therapy can be very effective in children if culprit food allergens are identified, and recent data show this to be effective including the elimination of offending agents (targeted elimination diet), or an allergen-free diet consisting only of an elemental formula (elemental diet)<sup>[76-78]</sup>.

The correction of steatorrhea is essential in CF. In the past, diets low in fat were recommended to try to reduce steatorrhea. Currently, restrictive diets have been replaced by hypercaloric diets rich in fat, which is a source of energy, are more economical and their intake should be encouraged<sup>[79]</sup>. The dose and timing should be followed very strictly, and patients should adhere to treatment. For infants, apple juice or small quantities of milk are consumed, and meals should be carried out in block in order to benefit from the bioavailability of the entire quantity of administered enzyme<sup>[49]</sup>.

Medium chain triglyceride fats should be included in the standard dietary regimen used in the management of any child with CF and failure to thrive. Their use is fully justified due to clinical improvement and alleviation of steatorrhea<sup>[80]</sup>.

In clinical practice, probiotics have been frequently prescribed for patients suffering from diarrhea to protect the body against pathogens<sup>[81]</sup>.

A probiotic is a "live microbial food ingredient that, when ingested in sufficient quantities, exerts health benefits on the consumer". Probiotics exert their benefits through several mechanisms; they prevent colonization, cellular adhesion and invasion by pathogenic organisms. The strongest evidence for their clinical effectiveness has been in their use for the prevention of symptoms of lactose intolerance, treatment of diarrhea, and attenuation of antibiotic-associated gastrointestinal side effects<sup>[81]</sup>.

Probiotics reduce the rate of pulmonary exacerbations in patients and may have preventive potential for

pulmonary deterioration in CF patients<sup>[82-84]</sup>.

To ensure a continuous effect, probiotics and prebiotics need to be ingested daily. Favorable changes in the composition of intestinal microbiota were observed at doses of 100 g of food product containing 109 colony forming units (cfu) of probiotic microorganisms and doses of 5 to 20 g inulin and/or oligofructose, usually during the administration period of 15 d. Thus, to be of physiological importance to the consumer, probiotics must reach populations greater than 10<sup>6</sup> to 10<sup>7</sup> cfu/g or mL bioproduct<sup>[85]</sup>.

The goal of nutritional therapy is to maintain the ideal weight, reduce malabsorption and digestion and control the intake of vitamins and minerals<sup>[1]</sup>. CF patients require diets with a high energetic rate (120% to 150% of the regular daily need for weight, height and age), hypercaloric, high-fat and high protein, divided into 5-6 meals a day and supplemented with vegetable oils such as medium chain triglycerides. In cases where dietary treatment does not result in weight gain, the diet can be offered in small volumes, several times a day or administered in the evening or through a nasogastric tube or gastrostomy. Enteral tube feeding has been evaluated in pediatric and mixed child and adult populations with CF, demonstrating positive outcomes post-insertion. The diet may be administered through an infusion pump or gravitational and it is recommended that the night diet reaches 40%-50% of the daily energy requirements so that there will be recovery or maintenance of the nutritional state<sup>[7,86,87]</sup>.

CF may include intestinal inflammation and CF patients have altered fatty acid metabolism characterized by an imbalance in the arachidonic/docosahexaenoic acid ratio in favor of the former, which can contribute to an increase in inflammation. Recent studies indicate that changes in fatty acid metabolism are responsible for abnormalities, and dietary supplementation with fish oils high in the omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid may have an anti-inflammatory effect<sup>[88-91]</sup>.

Various anti-inflammatory therapies, including dietary omega-3 polyunsaturated fatty acids supplementation, have been investigated in CF patients. The composition of dietary omega-3 and omega-6 influenced the inflammatory markers in CF and dietetic integration seems to improve clinical condition and the inflammatory pulmonary and intestinal state in patients suffering from CF<sup>[92,93]</sup>.

With a partial bowel obstruction, intestinal disimpaction is stimulated by hypertonic solutions, such as N-acetylcysteine, polyethylene glycol or hypertonic contrast, orally or by using probes. In cases of total obstruction the disimpaction is performed through enemas, while keeping the patient hydrated. After the disimpaction, pancreatic enzyme treatment should be included in the preventive treatment in obstructive conditions, administering lactulose, mineral oil, polyethylene glycol or N-acetylcysteine to the patient. Prokinetic drugs may also be helpful<sup>[34]</sup>.

It is recommended that, in the case of fibrosing colopathy, there is a reduction in the enzyme dose associated with nutritional support with either semi-elemental or elemental formulas according to the evaluation by the nutritionist for nutritional enteral therapy, and if necessary, associated with parenteral nutrition in the most severe cases. In the case of digestive bleeding, a surgical procedure is prescribed<sup>[34,46]</sup>.

The treatment of liver diseases focuses mainly on preventing disease progression which follows the sequence of cholestasis, fibrosis and cirrhosis. The maintenance of nutritional status is a part of this treatment, and aims to achieve and maintain the ideal weight of the patient, reduce malabsorption and maldigestion and control the intake of vitamins and minerals. However, nutritional treatment consists of enzyme replacement therapy, hypercaloric, high fat and micronutrient supplementation diets<sup>[1]</sup>.

Supplementation with taurine has also been suggested to improve the solubilization of lipid micelles by bile acids. Taurine is a conditionally essential amino acid that possibly improves the micellar phase of fat digestion. Patients with CF and severe steatorrhea, despite appropriate enzyme therapy, showed a significant improvement in the absorption of triglycerides, total fatty acids, and linoleic acid while receiving taurine supplements. Taurine supplementation could be a useful adjunct in the management of patients with CF with ongoing fat malabsorption and essential fatty acid deficiency<sup>[94,95]</sup>.

If CF patients also have taurine deficiency, this will result in malabsorption of bile acid and will require treatment with ursodeoxycholic acid (UDCA). The use of UDCA can increase the need for taurine administration for conjugation of bile acid<sup>[33]</sup>.

UDCA is the drug currently used in CF patients and aims to slow the progression of liver disease. UDCA is a hydrophilic drug and is not significantly concentrated in bile. It has a hepatoprotective effect with rare collateral effects reported<sup>[33]</sup> and is frequently used in CF. UDCA inhibits the hepatic synthesis of cholesterol and promotes the synthesis of bile acids, thereby restoring the necessary balance between cholesterol and bile salts. The suggested dose is 14-18 mg/kg per day, 2 to 3 times a day up to 30 mg/kg per day<sup>[3,96]</sup>.

Although it is one of the therapeutic options currently used for early changes in the liver, the use of UDCA as a preventive method requires further investigation as there are insufficient data on its long-term use, although adverse effects are rarely reported<sup>[97]</sup>.

Liver transplantation may be necessary in patients with progressive liver failure and/or evidence of major portal hypertension in the absence of significant pulmonary involvement<sup>[98,99]</sup>.

Careful monitoring and treatment should be offered to patients with CF associated liver disease (CFALD) and portal hypertension as they may require supplemental feeding by gastrostomy. However, this could lead to the development of stomal varices, which is an unwanted

complication. A recent study evaluated the risk of gastrostomy in a series of seven children with CFALD and portal hypertension. The research concluded that gastrostomy placement for poor nutrition in children with CFALD and portal hypertension is safe and contributes to improved nutritional and pulmonary outcome<sup>[100]</sup>.

CF is a multisystem disease and therefore requires different input from different professional reference centers for the treatment and monitoring of CF, supported by public health policies.

## CONCLUSION

CF has been extensively studied in Brazil and many other countries. Digestive manifestations significantly compromise the nutritional status of the patient and lead to numerous symptoms, organ deterioration, the need for transplantation and resections which can worsen the multisystem disease.

Reference Centers with up-to-date medical teams to monitor and treat CF patients and initiatives such as the Brazilian Cystic Fibrosis Research Group can contribute to the dissemination and standardization of information, in addition to improving the quality of treatment.

The scientific literature contains an important variety of drugs, including many that are available without charges through programs from the Unique Health System, Brazil.

The pharmaceutical assistance and the constant incentive to study digestive manifestations in CF patients are essential, as without them, there would be infinite clinical changes that would compromise patient survival.

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## Endoscopic tools for the diagnosis and evaluation of celiac disease

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**Core tip:** Celiac disease (CD) is an autoimmune disorder induced, in genetically predisposed people, by the ingestion of proteins rich in proline and glutamine. The aim of this review is to focus on the new endoscopic tools and techniques developed over the last years which can be useful in the diagnosis and the follow-up of CD.

### Abstract

Celiac disease (CD) is an autoimmune disease of the small bowel induced by ingestion of wheat, rye and barley. Current guidelines indicate histological analysis on at least four duodenal biopsies as the only way to diagnose CD. These indications are based on the conception of the inability of standard endoscopy to make diagnosis of CD and/or to drive biopsy sampling. Over the last years, technology development of endoscopic devices has greatly ameliorated the accuracy of macroscopic evaluation of duodenal villous pattern, increasing the diagnostic power of endoscopy of CD. The aim of this paper is to review the new endoscopic tools and procedures proved to be useful in the diagnosis of CD, such as chromoendoscopy, Fujinon Intelligent Chromo Endoscopy, Narrow Band Imaging, Optical Coherence Tomography, Water-Immersion Technique, confocal laser endomicroscopy, high-resolution magnification endoscopy, capsule endoscopy and I-Scan technology.

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**Key words:** Celiac disease; Malabsorption syndrome; Duodenum; Diagnostic techniques and procedures;

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### INTRODUCTION

Celiac disease (CD) is an autoimmune disorder induced, in genetically predisposed people, by the ingestion of proteins rich in proline and glutamine. It occurs in adults and children with an average prevalence of about 1% of the population. CD is characterized by an inflammatory reaction, primarily in the upper small intestine, with features of infiltration of the lamina propria and the epithelium with chronic inflammatory cells and progressive villous atrophy<sup>[1,2]</sup>. At the state of the art the role of serology is becoming more and more important, so that, according to the European Society for Paediatric Gastroenterology, Hepatology, and nutrition guidelines, diagnosis of celiac disease can be performed without histology in some selected situations-such as the presence, in children, of human leukocyte antigen-DQ2, high titers of

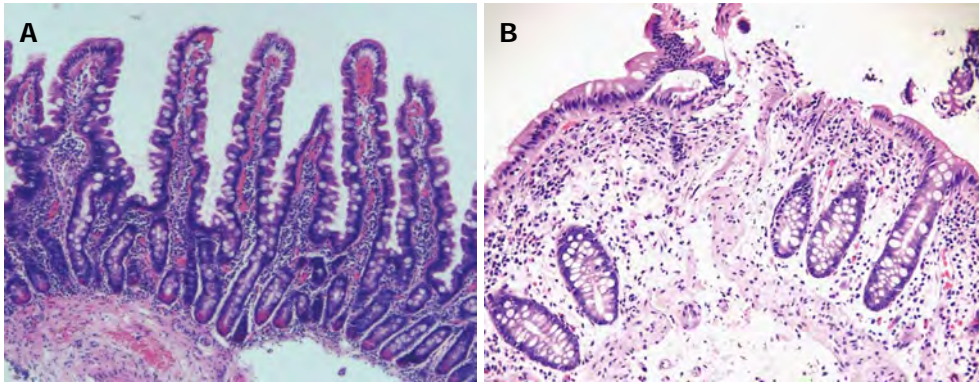


Figure 1 Histological appearance respectively. A: Normal duodenal pattern; B: Celiac disease.

anti-tissue transglutaminase antibodies and the positivity of anti-endomysial antibodies<sup>[3]</sup>. However, current guidelines indicate histological analysis as the gold standard for the diagnosis of CD: specific pathological features are infiltration of the lamina propria, crypt hyperplasia and villous atrophy, classified according to the Marsh classification and its modifications<sup>[4-8]</sup> (Figure 1). To perform a correct diagnosis, biopsy specimens have to be well oriented, and of good quality. From 4 to 6 duodenal biopsies, including a bulb biopsy, are required to make diagnosis of CD, even because villous atrophy can be unequally distributed -that is the so-called “patchy atrophy”<sup>[7,9-13]</sup>.

Anyway, the diagnosis of CD can also be missed if the disease is not suspected and biopsy sampling not performed. So, in such situations, the role of the endoscopist becomes crucial, because of the strong importance of the macroscopic appearance of the duodenum<sup>[14-16]</sup>.

## STANDARD ENDOSCOPIC FINDINGS

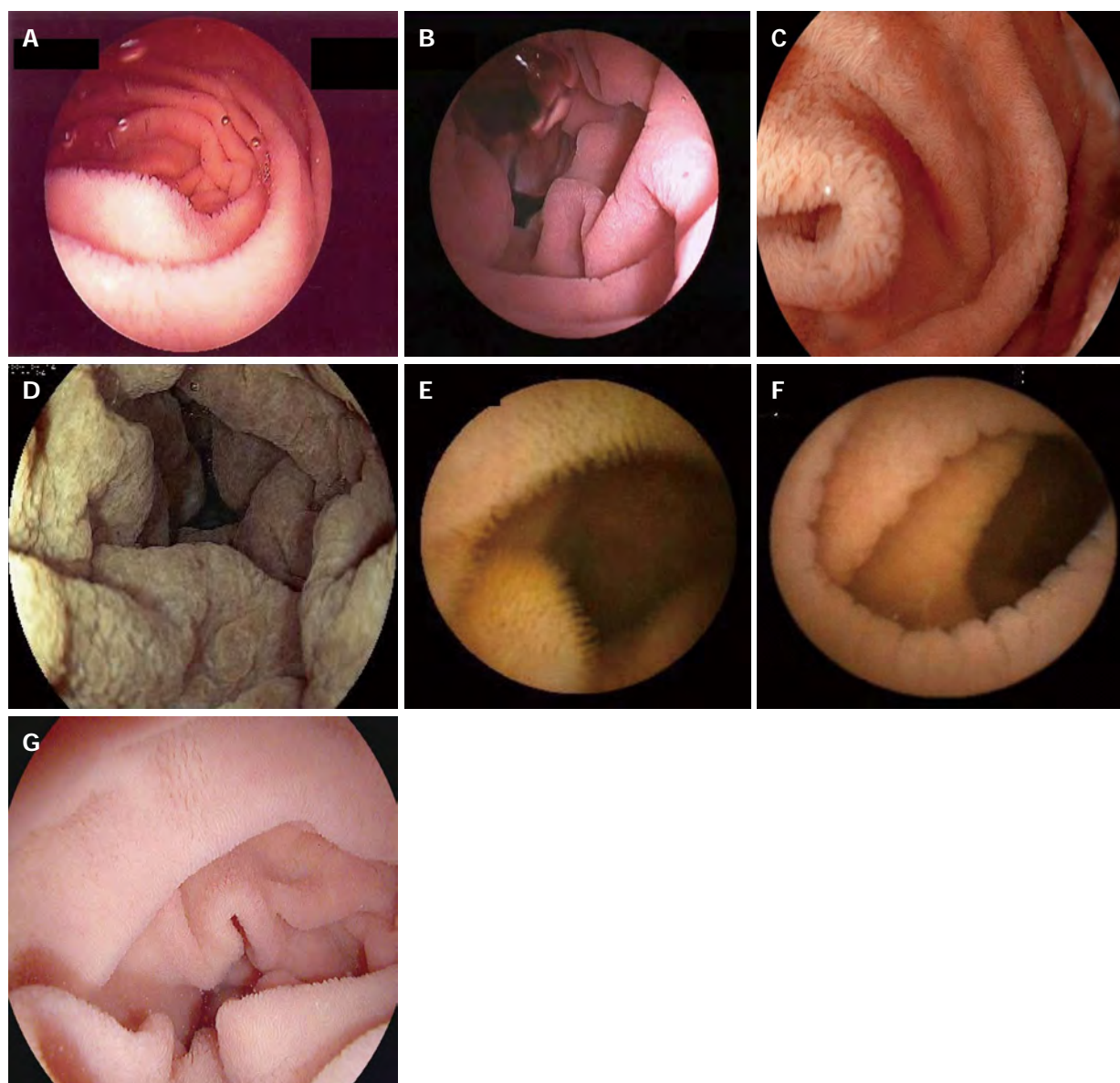
A number of macroscopic endoscopic markers of CD has been identified over the years, and they include the following: “scalloping” -that is a dented aspect- of the duodenal folds; an absence or a reduction in number of duodenal folds; evidence of submucosal vascular pattern; the so-called “mosaicism”, which is a micronodular look of the mucosa; finally, grooves and fissurations of the mucosa<sup>[9-10,14,15]</sup>. Results about the value of these markers, however, are conflicting: among different studies, the overall specificity and sensitivity sways from 83% to 100%, and from 6% to 94%, respectively<sup>[14,15,17-26]</sup>.

This happens probably because endoscopic markers cannot be present in milder degrees of the disease. (such as partial villous atrophy) and absent in case of patchy disease<sup>[12,18,19]</sup>. On the other hand, scalloped feature of duodenal folds has a positive predictive value of 69% for celiac disease and 96% for any duodenal mucosal disease<sup>[27]</sup>. So, the contradictory evidences and the low sensitivity of endoscopic markers implicates that bioptic sampling should always be performed when the disease is suspected, because their absence does not exclude the diagnosis<sup>[16]</sup>.

## WATER-IMMERSION TECHNIQUE

The water-immersion technique is a easy, prompt and safe procedure of enhancement of duodenal villous pattern during a conventional upper endoscopy. Our group developed this technique as a method to emphasize the visualization of duodenal villi<sup>[28]</sup>, and then modified it to make it helpful in clinical practice<sup>[29]</sup>. The mechanism of the water-immersion technique is very simple, comprising, at first, the removal of air from the duodenal lumen by suction, quickly followed by the injection of 90-150 mL of water<sup>[29]</sup>. The procedure requests about 25-30 s more than a standard upper endoscopy, resulting very fast. Our group proved the high accuracy of the water-immersion technique in highlighting the duodenal villous pattern in patients undergoing upper endoscopy for the investigation of dyspepsia<sup>[29]</sup>. This procedure was also trialed in the follow-up of celiac patients after gluten-free diet<sup>[30]</sup>, and also in cases with patchy villous atrophy or villous abnormality limited to the duodenal bulb<sup>[11,30]</sup>, and moreover in children with suspected CD, achieving the same optimal diagnostic accuracy for *in vivo* prediction of areas of the duodenum with villous damage<sup>[31]</sup>. The water-immersion technique has the potential to reduce the number of biopsy specimens, because of his power of enhancing visualization of areas with villous atrophy (Figure 2A, B); moreover, in patients strongly suspected from CD and with total villous atrophy at water-immersion visualization during upper endoscopy, the high specificity of the procedure could allow to avoid biopsy sampling, with a considerable cost saving<sup>[32]</sup>. Furthermore, water-immersion technique shows excellent results in terms of operator learning curve, safety, tolerability, and diagnostic accuracy<sup>[11,29-32]</sup>. In conclusion, for its facility and quickness of performance, and because of its high reliability in evaluating the duodenal villous pattern, the water-immersion technique could potentially be used as a routine procedure during conventional upper gastrointestinal endoscopy, potentially pulling down the number of misdiagnosis of CD, especially when not suspected. Trials with the water-immersion technique has not been replicated by other





**Figure 2** Evaluation of duodenal villous pattern with the water-immersion technique, Fujinon intelligent chromo endoscopy system, capsule endoscopy, I-scan technology. A: Presence of villi with the water-immersion technique; B: Total villous atrophy with the water-immersion technique; C: Presence of villi with Fujinon intelligent chromo endoscopy (FICE) system; D: Total villous atrophy with FICE system; E: Presence of villi with capsule endoscopy; F: Total villous atrophy with capsule endoscopy; G: Duodenal villous pattern with I-scan technology.

groups: therefore, further data, with larger population trials, including large multicenter studies, are required to strengthen this evidence.

## CHROMOENDOSCOPY AND HIGH-RESOLUTION MAGNIFICATION ENDOSCOPY

The efficacy of dye-staining chromoendoscopy with indigo carmine or methylene blue in enhancing the visualization of the mucosal surface is nowadays well known<sup>[33,34]</sup>. The usefulness of chromoendoscopy with indigo carmine for the evaluation of celiac disease was proved yet in 1976<sup>[35]</sup>. However, this evidence was not confirmed in a latter study<sup>[36]</sup>. A new generation of endoscopic tools-the “magnification” or “zoom” endoscopes-

can produce magnified, high-resolution images (up to 100-135 ×), enhancing details compared to conventional endoscopy<sup>[33,37]</sup>. They own charged computed device chips with a density of more than 850000 pixels; standard instruments, instead, have charged computer device chips with a density of 100000-300000 pixels. Video endoscopes can provide more and more details about the mucosal surface than conventional ones<sup>[38]</sup>. The association of indigo carmine-chromoendoscopy and magnification endoscopy in the evaluation of duodenal villous pattern was experienced by Siegel *et al*<sup>[39]</sup>: this combination showed a sensitivity and specificity of 94% and 88%, respectively for the detection of any villous alteration, and was especially helpful in documenting partial villous atrophy. In a following study, neither this combination technique nor each technology alone showed ad-



vantage compared to standard endoscopy in identifying duodenal lesions such as polyps or hyperplastic Brunner's glands, but anyway authors recognized the role of this combination in case of suspected CD<sup>[40]</sup>. The role of zoom endoscopy, with a total immersion technique (instillation of 10 mL of water), in the diagnosis of CD was analyzed in 2005<sup>[41]</sup>: a sensitivity of 90.7%, specificity of 62.9%, a positive predictive value of 83% and a negative predictive value of 77.2% for the diagnosis of any degree of villous atrophy resulted; diagnosis of total villous atrophy was better performed than diagnosis of partial villous atrophy. Cammarota *et al.*<sup>[42]</sup> investigated the combination of magnification endoscopy and water-immersion technique in subjects with suspected duodenal disease, showing a concordance of 100% with histopathology for detecting the absence or the presence of villi. The sensitivity, specificity, positive predictive value and negative predictive value for the detection of total villous atrophy were all 100%, and quite lower for the diagnosis of partial villous atrophy and normal villous patterns. According to other reports, magnification endoscopy could play a role in the detection of patchy villous atrophy<sup>[43,44]</sup>. In conclusion, enhanced magnification endoscopy, a technique that combines use of acetic acid instillation with magnification endoscopy, has showed a better accuracy in the evaluation of duodenal mucosal pattern than conventional endoscopy<sup>[45]</sup>.

## FUJINON INTELLIGENT CHROMO ENDOSCOPY SYSTEM

Fujinon intelligent chromo endoscopy system or optical band imaging (also known as multiband imaging) is able to assure the same contrast enhancement power of the standard chromoendoscopy, but in a virtual manner. This technology is based on the selection of particular wavelengths from a reflected light signal, resulting in an establishment of digitally created, enhanced images<sup>[46]</sup>. The usefulness of FICE technology has been successfully proved in Barrett's metaplasia, early gastric cancer, small colorectal tumors<sup>[47-49]</sup>; moreover, it has showed a great accuracy (100%) for the evaluation of duodenal villi and for the depiction of duodenal villous patterns in CD<sup>[50]</sup> (Figure 2C, D).

## NARROW BAND IMAGING

Narrow-band imaging (NBI) is a new endoscopic technique that allows evaluation of minimal mucosal alterations. NBI uses a narrowed wavelength of light, deriving from the narrowing of the bandwidths of the blue and green filters. This particular wavelength of light is greatly absorbed by hemoglobin, enhancing the visualization of microvascular pattern. It also has a quite deeper superficial penetration than standard white light<sup>[51,52]</sup>. The efficacy of NBI has been proved in the endoscopic evaluation of a number of diseases, among which also in CD<sup>[53,54]</sup>. According to Singh *et al.*<sup>[54]</sup>, NBI technique is able to detect and grade villous atrophy,

with a sensitivity and specificity in detecting villous atrophy of 93.3% and 97.8% respectively, and a sensitivity and specificity in grading villous atrophy of 83.3% and 100%.

## OPTICAL COHERENCE TOMOGRAPHY

Optical coherence tomography (OCT) had its debut in medicine in 1991, and nowadays is a cornerstone in ophthalmology, for the usefulness in the evaluation of the retina and atheromatic plaques<sup>[55]</sup>. The mechanism of OCT is very similar to that of B-mode ultrasonography: OCT detects the echo time delay and the magnitude of back-scattered light waves from various structural tissue features, using interferometry to measure back-scattered light because the delays of reflected light are too little for a direct electronic measurement<sup>[55-57]</sup>. The images performed by OCT resemble those generated by B-mode ultrasound and endoscopic ultrasonography; however, the resolution of OCT is better (5-10 mm)-because of the use of light instead of sound waves-, closer to the histological images<sup>[55,56,58]</sup>. So, OCT allows the study of the proximal layers of gastrointestinal (GI) wall, and may be helpful in the early diagnosis of neoplasms<sup>[57]</sup>. The usefulness of OCT has been proved yet in the study of GI malignancies<sup>[59,60]</sup>, Barrett's esophagus and dysplasia<sup>[61-67]</sup>, pancreatic and biliary ducts<sup>[68,69]</sup>, and other diseases. Preliminary reports from Masci *et al.*<sup>[70-72]</sup>, the use of OCT *in vivo* during real-time endoscopic imaging generated promising results for the evaluation of duodenal villous morphology. These authors, in fact, found total concordance between OCT and histology results for the evaluation of villous morphology in both patients with CD and healthy individuals, also in children, exactly identifying, furthermore, different degrees of villous atrophy.

## CONFOCAL LASER ENDOMICROSCOPY

Confocal laser endomicroscopy, or confocal endomicroscopy, is a novel technology that allows an *in-vivo* microscopy of the human gastrointestinal mucosa during upper or lower endoscopy<sup>[73,74]</sup>. Endomicroscopy has been applied in a number of gastrointestinal diseases, and also in CD<sup>[73-77]</sup>. In particular, in the experience of Zambelli *et al.*<sup>[76]</sup>, the images obtained by confocal endomicroscopy and histology were similar, both for negative subjects and for celiac patients; moreover, in celiac patients confocal endomicroscopy was able to identify moderate-to-severe villous atrophy, but quite less to visualize the crypt hyperplasia and flogistic infiltration. In a case report, CD was diagnosed *in vivo* by confocal endomicroscopy on the basis of the presence of complete villous atrophy and a rise of intraepithelial lymphocytes<sup>[77]</sup>.

## VIDEOCAPSULE ENDOSCOPY

Capsule endoscopy is a useful, patient-friendly method for the evaluation of the whole small bowel. Obscure

gastrointestinal bleeding is the strongest indication for capsule endoscopy<sup>[78]</sup>; however, recent evidences point out new, intriguing purposes and indications: in particular, regarding the object of this review, the role of capsule endoscopy in the diagnosis and follow-up of CD is growing up quickly<sup>[79-91]</sup>. The optical system of the capsule possesses a 8-folds magnification power, that allows to easily evaluate the duodenal villous pattern (Figure 2E, F). Moreover, it allows an evaluation of the small intestine along its whole length. Capsule endoscopy seems to be able to recognize the endoscopic markers of celiac disease described in the literature, such as scalloping and reduction in number of duodenal plicae, nodularity and mosaic pattern of mucosa<sup>[81,82,86,87]</sup>.

In an initial multicenter trial, capsule endoscopy had an excellent reported sensitivity and specificity of 87.5% and 90.9%, respectively, for the detection of villous atrophy as compared with the criterion standard of duodenal histology<sup>[84]</sup>, but such promising data have not been confirmed in the series presented by the same group<sup>[85]</sup>. Summarizing the most important studies about the role of capsule endoscopy in CD, it counts a high sensitivity (range, 70%-95.2%), a quite less high specificity (range, 63.6%-100%) and high positive predictive value (96.5%-100%), but a lower negative predictive value (71.4%-88.9%)<sup>[82,83,85,88]</sup>. These results are cheerful, but the relatively low negative predictive value indicates that CD can't be surely excluded by a negative evaluation at capsule endoscopy.

It should be noted that there is not an overall high degree of agreement between investigators (range 0.41-0.87), and it probably denotes a difficulty in evaluating correctly villous atrophy even if operators are well-experienced in video capsule enteroscopy.

However, the use of capsule endoscopy could be considered in patients with positive tissue transglutaminase or anti-endomysial antibodies who are unable or unwilling to perform an upper endoscopy<sup>[89]</sup>, and also for the evaluation of the whole small bowel in patients with positive antibodies and duodenal histology negative for CD, even if regarding evidences don't confirm this hypothesis<sup>[90]</sup>. More realistically, capsule endoscopy can be very useful in case of suspected refractory or complicated CD. In particular, capsule endoscopy can detect alterations such as malignancy or ulcerative jejunitis in refractory celiac disease (RCD) type II, but evidences are not so bright regarding RCD type I<sup>[91]</sup>.

## I-SCAN TECHNOLOGY

I-scan technology is an image enhanced endoscopy technology recently developed by Pentax Medical®, Japan<sup>[92]</sup>. It can be classified among digital contrast methods. It allows three different modalities of image enhancement: surface enhancement (SE), contrast enhancement (CE), and tone enhancement (TE). SE enhances light-dark contrast by obtaining luminance intensity data for each pixel. CE digitally adds blue color in relatively dark

areas, enhancing minute irregularities on the mucosal depressed areas. Both enhancement functions work in real time without impairing the original color of the organ. TE separates and analyzes the individual red, green and blue components of a normal image; the algorithm then alters the color frequencies of each component, recombining the components to a single, new color image. For SE and CE, it is possible to switch among three enhancement levels (low, medium and high). At now, three types of TE are available: TE-e (for esophagus), TE-g (for stomach) and TE-c (for intestine). Switching the levels or modes of enhancements can be done on a real-time basis, without any time lag, by pushing a relevant button.

I-scan technology has been applied to several field of interest in gastrointestinal endoscopy, such as colorectal lesions<sup>[93-97]</sup>, Whipple's disease<sup>[98]</sup>, gastroesophageal reflux disease<sup>[99-101]</sup>, Barrett's esophagus<sup>[102]</sup>. Recently, our group has experienced I-scan technology for the evaluation of duodenal villous pattern<sup>[103]</sup>, with the following results: I-scan system was demonstrated to have great accuracy (100%) in the detection of marked villous atrophy patterns and quite lower accuracy in determining partial villous atrophy or normal villous patterns (respectively, 90% for both items) (Figure 2G).

Therefore, I-scan technology seems to be a reliable tool also for the diagnosis of CD. Obviously, further, larger studies are needed to confirm this feeling.

## CONCLUSION

The recent advances in terms of technology and techniques of endoscopy, reviewed above, can certainly improve our diagnostic possibilities in the evaluation of CD, and should not be ignored, but accepted with wisdom. Surely, it is important to perform these tools in appropriate endoscopic centers, owning good equipment and enough expertise. Moreover, in a hypothetic world without biopsy sampling, but with a virtual histological analysis, a gastroenterologist can not absolutely brush aside a solid histological training. Therefore the most realistic scenario is not a replacement, but an interaction between endoscopic and histological analysis: a similar "joint-venture" might knock down misdiagnoses and reduce overall costs of diagnostic course of CD: large, randomized trials, also with cost analyses and clinical outcome evaluations, are needed to carry out this concept.

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## Oral manifestation in inflammatory bowel disease: A review

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### Abstract

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, not only affect the intestinal tract but also have an extraintestinal involvement within the oral cavity. These oral manifestations may assist in the diagnosis and the monitoring of disease activity, whilst ignoring them may lead to an inaccurate diagnosis and useless and expensive workups. Indurated tag-like lesions, cobblestoning, and mucogingivitis are the most common specific oral findings encountered in CD cases. Aphthous stomatitis and pyostomatitis vegetans are among non-specific oral manifestations of IBD. In differential diagnosis, side effects of drugs, infections, nutritional deficiencies, and other inflammatory conditions should also be considered. Treatment usually involves managing the underlying intestinal disease. In severe cases with local symptoms, topical and/or systemic steroids and immunosuppressive drugs might be used.

reserved.

**Key words:** Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Extra-intestinal manifestations; Pyostomatitis vegetans; Aphthous stomatitis; Cobblestoning; Mucogingivitis; Oral manifestation

**Core tip:** Although the gastrointestinal tract is the primary site of involvement in inflammatory bowel disease (IBD) patients, some cases might present with non-intestinal manifestations, such as oral lesions. These oral manifestations may aid in the diagnosis and the monitoring of disease activity, whilst ignoring them may lead to an inaccurate diagnosis and useless and expensive workups. Indurated tag-like lesions, cobblestoning, mucogingivitis, aphthous stomatitis, and pyostomatitis vegetans are the main oral presentations of IBDs. With the growing incidence of IBDs and the increased likelihood of encountering these particular manifestations, this review summarizes various oral findings seen in IBD cases by describing their unique morphologic description, treatment recommendations, and probable differential diagnosis.

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### INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases with primary intestinal involvement<sup>[1-5]</sup>. Although the exact underlying pathogenesis of IBD has not been clearly elucidated, it is postulated that dysregulated immunity is its basis<sup>[4,6-12]</sup>. Generally, it is assumed that IBD is a multifactorial disease in which immune system, genetics, and environmental factors all have a

role<sup>[2,8,13-17]</sup>. Other than the expected symptoms of gastrointestinal involvement, IBD patients may exhibit a wide range of non-intestinal signs and symptoms known as extraintestinal manifestations (EIMs), with prevalence rates ranging from 6%-47%<sup>[2,8,18,19]</sup>. Approximately one third of IBD patients develop EIMs in the course of their disease<sup>[1,20-25]</sup>. Joints, skin, eyes, and the biliary tract are among the most common organs involved in EIMs<sup>[22,26-28]</sup>. Oral involvement with different presentations may also be seen in IBD. Oral manifestations could also occur in these patients due to other causes, such as drug reactions, infections, and unrelated diseases<sup>[1,2,6,8,20,21]</sup>. Patients with IBD may present with these oral manifestations years before the appearance of intestinal disease<sup>[1,6]</sup>. Recognizing these patterns may assist physicians and other care givers in making a timely diagnosis of IBD while avoiding unnecessary workups<sup>[29]</sup>. The scope of this review is to describe various oral presentations in IBD and their differential diagnosis and treatment.

## EPIDEMIOLOGY OF ORAL MANIFESTATIONS IN IBD

In 1969, Dyes and colleagues initially described oral lesions in two patients with CD<sup>[7,30]</sup>. This was followed in the same year by Dudeney's report of another patient suffering from CD who had oral manifestation<sup>[51]</sup>. Oral lesions in the absence of intestinal findings in CD were initially described in 1972 by Varley<sup>[32]</sup>, and there have since been various reports on the incidence of oral lesions in CD<sup>[1-3,23,30,33-39]</sup>. The highest rate was reported from a study in a pediatric age group, indicating a rate of 48%<sup>[33]</sup>. The prevalence rate is estimated to be between 50% and 20% in most publications<sup>[1,33,38]</sup>.

This variation in rate might be related to the different ages of patients studied, their ethnicity and genetic background, whether they were receiving treatment while being investigated, the experience level of the examiners, and the variation in definition of specific lesions<sup>[34]</sup>.

In the majority of cases, intestinal involvement precedes the oral lesion<sup>[1]</sup>. Oral lesions are more common in CD compared to UC, more prevalent in children compared to adults, and with a male dominance<sup>[1,8,21,33,34,37,40,41]</sup>. The prevalence is also higher in CD patients with proximal gastrointestinal tract and/or perianal involvement<sup>[2,33,42]</sup>.

Oral lesions may be the primary presenting signs preceding gastrointestinal symptoms<sup>[43,44]</sup> in 5%-10% of affected patients<sup>[39]</sup>. This figure has been reported to be as high as 60% in patients with CD<sup>[37]</sup>.

Although the lesions might be more severe at the time of active disease, the correlation is not universal, and up to 30% of affected patients may continue to manifest oral lesions (especially in the pediatric age group) despite disease control<sup>[34,45]</sup>.

## ORAL LESIONS IN CROHN'S DISEASE

Dudeney's report of oral Crohn's disease in 1969 de-

scribed it as a raised, edematous, pink granulation tissue in the buccal mucosa<sup>[31]</sup>. It is now known that the lips are the most frequent site of oral Crohn's disease (OCD) lesions<sup>[37]</sup>. Oral lesions may be painful, impair proper oral function, or lead to psychological disorders due to disfigurement<sup>[8,46]</sup>. Oral manifestations of CD can be specific or non-specific, based on the presence of granulomas noted on the histopathology reports<sup>[1]</sup>.

## SPECIFIC ORAL CROHN'S DISEASE LESIONS

These specific lesions contain granulomatous changes noted upon histopathological examination. They are less common than non-specific lesions, and can occur either concomitantly with intestinal symptoms or before gut presentation by several years<sup>[47,48]</sup>. The most affected portions in the mouth are the buccal mucosa, gingiva, lips, vestibular, and retromolar areas<sup>[32]</sup>. There are four main lesions, as described below and shown in Table 1.

### INDURATED TAG-LIKE LESIONS

These are white reticular tags<sup>[35]</sup> referred to as mucosal tags, epithelial tags, or folds<sup>[49]</sup>. These lesions are mostly discovered in the labial and buccal vestibules, and in the retromolar regions<sup>[21]</sup>. Up to 75% of these lesions may show non-caseating granulomas on histopathology<sup>[33,42]</sup>. There has been no specific direct association of these lesions with intestinal CD activity reported<sup>[1]</sup>. Treatment is described in the later section on general treatments of OCD lesions.

### COBBLESTONING

Fissured swollen buccal mucosa with corrugation and hyperplastic appearance of the mucosa are called cobblestoning<sup>[1,42,50,51]</sup>. These lesions are usually seen in the posterior buccal mucosa and may be associated with succulent mucosal folds with normal epithelium<sup>[21]</sup>. The lesions usually consist of mucosal-colored papules that produce firm plaques on the buccal mucosa and palate. Such lesions may cause pain and make speaking and eating normally difficult<sup>[52]</sup>. These lesions, along with mucosal tags, are considered pathognomonic for CD<sup>[35]</sup>, but are not associated with intestinal CD activity<sup>[1]</sup>. Treatment consists of topical steroids in addition to the treatment of intestinal involvement. In more severe presentations, systemic steroids could be used<sup>[53]</sup>.

### MUCOGINGIVITIS

The gingiva may become edematous, granular, and hyperplastic in Crohn's disease, with or without ulceration. The whole gingiva up to the mucogingival line might be involved<sup>[7,30]</sup>. As with other specific lesions of the oral cavity, this lesion has no association with intestinal CD activity. Treatment is discussed in the section on general



**Table 1 Summary of specific and non-specific oral lesions in Crohn's disease**

	Lesion	Relation with CD activity	Frequency	Treatment options
Specific oral lesions	Indurated tag-like lesions	No specific direct association reported	Common in OCD patients	See general points on the treatment of OCD in the text
	Cobblestoning	No specific direct association reported	Common in OCD patients	Topical steroids for less severe cases and systemic steroids for others
	Mucogingivitis	No specific direct association reported	Common in OCD patients	See general points on the treatment of OCD in the text
	Others: Lip swelling with vertical fissures Deep linear ulcerations	No specific direct association reported		Topical tacrolimus, intra-lesional injection of steroids, immunosuppressive agents Topical analgesics, 5-ASA, or steroids, intra-lesional steroids, topical tacrolimus, other medications used in PV treatment
Non-specific oral lesions	Aphthous stomatitis	No specific direct association reported	10% of patients with UC and 20%-30% of those with CD	Topical agents (lidocaine 2%, triamcinolone 0.1%, dexamethasone elixir), non-steroidal anti-inflammatory pastes, systemic steroids, intra-lesional steroids
	Pyostomatitis vegetans	Associated with active CD	Rare	Antiseptic mouthwashes/topical steroids (though less effective), systemic steroids, azathioprine and sulfamethoxypyridazine, dapsone, cyclosporine A, injections of infliximab pursued by maintenance therapy with MTX, adalimumab, surgical colectomy in UC
	Others: Angular cheilitis	No specific direct association reported	Unknown	5-ASA mouthwashes, topical steroids (1% hydrocortisone), vitamin supplements, intra-lesional steroids
	Persistent submandibular lymphadenopathy Recurrent buccal abscesses Perioral erythema with scaling Glossitis			See general points on the treatment of OCD in the text Antibiotics, infliximab, methotrexate, thalidomide

CD: Crohn's disease; OCD: Oral Crohn's disease; MTX: Methotrexate; UC: Ulcerative colitis.

treatments of OCD lesions below.

## OTHER SPECIFIC LESIONS

Lip swelling with vertical fissures, deep linear ulcerations (usually in the buccal sulci with hyperplastic folds), and midline lip fissuring may also occur in CD<sup>[1,2,7,8,22,30,33,35,39,42,49,54]</sup>. These lesions also have no association with intestinal CD activity<sup>[1]</sup>.

While these lesions may be very incommensurate for patients, they can be treated with topical tacrolimus at low concentration (0.5 mg/kg) and intra-lesional steroid injection with or without mandibular blockade<sup>[34,55,56]</sup>. In more severe cases with persistent pain and cosmetic disfigurement, more aggressive therapy with immunosuppressive agents is recommended<sup>[34]</sup>.

## NON-SPECIFIC ORAL LESIONS IN CD

Table 1 provides details of various non-specific oral lesions that occur with Crohn's disease.

## APHTHOUS STOMATITIS

Aphthae are shallow round ulcerations with central fi-

brinous exudate and an erythematous border<sup>[23,57]</sup>. These lesions may occur in 20%-25% of the general population<sup>[3,58]</sup>, up to 10% of patients with UC, and 20%-30% of those with CD that have oral aphthosis<sup>[4]</sup>. In a survey conducted in Iran, oral aphthous lesions were found in approximately 13% of CD *vs* 6% of UC patients<sup>[13]</sup>. The association of oral aphthosis and disease activity is not clear. While it may become more severe in active disease, its presence does not correlate with disease activity.

Patients with IBD and other EIMs may suffer recurrent aphthous stomatitis more often than others<sup>[4]</sup>. Aphthous stomatitis has been associated with ankylosing spondylitis, uveitis, peripheral arthritis, and erythema nodosum<sup>[59]</sup>. Aphthous eruptions are not specific for IBD and may be observed in several other disorders including celiac sprue, HIV/AIDS, Behçet's disease, and Reiter's syndrome, as well as common aphthae seen in the normal population<sup>[23,60-66]</sup>.

Management of CD is usually sufficient for control of oral aphthosis. For control of pain, topical agents (such as lidocaine) and/or topical steroids (such as triamcinolone 0.1%) up to three times per day can be used. Dexamethasone elixir (0.5 mg/5 mL spit or swish) or ointment up to three times per day is also efficacious. Non-steroidal anti-inflammatory pastes are effective in

relieving pain and promoting healing. Systemic or intra-lesional steroids should be reserved for severe refractory and/or persistent cases<sup>[4,13,21,32,67-70]</sup>.

## PYOSTOMATITIS VEGETANS AND OTHER NON-SPECIFIC LESIONS

Pyostomatitis vegetans can occur in both UC and CD, but is more common in the former and will be discussed in more detail in the later section addressing oral manifestations of UC.

Other non-specific oral findings of CD include angular cheilitis, persistent submandibular lymphadenopathy, sicca syndrome and reduced salivation, halitosis, dental caries and periodontal involvement, candidiasis, odynophagia, dysphagia, minor salivary gland enlargement, perioral erythema with scaling, recurrent buccal abscesses, glossitis, mucosal discoloration, lichen planus, and metallic dysgeusia<sup>[2,7,21,32,34,35,40,54,71]</sup>. For the management of angular cheilitis, 5-ASA mouthwashes, topical steroids (1% hydrocortisone), vitamin supplements, and intra-lesional steroids may be effective. Antibiotics are the first step in treating recurrent buccal abscesses. For more severe cases, immunomodulating agents including chimeric anti-tissue necrosis factor (TNF) alpha monoclonal antibody-infliximab, methotrexate, and thalidomide have been used<sup>[7,21]</sup>.

## GENERAL POINTS ON THE TREATMENT OF OCD

In the majority of patients with OCD, the oral findings are asymptomatic and clinically silent. In these patients, no peculiar treatment is needed for oral lesions and the latter will resolve over time in association with the treatment of gastrointestinal disease using anti-inflammatory drugs (5-ASA), immunosuppressive agents, and finally biological agents, whichever are indicated<sup>[8,21,34,40,72]</sup>.

The treatment armamentarium includes topical and systemic steroids, 5-ASA compounds, immunosuppressive agents, biologic treatments, and even antibiotics such as tetracycline<sup>[2,73]</sup>.

The first and foremost step in treating oral lesions is to control colonic disease<sup>[74]</sup>. Food restriction, which is discussed later in the management of orofacial granulomatosis (OFG), could also be tried in OCD<sup>[75,76]</sup>.

## ORAL LESIONS IN UC

There are many similarities between the oral manifestations of CD and UC. Although oral lesions are more common in CD, almost all of the so-called non-specific oral lesions described in CD can also occur in UC. Among these lesions, pyostomatitis vegetans occurs more commonly in UC than in CD and will be discussed here in more detail<sup>[1,2,74,77,78]</sup>.

The term pyostomatitis vegetans (PV) was first in-

troduced by McCarthy in 1949<sup>[38]</sup>, but its association with IBD was not initially recognized<sup>[38]</sup>. PV is a chronic mucocutaneous ulcerative disorder consisting of multiple milium white or yellow pustules with an erythematous and edematous mucosal base<sup>[1,23,77,79]</sup>. The pustules can rupture and coalesce to form linear or "snail-track" ulcers<sup>[1,23,38,77,78,80]</sup>. The most frequently involved regions of the oral cavity are the labial gingiva, labial, and buccal mucosa<sup>[78]</sup>. The least damaged portions are the tongue and floor of the mouth<sup>[1]</sup>, but pustules can involve almost all parts of the oral cavity<sup>[78]</sup>.

PV can be seen at any age, but is more prevalent in patients aged between 20 and 59 years, with an average age of 34 years. Males are affected more frequently than females, with a ratio of 2:1-3:1<sup>[81,82]</sup>. PV is considered to be the oral equivalent of pyodermitis vegetans on the skin<sup>[77,78]</sup>. There is a strong association between PV and IBD, and the former is a specific marker of disease activity in UC<sup>[1,2,38,39,78,83,84]</sup>. Intestinal involvement usually predates the onset of PV in IBD, although this could be asymptomatic and mild<sup>[23,85]</sup>. Despite every effort, no bacterial, fungal, or viral cause has yet been found for this manifestation and its pathogenesis remains obscure<sup>[77]</sup>. The principal histological features on microscopy are intra-epithelial and/or sub-epithelial micro-abscesses with neutrophils and eosinophils. Furthermore, hyperkeratosis, acanthosis, and acantholysis are seen in histology examination<sup>[1,38,40,78,86]</sup>. Direct immunofluorescence in PV is negative for deposits of IgA, IgG and C3 and this result is helpful in distinguishing PV from pemphigus vulgaris<sup>[1,87]</sup>. Clinically, the patient may have fever, enlarged and tender submandibular lymph nodes, and pain. Pain intensity is variable; some patients with extensive oral lesions may not have any pain<sup>[78]</sup>. Peripheral eosinophilia is seen in up to 90% of cases and is the main laboratory finding in many patients<sup>[87]</sup>.

The diagnosis of PV is based on a constellation of clinical features that include concurrent IBD, peripheral eosinophilia, histological study, and negative culture results of the lesion exudate. As mentioned above, a negative immunofluorescence study is also helpful<sup>[1,77,78]</sup>.

The main differential diagnoses of PV include vesicular disorders involving both the skin and oral cavity; similar to pemphigus vulgaris in particular, as well as other diseases like bullous pemphigoid, acquired epidermolysis bullosa, bullous drug eruptions, herpetic infection, Behçet's disease, and erythema multiforme<sup>[1,77,80,88]</sup>. Herpetic infections should be excluded by Tzanck smear, antigen detection, and culture of the virus, or PCR for HSV virus<sup>[23]</sup>. The mainstay in the management of PV is the treatment of underlying IBD. Topical steroids and antiseptic mouthwashes alone are effective in only a few instances. Systemic steroids are usually prescribed for these patients and are considered as the treatment of choice. Azathioprine and sulfamethoxypyridazine can be used in parallel with steroids as sparing agents<sup>[3,21,23,38,77,78]</sup>. Dapsone is another option, but should be used as a second line agent, especially in relapsing cases. Hemolytic ane-

mia, hepatitis, agranulocytosis, and drug-induced allergic reactions are the major side effects of dapsone requiring attention<sup>[3,78]</sup>.

Calcineurin blockers like cyclosporine A have been successfully used, as described in case reports in the treatment of PV<sup>[89]</sup>. Injections of infliximab followed by maintenance therapy with methotrexate have been also effective, especially in PV associated with CD<sup>[77]</sup>. Systemic use of newer humanized anti-TNF agents like adalimumab has also proven effective in inducing remission of both oral and gastrointestinal manifestations<sup>[77]</sup>. Surgical colectomy produces promising results in PV associated with UC<sup>[3,78,90]</sup>.

Other non-specific findings in UC include oral aphthae, glossitis, cheilitis, stomatitis, lichen planus, mucosal ulcers, diffuse pustules, and non-specific gingivitis<sup>[1-3,23,42]</sup>.

In a report of patients with UC, 4.3% had aphthous stomatitis during intestinal disease flare-ups<sup>[2]</sup>, thus the presence of this non-specific manifestation may have some correlation with disease activity in UC.

## DIFFERENTIAL DIAGNOSES

Because CD is a granulomatous disorder, all other diseases capable of inducing granulomatous reaction in the oral cavity are included in the differential diagnosis (DDX) list. The most common cause of developing oral granulomas is a response to foreign bodies, primarily dental materials such as retained amalgams or endodontic sealers<sup>[91]</sup>. The second important DDX to be considered is tuberculosis bacilli. Special staining processes for acid-fast bacilli, PPD skin test, sputum culture, and chest X-ray are often used to diagnose oral tuberculosis<sup>[2,80,92]</sup>.

Fungal infections such as candidiasis, histoplasmosis, cryptococcosis, paracoccidioidomycosis, and blastomycosis can all trigger granulomatous involvement of the mouth. The presence of these infections could be confirmed by special stains including applying PAS or Gömöri trichrome stain and, more specifically, with molecular studies<sup>[2,21,80]</sup>.

Oral sarcoidosis should always be considered in DDX, and an appropriate workup should include measuring serum angiotensin converting enzyme, IL-2 receptor level, IL-8 level, and chest X-ray in suspected cases<sup>[2,6,21,39,93]</sup>.

Leprosy, cat scratch disease, tertiary syphilis, orofacial granulomatosis, T-cell lymphoma, and Wegener's disease can all produce a granulomatous reaction in the oral cavity, but are much rarer and usually have other prominent features leading to diagnosis<sup>[21,39]</sup>.

Considering the role of nutritional deficiencies is of utmost importance as stomatitis, glossitis, aphthous ulcers, cheilitis, or perioral dermatitis may occur with nutrient deficiencies resulting from an insufficient supply of the vitamin B family, albumin, iron, folate, zinc, niacin, and/or other essential elements<sup>[8,41,94-97]</sup>. Nutrient deficiencies may be the result of intestinal involvement or may be caused by the medications used in the treatment of

IBD<sup>[98,99]</sup>. Sulfasalazine and azathioprine, for instance, may cause folate and niacin deficiency, respectively<sup>[2]</sup>.

Other non-specific oral manifestations may also be related to the side effects of drugs. As an example, oral aphthosis has been reported in association with non-steroidal anti-inflammatory agents, nicorandil<sup>[100]</sup>, and bupropion<sup>[101]</sup>; gingival hyperplasia with cyclosporine<sup>[102]</sup>, amlodipine<sup>[103]</sup>, and anticonvulsants such as phenytoin<sup>[104]</sup>; and reversible lichen planus with sulfasalazine<sup>[54]</sup>.

## OROFACIAL GRANULOMATOSIS

Gibson *et al.*<sup>[40]</sup> used the term OFG in 1985 to define a constellation of oral signs similar to those seen in OCD, but in the absence of evidence of intestinal CD. In this rare syndrome, chronic swelling of the lips and lower half of the face is prominent, in association with oral ulcers and hyperplastic gingivitis. Granulomatous cheilitis is the most common sign seen in OFG<sup>[105]</sup>. The most frequent sites of involvement in OFG are the lips, which may be individually or both involved<sup>[80]</sup>. Lip swelling usually leads to painful vertical fissures<sup>[2]</sup>. Three forms of ulcers are found in OFG: deep buccal ulcers with raised peripheral mucosa, aphthous-type ulcers, and micro-abscesses located commonly on the gingival margin or soft palate<sup>[21]</sup>. In general, the ulcers are mainly superficial and the gingivae are erythematous with patchy distribution, mostly affecting the anterior portion. These alterations extend from the free gingival margin to the non-keratinized mucosa of the sulci, developing a full-thickness gingivitis pattern<sup>[40]</sup>.

In the largest series of studies involving OFG reported to date, the mean age of those affected at presentation was 20 years with no gender predilection. With the pathogenesis unknown, allergic, infectious, and genetic causes have also been postulated<sup>[40,106]</sup>. Unlike OCD in which Th1 CD4<sup>+</sup> lymphocytes are the dominant population, in OFG the overstimulation of Th2 CD4<sup>+</sup> lymphocytes is detected in biopsy specimens, where it is shown as infiltrating cells<sup>[21]</sup>.

Granulomas noted upon histology examination are the hallmark in both OFG and OCD. The only way to exclude CD is by clinical presentation<sup>[21]</sup>. As mentioned previously, oral manifestations may precede gastrointestinal involvement in CD for many years. Thus, cases labeled as OFG may later progress to being diagnosed as CD<sup>[21,34]</sup>. Recently, it has been reported that 4 out of 6 children with OFG in early childhood were reported as having developed CD on follow-up<sup>[34]</sup>.

A rare presentation of OFG seen in adults is Melkersson-Rosenthal syndrome; a triad of orofacial swelling, intermittent facial paralysis, and a fissured tongue<sup>[21,34,107]</sup>.

Observational studies in pediatric patients with OFG have demonstrated that dietary elimination of some triggering elements (encompassing cinnamaldehyde, benzoate additives, carnosine, monosodium glutamate, cocoa, and sunset yellow) are effective in the treatment of oral lesions<sup>[75,76]</sup>. Analgesia and topical agents like beclomethasone mouthwash and 5-ASA spray or ointments can be

used as basic therapies. In unresponsive cases, treatment with systemic steroids and immunosuppressive medications can be used<sup>[21]</sup>. Clofazimine, a drug used in the treatment of leprosy, is occasionally effective in OFG<sup>[37]</sup>.

## CONCLUSION

Oral manifestations of inflammatory bowel diseases are diverse. Although they are generally more common in patients with Crohn's disease, specific manifestations like PV occur more commonly in ulcerative colitis, which is associated with disease activity in most instances. Most other manifestations have no correlation with disease activity. In differential diagnosis of these oral manifestations, side effects of drugs, nutritional deficiencies, infections, as well as other granulomatous diseases with oral involvement should all be considered. There is usually no need for specific treatment for these lesions, but when indicated it may comprise topical and systemic steroids, immunosuppressive drugs, antibiotics, and even biological treatment in more severe cases.

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## Endoscopic papillary large balloon dilation for the removal of bile duct stones

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### Abstract

Endoscopic papillary large balloon dilation (EPLBD) with endoscopic sphincterotomy (EST) has been widely used as the alternative to EST along with endoscopic mechanical lithotripsy (EML) for the removal of large or difficult bile duct stones. Furthermore, EPLBD without EST was recently introduced as its simplified alternative technique. Thus, we systematically searched PubMed, Medline, the Cochrane Library and EMBASE, and analyzed all gathered data of EPLBD with and without EST, respectively, by using a single standardized definition, reviewing relevant literatures, published between 2003 and June 2013, where it was performed with large-diameter balloons (12-20 mm). The outcomes, including the initial success rate, the rate of needs for EML, and the overall success rate, and adverse events were assessed in each and compared between both of two procedures: "EPLBD with EST" and "EPLBD without EST". A total of 2511 procedures from 30 published articles were included in EPLBD with EST, while a total of 413 procedures from 3 published articles were included in EPLBD without EST. In the results of outcomes, the

overall success rate was 96.5% in EPLBD with EST and 97.2% in EPLBD without EST, showing no significant difference between both of them. The initial success rate (84.0% vs 76.2%,  $P < 0.001$ ) and the success rate of EPLBD without EML (83.2% vs 76.7%,  $P = 0.001$ ) was significantly higher, while the rate of use of EML was significantly lower (14.1% vs 21.6%,  $P < 0.001$ ), in EPLBD with EST. The rate of overall adverse events, pancreatitis, bleeding, perforation, other adverse events, surgery for adverse events, and fatal adverse events were 8.3%, 2.4%, 3.6%, 0.6%, 1.7%, 0.2% and 0.2% in EPLBD with EST and 7.0%, 3.9%, 1.9%, 0.5%, 0.7%, 0% and 0% in EPLBD without EST, respectively, showing no significant difference between both of them. In conclusion, recent accumulated results of EPLBD with or even without EST suggest that it is a safe and effective procedure for the removal of large or difficult bile duct stones without any additional risk of severe adverse events, when performed under appropriate guidelines.

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**Key words:** Balloon dilation; Endoscopic sphincterotomy; Common bile duct gallstones; Lithotripsy; Complications; Assessment; Patient outcomes

**Core tip:** We systematically analyzed all gathered data of endoscopic papillary large balloon dilation (EPLBD) with and without endoscopic sphincterotomy (EST), respectively, by using a single standardized definition, to evaluate their outcomes, reviewing relevant literatures. Thirty studies involving 2511 procedures of EPLBD with EST and 3 studies involving 413 procedures of EPLBD without EST were enrolled in this review. The results of EPLBD with or even without EST suggest that it is a safe and effective procedure for the removal of large or difficult bile duct stones without any additional risk of severe adverse events, when performed under appropriate



priate guidelines.

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## INTRODUCTION

Ever since its introduction in 1974<sup>[1,2]</sup>, endoscopic sphincterotomy (EST) has become the standard procedure for the removal of common bile duct stones. However, it still runs the risk of various adverse events, such as bleeding, perforation, pancreatitis and cholangitis<sup>[3-6]</sup>, and large bile duct stones may require endoscopic mechanical lithotripsy (EML) as an adjunctive procedure to facilitate stone clearance<sup>[7-11]</sup>. Endoscopic papillary balloon dilation (EPBD) was first proposed as an alternative to EST in 1982<sup>[12]</sup>. Initially it was widely performed in the belief that it had more advantages over EST such as the reduction of bleeding and perforation risks and functional preservation of the biliary sphincter<sup>[13-17]</sup>. However, it has been proven that EPBD is significantly less successful in removing bile duct stones compared to EST, because dilating balloons with a range of 6- to 10-mm in diameter are inadequate in achieving an ampullary opening wide enough<sup>[18,19]</sup>. More importantly the risk of pancreatitis is significantly higher than EST to the extent of an increased mortality rate<sup>[7,18,20]</sup>.

Endoscopic papillary large balloon dilation (EPLBD) combined with EST was initially introduced to facilitate in the removal of large bile duct stones in 2003<sup>[21]</sup>, where large-diameter balloons (12- to 20-mm balloon) are used to remove large or difficult bile duct stones<sup>[22-26]</sup>. It was initially presumed that this new technique would cause higher incidence rates of potential serious adverse events such as pancreatitis and bile duct perforation<sup>[27-31]</sup>. However, recent results on EPLBD with EST have quashed these presumptions<sup>[32-36]</sup>, therefore it is rapidly and widely being adopted as a useful technique for the removal of large or difficult bile duct stones<sup>[37-50]</sup>. As an alternative technique, EPLBD without EST was formally incorporated as a simplified technique in 2009<sup>[51]</sup>. A number of studies have recently been conducted in South Korea and Taiwan<sup>[43,45,52,53]</sup>, concurring that it is also as safe and effective in patients with large bile duct stones without any additional risk of severe pancreatitis or perforation. Nevertheless, it was very difficult to get a precise analysis of the outcomes of EPLBD, because the results from each article were based on different definitions. Thus, we analyzed all gathered data of EPLBD with and without EST, respectively, by using a single standardized definition, reviewing relevant literatures.

## LITERATURE SEARCH AND REVIEW

A search of literatures on EPLBD was initially performed

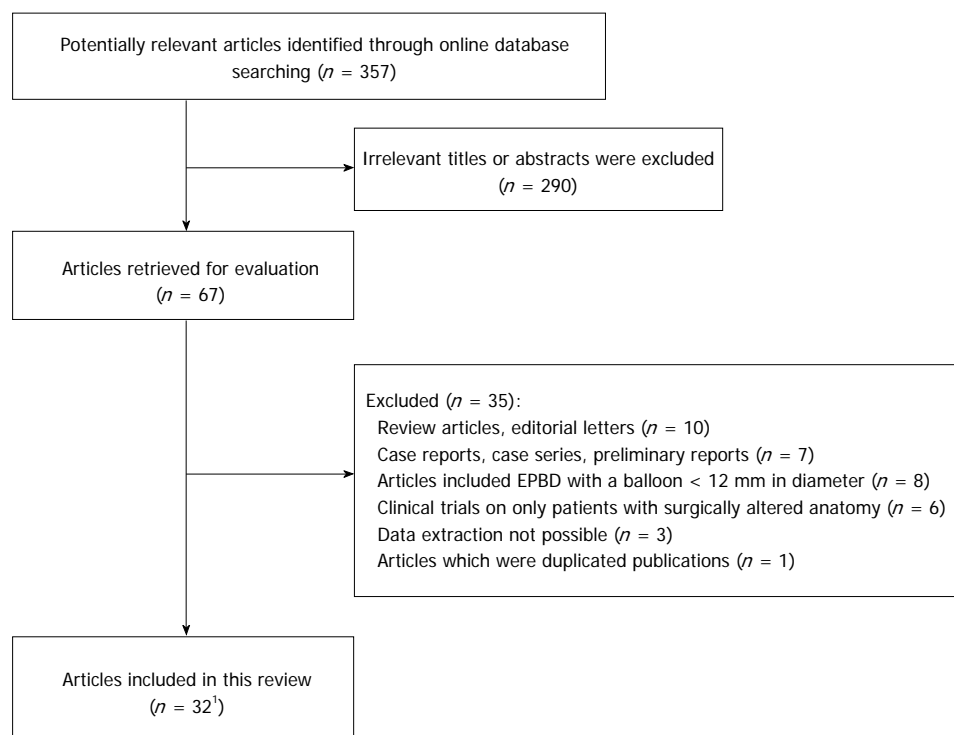
under title and abstract with the search terms “large balloon”, “balloon dilation”, “sphincteroplasty” and “endoscopic papillary large balloon dilation” by means of the commonly used online databases; PubMed, Medline, the Cochrane Library and EMBASE. After reviewing the corresponding abstracts of the retrieved articles, those that showed relevance to this review were downloaded in full text. Additional articles were then searched by tracing back on their references. Details of literature search and evaluation process are shown in Figure 1.

The following inclusion criteria were employed in this review: (1) original articles about clinical trials in humans published between 2003 and 2013 June, since EPLBD was first reported on in 2003<sup>[21]</sup>; (2) the language filtering system was not used in online databases; (3) EPLBD performed with large-diameter balloons (12-20 mm) whether preceding EST was done or not; and (4) EPLBD performed when the standard balloon and basket techniques after EST failed even though the stone size was under 10 mm. Exclusion criteria of patients or articles were as follows: (1) review articles<sup>[54-58]</sup>, editorial letters<sup>[59-63]</sup>, case reports<sup>[64-68]</sup>, case series<sup>[69]</sup> and preliminary reports<sup>[70]</sup>; (2) articles which included EPBD with a dilating balloon less than 12 mm in diameter<sup>[52,53,71-76]</sup>; (3) articles about clinical trials on only patients with surgically altered anatomy of the upper gastrointestinal tract, such as Billroth II surgery and Roux-en-Y anastomosis<sup>[77-82]</sup>; (4) articles where data extraction were not possible<sup>[83-86]</sup>; and (5) articles which contained duplicated patient data from another publication<sup>[87]</sup>.

Patient data from the relevant articles was independently extracted by two reviewers and is as follows; baseline clinical characteristics of the patients, study design, study inclusion criteria, a history of gastrointestinal surgery, perampullary diverticulum, largest stone size, range of stones, number of stones, treatment naïve, performance of EST, size of EST, prior EST, balloon diameter, time duration of inflated balloon, initial success rate, success rate of EPLBD without EML, rate of use of EML, overall success rate, number of sessions needed for complete stone removal, rates of adverse events and rate of surgery and mortality due to adverse events. An article of a large scaled multicenter study<sup>[43]</sup>, that included our institute, where the data of both the patients who had EPLBD with EST and those without EST were calculated as one, was re-analyzed using its raw data in order to re-group both of them separately. Any discrepancies between the two reviewers' results were resolved through discussion.

## DEFINITION

Because data from each article, such as size of EST, initial success rate, success rate of EPLBD without EML, rate of use of EML, overall success rate, and rate of adverse events, was based on different definitions, we re-analyzed all gathered data by using a single standardized definition, in order to get a precise analysis of the outcomes. The size of the EST used before performing EPLBD was classified into 2 groups based on the extent of ampullary



**Figure 1** Flow-chart of literature search and evaluation. Data from one article of a large scaled multi-center study was re-grouped into two; endoscopic papillary large balloon dilation with and without endoscopic sphincterotomy, and the outcomes were re-analyzed separately.

incision: (1) “large” if EST was completed to anywhere between two-thirds of the total length of the ampulla and up until the major horizontal fold crossing the intramural portion of the bile duct or if the extent of EST was described under such terms as “full incision EST”<sup>[40,46]</sup>, “full-EST”<sup>[43]</sup>, “maximum EST”<sup>[23]</sup>, “major EST”<sup>[49]</sup>, “complete EST”<sup>[29]</sup>, “standard EST”<sup>[30,36]</sup> or “normal EST”<sup>[44]</sup>; and (2) “limited” if EST was made from the orifice of the ampulla proximally up to, but not exceeding two-thirds of the ampulla or if the extent of EST was described under such terms as “mid-incision EST”<sup>[22,28,38]</sup>, “medium EST”<sup>[28]</sup>, “middle EST”<sup>[48]</sup>, “mid-EST”<sup>[43]</sup>, “small EST”<sup>[24,42]</sup>, “minor EST”<sup>[25]</sup> or “limited EST”<sup>[41,47,48]</sup>.

Initial success was defined as complete bile duct stone clearance when only one session of EPLBD was performed whether EML as an adjunctive procedure was used or not. Overall success was defined as overall complete bile duct stone clearance by using EPLBD whether EML as an adjunctive procedure was used or not, with the exception of using other lithotripsies such as electrohydraulic lithotripsy and laser lithotripsy, irrespective of the number of EPLBD sessions. Success of EPLBD without EML was defined as complete stone clearance without the assistance of EML by using EPLBD irrespective of the number of EPLBD sessions. The rate of use of EML was defined as the rate for using EML as an adjunctive procedure to remove bile duct stones in all cases irrespective of the number of EPLBD sessions. Adverse events were classified and graded according to the consensus criteria proposed by Cotton *et al*<sup>[3]</sup>.

## STATISTICAL ANALYSIS

Statistical analyses were done using SPSS version 18.0 software (SPSS Inc., Chicago, Illinois, United States). The

significance of difference for categorical variables was determined using either chi-square test or Fisher’s exact test and a logistic regression analysis was performed for multiple comparisons in the statistically significant categorical variables that have more than two subgroups. Quantitative data were analyzed by either unpaired Student’s *t* test or Mann-Whitney test, and presented as the mean  $\pm$  SD. A *P* value below 0.05 was regarded as statistically significant.

## EPLBD COMBINED WITH EST

A total of 2511 procedures in 2503 patients were included in this review from 30 published original articles, made up of 23 retrospective studies, 4 prospective studies and 3 prospective randomized controlled studies. The baseline clinical characteristics of the patients are described in Table 1. Periampullary diverticulum, which was provided in 25 studies, was noted in 36.7%. Prior EST, which was provided in 28 studies, was done in 20.2%. Patients with surgically altered anatomy of the upper gastrointestinal tract, such as Billroth I or II surgery and Roux-en-Y anastomosis were included in 2.4% from 20 studies.

### Patient outcomes

Based on the size of EST, EPLBD was performed in 10 studies mainly when stone removal had failed with the standard techniques after a large EST, in 13 studies after a limited EST mainly if it is speculated that the stone size is too large to be removed using the standard techniques, in 4 studies without additional EST if they had a previous history of EST, and in one multi-center study after variable sizes of EST. Twenty four studies described time duration of inflated balloon using a dilating balloon with a diameter of 12 to 20 mm which varied from 10 to 180 s, most of which were less than 60 s with the exception

**Table 1** Baseline clinical characteristics of the patients undergoing endoscopic papillary large balloon dilation with endoscopic sphincterotomy *n* (%)

Ref.	Study design	No. of procedures No. of patients	Mean age, year	No. of periampullary diverticulum	Mean size of largest stone, mm	Range of stone size, mm	Prior EST	Altered anatomy
Ersoz <i>et al</i> <sup>[21]</sup>	R	58	NA	4 (6.9)	NA	12-28	14	0
Hwang <i>et al</i> <sup>[22]</sup>	R	30	71.3	6 (20.0)	21.6	15-35	0	NA
Maydeo <i>et al</i> <sup>[23]</sup>	P	60	58.0	0 (0.0)	16.0	12-20	0	0
Minami <i>et al</i> <sup>[24]</sup>	R	88	74.0 <sup>1</sup>	NA	14.0	> 12	0	NA
Heo <i>et al</i> <sup>[25]</sup>	RCT	100	64.4	49 (49.0)	16.0	NA	0	0
Lee <i>et al</i> <sup>[26]</sup>	R	55	70.8	16 (29.1)	20.8	15.4-35.5	0	B-II :2
Kim <i>et al</i> <sup>[27]</sup>	R	35	66.9	9 (25.7)	26.1	12-50	14	NA
Lee <i>et al</i> <sup>[28]</sup>	R	41	72.2	21 (51.2)	18.2	10-45	0	B-II :2, R-Y:2
Misra <i>et al</i> <sup>[29]</sup>	R	50	40.1	NA	NA	< 15-25	0	NA
Attasaranya <i>et al</i> <sup>[30]</sup>	R	107, 103	70.1	36 (35.0)	13.0 <sup>1</sup>	10-30	50	B-II :6
Espinel <i>et al</i> <sup>[31]</sup>	P	93	76.5	30 (32.2)	13.4	5-30	42	B-II :4
Itoi <i>et al</i> <sup>[32]</sup>	R	53	75.3	25 (47.2)	14.8	10-28	0	0
Kim <i>et al</i> <sup>[33]</sup>	RCT	27	70.3	9 (33.3)	20.8	15-38.3	0	0
Itoi <i>et al</i> <sup>[34]</sup>	R	18	79.1	9 (50.0)	16.7	13-21	0	B-I :1
Kurita <i>et al</i> <sup>[35]</sup>	R	24	82.0 <sup>1</sup>	18 (75.0)	16.5 <sup>1</sup>	12-33	24	NA
Ghazanfar <i>et al</i> <sup>[36]</sup>	P	84	48.4	NA	14.7	10-32	0	NA
Kim <i>et al</i> <sup>[37]</sup>	R	70	68.7	24 (34.3)	12.5	5-30	70	NA
Youn <i>et al</i> <sup>[38]</sup>	R	101	69.1	12 (11.9)	21.8	7-52	0	B-I :2, B-II:3
Kim <i>et al</i> <sup>[39]</sup>	R	72	69.3	41 (56.9)	NA	> 10	0	0
Stefanidis <i>et al</i> <sup>[40]</sup>	RCT	45	69.4	NA	NA	12-20	0	0
Rebello <i>et al</i> <sup>[41]</sup>	R	30	68.0	7 (23.3)	17.0 <sup>1</sup>	12-30	4	NA
Sakai <i>et al</i> <sup>[42]</sup>	R	59	76.7	27 (45.8)	15.0	10-28	21	B-I :3, B-II :2
Park <i>et al</i> <sup>[43]</sup>	R	633	72.7	246 (39.1)	15.4	10-38.4	NA <sup>2</sup>	B-II :20
Poincloux <i>et al</i> <sup>[44]</sup>	R	64, 62	77.0	15 (24.2)	NA	NA	0	NA
Hwang <i>et al</i> <sup>[45]</sup>	R	69	68.2	33 (47.8)	16.5	NA	0	0
Paspatis <i>et al</i> <sup>[46]</sup>	RCT	124	74.9	21 (16.9)	15.7	NA	NA <sup>2</sup>	0
Rosa <i>et al</i> <sup>[47]</sup>	R	68	70.8	NA	16.8	NA	0	0
Yang <i>et al</i> <sup>[48]</sup>	R	171, 169	69.3	73 (43.2)	15.0 <sup>1</sup>	10-45	32	B-II :1
Yoon <i>et al</i> <sup>[49]</sup>	P	52	68.1	19 (36.5)	20.1	12-40	52	0
Harada <i>et al</i> <sup>[50]</sup>	R	30	78.0	23 (76.7)	18.0	10-39	30	NA
Total		2511, 2503		773 (36.7)		5-45	353 (20.2)	48 (2.4)

<sup>1</sup>Median value; <sup>2</sup>Studies that included patients with a history of prior endoscopic sphincterotomy, but their exact numbers were not described. EST: Endoscopic sphincterotomy; R: Retrospective; P: Prospective; RCT: Randomized controlled trial; NA: Not available; B-I : Billroth- I anastomosis; B-II : Billroth-II anastomosis; R-Y: Roux-en-Y anastomosis.

of 3 studies<sup>[27,43,49]</sup> (Table 2). The initial success rate was 84.0% (range 61.9%-100%), which was provided in only 24 studies, thirteen of which studies were designed to include cases where EML was performed along with the first session of EPLBD. The mean number of EPLBD sessions for complete stone clearance was 1.2. The success rate of EPLBD without EML, the rate of use of EML, and the overall success rate, which were provided from all 30 studies, were 83.2% (59.6%-100%), 14.1% (0%-38.6%) and 96.5% (79.7%-100%), respectively (Table 2).

### Adverse events

The overall rate of adverse events following EPLBD with EST was 8.3% (0%-17.0%), the majority of which were of mild to moderate severity. Adverse events were classified as pancreatitis, bleeding, perforation, and others (Table 3), and graded accordingly to severity as found in Table 4. Pancreatitis occurred in 2.4% (0%-13.2%), all cases of which were of mild to moderate severity (98.4%), except for one fatal case who had had a history of severe pancreatitis<sup>[46]</sup>. Bleeding occurred in 3.6% (0%-8.6%), but it mostly was of mild to moderate severity (94.5%). Four problematic bleedings, including 2 severe and 2

fatal cases, were reported in 4 studies<sup>[29,30,36,43]</sup>; two were successfully managed with angiography and surgery, respectively, and the other two had expired due to post-EPLBD massive bleeding. Perforation occurred in 0.6% (0%-2.8%). Six problematic perforations (5 duodenum and 1 cystic duct), including 3 severe and 3 fatal cases, were reported in 3 studies<sup>[30,43,45]</sup>; two with duodenal perforation were successfully managed with surgery and one with cystic duct perforation with percutaneous drainage, and the other three expired due to septic shock and multi-organ failure (2) and cardiogenic shock (1). Other adverse events were noted in 1.7% (0%-14.8%), including cholangitis (14), hypotension (10), pain (4), intramural dissection (3), pneumonia (3), basket impaction (2), sepsis (2), cholecystitis (1), injured bile duct (1), and hypoxia (1). All of these cases were successfully managed with conservative treatment, except for all basket impaction cases who received surgery.

### EPLBD WITHOUT EST

A total of 413 patients who each received EPLBD without EST were included in this review from 3 published

**Table 2 Procedure characteristics and outcomes of endoscopic papillary large balloon dilation with endoscopic sphincterotomy *n* (%)**

Ref.	Size of EST	Balloon size, mm	Duration of inflated balloon, s	Initial success	No. of sessions, mean	Success without EML	Use of EML	Overall success
Ersoz <i>et al</i> <sup>[21]</sup>	Large	12-20	20-45	48 (82.8)	1.17	54 (93.1)	4 (6.9)	58 (100)
Hwang <i>et al</i> <sup>[22]</sup>	Limited	15-18	30-60	NA	NA	30 (100.0)	0 (0.0)	30 (100)
Maydeo <i>et al</i> <sup>[23]</sup>	Large	12-20	30	57 (95.0)	1.05	57 (95.0)	3 (5.0)	60 (100)
Minami <i>et al</i> <sup>[24]</sup>	Limited	20	NA	87 (98.9)	1.00	87 (98.9)	1 (1.1)	88 (100)
Heo <i>et al</i> <sup>[25]</sup>	Limited	12-20	60	83 (83.0)	1.12	90 (90.0)	8 (8.0)	97 (97.0)
Lee <i>et al</i> <sup>[26]</sup>	Limited	15-20	30-60	NA	NA	52 (94.5)	3 (5.5)	55 (100)
Kim <i>et al</i> <sup>[27]</sup>	Limited	12-20	60-90	NA	NA	22 (63.1)	9 (25.7)	31 (88.6)
Lee <i>et al</i> <sup>[28]</sup>	Limited	13-20	20-60	35 (85.3)	1.20	37 (90.3)	4 (9.8)	41 (100)
Misra <i>et al</i> <sup>[29]</sup>	Large	15-20	30-45	NA	NA	45 (90.0)	5 (10.0)	50 (100)
Attasaranya <i>et al</i> <sup>[30]</sup>	Large	12-18	NA	102 (95.3) <sup>1</sup>	1.00	78 (72.9)	29 (27.1)	102 (95.3)
Espinell <i>et al</i> <sup>[31]</sup>	Large	12-20	30-45	93 (100.0) <sup>1</sup>	1.00	90 (96.8)	3 (3.2)	93 (100)
Itoi <i>et al</i> <sup>[32]</sup>	Large	15-20	15-30	51 (96.2) <sup>1</sup>	1.04	50 (94.3)	3 (5.7)	53 (100)
Kim <i>et al</i> <sup>[33]</sup>	Limited	15-18	NA	23 (85.2) <sup>1</sup>	1.27	18 (66.7)	9 (33.3)	27 (100)
Itoi <i>et al</i> <sup>[34]</sup>	Large	15-18	10	17 (94.4)	1.06	14 (77.8)	4 (22.2)	18 (100)
Kurita <i>et al</i> <sup>[35]</sup>	Prior	15-20	30	23 (95.8)	1.00	23 (95.8)	1 (4.2)	23 (95.8)
Ghazanfar <i>et al</i> <sup>[36]</sup>	Large	15-18	NA	52 (61.9)	1.28	67 (79.7)	0 (0.0)	67 (79.7)
Kim <i>et al</i> <sup>[37]</sup>	Prior	12-18	20-60	68 (97.1)	1.02	69 (98.6)	1 (1.4)	70 (100)
Youn <i>et al</i> <sup>[38]</sup>	Limited	15-20	30-60	93 (92.1) <sup>1</sup>	1.08	94 (93.1)	7 (6.9)	101 (100)
Kim <i>et al</i> <sup>[39]</sup>	Limited	12-20	30	63 (87.5) <sup>1</sup>	1.14	64 (88.9)	6 (8.3)	70 (97.2)
Stefanidis <i>et al</i> <sup>[40]</sup>	Large	15-20	10-12	44 (97.7)	1.00	44 (97.7)	0 (0.0)	44 (97.7)
Rebello <i>et al</i> <sup>[41]</sup>	Limited	12-18	60	25 (83.3) <sup>1</sup>	1.14	23 (76.7)	6 (20.0)	29 (96.7)
Sakai <i>et al</i> <sup>[42]</sup>	Limited	12-20	NA	49 (83.1) <sup>1</sup>	1.30	51 (86.4)	8 (13.6)	57 (96.6)
Park <i>et al</i> <sup>[43]</sup>	Variable	12-20	30-180	357 <sup>3</sup> (65.4) <sup>1</sup>	1.46	484 <sup>4</sup> (78.4)	123 <sup>4</sup> (19.9)	602 <sup>4</sup> (97.6)
Poincloux <i>et al</i> <sup>[44]</sup>	Large	15-20	30-60	62 (96.9)	1.05	61 (95.3)	3 (4.7)	64 (100)
Hwang <i>et al</i> <sup>[45]</sup>	Limited	12-20	60	65 (94.2) <sup>1</sup>	1.02	51 (73.9)	18 (26.1)	66 (95.7)
Paspatis <i>et al</i> <sup>[46]</sup>	Large	15-20	30-60	NA	NA	102 (81.8)	4 (3.2)	106 (85.0)
Rosa <i>et al</i> <sup>[47]</sup>	Limited	12-18	60	56 (82.4) <sup>1</sup>	1.10	55 (80.9)	10 (14.7)	65 (95.6)
Yang <i>et al</i> <sup>[48]</sup>	Limited	12-18	NA	163 (95.3) <sup>1</sup>	1.00	102 (59.6)	66 (38.6)	163 (95.3)
Yoon <i>et al</i> <sup>[49]</sup>	Prior	12-20	60-120	NA	1.70	36 (69.2)	12 (23.1)	48 (92.4)
Harada <i>et al</i> <sup>[50]</sup>	Prior	15-20	30	29 (96.7) <sup>1</sup>	1.00	27 (90.0)	3 (10.0)	29 (96.7)
Total		12-20	10-180	1745 (84.0)	1.20 <sup>2</sup>	2077 (83.2)	353 (14.1)	2407 (96.5)

<sup>1</sup>Studies which were designed to include cases where endoscopic mechanical lithotripsy was performed along with the first session of endoscopic papillary large balloon dilation; <sup>2</sup>Calculated by dividing total number of procedures into total number of sessions which was calculated by multiplying each mean number of session with each number of procedures; <sup>3</sup>Total number of procedures was 546 due to missing data; <sup>4</sup>Total number of procedures was 617 due to missing data. EST: Endoscopic sphincterotomy; EML: Endoscopic mechanical lithotripsy; Prior: Prior endoscopic sphincterotomy; NA: Not available.

original articles, all of which were retrospective studies. The baseline clinical characteristics of the patients are described in Table 5. Mean age was 71.8, periampullary diverticulum was noted in 33.2% of the patients, the mean size of the largest stone was 15.4 mm, the range of stone size was 10 mm up to 37 mm, and patients with Billroth II surgery were included in 2.7%.

### Patient outcomes

EPLBD without EST was performed using a dilating balloon with a diameter of 12 to 20 mm in all 3 studies with time duration of inflated balloon of 30 s up to 180 s. The initial success rate was 76.2% (74.1%-91.9%), but two of the 3 studies were designed to include cases where EML was performed along with the first session of EPLBD. The mean number of EPLBD sessions for complete stone clearance was 1.27. The success rate of EPLBD without EML, the rate of use of EML, and the overall success rate were 76.7% (76.0%-80.6%), 21.6% (19.4%-21.7%), and 97.2% (96.8%-97.4%), respectively (Table 6).

### Adverse events

The overall rate of adverse events following EPLBD without EST was 7.0% (2.6%-7.7%), the majority of which were of mild to moderate severity. Adverse events were classified as pancreatitis, bleeding, perforation, and others (Table 7), and graded accordingly to severity as found in Table 4. No cases of severe or fatal adverse events were reported. Pancreatitis and bleeding occurred in 3.9% (2.6%-6.4%) and 1.9% (0%-2.6%), respectively, all cases of which were of mild to moderate severity. Perforation occurred in two cases, 0.5% (0%-0.6%), both of which were of moderate severity, which were successfully managed with conservative management. As other adverse events, only 3 cases of mild cholangitis were reported from one multicenter study<sup>[43]</sup>.

## COMPARISON BETWEEN EPLBD WITH EST AND EPLBD WITHOUT EST

Comparison between patients who received EPLBD with



**Table 3** Adverse events of endoscopic papillary large balloon dilation with endoscopic sphincterotomy *n* (%)

Ref.	Overall AEs	Pancreatitis	Bleeding	Perforation	Others	AE-related surgery	AE-related death
Ersoz <i>et al</i> <sup>[21]</sup>	9 (15.5)	2 (3.4)	5 (8.6)	0 (0.0)	2 (3.4)	0 (0.0)	0 (0.0)
Hwang <i>et al</i> <sup>[22]</sup>	1 (3.3)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Maydeo <i>et al</i> <sup>[23]</sup>	5 (8.3)	0 (0.0)	5 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Minami <i>et al</i> <sup>[24]</sup>	15 (17.0)	1 (1.1)	1 (1.1)	0 (0.0)	13 (14.8)	0 (0.0)	0 (0.0)
Heo <i>et al</i> <sup>[25]</sup>	5 (5.0)	4 (4.0)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)
Lee <i>et al</i> <sup>[26]</sup>	2 (3.6)	0 (0.0)	2 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kim <i>et al</i> <sup>[27]</sup>	1 (2.8)	0 (0.0)	0 (0.0)	1 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)
Lee <i>et al</i> <sup>[28]</sup>	3 (7.2)	2 (4.8)	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Misra <i>et al</i> <sup>[29]</sup>	7 (14.0)	4 (8.0)	3 (6.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
Attasaranya <i>et al</i> <sup>[30]</sup>	6 (5.6)	0 (0.0)	2 (1.9)	1 (0.9)	3 (2.8)	1 (0.9)	0 (0.0)
Espinell <i>et al</i> <sup>[31]</sup>	2 (2.2)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Itoi <i>et al</i> <sup>[32]</sup>	2 (3.8)	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.9)	0 (0.0)	0 (0.0)
Kim <i>et al</i> <sup>[33]</sup>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Itoi <i>et al</i> <sup>[34]</sup>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kurita <i>et al</i> <sup>[35]</sup>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ghazanfar <i>et al</i> <sup>[36]</sup>	6 (7.1)	3 (3.6)	3 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.2)
Kim <i>et al</i> <sup>[37]</sup>	1 (2.3)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Youn <i>et al</i> <sup>[38]</sup>	7 (6.9)	2 (2.0)	2 (2.0)	1 (1.0)	2 (2.0)	0 (1.0)	0 (1.0)
Kim <i>et al</i> <sup>[39]</sup>	6 (8.3)	5 (6.9)	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	0 (0.0)
Stefanidis <i>et al</i> <sup>[40]</sup>	2 (4.4)	1 (2.2)	1 (2.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Rebello <i>et al</i> <sup>[41]</sup>	4 (13.3)	1 (3.3)	0 (0.0)	0 (0.0)	3 (10.0)	0 (0.0)	0 (0.0)
Sakai <i>et al</i> <sup>[42]</sup>	4 (6.8)	0 (0.0)	1 (1.7)	1 (1.7)	2 (3.4)	0 (0.0)	0 (0.0)
Park <i>et al</i> <sup>[43]</sup>	71 (11.2)	13 (2.1)	48 (7.6)	7 (1.1)	3 (0.4)	2 (0.3)	4 (0.6)
Poincloux <i>et al</i> <sup>[44]</sup>	9 (14.1)	2 (3.1)	5 (7.8)	0 (0.0)	2 (3.1)	0 (0.0)	0 (0.0)
Hwang <i>et al</i> <sup>[45]</sup>	5 (7.2)	3 (4.3)	0 (0.0)	1 (1.4)	1 (1.4)	2 (2.9)	0 (0.0)
Paspatis <i>et al</i> <sup>[46]</sup>	17 (13.7)	4 (3.2)	6 (4.8)	2 (1.6)	5 (4.1)	0 (0.0)	1 (0.8)
Rosa <i>et al</i> <sup>[47]</sup>	10 (14.7)	9 (13.2)	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Yang <i>et al</i> <sup>[48]</sup>	8 (4.7)	2 (1.2)	4 (2.4)	1 (0.6)	1 (0.6)	0 (0.0)	0 (0.0)
Yoon <i>et al</i> <sup>[49]</sup>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Harada <i>et al</i> <sup>[50]</sup>	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)
Total	809 (8.3)	61 (2.4)	91 (3.6)	15 (0.6)	42 (1.7)	6 (0.2)	6 (0.2)

AE: Adverse event.

**Table 4** Comparison between endoscopic papillary large balloon dilation with and without endoscopic sphincterotomy *n* (%)

	EPLBD with EST	No. of studies	EPLBD without EST	No. of studies	<i>P</i> value
No. of procedures	2511	30	413	3	
Mean of mean age, yr	69.6 ± 8.6 <sup>1</sup>	29	70.3 ± 2.3 <sup>1</sup>	3	0.808
Periampullary diverticulum	773 (36.7)	23	122 (33.2)	2	0.186
Initial success	1745 (84.0)	24	285 (76.2)	3	< 0.001
Success without EML	2077 (83.2)	30	306 (76.7)	3	0.001
Use of EML	353 (14.1)	30	86 (21.6)	3	< 0.001
Overall success	2407 (96.5)	30	388 (97.2)	3	0.432
Overall adverse events	209 (8.3)	30	29 (7.0)	3	0.370
Pancreatitis, total; M/Mod/S/F	61; 51/9/0/1 (2.4)	30	16; 14/2/0/0 (3.9)	3	0.089
Bleeding, total; M/Mod/S/F	91; 75/11/2/2 (3.6) <sup>2</sup>	30	8; 7/1/0/0 (1.9)	3	0.079
Perforation, total; M/Mod/S/F	15; 3/6/3/3 (0.6)	30	2; 0/2/0/0 (0.5)	3	1.000
Other adverse events	42 (1.7)	30	3; 3/0/0/0 (0.7)	3	0.148
AE-related surgery	6 (0.2)	30	0 (0)	3	1.000
AE-related death	6 (0.2)	30	0 (0)	3	1.000

<sup>1</sup>mean ± SD; <sup>2</sup>One case of bleeding was not graded for severity. M: Mild; Mod: Moderate; S: Severe; F: Fatal; EPLBD: Endoscopic papillary large balloon dilation; EST: Endoscopic sphincterotomy; EML: Endoscopic mechanical lithotripsy; AE: Adverse event.

EST and those who received EPLBD without EST were summarized in Table 4. Mean age and the rate of periampullary diverticulum showed no significant difference between both procedures. Mean number of EPLBD session and the overall success rate were not significantly different between both procedures, but the initial success rate (84.0% *vs* 76.2%, *P* < 0.001) and the success rate of

EPLBD without EML (83.2% *vs* 76.7%, *P* = 0.001) were significantly higher in patients who received EPLBD with EST than in those who received EPLBD without EST, while the rate of use of EML (14.1% *vs* 21.6%, *P* < 0.001) were significantly lower in patients who received EPLBD with EST. Overall adverse events, pancreatitis, bleeding, perforation, other adverse events, the rate of surgery for

**Table 5 Baseline clinical characteristics of the patients on endoscopic papillary large balloon dilation without endoscopic sphincterotomy *n* (%)**

Ref.	Study design	No. of procedures	Mean age, yr	No. of periampullary diverticulum	Mean size of largest stone, mm	Range of stone size, mm	Altered anatomy
Jeong <i>et al</i> <sup>[21]</sup>	R	38	68	NA	17.7	12-31	0
Hwang <i>et al</i> <sup>[45]</sup>	R	62	70.4	16 (25.8)	15.7	12-26	0
Park <i>et al</i> <sup>[43]</sup>	R	313	72.6	106 (34.6)	15.0	10-37	B-II :11
Total		413	71.8 <sup>1</sup>	122 (33.2)	15.4 <sup>2</sup>	10-37	11 (2.7)

<sup>1</sup>Calculated by dividing total number of procedures into total number of the parameter which was calculated by multiplying each mean value with each number of procedures; <sup>2</sup>A retrospective multicenter study where missing data are present in each analyzed variable. R: Retrospective; B-II: Billroth-II anastomosis.

**Table 6 Procedure characteristics and outcomes of endoscopic papillary large balloon dilation without endoscopic sphincterotomy *n* (%)**

Ref.	Balloon size, mm	Duration of inflated balloon, s	Initial success	No. of sessions, mean	Success without EML	Use of EML	Overall success
Jeong <i>et al</i> <sup>[21]</sup>	15-18	60	25 (65.8)	1.20	29 (76.3)	9 (23.7)	37 (97.4)
Hwang <i>et al</i> <sup>[45]</sup>	12-20	60	57 (91.9) <sup>1</sup>	1.05	50 (80.6)	12 (19.4)	60 (96.8)
Park <i>et al</i> <sup>[43]</sup>	12-20	30-180	203 <sup>3</sup> (74.1) <sup>1</sup>	1.33	227 <sup>4</sup> (76.0)	65 <sup>4</sup> (21.7)	291 <sup>4</sup> (97.3)
Total	12-20	30-180	285 (76.2)	1.27 <sup>2</sup>	306 (76.7)	86 (21.6)	388 (97.2)

<sup>1</sup>Studies which were designed to include cases where endoscopic mechanical lithotripsy was performed along with the first session of endoscopic papillary large balloon dilation; <sup>2</sup>Calculated by dividing total number of procedures into total number of sessions which was calculated by multiplying each mean number of session with each number of procedures; <sup>3</sup>Total number of procedures was 274 due to missing data; <sup>4</sup>Total number of procedures was 299 due to missing data. EML: Endoscopic mechanical lithotripsy.

**Table 7 Adverse events of endoscopic papillary large balloon dilation without endoscopic sphincterotomy *n* (%)**

Ref.	Overall AEs	Pancreatitis	Bleeding	Perforation	Others	AE-related surgery	AE-related death
Jeong <i>et al</i> <sup>[21]</sup>	1 (2.6)	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hwang <i>et al</i> <sup>[45]</sup>	4 (6.4)	4 (6.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Park <i>et al</i> <sup>[43]</sup>	24 (7.7)	11 (3.5)	8 (2.6)	2 (0.6)	3 (1.0)	0 (0.0)	0 (0.0)
Total	29 (7.0)	16 (3.9)	8 (1.9)	2 (0.5)	3 (0.7)	0 (0.0)	0 (0.0)

AE: Adverse event.

**Table 8 Comparison of adverse events among endoscopic papillary large balloon dilation with large, limited and without endoscopic sphincterotomy *n* (%)**

	EPLBD with large EST	EPLBD with limited EST	EPLBD without EST	<i>P</i> value
No. of procedures	756	946	413	
Overall adverse event	65 (8.6)	71 (7.5)	29 (7.0)	0.568
Pancreatitis	18 (2.4)	29 (3.1)	16 (3.9)	0.349
Bleeding	31 (4.1)	12 (1.3)	8 (1.9)	0.001 <sup>1</sup>
Perforation	3 (0.4)	5 (0.5)	2 (0.5)	1.000
Other adverse events	13 (1.7)	25 (2.6)	3 (0.7)	0.054
AE-related surgery	2 (0.3)	2 (0.2)	0 (0.0)	0.832
AE-related death	2 (0.3)	0 (0.0)	0 (0.0)	0.166

<sup>1</sup>EPLBD with large EST *vs* EPLBD with limited EST, *P* < 0.001; EPLBD with large EST *vs* EPLBD without EST, *P* = 0.049; EPLBD with limited EST *vs* EPLBD without EST, *P* = 0.35. EPLBD: Endoscopic papillary large balloon dilation; EST: Endoscopic sphincterotomy; AE: Adverse event.

adverse events, and fatal adverse events were not significantly different between both procedures.

We compared the rates of adverse events among 3 kinds of EPLBD procedures which we classified based on the extent of ampullary incision of the EST; large EST, limited EST and no EST (Table 8). There were no significant differences among the 3 EPLBD procedures

in the rates of the overall adverse events, pancreatitis, perforation, other adverse events, and adverse events related to surgery and death, but the rate of bleeding was significantly higher in EPLBD with large EST, compared with EPLBD with limited EST (*P* < 0.001, OR = 3.33) or without EST (*P* = 0.049, OR = 2.17), but no significant difference between EPLBD with limited EST and

**Table 9** Comparison among endoscopic sphincterotomy, endoscopic papillary balloon dilation, and endoscopic papillary balloon dilation with endoscopic sphincterotomy *n* (%)

	EST <sup>1</sup>	EPBD <sup>1</sup>	No. of studies	EPLBD with EST	No. of studies	<i>P</i> value
No. of procedures	890	878	15	2511	30	
Mean age, range, yr	47-71	49-75	15	40-82	29	
Mean stone size, range, mm	7.3-16.9	7-15.6	15	5-45	25	
Initial success	322 (80.9)	285 (73.5)	7	1745 (84.0)	24	< 0.001
Use of EML	121 (13.3)	162 (19.6)	13	353 (14.1)	30	< 0.001
Overall success	776 (95.3)	733 (90.1)	13	2407 (96.5)	30	< 0.001
Overall adverse events	113 (12.7)	106 (12.1)	15	209(8.3)	30	< 0.001
Pancreatitis	36 (4.3)	71 (8.6)	14	61 (2.4)	30	< 0.001
Bleeding	33 (4.8)	1 (0.1)	12	91 (3.6)	30	< 0.001
Perforation	3 (0.5)	2 (0.3)	9	15 (0.6)	30	0.941
AE-related death	2 (0.3)	4 (0.7)	7	6 (0.24)	30	0.152

<sup>1</sup>Results of a meta-analysis by Weinberg *et al*<sup>[18]</sup>. EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilation; EPLBD: Endoscopic papillary large balloon dilation; EML: Endoscopic mechanical lithotripsy.

without EST ( $P = 0.35$ ).

## DISCUSSION

Standard basket and balloon techniques after EST are most commonly used for the removal of bile duct stones with overall success rates of more than 80% to 90%<sup>[88-92]</sup>. When it fails due to the stone size being larger than the widened ampullary orifice by performing EST or the distal common bile duct, additional endoscopic procedures, mainly EML, are usually required for complete stone clearance<sup>[93-97]</sup>. However, EML proved to be a time-consuming and challenging technique<sup>[11,98,99]</sup>. EPLBD has been widely used as the alternative to EST with EML for the removal of large or difficult bile duct stones. EPLBD was initially performed when the standard techniques failed after a large EST<sup>[21,23]</sup>, but recently it has been performed after a limited EST or sometimes without EST, even before attempting trials the standard technique with a large EST. Such procedure is usually performed when it is speculated that the size of the stone is too large for it to be removed using the standard techniques after a large EST and on the assumption that it would reduce the incidence rate of potential serious adverse events of a large EST such as bleeding and bile duct perforation.

The initial success rate and the overall success rate were 84.0% and 96.5%, respectively, in EPLBD with EST in this review, while the results showed 80.9% and 95.3% in EST alone and 73.5% and 90.1% in EPBD alone, respectively, in a previous meta-analysis<sup>[18]</sup> (Table 9). When we compared these results, the initial success rate was significantly lower in EPBD alone than EPLBD with EST ( $P < 0.001$ , OR = 1.89) and EST alone ( $P = 0.013$ , OR = 1.53), but showing no significant differences between EPLBD with EST and EST alone ( $P = 0.131$ ); the overall success rate was also significantly lower in EPBD alone than EPLBD with EST ( $P < 0.001$ , OR = 2.72) and EST alone ( $P = 0.001$ , OR = 2.03), and showing also no significant differences between EPLBD with EST and EST alone ( $P = 0.141$ ). However, a comparison between these meta-analysis results and ours is somewhat contradictory

because their meta-analysis was of relatively small bile duct stones. Furthermore, the initial success rate in this review was statistically flawed, because studies included were designed heterogeneously based on different definitions, some of which included cases where EML was performed along with the first session of EPLBD. There were only 4 comparison studies, including 2 prospective randomized studies<sup>[25,33]</sup> and 2 retrospective studies<sup>[32,39]</sup>, done for the evaluation of outcomes between EPLBD with EST and EST alone with the assistance of EML in patients with large or difficult bile duct stones. However, these studies failed to show any differences in the initial success rate and the overall success rate between both procedures, except one retrospective study<sup>[32]</sup>, where EPLBD with EST was superior to EST alone only in the initial success rate, not the overall success rate<sup>[32]</sup>. The initial success rate in EPLBD without EST in this review was significantly lower, compared with that in EPLBD with EST, most likely due to the opening of the orifice retracting almost immediately back to its original size which is commonly seen in EPBD alone. However, the overall success rate showed no significant difference between both of them.

The intended purpose of EPLBD was to simplify removing large or difficult bile duct stones without additional adverse events to EST alone or EPBD alone, and contemplated major advantages were that it would reduce both the need of EML and the procedure time, increasing the success rates of stone removal, compared with EST alone and EPBD alone. This is believed to be because the wider ampullary orifice, made when using EPLBD, would facilitate in the easier extraction of relatively large bile duct stones. In addition, it may also reduce potential EML-related adverse events, such as basket impaction and bile duct injury. However, the frequency of EML use in EPLBD might be related to various factors, such as the diameter of dilating balloon used, discrepancy in the size between the stone and the ampullary orifice or the distal bile duct, and the shape of the stone and the bile duct. The rate of use of EML was 14.1% in EPLBD with EST with a wide range of 0% to 38.6% in this review. It

showed similar results in EST alone of 13.3% in a previous meta-analysis<sup>[18]</sup>, but significantly lower than EPLBD without EST of 21.6% from this review and EPBD alone of 19.6% in a previous meta-analysis<sup>[18]</sup>. In 4 comparison studies between EPLBD with EST and EST alone, there were conflicting results concerning the use of EML for the removal of large or difficult bile duct stones; two prospective randomized studies reported no significant difference in the use of EML<sup>[25,33]</sup>, on the contrary to two retrospective studies<sup>[32,39]</sup>. These studies overlooked one important fact that the rate of use of EML when tallied against the number of patients requiring EML, could not help but be similar between both procedures, because EML was still needed in patients where the size of the stones exceeded the size of the widened ampullary orifice even after EPLBD. However, the need for repeated EML would be reduced due to a wider ampullary orifice, if the stones were fragmented mostly by one session of EML following EPLBD. Thus, for a more accurate evaluation about the rate of use of EML, it should be calculated based on the frequency of EML use in each patient who underwent EPLBD, not the number of patients requiring EML. Mean procedure time was evaluated in two of these 4 comparison studies; one prospective randomized study failed to show any difference between EPLBD with EST and EST alone<sup>[33]</sup>, while the other retrospective study showed a shorter procedure time in EPLBD with EST<sup>[32]</sup>. Large-scale, prospective multicenter comparison studies will be needed to confirm advantages of EPLBD in the frequency of EML use and procedure time.

In results of adverse events following EPLBD in this review, adverse events in EPLBD without EST showed no significant difference compared with those in EPLBD with EST. The most common adverse event in each procedure was bleeding with a mean rate of 3.6% in EPLBD with EST and pancreatitis with a mean rate of 3.9% in EPLBD without EST. Our results showed definite evidence that EPLBD with and even without EST, did not increase the risk of serious pancreatitis, as more frequently seen in EPBD using small-diameter balloons ( $\leq 10$  mm)<sup>[18-20]</sup>. It is no doubt that the mechanism of pancreatitis would be different in EPLBD, compared with EPBD, although its mechanism still remains unclear, a major etiologic factor of pancreatitis. The most serious adverse event was bile duct perforation in EPLBD with EST. The following shows the comparison of adverse events between results of a previous meta-analysis<sup>[18]</sup> in EST alone and EPBD alone and those of our review in EPLBD with EST (Table 9); the rate of overall adverse events was significantly lower in EPLBD with EST than EST alone ( $P < 0.001$ , OR = 1.60) and EPBD alone ( $P = 0.001$ , OR = 1.51); the rate of pancreatitis was significantly lower in EPLBD with EST than EST alone ( $P = 0.006$ , OR = 1.80) and EPBD alone ( $P < 0.001$ , OR = 3.77); the rate of bleeding was not significantly different between the EPLBD with EST and EST alone ( $P = 0.164$ ) and was significantly lower in EPBD alone than EPLBD with EST ( $P = 0.001$ , OR = 25.27) and EST alone ( $P =$

0.001, OR = 33.75); the rate of perforation and the rate of adverse event-related death showed no significant differences among the 3 procedures ( $P = 0.941$  and  $P = 0.152$ , respectively). However, within 4 comparison studies on adverse events between EPLBD with EST and EST alone, each study showed no significant differences between both of them<sup>[25,32,33,39]</sup>.

Major risk factors which are related to adverse events include procedure-related factors such as size of balloon, size of EST, and time duration of inflated balloon, and patient-related factors such as the existence of bile duct strictures, periampullary diverticulum, surgically altered anatomy, and a bleeding tendency. Park *et al.*<sup>[43]</sup> reported that larger stone size more than 16mm in diameter, underlying cirrhosis, and full-length EST were independently associated with an increase in adverse events. The size of the balloon is the most important major factor in ensuring a success of EPLBD and a reduction of adverse events<sup>[53]</sup>. As the ampullary orifice becomes wider as a result of balloon dilation, stone removal becomes easier. However, choosing an inappropriately oversized balloon increases the risk of adverse events, such as perforation or bleeding due to blood vessel injury<sup>[53]</sup>. Interestingly, a multicenter study by Park *et al.*<sup>[43]</sup> reported that balloons larger than 14 mm in diameter were independently associated with a decreased risk of pancreatitis, projecting that only simple stretching of the ampullary orifice or direct blockage of the pancreatic orifice by compression of large-diameter balloons is not a major etiologic factor of pancreatitis following EPLBD.

The intended maximal target diameter of a dilating balloon for EPLBD should be determined based on the size of the stone and the size of the distal bile duct proximal to the tapered segment<sup>[26,55,100]</sup>, but must never exceed the diameter of the distal bile duct to prevent bile duct perforation<sup>[43,53]</sup>. A 12- to 20-mm diameter balloon for pyloric use (CRE<sup>TM</sup> wire-guided balloon dilator, Boston Scientific, Natick, Massachusetts, United States) is mostly used to dilate the duodenal ampulla during EPLBD, each of which gradually inflates in 3 different diameter steps by increasing balloon inflation pressure. The balloon used should be selected with the 2<sup>nd</sup> or the 3<sup>rd</sup> diameter step being the intended maximal target diameter, and be inflated gradually, starting from a smaller diameter step of the balloon than the intended maximal target diameter. The balloon is slowly dilated until it reaches its 1<sup>st</sup> diameter step with gradual increment of balloon pressure to prevent sudden tearing of the ampullary roof. If the balloon is dilated without any difficulty with the disappearance of its central waist, it is then dilated gradually to its 2<sup>nd</sup> diameter step and then further up to its 3<sup>rd</sup> diameter step till its diameter reaches the intended maximal target diameter. If the central waist of the balloon does not disappear against the marked resistance of the bile duct or the patient indicates severe pain during balloon inflation at any step, further balloon inflation must be ceased for the prevention of bile duct perforation<sup>[43]</sup>. Lee *et al.*<sup>[55]</sup> recommended based on personal experience that balloon



inflation should be discontinued if the balloon waist does not disappear even once it reaches 75% of the recommended maximal inflation pressure. In patients who are known to have obvious distal bile duct strictures, EPBLD should be avoided to prevent bile duct perforation<sup>[43,55]</sup>. If there is a suspicion of strictures, based on personal experience, we recommend pulling back a large retrieval balloon, that should be inflated up to approximately the same size as the distal bile duct, just up until the inside of the ampullary orifice. If there is no existence of a stricture, the suspected site of stricture should easily expand allowing the balloon to pass through without any resistance.

The extent of ampullary incision is another important major factor to prevent adverse events, such as bleeding and perforation. Theoretically, EPLBD with limited EST would have combined advantages to minimize major adverse events of both EST alone and EPBD alone, such as bleeding and perforation mainly in a large EST and pancreatitis mainly in EPBD<sup>[54]</sup>. In comparison of the 3 different EPLBD procedures based on the extent of ampullary incision of the preceding EST, which were classified into large, limited and no EST, it showed no significant differences among them in the rates of overall adverse events, pancreatitis, perforation and other adverse events. However, the rate of bleeding was significantly higher in EPLBD with large EST than in EPLBD with limited EST or without EST, but there was no significant difference between EPLBD with limited EST and without EST. Delayed fatal bleeding was noted in 2 patients who underwent a full-incision EST before EPLBD in this review. Delayed serious bleeding may occur if a large blood vessel located at the proximal part of the ampullary roof is severed during full-incision EST, not injury caused by stretching of the ampullary orifice using a large-diameter balloon. Therefore, EPLBD with large, especially full-incision EST should be avoided to prevent serious bleeding. In patients with prior EST, it is known that extended incision of the previous EST site can increase the risk of adverse events such as bleeding or perforation<sup>[3,6,8]</sup>. Therefore, almost all patients with prior EST did not receive repeated EST in this review. There were 3 retrospective studies and one prospective study about clinical trials of EPLBD using 12- to 20-mm large balloons on only patients with prior EST but without repeated EST, showing similar results in stone clearance and adverse events, compared with their counterpart studies in which all patients underwent no prior EST<sup>[35,37,49,50]</sup>.

The main purpose of EST during EPLBD is not to make an incision of the duodenal ampulla long, but to control the direction of tearing during balloon dilation. A probable mechanism of a reduced pancreatitis rate in EPLBD with EST is believed to be that the radial force exerted by the dilating balloon shifts along the cutting direction made during EST toward the bile duct away from the pancreatic orifice, resulting in less periampullary injury around the pancreatic duct with a decreased risk of pancreatitis<sup>[21,24,43,101]</sup>. However, EST may be a limited

role in preventing pancreatitis in EPLBD, because there was no evidence that EPLBD without EST increased the risk of pancreatitis in this review. So to explain this, we suggest the following hypothesis surrounding the mechanism of pancreatitis after EPLBD; manipulation of Dormia basket and retrieval balloon catheter as well as the frequency of EML in EPLBD with, or even without, EST, may be reduced due to a sufficiently widened ampullary orifice, resulting in less periampullary trauma or edema that occurs during stone extraction and eventually leading to a low risk of pancreatitis. On the contrary, its frequency in EPBD using small-diameter balloons is increased due to the ampullary orifice not widening enough, increasing the risk of pancreatitis<sup>[100]</sup>.

Time duration of inflated balloon of the duodenal ampulla during EPLBD is mostly around 1 min in this review, after the intended maximal target diameter of the balloon was reached. One prospective randomized study revealed that 30 s of duration of inflated balloon was not different in adverse events, including pancreatitis, bleeding and perforation, to 60 s in EPLBD with EST<sup>[46]</sup>. The longer the time duration of inflated balloon did not seem to be related to an increase of the risk of adverse events, and the shorter the time duration of inflated balloon seems to be related to an increase of the risk of serious bleeding, due to insufficient compression by the balloon. Further studies are warranted to determine the optimal time duration of inflated balloon during EPLBD.

The patient-related factors related to adverse events include periampullary diverticulum, surgically altered anatomy, and a bleeding tendency. Patients with periampullary diverticulum were suitable for EPLBD. A retrospective comparison study in patients between with and without periampullary diverticulum, showed similar stone clearance rates and adverse events in both, following EPLBD with limited EST<sup>[76]</sup>, and several studies reported that the presence of a periampullary diverticulum was not associated with a significant increased rate of adverse events such as pancreatitis, bleeding, or perforation<sup>[43,46,48,86]</sup>. There were 6 studies about clinical trials of EPLBD on only patients with surgically altered anatomy, such as Billroth II surgery (5)<sup>[77-81]</sup> and Roux-en-Y anastomosis (1)<sup>[82]</sup>, resulting in complete stone clearance in all patients with a low incidence of pancreatitis and bleeding. In patients with coagulopathy, EPLBD without EST may be useful, but should be undertaken cautiously<sup>[43,100]</sup>, even though further studies are warranted. Park *et al*<sup>[43]</sup> reported that the size of the bile duct stone ( $\geq 16$  mm) and presence of cirrhosis might be independent factors of bleeding. If serious bleeding from the ampulla occurs after balloon deflation, compression of the ampulla with re-balloon can be done for several minutes till the bleeding stops.

Our recommendations for a successful EPLBD are as follows, based on the results in this review and personal experiences: (1) EPLBD with large, especially full-incision EST should be avoided to prevent serious bleeding; (2) EPLBD with limited EST is recommended to be

**Table 10 Recommendations for successful endoscopic papillary large balloon dilation**

1 EPLBD with large, especially full-incision EST should be avoided
2 EPLBD with limited EST is recommended to be performed, even before attempting trials of a standard technique with large EST, when the stone is seen to be too large on cholangiogram
3 EPLBD without EST may be useful in some patients with coagulopathy, periampullary diverticulum, or surgically altered anatomy
4 In patients with obvious distal bile duct strictures, EPBLD should be avoided. If there is a suspicion of strictures, using the pulling method of a large inflated retrieval balloon through the site is recommended to confirm an existence
5 The intended maximal target diameter of the balloon should be determined based on the diameter of the largest stones, but should not exceed the diameter of the distal bile duct
6 The balloon should always be inflated gradually, starting from a smaller diameter step of the balloon than the intended maximal target diameter
7 Further balloon inflation must be ceased, if the central waist of the balloon does not disappear or the patient indicates severe pain during balloon inflation at any step

EST: Endoscopic sphincterotomy; EPLBD: Endoscopic papillary large balloon dilation.

performed to reduce the risk of bleeding and perforation even before attempting trials of a standard technique with large EST, when the stone is seen to be too large on cholangiogram; (3) EPLBD without EST may be useful in some patients with coagulopathy, periampullary diverticulum, or surgically altered anatomy to reduce the risk of serious bleeding and perforation; (4) In patients with obvious distal bile duct strictures, EPBLD should be avoided to prevent perforation. If there is a suspicion of strictures, using the pulling method of a large inflated retrieval balloon through the site is recommended to confirm an existence; (5) The intended maximal target diameter of the balloon should be determined based on the diameter of the largest stones, but should not exceed the diameter of the distal bile duct to reduce the risk of perforation; (6) The balloon should always be inflated gradually, starting from a smaller diameter step of the balloon than the intended maximal target diameter; and (7) Further balloon inflation must be ceased to prevent perforation, if the central waist of the balloon does not disappear or the patient indicates severe pain during balloon inflation at any step (Table 10).

There are several limitations to this review. It was very difficult to analyze systematically the outcomes of EPLBD, because the results from each relevant article were based on different definitions. So we re-analyzed all gathered data by using a single standardized definition. An article of a large scaled multicenter study<sup>[43]</sup> that included our institute, where the data of the patients undergoing EPLBD with and without EST were calculated as one, was re-analyzed using its raw data in order to re-group both of them separately. Another limitation is that most articles included in this review are not prospective controlled studies, but retrospective studies. Therefore, further large-scale prospective randomized controlled studies will be needed not only to confirm our claims on effectiveness of EPLBD with or without EST, compared with both of EST alone and EPBD alone, but to assess the facts which affect the outcome and adverse events of EPLBD. In conclusion, recent accumulated results of EPLBD with or even without EST suggest that it is a safe and effective procedure for the removal of large or difficult bile duct stones without any additional risk of

severe adverse events, when performed under appropriate guidelines.

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## Intraductal papillary neoplasm of the bile duct

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### Abstract

Intraductal papillary neoplasm of the bile duct (IPNB) is a variant of bile duct carcinoma that is characterized by intraductal growth and better outcomes compared with common cholangiocarcinoma. IPNBs are mainly found in patients from Far Eastern areas, where hepatolithiasis and clonorchiasis are endemic. According to the immunohistochemical profiles of the mucin core proteins, IPNBs are classified into four types: pancreaticobiliary, intestinal, gastric, and oncocytic. Approximately 40%-80% of IPNBs contain a component of invasive carcinoma or tubular or mucinous adenocarcinoma, suggesting that IPNB is a disease with high potential for malignancy. It is difficult to make

an accurate preoperative diagnosis because of IPNB's low incidence and the lack of specificity in its clinical manifestation. The most common abnormal preoperative imaging findings of IPNB are intraductal masses and the involvement of bile duct dilation. Simultaneous proximal and distal bile duct dilation can be detected in some cases, which has diagnostic significance. Cholangiography and cholangioscopy are needed to confirm the pathology and demonstrate the extent of the lesions. However, pathologic diagnosis by biopsy cannot reflect the actual stage in many cases because different foci may be of different stages and because mixed pathologic findings may exist in the same lesion. Surgical resection is the major treatment. Systematic cholangioscopy with staged biopsies and frozen sections is recommended during resection to ensure that no minor tumors are left and that curative resection is achieved. Staging, histologic subtype, curative resection and lymph node metastasis are factors affecting long-term survival.

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**Key words:** Intraductal neoplasm; Papillary cholangiocarcinoma; Biliary papillomatosis; Mucinous; Prognosis

**Core tip:** In this review, we have provided a more comprehensive understanding of "intraductal papillary neoplasm of the bile duct" than in other research articles. We found that preoperative pathologic diagnosis by biopsy could not reflect the actual stage in many cases because different foci might be of different stages and because mixed pathologic findings might exist in the same lesion. Staging, histologic subtype, curative resection and lymph node metastasis were factors affecting long-term survival.

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## INTRODUCTION

In the biliary system there is a class of tumor that is characterized by predominant intraductal papillary growth, which can occur anywhere along the biliary tree. Growths are usually multifocal, with or without macroscopically visible mucin secretion, and can be of any type of pathological transformation, from low-grade dysplasia to invasive carcinoma. According to these features, these growths used to be identified by various names such as biliary papillomatosis, mucin-producing cholangiocarcinoma, mucin-hypersecreting bile duct tumor, and biliary intraductal papillary mucinous neoplasm. They share common phenotypic changes in tumorigenesis or tumor-progression and show more favorable prognoses compared with non-papillary cholangiocarcinoma. The clinical entity intraductal papillary neoplasm of the bile duct (IPNB) was added to the 2010 World Health Organization (WHO) classification, and it includes intraductal papillary cholangiocarcinoma and its precursor lesions<sup>[1]</sup>. Previous studies have usually focused on one or several aspects of this disease and have added to our knowledge about their clinicopathological features. This review aims to summarize this knowledge and provide a more comprehensive understanding of IPNB.

## EPIDEMIOLOGY

IPNB is mainly found in patients in Far Eastern areas, such as Taiwan, Japan, and Korea, where hepatolithiasis and clonorchiasis are endemic. It is a rare disease, and papillary cholangiocarcinoma accounts for approximately 4%-38% of all bile duct adenocarcinomas. Current reports are of sporadic cases without a tendency to familial aggregation. IPNB most commonly develops in patients between 50 and 70 years of age and shows different sex preponderances in different regions such that the male-to-female ratio is nearly 1:2 in Taiwan, 1:1 in Japan, and 2:1 in Korea and Western countries<sup>[2-5]</sup>.

## ETIOLOGY AND PATHOGENESIS

Although the specific etiology and pathogenesis are unclear, IPNB is known to have two major risk factors: hepatolithiasis and clonorchiasis. Yeh *et al*<sup>[6]</sup> reported that nearly 87% of patients with IPNB had hepatolithiasis in Taiwan. Another study<sup>[7]</sup> from Korea suggested that 31% of IPNB patients had hepatolithiasis and 18% had clonorchiasis, but there was no such association in Western countries<sup>[2]</sup>. This phenomenon suggests that racial and environmental factors may play a role in the development of IPNB in addition to the two major risk factors.

The time lag between the development of hepatolithiasis and IPNB is 6-8 years, and intraductal carcinoma

*in situ* can take 1-2 years to evolve into an invasive lesion. Considering the findings of mixed pathologic types in each case, as well as papillary adenocarcinoma in the background of papillary adenoma, a process of inflammatory cell-repair dysplasia and malignant transformation is likely<sup>[6,8,9]</sup> (Figure 1). Approximately 40%-80% of IPNBs contain a component of invasive carcinoma or tubular or mucinous adenocarcinoma, suggesting that IPNB is a disease with a high potential for malignancy<sup>[2,3,6,8,9]</sup>. Immunohistochemical study of surgically removed specimens shows that almost all IPNBs express CK7, CK20, and mucin (MUC)5AC, which are markers of biliary, intestinal, and gastric epithelium, respectively. This finding indicates that IPNB tumor cells retain a biliary immunophenotype and obtain intestinal and gastric immunophenotypes during carcinogenesis. MUC1 expression is frequently associated with the development of invasive lesions<sup>[2,4,6,10-14]</sup>, especially tubular adenocarcinoma, while mucinous carcinoma is usually associated with negativity for MUC1 but positivity for MUC2. Sasaki *et al*<sup>[15]</sup> found that overexpression of enhancer of zeste homolog 2 might be associated with malignant behavior in IPNB, in parallel with up-regulated MUC1 expression and down-regulated MUC6 expression. Recently Nakanuma *et al*<sup>[16]</sup> provided evidence that peribiliary glands (PBGs) contain cells implicated in the origin of IPNB. Cardinale *et al*<sup>[17]</sup> suggested that IPNB might arise from biliary tree stem/progenitor cells (BTSCs) located in PBGs. In response to risk factors such as inflammation, BTSCs might undergo a series of genetic changes and progress from dysplasia to invasive carcinoma.

## PATHOLOGY

IPNB usually appears as singular or multiple grayish-tan to yellow, friable, papillary masses anywhere along the biliary tree, and small lesions may at times be remote from the main tumor. Histologically, IPNB is defined by tumors that show papillary proliferation of neoplastic biliary epithelial cells with delicate fibrovascular stalks within the bile duct, the macroscopic or microscopic existence of mucin, and dilation of the proximal or remote bile duct. Hematoxylin and eosin staining and immunohistochemical profiling of the mucin core proteins are used to classify IPNB into four types<sup>[7]</sup> (Table 1). The pancreaticobiliary type consists of columnar cells with eosinophilic cytoplasm and round nuclei. This type is often positive for MUC1 but negative for MUC2. The intestinal type resembles an intestinal villous neoplasm, and the neoplastic cells consistently express MUC2 and MUC5AC but not MUC1. The gastric type consists of columnar cells resembling gastric foveae that express MUC5AC but are negative for MUC1 and MUC2. The oncocytic type consists of cells with abundant, intensely eosinophilic cytoplasm that consistently express MUC5AC with focal expression of MUC1 and/or MUC2. The pancreaticobiliary type is the most common and is usually associated with invasive lesions, while the onco-



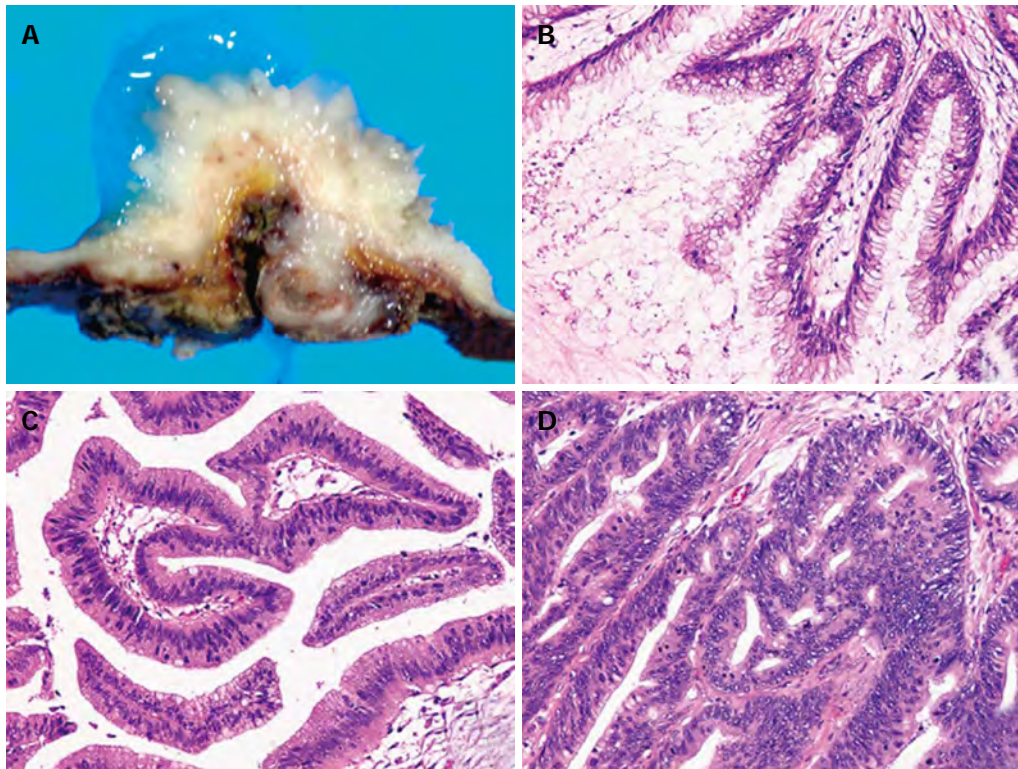


Figure 1 A representative case of intraductal papillary neoplasm of the bile duct with macroscopically visible mucin secretion. Within a single tumor (A), the coexistence of adenoma (B), borderline lesion (C), and adenocarcinoma (D) was found (hematoxylin and eosin stain,  $\times 200$ ).

**Table 1** Histologic subtypes classified by mucin core protein and cytokeratins

Histologic subtype	Profile of MUCs			Cytokeratin	
	MUC1	MUC2	MUC5AC	CK7	CK20
Pancreaticobiliary	+	-	+	+	+
Intestinal	-	+	+	+	+
Gastric	-	-	+	+	+
Oncocytic	Focal+	Focal+	+	+	+

MUC: Mucin core proteins; CK: Cytokeratin.

cytic and gastric types are rare. According to the degree of dysplasia and depth of invasion, IPNB is classified into four stages: IPNB with low-to-intermediate grade dysplasia; IPNB with high-grade dysplasia; intraductal growth-type cholangiocarcinoma, AJCC stage T1; and intraductal growth-type cholangiocarcinoma, AJCC stage T2 or higher.

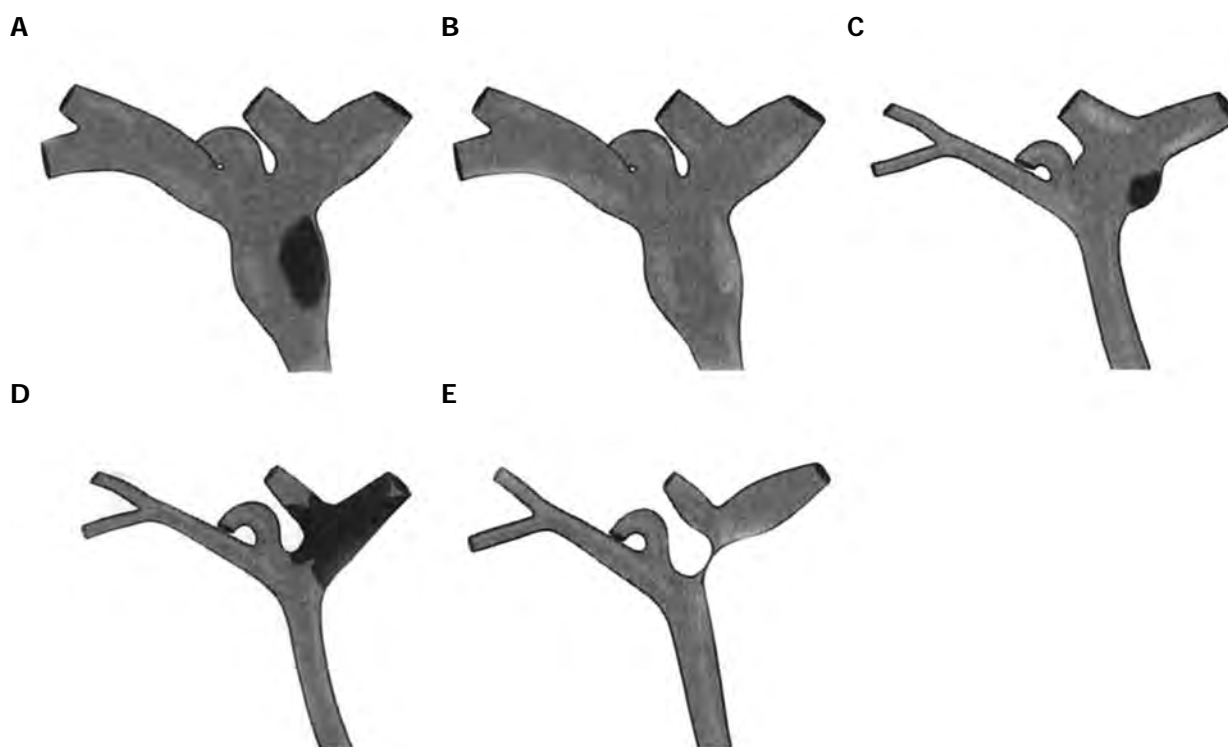
## CLINICAL MANIFESTATION

The most common clinical manifestations of patients with IPNB are right hypochondrialgia (35%-88.5%), repeated episodes of acute cholangitis (5%-59%), and obstructive jaundice (20%-36%). Anemia and loss of body weight are relatively less common. Some patients are asymptomatic<sup>[2,3,5-9]</sup>. Acute cholangitis, which is not a common presentation of conventional cholangiocarcinoma, is the second most common manifestation of IPNB. First, friable tumor emboli can easily detach

from their origins, leading to acute obstruction of the bile duct. Second, more patients are diagnosed with biliary stones in IPNB than in typical cholangiocarcinoma. Third, macroscopic mucin hypersecretion can be observed in nearly one third of IPNB patients. Theoretically, abundant mucin discharge into the bile duct may intermittently impede bile flow, leading to obstructive jaundice and cholangitis, which can also cause volatile jaundice. The majority of IPNBs are found in the hilum and left liver; however, despite these variable locations, the primary site does not affect the course of the disease or prognosis<sup>[2]</sup>.

## LABORATORY TESTS

Laboratory data show common manifestations of obstruction of the bile duct such as elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin, gamma-glutamyl transpeptidase, alkaline phosphatase, *etc.* Yeh *et al.*<sup>[18]</sup> found that an increased ALT level ( $> 36$  U/L,  $P = 0.022$ ) in IPNB was the only independent factor that could differentiate it from conventional cholangiocarcinoma. However, the specific relationship was not clearly elaborated, and this finding has not been supported by any other reports. Lee *et al.*<sup>[8]</sup> observed elevation of CA19-9 in 20 of 50 IPNB patients, and the mean level was higher in patients with mucin hypersecretion. Yeh *et al.*<sup>[6]</sup> found that an elevated serum CA19-9 level was detected in 35% of benign lesions, while 61% of malignant lesions



**Figure 2** Schematic drawings of intraductal papillary neoplasms of the bile duct showing the five imaging patterns. A: Type 1: Diffuse duct ectasia with a grossly visible papillary mass; B: Type 2: Diffuse duct ectasia without a visible mass; C: Type 3: An intraductal polypoid mass within localized duct dilation; D: Type 4: Intraductal cast-like lesions; E: Type 5: A focal stricture-like lesion with mild proximal ductal dilation.

had elevated levels; however, there was no significant difference. Thus, the elevation of CA19-9 may be due to common cholestasis or cholangitis associated with mucin overproduction<sup>[19-22]</sup>. Additionally, an elevated CEA level was detected in nearly 25% of malignant IPNBs, so CEA may be of some value in differentiating intraductal papillary cholangiocarcinoma from its precursor lesions.

## IMAGING FEATURES

The most common abnormal preoperative imaging findings for IPNB are intraductal masses and the involvement of bile duct dilation. Simultaneous proximal and distal bile duct dilation can be detected in some cases, which has diagnostic significance. Imaging patterns can be specifically classified into five subtypes<sup>[7,23]</sup> (Figure 2). Type 1 shows diffuse duct dilation with a grossly visible intraductal mass (45.4%). Type 2 shows diffuse and marked duct ectasia as in type 1 but without a grossly visible mass (23.7%). Type 3 shows an intraductal papillary mass with localized duct dilation (19.6%). Type 4 shows mild ductal dilation filled with intraductal cast-like lesions (4.1%). Type 5 shows a focal stricture-like lesion with mild proximal duct dilation (7.2%).

### Ultrasound and ultrasonography

Ultrasound is sensitive for the detection of bile duct dilation, but it is only able to detect a low-echoic mass in nearly 41.2% of cases<sup>[8]</sup>. Although it helps to differenti-

ate a stone from a tumor in most cases, the accuracy of ultrasound depends on the skill of the investigator. In addition, the presence of mucin cannot be detected on ultrasound because it is equally anechoic as bile. Endoscopic ultrasonography (EUS) and intraductal ultrasonography (IDUS) are useful for assessing the depth of invasion and involvement of the lymph nodes even in the presence of thick mucin, which is important to judge the resectability and predict prognosis. Therefore, it is difficult to distinguish between inflammatory wall thickness and the superficial spread of a tumor using EUS or IDUS<sup>[24,25]</sup>.

### Computed tomography and magnetic resonance image

Computed tomography (CT) can detect tumors larger than 1 cm and dilated bile ducts, and its sensitivity is 50%. The enhancement pattern of a tumor is related to the tumoral blood volume and blood flow as well as the prevalence of stromal space. IPNB is usually confined to the mucosa of the bile duct and suspended on small fibrovascular stalks, so it more often shows washout in enhancement scanning rather than the gradually persistent or progressive enhancement observed for conventional cholangiocarcinoma. IPNB appears as a slightly lower signal than hepatic parenchyma in T1WI and as a slightly higher signal in T2WI on magnetic resonance image (MRI) axial scanning. The enhancement pattern on MRI is similar to CT scan<sup>[26-28]</sup>. Neither CT nor MRI can detect the presence of mucin.



Figure 3 Endoscopic retrograde cholangiography showing an amorphous filling defect, suggesting the presence of mucobilia.

Table 2 Major utility of different imaging techniques

Techniques	Utility
Ultrasound	Detection of bile duct dilation Differentiation from a stone
EUS/IDUS	Assessing the depth of invasion and lymph node involvement
CT/MRI	Detect tumors larger than 1 cm and bile duct dilation Differentiation from conventional cholangiocarcinoma
Cholangiography	Define the extent of tumors Detection and drainage of mucin in ERC and PTC
Cholangioscopy	Confirm the histology and extent of lesions Adjuvant treatments
PET/CT	Detection of unsuspected distant metastases

EUS: Endoscopic ultrasonography; IDUS: Intraductal ultrasonography; ERC: Endoscopic retrograde cholangiography; PTC: Percutaneous transhepatic cholangiography; CT: Computed tomography; MRI: Magnetic resonance image; PET: Positron emission tomography.

### Cholangiography

IPNB often involves the biliary epithelium either diffusely or multifocally, and the actual extent of the lesions usually exceeds CT, MRI and other conventional imaging findings. Cholangiography, including indirect (magnetic resonance cholangiography, MRC) and direct [endoscopic retrograde cholangiography (ERC), percutaneous transhepatic cholangiography (PTC)] cholangiography, is useful for showing the entire bile duct to define the extent of the IPNB<sup>[5,25]</sup>. MRC is noninvasive and can demonstrate the extent of narrowing or dilation of the bile duct and multifocal intraductal tumors, but it cannot detect the presence of overproduced mucin. ERC and PTC can show multiple small irregular filling defects in the bile duct wall. In patients with mucin overproduction, hypersecreted mucin draining through the ampulla and a patulous ampulla are the characteristic findings. On cholangiography, diffuse bile duct dilation and amorphous filling defects in the bile duct are characteristic<sup>[5]</sup> (Figure 3). However, a large amount of mucin secretion and obstruction by the tumor may prevent the complete opacification of the entire biliary system to locate the tumors. Cholangiography cannot detect multiple small tumors or lesions confined to the

mucosa, and it cannot differentiate tumors from stones or benign strictures of the bile duct.

### Cholangioscopy

Peroral cholangioscopy (POCS) and percutaneous transhepatic cholangioscopy (PTCS) can visualize the bile duct directly and confirm the histology and extent of the lesions to ensure that appropriate treatment is provided. In a study by Lee *et al*<sup>[8]</sup>, PTCS revealed additional lesions in nearly one third of IPNB patients after radiologic imaging examinations including ERC and MRC. Therefore, preoperative cholangioscopy has been suggested to be essential. If the papillary orifice is dilated with or without mucin secretion, POCS can be performed immediately after ERC, resulting in an accurate early diagnosis of IPNB. This approach avoids the complications caused by PTCS, such as catheter dislodgement, hemobilia, and tumor seeding of the sinus tract<sup>[25,29]</sup>.

### Position-emission tomography/computed tomography

Malignant IPNB with a large mural nodule will present an increased fludeoxyglucose uptake. FDG PET has advantages in the detection of unsuspected distant metastases and in patients with renal dysfunction<sup>[24,30,31]</sup>.

## DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

It is difficult to make an accurate diagnosis preoperatively because of IPNB's low incidence and lack of a specific clinical manifestation. The combined application of different imaging techniques is very helpful (Table 2). Noninvasive imaging modalities such as CT and MRI usually fail to detect minor tumors and mucin; thus, cholangiography and cholangioscopy are needed to confirm pathology and demonstrate the extent of the lesions. Kang *et al*<sup>[32]</sup> reported that the accuracy of predicting macroscopic multiplicity based on preoperative radiologic imaging findings was 53.5%, with a false positive rate of 25.8% and a false-negative rate of 37.7%. In multifocal IPNB, different foci may be of different stages, and mixed pathologic findings may exist within the same lesion. This phenomenon suggests that pathologic diagnosis by biopsy cannot reflect the actual stage in many cases<sup>[8]</sup>.

The differential diagnoses of IPNB includes recurrent pyogenic cholangitis with bile duct stones<sup>[31]</sup>, mass-forming cholangiocarcinomas<sup>[18,27]</sup>, and biliary mucinous cystic neoplasms (MCNs) (cystadenoma/cystadenocarcinoma)<sup>[33-36]</sup>. Both IPNB and recurrent pyogenic cholangitis with bile duct stones involve intermittent and incomplete biliary obstruction and intraluminal masses or filling defects on imaging. Mucin plugs or sloughed masses may be confused with stones. Invasive methods such as ERC or cholangioscopy may be necessary to differentiate these diseases. Mass-forming cholangiocarcinoma often appears as a single intrahepatic mass with upstream bile duct dilation and gradually persistent



or progressive enhancement on CT and MRI imaging. However, IPNB usually appears as multifocal papillary lesions with both upstream and downstream bile duct dilation with or without visible mucin overproduction that shows washout on enhancement scanning. The vast majority of MCN patients are female; 90% of cases are histologically benign but have the potential to recur and undergo malignant transformation. Multilocular cysts with separation or a cyst-in-cyst appearance are distinctive. Mucin produced by MCNs is confined and does not enter the biliary duct. Ovarian-like stroma is the characteristic microscopic finding. On the contrary, there is no such sex preponderance in IPNB, and 40%-80% of IPNBs are malignant. IPNBs communicate with the bile duct, and there is no ovarian-like stroma pathologically.

## TREATMENTS

### *Surgical resection*

Patients without distant metastasis are considered for surgical resection<sup>[37-39]</sup>. Preoperative IDUS or EUS is used for extrahepatic bile duct assessment and to look for the presence of lymph nodes. Cholangioscopy should be performed to determine the extent of the lesions and to draw up the optimal surgical strategy. During resection, systematic cholangioscopy is performed with staged biopsies and frozen sections. Patients with IPNBs localized to the intrahepatic bile duct are treated with hepatectomy. Patients with IPNBs involving one of the two intrahepatic bile ducts are treated with resection of the affected hemiliver and the common bile duct. For IPNBs localized to the extrapancreatic portion of the bile duct, complete resection of the bile duct from the biliary confluence to the intrapancreatic portion with extended lymphadenectomy is recommended. In cases of positive distal frozen sections, resection of the bile duct is performed with or without pancreatic resection (transduodenal resection). A partial liver resection can be performed when a proximal frozen section is positive in a single intrahepatic duct. Hilar lymphadenectomy has been suggested to be essential for tumors localized to the hilum or common bile duct, but a policy of selective application of caudate lobectomy and portal vein resection can be applied when it is necessary to achieve tumor clearance<sup>[40]</sup>.

### *Liver transplantation*

Surgical resection may remain incomplete due to the high risk of recurrence given positive margins in cases with superficial mucosal spread or recurrence on the remnant bile duct because of the high incidence of multifocal involvement. Resection of the entire biliary tree by liver transplantation and duodenopancreatectomy can be theoretically regarded as the only curative treatment. So far, case reports<sup>[41-44]</sup> on this approach indicate that patients with positive lymph nodes or major tumor invasion or associated severe comorbidities have not been eligible for liver transplantation. However, the preoperative assessment is usually insufficient for the majority

of IPNBs. Thus, a strategy of initial resection to select patients without positive lymph nodes or advanced tumor invasion by definitive analysis of the specimen who would actually benefit from a subsequent liver transplantation seems to be reasonable<sup>[37]</sup>.

### *Palliative treatment*

In case major surgery is not indicated, palliative treatments are recommended<sup>[34]</sup>. Palliative intrahepatic tubing or percutaneous transhepatic biliary drainage can alleviate jaundice and cholangitis to prolong survival. Recently, new approaches such as percutaneous cholangioscopic laser ablation, cholangioscopic electrocoagulation, iridium-192 intraluminal therapy, and argon plasma coagulation are also useful for improved survival<sup>[45]</sup>.

## PROGNOSIS AND FOLLOW-UP

The prognosis of patients with IPNB has been consistently better than of those with conventional bile duct cholangiocarcinoma<sup>[40,46-49]</sup>, and this finding may be related to the inherent biology of IPNB or its primarily intraductal growth pattern. Likewise, there is significant heterogeneity among these tumors. We summarize several factors affecting IPNB patient survival below.

### *Staging*

There was no uniform staging system for IPNB before the 2010 WHO classification. However, it seems to be clear that the overall survival and recurrence-free survival of patients with IPNB is worse as one progresses from low-grade dysplasia to invasive carcinoma on the pathological scale<sup>[5,6]</sup>. Rocha *et al*<sup>[2]</sup> found that both depth of invasion and percentage of invasive carcinoma components correlated with survival (Figure 4). The depth of invasion, graded as  $\geq 5$  mm,  $< 5$  mm, or none, was associated with survivals of 39, 128, and 144 mo, respectively ( $P < 0.007$ ). In addition, the percentage of invasive carcinoma components, graded as  $\geq 10\%$ ,  $< 10\%$ , or none, was associated with survivals of 42 mo, 128 mo and 144 mo, respectively ( $P < 0.03$ ).

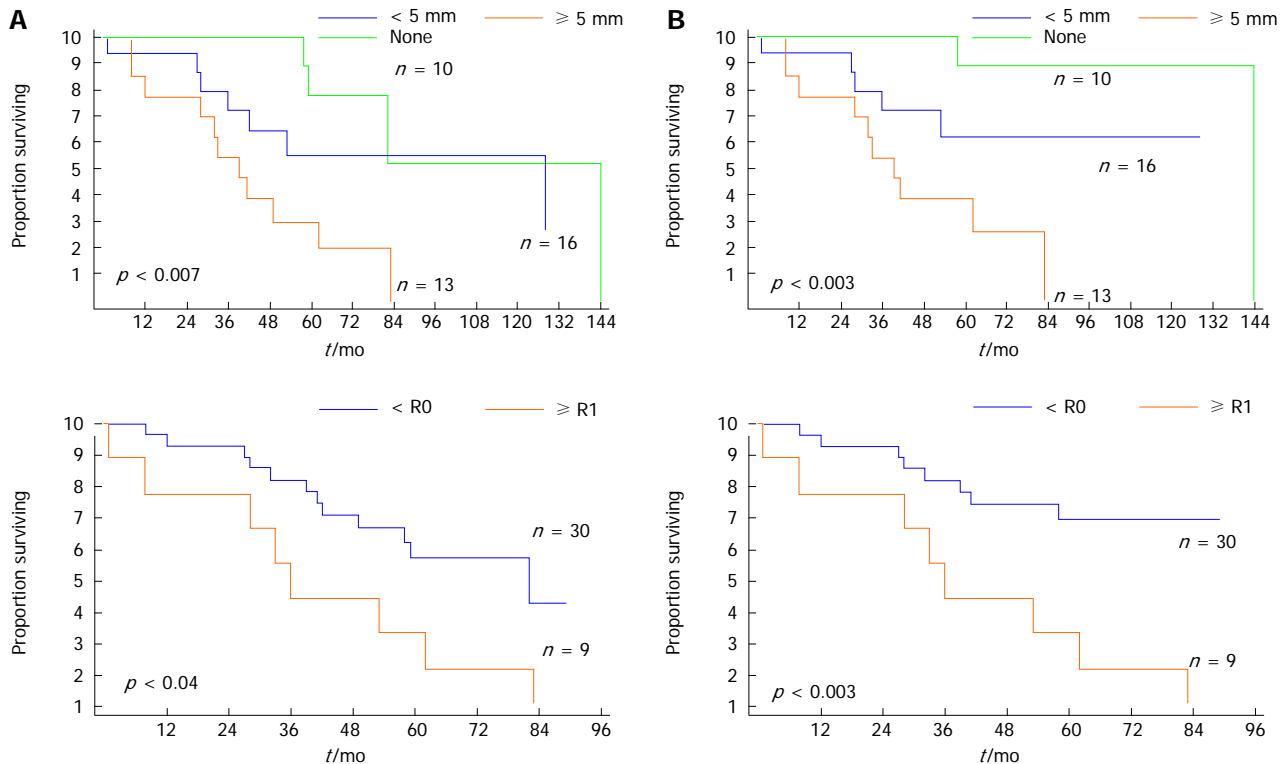
### *Histologic subtypes*

Kim *et al*<sup>[7]</sup> studied a group of 97 patients with IPNB and found that the histologic subtypes of IPNB were associated with different clinicopathologic features and prognoses. Specifically, the pancreaticobiliary type was distinct from the gastric and intestinal types with respect to higher histologic grades, more lymph node metastases, more postoperative recurrences, and worse clinical outcomes. The MUC1-high expressing group showed a shorter disease-specific and recurrence-free survival than the MUC1-low expressing group. In addition, patients with mucinous carcinoma showed a better prognosis compared with patients with tubular adenocarcinoma<sup>[3,13]</sup>.

### *Curative resection*

Lee *et al*<sup>[8]</sup> reported that the disease-free survival rate for patients who underwent curative resection was 81% at 5





**Figure 4** Kaplan-Meier survival estimates of overall survival (A) and disease-specific survival (B) according to the depth of extraductal invasion (none, < 5 mm, and  $\geq 5$  mm) and resection type (R0 vs R1).  $P < 0.05$  was considered significant.

years in a group of 58 cases of IPNB, and the mean survival period was  $60.87 \pm 5.86$  mo (95%CI: 49.38-72.36), while it was  $36.72 \pm 6.61$  mo (95%CI: 23.77-49.67) in patients who underwent palliative treatments such as percutaneous transhepatic biliary drainage (PTBD). Rocha *et al*<sup>[2]</sup> found that R0 resection was associated with an improved median survival time of 82 mo compared with 36 mo in the R1 resection group (Figure 4). Positive resection margins were strongly associated with shorter overall and recurrence-free survival rates, even for low-to-intermediate grade dysplasia<sup>[3]</sup>.

### Lymph node metastasis

Lymph node metastasis is rare in early-stage IPNB. Even in patients with invasive carcinoma, it is relatively less common than conventional cholangiocarcinoma. Yeh *et al*<sup>[5]</sup> found that the mean survival times with malignant IPNB with and without lymph node metastasis were  $12.1 \pm 5.1$  mo (95%CI: 2.0-22.0) and  $39.0 \pm 6.7$  mo (95%CI: 25.9-52.1), respectively.

A high rate of recurrence after surgical resection has been noticed. Incomplete preoperative assessment of the extent of IPNB might be an important contributing factor. Because small papillary tumors may not be detected by conventional radiologic methods, these undetected lesions, which are usually remote from the main tumor, may be the foci of recurrence<sup>[50,51]</sup>. In addition, positive margins related to the superficial spreading pattern of IPNB may be the reason for recurrence in many cases<sup>[8]</sup>. The recurrence rate at 5 years in benign IPNBs

has been reported at nearly 20%, which rises to 60% in malignant cases, and most recurrences are locoregional<sup>[2,7]</sup>. Therefore, to improve the prognosis, full preoperative evaluation of the extent of disease is essential; and to detect recurrences, follow-up appointments scheduled every 3 mo for the first year and every 6 mo beginning in the second year are recommended. MRC is the optimal imaging method, while cholangioscopy can also be performed percutaneously or through the jejunal loop<sup>[34]</sup>.

### DISCUSSION

Recent studies<sup>[2,4,12,13,16,52]</sup> have revealed striking similarities between IPNB and pancreatic intraductal papillary mucinous neoplasm (IPMN). In both organs, these neoplasms arise within the duct system and show a predominantly intraductal growth pattern macroscopically and papillary proliferation with delicate fibrovascular cores and four types of tumor cells microscopically, occasional association with multiple lesions, possible progression to tubular adenocarcinoma and mucinous carcinoma, and more favorable biological behaviors and clinical outcomes. Based on these similarities, IPNB is recognized as a biliary counterpart of IPMN and can be differentiated from conventional cholangiocarcinoma. However, there are several differences between IPNB and IPMN. The most frequent phenotype is intestinal in IPMN but pancreaticobiliary in IPNB, which is more often associated with invasive carcinoma. The other important difference between IPNB and IPMN is with respect to mucin

hypersecretion. Mucin is macroscopically identifiable in most cases of IPMN but only in one third of IPNB cases. Considering the existence of goblet cells (one of the mucin-producing cells) and the expression of secretory-type mucin core proteins such as MUC2 and MUC5AC, this difference might be caused by the amount of mucin production.

Ohtsuka *et al*<sup>[9]</sup> separated IPNB with or without hypersecretion of mucin into two groups, and found that they were similar in terms of clinical features but somewhat different in pathological findings. IPNB without mucin hypersecretion showed a tubulopapillary growth pattern and uniform degree of cytoarchitectural atypia throughout the neoplasm, which was different from the mixed pathologic transformations in IPNB with mucin hypersecretion. Therefore, whether IPNB with and without mucin hypersecretion are different subtypes or they are distinct clinical entities needs further study.

In conclusion, intraductal papillary neoplasm of the bile duct is a rare biliary tumor with a better prognosis than conventional cholangiocarcinoma. Its specific mechanism of pathogenesis and progression has not yet been well defined<sup>[14,15,53-55]</sup>, and its clinicopathologic features are similar to IPMN. Curative resection is the major treatment and an important favorable factor for long-term survival, especially in patients with early-stage IPNB.

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## Cognitive-behavioral therapy for the management of irritable bowel syndrome

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**Core tip:** There is increasing evidence to suggest that cognitive-behavioral therapy (CBT) is effective for the management of irritable bowel syndrome (IBS). CBT can alleviate the physical and psychological symptoms of IBS, and has thus been recommended as a treatment option for the syndrome.

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### Abstract

Irritable bowel syndrome (IBS) is a common disorder, reported to be found in 5%-20% of the general population. Its management accounts for up to 25% of a gastroenterologist's workload in the outpatient department, and the main symptoms are abdominal pain, bloating, and altered bowel habits. Despite a great amount of available pharmacological treatments aimed at a wide variety of gastrointestinal and brain targets, many patients have not shown adequate symptom relief. In recent years, there has been increasing evidence to suggest that psychological treatments, in particular cognitive-behavioral therapy (CBT), are effective for the management of IBS. This review discusses CBT for the management of IBS. CBT has proved to be effective in alleviating the physical and psychological symptoms of IBS and has thus been recommended as a treatment option for the syndrome.

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### INTRODUCTION

The prevalence of irritable bowel syndrome (IBS), a functional gastrointestinal (GI) disorder defined as discomfort or pain specifically associated with an abnormal bowel habit without structural or anatomical explanation, is reported to be between 5% and 20% in the general population<sup>[1]</sup>, and its management accounts for up to 25% of a gastroenterologist's workload in the outpatient department<sup>[2]</sup>. IBS affects 10%-20% of the population in developed countries<sup>[3]</sup>. It also poses a huge burden to society due to direct and indirect costs, and reduced social functioning<sup>[4-6]</sup>. The cost of health care utilization and financial loss because of work absenteeism as a result of IBS is enormous in developed countries<sup>[5,7-9]</sup>. IBS is one of the most common diseases seen in primary care and specialty GI practices<sup>[10]</sup>. An estimated 12% of primary care patients and up to half of consultations in secondary gastroenterology practices are due to IBS-related symptoms<sup>[11,12]</sup>. It was observed by a tertiary care center that 38% of IBS patients had considered suicide because of their symptoms, highlighting the severe effect of IBS

on those patients<sup>[13]</sup>. Most patients with IBS suffer from coexistent mood disorder, anxiety, and neuroticism, and are reported to have a lower quality of life than other patients with serious chronic medical conditions such as diabetes mellitus or end-stage renal disease<sup>[14,15]</sup>. The diagnosis of IBS can be made on the basis of a series of symptoms fulfilling Rome III criteria, but in clinical practice it is still frequently made by exclusion of an organic disorder after investigation<sup>[16]</sup>. There is a multifactorial etiology<sup>[17]</sup>, altered pain perception, involvement of altered gut reactivity and motility, and alteration of the brain-gut axis in IBS<sup>[18]</sup>. Psychological and social factors can influence digestive function, symptom perception, illness behavior, and outcome<sup>[19]</sup>. Therefore, effective therapies for IBS are required in order to alleviate symptoms, and to reduce consultation behavior and consumption of other valuable medical resources.

Although pharmacological therapies can temporarily relieve symptoms, they are often costly and may result in negative side effects<sup>[20]</sup>. A substantial proportion of patients with IBS do not attain adequate relief through conventional medical approaches<sup>[21]</sup>. In recent years, there has been increasing evidence to suggest that psychological treatments, in particular cognitive-behavioral therapy (CBT), are effective for the management of IBS<sup>[22]</sup>. The cognitive-behavioral model was developed in the 1960s by the American psychiatrist and psychotherapist Rush *et al.*<sup>[23]</sup>, who applied it first to depression and then to anxiety disorders<sup>[24]</sup>. The model aims to identify patterns of thinking and behavior which deal with problems leading to negative emotions and hindering progress towards goals. When it is applied to physical health problems, it can reduce physical symptoms by addressing behavior patterns and physiological responses. There is excellent evidence for the efficacy of CBT in reducing symptoms in patients with IBS<sup>[25]</sup>.

This review provides clinicians with an updated and predominantly evidence-based review of CBT for the management of IBS. Several systematic reviews and meta-analyses recently published in high impact factor journals and some randomized controlled trials are included. A better understanding of the recommended therapeutic approaches can lead to increased patient satisfaction, as well as reduced health-care costs.

## CBT AND APPLICATION TO IBS

The idea that emotions can influence the sensorimotor function of the GI tract emerged at the beginning of the 19<sup>th</sup> century, and evidence from research conducted during that period is still valid<sup>[26]</sup>. Psychological disturbance, especially in referred patients, includes psychiatric disorders (*e.g.*, panic disorder, generalized anxiety disorder, mood disorder, and post-traumatic stress disorder), sleep disturbance, and dysfunctional coping<sup>[27]</sup>. A history of childhood abuse is common<sup>[19]</sup>. It has been indicated that up to two-thirds of patients with IBS in tertiary care centers have demonstrable psychiatric illness<sup>[28-30]</sup>, and

that these patients have a worse prognosis than those who are psychologically normal<sup>[31]</sup>. Approximately 50% of patients with a psychiatric disorder develop the condition before the onset of gastrointestinal symptoms, and psychiatric symptoms start at the same time in most of the remaining 50%<sup>[27]</sup>. Recently, it has been demonstrated that psychosocial factors, as an indication of the process of somatization, are independent risk markers for the development of IBS in a group of subjects previously free of IBS<sup>[32]</sup>, and that the effect of psychosocial factors is strongest in severely affected IBS patients<sup>[33]</sup>. On the whole, IBS patients have been reported to have more psychological disturbance than control groups with organic gastrointestinal disease or general populations<sup>[24]</sup>.

There is an increasing evidence for the effectiveness of CBT in alleviating the physical and psychological symptoms of IBS<sup>[2,25,34,35]</sup> and it has thus been recommended as a treatment option for the syndrome<sup>[17,19]</sup>. CBT has matured into a creative and rigorous synergy from empirical evidence and clinical innovation<sup>[36]</sup>. In the 1970s, a group of cognitive therapists in Philadelphia led by Aaron T Beck listened cautiously to what their clients were saying and turned to learning theory and the cognitive revolution to formulate a new theoretical account and therapeutic approach to depression<sup>[23]</sup>. CBT, from its inception growing out of basic and applied research<sup>[37]</sup>, remains closely tied to ongoing research<sup>[38]</sup>, and is used to deal with IBS. It was designed to educate participants about physical, cognitive, and behavioral factors which contribute to IBS; thus teaching them methods of enhancing self-control over stress, anxiety, and IBS symptoms; to correct dysfunctional thoughts and to prevent symptom relapse<sup>[39]</sup>. This is helpful for refractory IBS, as it blocks the vicious circle between psychological factors and symptoms. Thus, CBT that targets psychological disturbance may alleviate IBS symptoms<sup>[40]</sup>.

## COMPONENTS OF CBT FOR IBS

CBT is an extremely broad concept and the psychotherapy methods described in the literature have differed in their composition. However, each of the following components are generally included.

### Education about IBS

IBS is presented as a functional bowel disorder, which is more ordinary than it appears, associated with bowel function, and as a distinct disorder with real physical symptoms, including abdominal pain, distress, anxiety, disruption to lifestyle, and embarrassment. Information is provided about intestinal function in general, such as the range of normal bowel frequency, the negative effects of straining to pass a motion or ignoring the urge, ways of dealing with constipation and diarrhea, pathogenesis, and treatment and clinical efficacy of IBS. IBS is considered a biopsychological disorder in which an association between life stress described as a normal part of life and an interaction between individuals and their environment,

and physiological changes leading to bowel irregularity is present. The impact of life stress on the gastrointestinal tract is characterized, with reference to the roles of central and autonomic nervous systems and the idea of “fight or flight” responses, including bowel muscle spasm<sup>[24]</sup>. The effect of psychological factors is discussed, which clarifies that pain signals from the site of physiological disturbance or damage passing through a special mechanism to the brain, which then interprets them by combining information from various stems. Abdominal pain is experienced, and this experience is influenced by current physiological arousal, focus of attention, mood, and beliefs about abdominal pain. For example, a patient who believes that eating food in a public place will always produce diarrhea symptoms might lead to an avoidance of social interactions, as well as anxiety when dining in a restaurant. The anxiety caused by this maladaptive thought may trigger diarrhea. The therapist aims to help the patient to recognize that a maladaptive idea adversely affects normal life functioning and symptom experience.

#### Good maintenance of a physician-patient relationship

Effectiveness of the therapy depends on maintaining a good relation between patients and medical personnel, forming a good working relationship. Experienced physicians know that maintaining a positive therapeutic physician-patient relationship for patients with IBS is of great importance; patients who experience this positive interaction with their physician have fewer IBS-related follow-up visits than patients who do not have this interaction<sup>[41]</sup>. Patients are encouraged to speak out about their own doubts and fears, and communicate with physicians; according to the patients’ problems, physicians should be able to give a detailed answer in simple terms. In fact, most patients are conscious of the origin which has caused the symptoms of IBS, but the lack of proper cognitive meaning with symptoms is common. Patients are often organized to participate in discussions, and good experiences can be shared and improves their confidence in beating IBS. During the period, physicians and nurses can detect the patient’s cognitive errors, correct them in an appropriate manner, and ensure smooth treatment progression.

#### Stress management

It is necessary for patients with IBS to understand that it is normal that the stress response appears when people meet stress. Identifying sources of stress for the individual concerned, working with them, and developing more helpful strategies for coping with them are prerequisite. Behavioral strategies made to ease the psychological pressure caused by cognitive behavioral efforts made in the face of stress. Positive behavior can mitigate stress and be beneficial to health, while a negative response will have the opposite effect.

#### Planning activities and training

An increased level of planning activity, including where

**Table 1 Randomized controlled trials reviewed by Khan *et al*<sup>[17]</sup>**

Ref.	Country	Sample size	Psychological therapy used
Lackner <i>et al</i> <sup>[35]</sup>	United States	75	CBT
Lackner <i>et al</i> <sup>[42]</sup>	United States	71	CBT
Reme <i>et al</i> <sup>[43]</sup>	United States	149	CBT

CBT: Cognitive-behavioral therapy.

**Table 2 Randomized controlled trials reviewed by Ford *et al*<sup>[21]</sup> (not including the trials described in Table 1)**

Ref.	Country	Sample size	Psychological therapy used
Greene <i>et al</i> <sup>[45]</sup>	United States	20	CBT
Payne <i>et al</i> <sup>[46]</sup>	United States	22	CBT
Vollmer <i>et al</i> <sup>[48]</sup>	United States	34	CBT
Boyce <i>et al</i> <sup>[50]</sup>	Australia	105	CBT
Drossman <i>et al</i> <sup>[44]</sup>	United States	169	CBT
Tkachuk <i>et al</i> <sup>[47]</sup>	Canada	28	CBT
Kennedy <i>et al</i> <sup>[49]</sup>	England	149	CBT

CBT: Cognitive-behavioral therapy.

and when certain foods should be eaten, also lifts mood and provides more distraction from the symptoms of IBS. Self-discipline training is an effective integrated relaxation technique, as there are some physiological changes in training in accordance with wishes.

## EVIDENCE FOR TREATMENT EFFICACY OF CBT

Khan *et al*<sup>[17]</sup> provide a useful review of the literature. Of the three controlled studies of patients with severe IBS, they noted that those in the CBT group showed reduced gastrointestinal symptoms and psychological distress to a greater extent than those in the control group. More details are given in Table 1.

A systematic review and meta-analysis carried out by Ford *et al*<sup>[21]</sup> was not included in this review. There were seven studies which compared CBT with control therapy or physicians’ “usual management” in 491 patients<sup>[44-50]</sup>. IBS symptoms persisted in 118 of 279 individuals assigned to CBT, compared to 130 of 212 allocated to control therapy or physicians’ “usual management”. There was statistically significant heterogeneity and evidence among those studies, with small-sample studies showing no effect of CBT on IBS symptoms. When three studies conducted in the same center were excluded from the meta-analysis, the beneficial effect of CBT on IBS symptoms disappeared. More details are given in Table 2. Finally, they demonstrated that a range of different psychological therapies could significantly improve physical symptoms in IBS patients, with studies on CBT providing the greatest evidence.

For IBS, CBT has been studied more than any other form of psychological intervention in randomized controlled trials. In a recent review by Palsson *et al*<sup>[51]</sup>, CBT outcomes for IBS treatment were compared with control

**Table 3** Randomized controlled trials reviewed by Pålsson *et al.*<sup>[51]</sup> (not including the trials described in Tables 1 and 2)

Ref.	Country	Sample size	Psychological therapy used
Lynch <i>et al.</i> <sup>[52]</sup>	Canada	21	CBT
Heymann-Monnikes <i>et al.</i> <sup>[53]</sup>	Germany	20	CBT
Sanders <i>et al.</i> <sup>[54]</sup>	United States	28	CBT
Hunt <i>et al.</i> <sup>[55]</sup>	United States	54	CBT
Moss-Morris <i>et al.</i> <sup>[56]</sup>	England	64	CBT
Craske <i>et al.</i> <sup>[40]</sup>	United States	110	CBT
Ljotsson <i>et al.</i> <sup>[57]</sup>	Sweden	195	CBT
Ljotsson <i>et al.</i> <sup>[58]</sup>	Sweden	61	CBT
Oerlemans <i>et al.</i> <sup>[59]</sup>	The Netherlands	75	CBT

CBT: Cognitive-behavioral therapy.

groups receiving usual medical care or on waiting lists for treatment, antidepressant or antispasmodic medication, placebo, or active psychological interventions such as supportive therapy, education, or stress management treatment. The substantial body of those studies demonstrates that CBT is an effective therapy for improving IBS. In the positive trials, gastrointestinal symptoms were almost uniformly found to be significantly ameliorated after treatment, sometimes substantially more than in control groups. Michelle *et al.*<sup>[40]</sup> examined the efficacy of a CBT protocol for the treatment of IBS, which directly targeted visceral sensations. Participants ( $n = 110$ ) were randomized to receive 10 sessions of either: (1) CBT with interoceptive exposure to visceral sensations (IE); (2) stress management (SM); or (3) an attention control (AC), and were evaluated at baseline, mid-treatment, post-treatment, and follow-up sessions. The results showed that the IE group outperformed AC on several indices of outcome, and outperformed SM in some domains. There was no difference observed between SM and AC. The results suggested that IE might be a particularly efficacious treatment for IBS. In spite of the fact that the majority of studies did not include any follow-up longer than 3 mo after medical treatment, there is some evidence that the therapeutic benefit of CBT for IBS can last 8 mo to 2 years after treatment termination. Apart from gastrointestinal symptom improvement, quality of life and emotional well-being are often documented to improve significantly from such therapy as well. More details are given in Table 3.

## POTENTIAL PROBLEMS

Although CBT is considered the most well-studied psychological treatment for IBS<sup>[10]</sup>, it is rarely available in routine care of IBS<sup>[60]</sup>, and delivering the treatment may be cumbersome<sup>[12]</sup>. There is no evidence that patients' attributions for their illness and expectations/preferences for intervention influence the efficacy in any treatment. It is suggested that some patients do not understand the cognitive behavior model as applying to them and are thus unlikely to engage in CBT<sup>[61]</sup>. As this is a therapy which makes significant demands on patients' time, some

will not feel able to make this commitment. Several problematic factors are a lack of trained therapists, high costs of delivering the treatment, and the practical difficulties for patients of scheduling weekly visits at a clinic<sup>[62,63]</sup>. Some modifications to the traditional CBT format have been evaluated by researchers, and these studies have demonstrated that CBT-based interventions can be delivered in different, and more cost-effective, formats<sup>[63,64]</sup>. Some clinicians have conducted studies investigating CBT for IBS where participants had a therapist contact *via* the internet (ICBT), defined as a web-based bibliotherapy with an online therapist contact. ICBT proved to be a promising cost-effective treatment modality for IBS, as it can be offered to IBS patients on a much larger scale than conventional psychological treatments<sup>[58,65]</sup>. Among gastroenterologists, development and testing of a CBT program for IBS has the potential to make it more widely available for IBS.

## CONCLUSION

IBS is a prevalent chronic relapsing condition that is regularly associated with significant disability and has a considerable financial burden for the health service due to the consumption of resources including physician time, investigations, and costs of treatment<sup>[66]</sup>. The presence of clinically significant psychiatric symptoms in patients with IBS is an indication for psychotherapy, especially CBT. Although the availability of therapists who are trained in CBT and have specialist experience in IBS is limited, even when specialist referral is not an option, CBT has implications for gastroenterologists' own clinical practice. There is increasing evidence for the efficacy of CBT in alleviating the physical and psychological symptoms of IBS, and it has been recommended that it should be considered as a treatment option for the syndrome<sup>[28]</sup>. CBT is most appropriately offered to patients who have already had reasonable medical investigations but still have significant physical discomfort and psychological distress, and are interested in taking an active part in achieving greater control over their symptoms.

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## Prognosis of patients with gastric cancer and solitary lymph node metastasis

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### Abstract

**AIM:** To investigate the relationship of solitary lymph node metastasis (SLNM) and age with patient survival in gastric cancer (GC).

**METHODS:** The medical records databases of China's Beijing Cancer Hospital at the Peking University School of Oncology and Shanghai Tenth People's Hospital affiliated to Tongji University were searched retrospectively to identify patients with histologically proven GC and SLNM who underwent surgical resection between October 2003 and December 2012. Patients with distant metastasis or gastric stump carcinoma following resection for benign disease were excluded from the

analysis. In total, 936 patients with GC + SLNM were selected for analysis and the recorded parameters of clinicopathological disease and follow-up (range: 13-2925 d) were collected. The Kaplan-Meier method was used to stratify patients by age ( $\leq 50$  years-old,  $n = 198$ ; 50-64 years-old,  $n = 321$ ;  $\geq 65$  years-old,  $n = 446$ ) and by metastatic lymph node ratio [MLR  $< 0.04$  (1/25),  $n = 180$ ; 0.04-0.06 (1/25-1/15),  $n = 687$ ;  $\geq 0.06$  (1/15),  $n = 98$ ] for 5-year survival analysis. The significance of intergroup differences between the survival curves was assessed by a log-rank test.

**RESULTS:** The 5-year survival rate of the entire GC + SLNM patient population was 49.9%. Stratification analysis showed significant differences in survival time (post-operative days) according to age:  $\leq 50$  years-old:  $950.7 \pm 79.0$  vs 50-64 years-old:  $1697.8 \pm 65.9$  vs  $\geq 65$  years-old:  $1996.2 \pm 57.6$ , all  $P < 0.05$ . In addition, younger age ( $\leq 50$  years-old) correlated significantly with mean survival time ( $r = 0.367$ ,  $P < 0.001$ ). Stratification analysis also indicated an inverse relationship between increasing MLR and shorter survival time:  $< 0.04$ : 52.8% and 0.04-0.06: 51.1% vs  $\geq 0.06$ : 40.5%,  $P < 0.05$ . The patients with the shortest survival times and rates were younger and had a high MLR ( $\geq 0.06$ ):  $\leq 50$  years-old:  $496.4 \pm 133.0$  and 0.0% vs 50-65 years-old:  $1180.9 \pm 201.8$  and 21.4% vs  $\geq 65$  years-old:  $1538.4 \pm 72.4$  and 37.3%, all  $P < 0.05$ . The same significant trend in shorter survival times and rates for younger patients was seen with the mid-range MLR group (0.04-0.06), but the difference between the two older groups was not significant. No significant differences were found between the age groups of patients with MLR  $< 0.04$ . Assessment of clinicopathological parameters identified age group, Borrmann type, histological type and tumor depth as the most important predictors of the survival rates and times observed for this study population.

**CONCLUSION:** GC patients below 51 years of age with MLR of SLNM above 0.06 have shorter life expect-



tancy than their older counterparts.

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**Key words:** Gastric cancer; Solitary lymph node metastasis; Age; Metastatic lymph node ratio; Survival

**Core tip:** Among patients with gastric cancer and single lymph node metastasis, younger patients ( $\leq 50$  years-old) tend to have less and shorter survival than their older counterparts; in particular, younger patients with the highest metastatic lymph node ratio have the worse prognosis.

Chen CQ, Wu XJ, Yu Z, Bu ZD, Zuo KQ, Li ZY, Ji JF. Prognosis of patients with gastric cancer and solitary lymph node metastasis. *World J Gastroenterol* 2013; 19(46): 8611-8618 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8611.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8611>

## INTRODUCTION

Gastric carcinoma (GC) is one of the most commonly diagnosed cancers in China. The elderly (65 years and older) represent over one-half of these cases, and occurrence of GC in individuals younger than 40-years-old is relatively rare (approximately 5% of total reported cases)<sup>[1,2]</sup>. Studies of age-related GC progression and prognosis have yielded inconsistent findings. The collective data in the literature indicate both distinctly unfavorable outcomes for the younger patient population (citing more advanced disease at diagnosis and/or faster disease progression) and a seemingly paradoxical favorable overall survival (with early disease stage cases possibly providing a confounding subgroup effect); in addition, other studies have demonstrated a lack of age-related impact on GC prognosis<sup>[3-8]</sup>.

Regardless of patient age, lymph node metastasis is a well-established critical prognostic factor and predictor of recurrence in GC. Histological detection of metastatic lymph nodes (MLNs) is strongly correlated with a high risk of recurrence, and this is an especially critical clinical finding for patients diagnosed in the early stages of GC to help design effectively robust, but safe, clinical management strategies<sup>[9-12]</sup>. While calculation of the metastatic lymph node ratio (MLR; single positive lymph nodes per total number of lymph nodes harvested) improves the sensitivity of predicting GC recurrence, minimizing the number of lymph node dissection is necessary to reduce the corresponding side effects, such as lymphedema<sup>[13-15]</sup>.

A major clinical challenge in GC evaluation is determining the appropriate extent of lymph node dissection that is capable of detecting single lymph node metastasis (SLNM). The gastric lymphatic drainage system is particularly complex, and not all cases of SLNM are localized to the perigastric node area and are detectable by

standard D2 lymphadenectomy. MLR, however, can help to overcome this limitation.

In this study, the differential prognostic features of younger and older GC patients with SLNM were investigated using MLR to gain further insights into the particular clinicopathological features and surgical outcomes of these two patient populations.

## MATERIALS AND METHODS

### Patients

The clinical records databases of two large metropolitan hospitals - Beijing Cancer Hospital of the Peking University School of Oncology, and the Shanghai Tenth People's Hospital Affiliated to Tongji University - were searched retrospectively to identify patients with histologically proven GC and SLNM who underwent resection surgery between October 2003 and December 2012. Patient records were selected for study inclusion according to the following eligibility criteria: available histological and pathological data related to diagnosis, including total number of resected lymph nodes. Of the 965 patients identified, 29 were excluded from analysis according to the presence of distant metastasis or development of gastric stump tumors following resection for benign disease.

In total, 936 patients with GC + SLNM were selected for analysis and the recorded parameters of clinicopathological disease and follow-up were collected. The patients were stratified by age ( $\leq 50$ , 50-64, and  $\geq 65$  years-old) and by MLR [ $< 0.04$  (1/25) with  $> 25$  lymph nodes sampled, 0.04-0.06 (1/25-1/15) with 15-25 lymph nodes sampled, and  $\geq 0.06$  (1/15) with  $\geq 15$  lymph nodes sampled].

### Statistical analysis

All statistical analyses were carried out by the SPSS statistical software package, version 13.0 (SPSS Inc., Chicago, IL, United States). The Kaplan-Meier method, determined the overall and subgroups' 5-year survival rates, with the "event" endpoint being defined as death by any cause. The significance of differences between the various survival curves was assessed by a log-rank test. The chi-square test was used to evaluate differences between the clinicopathological disease and follow-up categorical variables. A *P*-value of  $< 0.05$  was set as the threshold for statistical significance.

## RESULTS

### Characteristics of GC + SLNM patients

The study population's demographic and clinicopathological characteristics related to diagnosis and treatment, and stratified by age, are presented in Table 1. The median age was 68.6 years old (range: 26-86 years), with a relatively similar representation among the three age groups (20.5%,  $\leq 50$  years-old; 33.3%, 50-64 years-old; 46.2%,  $\geq 65$  years-old) but with a remarkably high proportion



**Table 1** Characteristics of gastric cancer + solitary lymph node metastasis during clinical management, stratified by age *n* (%)

Parameter	Age (yr)			All patients ( <i>n</i> = 965)
	≤ 50 ( <i>n</i> = 198)	50-64 ( <i>n</i> = 321)	≥ 65 ( <i>n</i> = 446)	
Sex				
Male	117 (59.1)	207 (64.5)	272 (61.0)	596 (61.8)
Female	81 (40.9)	114 (35.5)	174 (39.0)	369 (38.2)
Tumor location				
Upper stomach	15 (7.6)	28 (8.7)	40 (9.0)	83 (8.6)
Middle stomach	79 (39.9)	131 (40.8)	183 (41.0)	393 (40.7)
Lower stomach	104 (52.5)	162 (50.5)	223 (50.0)	489 (50.7)
Gross type (Borrmann)				
I	5 (2.5)	10 (3.1)	19 (4.3)	34 (3.5)
II	51 (25.8)	59 (18.4)	103 (23.1)	213 (22.1)
III	78 (39.4)	182 (56.7)	205 (45.9)	465 (48.2)
IV	64 (32.3)	70 (21.8)	119 (26.7)	253 (26.2)
Histological type				
High differentiation	1 (0.5)	2 (0.6)	4 (0.9)	7 (0.7)
Moderate differentiation	27 (13.6)	79 (24.6)	119 (26.7)	225 (23.3)
Low differentiation	170 (85.9)	240 (74.8)	323 (72.4)	733 (76.0)
Tumor status				
T1	16 (8.1)	29 (9.1)	42 (9.4)	87 (9.0)
T2	36 (18.2)	53 (16.5)	87 (19.5)	176 (18.2)
T3	75 (37.9)	125 (38.9)	194 (43.5)	394 (40.8)
T4	71 (35.8)	114 (35.5)	123 (27.6)	308 (32.0)
Metastasis lymph node ratio				
≥ 0.06	12 (6.1)	32 (10.0)	54 (12.1)	98 (10.2)
0.04-0.06	155 (78.3)	230 (71.7)	302 (67.7)	687 (71.2)
< 0.04	31 (15.6)	59 (18.3)	90 (20.2)	180 (18.6)
Postoperative chemotherapy				
No	4 (2.0)	11 (3.4)	29 (6.5)	43 (4.5)
Yes	194 (98.0)	310 (96.6)	417 (93.5)	922 (95.5)
Surgery				
Subtotal gastric resection	113 (57.1)	181 (56.4)	238 (53.4)	532 (55.1)
Total gastrectomy	85 (42.9)	140 (43.6)	208 (46.6)	433 (44.9)

in the mid MLR (0.04-0.06) group (71.2%). The median number of dissected lymph nodes was 24.3 (range: 5-71) and almost all patients received postoperative chemotherapy (with similar representation among the three age groups). The three age groups also showed statistically similar ( $P > 0.05$ ) patient distribution for sex and tumor location, gross (Borrmann) type, histological (differentiation) type and status.

#### GC + SLNM patient outcome and predictors of survival

The study population's demographic and clinicopathological characteristics related to follow-up, stratified by age, are presented in Table 2. Twenty-nine of the patients were lost to follow-up and were excluded from further analysis. For the remaining overall study population, the median follow-up was 957 d (range: 13-2925 d) and the 5-year survival rate was 49.9%. Comparative analysis of the survival curves indicated significant differences among groups according to age (all three categories), Borrmann type (I *vs* II *vs* III *vs* IV), histological type (high *vs* moderate *vs* low), and tumor depth (T1 *vs* T2 *vs* T3 *vs* T4) (all  $P < 0.0001$ ); thus, all four of these variables were characterized as important predictors of survival.

#### Correlation of age with survival of GC + SLNM patients

Comparative analysis of the cumulative survival rates

between the age categories ( $\leq 50$  years-old:  $950.7 \pm 79.0$ , 50-64 years-old:  $1697.8 \pm 65.9$ , and  $\geq 65$  years-old:  $1996.2 \pm 57.6$ ) showed statistically significant differences among all three ( $\leq 50$  *vs* 50-64,  $P < 0.001$ ;  $\leq 50$  *vs*  $\geq 65$   $P < 0.001$ ; 50-64 *vs*  $\geq 65$ ,  $P = 0.020$ ) (Figure 1). The group of patients  $\geq 65$  years old had the best survival, and younger age ( $\leq 50$  years-old) was found to correlate significantly with mean survival time ( $r = 0.367$ ,  $P < 0.001$ ) (Figure 2).

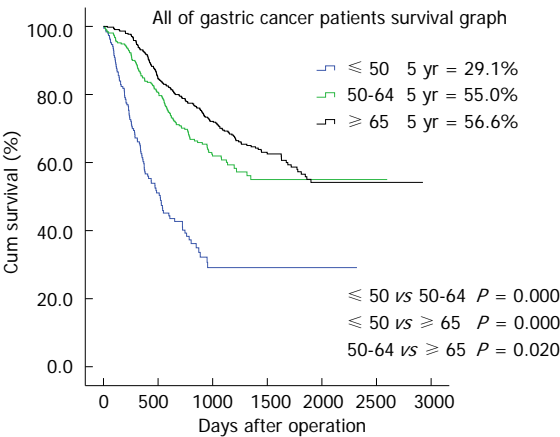
#### Correlation of MLR with survival of GC + SLNM patients

Comparative analysis of the cumulative survival rates between the MLR categories ( $< 0.04$ :  $1527.0 \pm 67.6$ , 0.04-0.06:  $1851.1 \pm 1527.0$ , and  $\geq 0.06$ :  $1352.1 \pm 111.8$ ) indicated that high MLR was associated with shorter survival (0.04-0.06 *vs*  $\geq 0.06$ ,  $P = 0.030$ ;  $< 0.04$  *vs*  $\geq 0.06$   $P = 0.028$ ). Comparison of the lower MLR categories showed no significant difference between the two ( $< 0.04$  *vs* 0.04-0.06,  $P = 0.681$ ). The high MRL group also showed a significantly lower 5-year survival rate than the other two groups ( $< 0.04$ : 52.8%, 0.04-0.06: 51.1%, and  $\geq 0.06$ : 40.5%) (Figure 3).

Age-based stratification analysis of the MLR categories indicated that the younger patients with higher MLR had the shortest survival rate (Figure 4). In particular, the cumulative survival curves for patients with MLR of  $\geq$

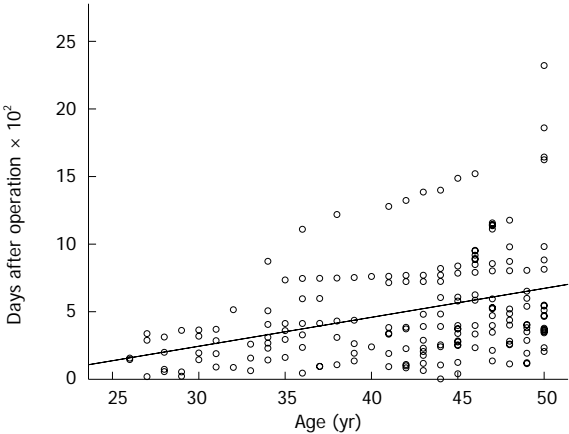
**Table 2** Characteristics of gastric cancer + solitary lymph node metastasis during follow-up, stratified by age

Parameter	All patients ( <i>n</i> = 936)	5-yr survival	<i>P</i> value
Age (yr)			< 0.0001
≤ 50	188	29.10%	
50-64	311	55.00%	
≥ 65	437	56.60%	
Borrmann type			< 0.0001
I	33	92.40%	
II	208	90.00%	
III	455	40.40%	
IV	240	19.20%	
Histological type			< 0.0001
High differentiation	7	100.00%	
Moderate differentiation	215	74.60%	
Low differentiation	714	42.00%	
Tumor status			< 0.0001
T1	84	94.80%	
T2	170	82.10%	
T3	382	40.20%	
T4	300	30.40%	

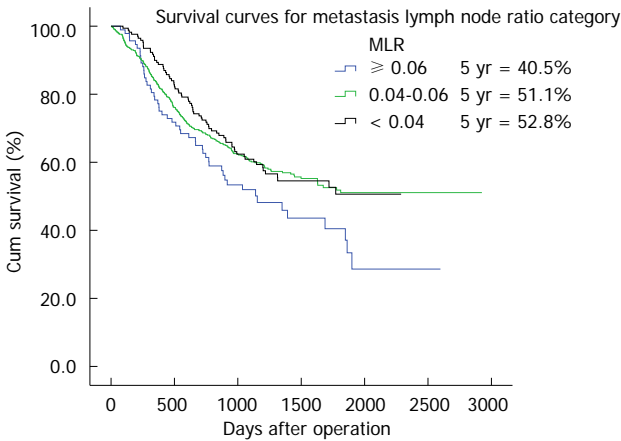


**Figure 1** Cumulative survival of patients with gastric cancer + solitary lymph node metastasis according to age category.

0.06 ( $\leq 50$ :  $496.4 \pm 133.0$ , 50-65:  $1180.9 \pm 201.8$ , and  $\geq 65$ :  $1538.4 \pm 72.4$ ) were significantly different among the three age categories ( $\leq 50$  vs 50-65,  $P = 0.03$ ;  $\leq 50$  vs  $\geq 65$ ,  $P = 0.000$ ; 50-65 vs  $\geq 65$ ,  $P = 0.005$ ). The 5-year survival rates followed a similar trend:  $\leq 50$ , 0.0%; 50-65, 21.4%;  $\geq 65$ , 37.3% (Figure 4A). The cumulative survival curves for patients with MLR of 0.04-0.06 ( $\leq 50$ :  $847.3 \pm 85.1$ , 50-65:  $1410.1 \pm 53.4$ , and  $\geq 65$ :  $2140.7 \pm 68.1$ ) were also significantly different from the lowest age category ( $\leq 50$  vs 50-65,  $P = 0.000$ ;  $\leq 50$  vs  $\geq 65$ ,  $P = 0.000$ ); however, no difference was observed between the two older groups. The 5-year survival rates followed a similar trend:  $\leq 50$ , 23.9%; 50-65: 60.0%;  $\geq 65$ : 66.0% (Figure 4B). The cumulative survival curves of patients with MLR of  $< 0.04$  showed no significant differences among the age categories, and the 5-year survival rates were also similar ( $\leq 50$ : 50.8%, 50-65: 56.3%, and  $\geq 65$ : 46.0%) (Figure 4C).



**Figure 2** Correlation between age ( $\leq 50$  years-old) and mean survival days after surgery in patients with gastric cancer + solitary lymph node metastasis ( $r = 0.367$ ;  $P < 0.001$ ).

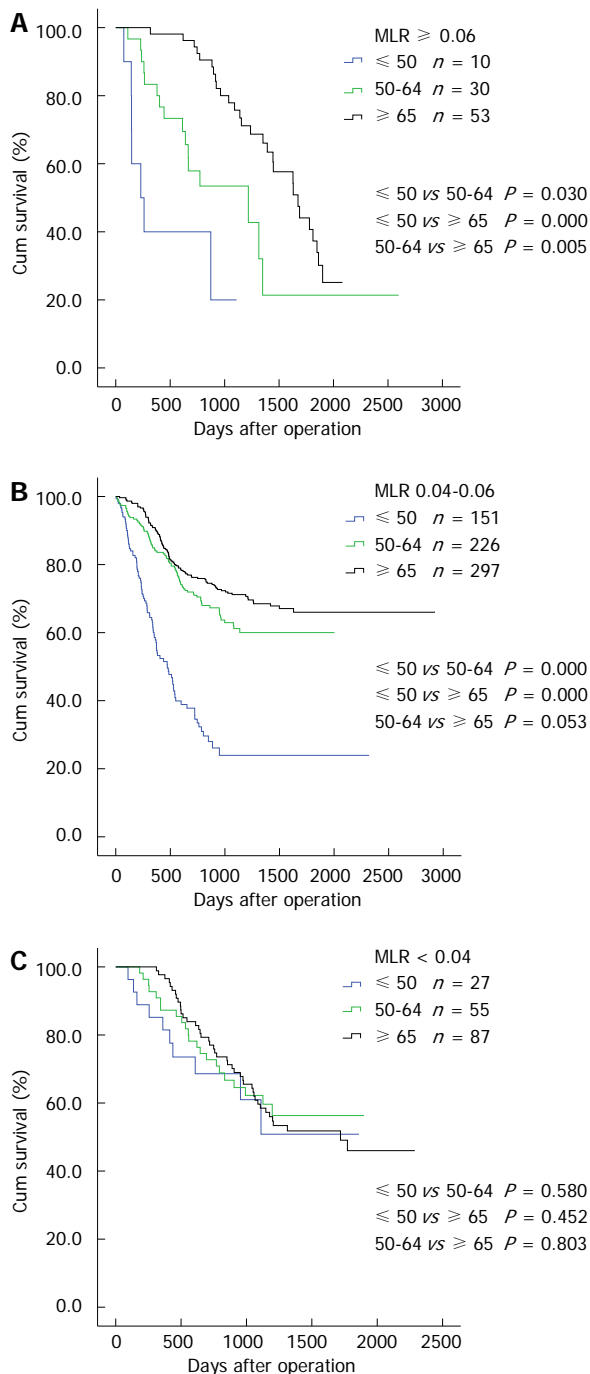


**Figure 3** Survival curves for patients with gastric cancer + solitary lymph node metastasis according to metastatic lymph node ratio category. Survival is shown to be inversely associated with the ratio of positive nodes to lymph nodes harvested during surgery.

## DISCUSSION

Some studies have indicated that a younger age of diagnosis may correspond to worse prognosis of GC<sup>[16]</sup>. From a histological perspective, GC in younger patients is more likely to be diffuse type, rather intestinal or mixed, and with disseminated morphology<sup>[17-19]</sup>. These features may underlie a more aggressive behavior of GC in this patient population or merely reflect a trend in diagnosis being made at a later disease state<sup>[4]</sup>; nevertheless, both of these issues are associated with poorer prognosis and may help to explain a key pathological difference between younger and older GC patients.

As cited in the Introduction, the collective research to date has yet to define the precise age-related epidemiological and clinicopathological characteristics of GC. For example, in a study of old and young GC patients matched by tumor stage, Moreira *et al*<sup>[20]</sup> found that younger age was associated with a more favorable out-



**Figure 4** Cumulative survival of patients with gastric cancer + solitary lymph node metastasis and different metastatic lymph node ratio according to age category. A:  $P < 0.05$  for comparisons among all three groups; B: Younger patients have significantly worse survival than the two older patient groups ( $P < 0.01$ ); C: There are no significant differences among the three groups. Cumulative survival of patients with metastatic lymph node ratio (MLR)  $\geq 0.06$  (A), 0.04-0.06 (B), and  $< 0.04$  (C) by age.

come. Similarly, in a study of elderly and middle-aged GC patients matched for tumor extension, Kitamura *et al.*<sup>[21]</sup> found that older age was associated with poorer overall survival and death within 30 d after surgery. Indeed, the increased risk of complications and death from surgery in general is well recognized, and a study of surgically-treated GC patients showed markedly better 5-year post-

operative survival at all tumor stages<sup>[22]</sup>.

It has been reported that younger (middle-aged) patients have better prognosis following curative resection of stage I tumors than their elderly counterparts<sup>[6]</sup>. Other reports, however, have demonstrated that younger age provides no benefit to survival when the GC is present in more advanced stages and that the most important prognostic factor in young patients is advanced nodal involvement<sup>[23-25]</sup>. Regardless of whether or not there is a distinctive malignant trait related to age, a key means towards improving survival rates is early diagnosis and timely application of curative resection.

The results from the current study confirmed the view that the relationship between GC prognosis and patient age is complicated. In general, the mean age of GC diagnosis falls within the 5<sup>th</sup> decade of life and cases younger than 50 years old are relatively rare<sup>[26,27]</sup>; however, by searching a large patient database we were able to analyze a patient population that equally represented young and old GC sufferers. The current study population showed a poorer prognosis for younger ( $\leq 50$  years old) patients, as evidenced by both cumulative and 5-year survival rates. Moreover, the younger patients had fewer surgery-related complications (data not shown) that may have benefited their recovery and prognosis.

Another important finding for the current study's population of patients with GC + SLNM was the relationship between age and mean survival days after surgery. The younger patients, who also had more aggressive tumors by histological analysis, survived for a significantly shorter duration following the resection treatment. This finding agrees with another study of GC patients that found diffuse cancers more likely to occur in younger patients and to be associated with poorer prognosis.

Depth of invasion and presence of MLNs are well-established and essential prognostic factors of GC<sup>[28]</sup>, and nodal involvement is considered an especially significant clinical finding in early GC. Ten-year overall survival in node-positive patients has been reported at 27%, compared to the estimated 92% for node-negative patients<sup>[29]</sup>. Incomplete removal of MLNs, which harbor residual tumor cells, represents an increased risk of disease spread or recurrence. Indeed, studies of post-gastrectomy survival in GC patients have shown that survival rates improve in conjunction with number of lymph nodes removed for examination<sup>[30-32]</sup>.

The benefit of lymphadenectomy was related to extent; however, it remains to be precisely determined. Both the Union for International Cancer Control and the American Joint Commission for Cancer have recommended that at least 15 lymph nodes be examined for correct assessment node metastatic status in GC (7<sup>th</sup> edition TNM system). Moreover, dissection of  $\geq 15$  lymph nodes in resections with curative intent has been reported to significantly improve prognosis of patients with GC + MLN<sup>[33]</sup>. Yet, while the useful prognostic impact of this lower-limit criteria has been validated in several large clinical studies<sup>[34,35]</sup>, no study to date has systematically

evaluated the risk to benefit ratio of precise numbers of lymph nodes for GC or its myriad of histological parameters.

The association of SLNM with depth of tumor invasion and its prognostic significance in GC are well established. Furthermore, it is generally accepted that GC patients with SLNM have a worse survival rate than those without SLNM (zero positive lymph nodes). The estimates of GC cases with single nodal metastasis distributed beyond the perigastric area range from 12.6%-29.0%, and it is hypothesized that this feature may be related to (caused by) complicated lymphatic drainage from the stomach<sup>[36-38]</sup>. However, a comparative study of patients with and without skip metastasis after standard D2 lymphadenectomy found no significant difference in survival between the two groups<sup>[39]</sup>. Sentinel node mapping with a visible tracer or radio-guided approach has limited accuracy in GC patients. Therefore, the current study evaluated the age-related 5-year survival rates of follow-up GC + SLNM patients using an array of clinicopathological parameters, and found that Borrmann type, histological type, and tumor status were significantly different among the groups and were related to patient survival.

MLR calculation is considered a simpler and (possibly) more effective method for prognosing patients with GC who undergo curative or radical resections<sup>[40-42]</sup>, compared to the conventional lymph node staging systems. In particular, MLR could supplement the conventional N staging system when a limited number of lymph nodes are obtained, thus providing more accurate prognostic stratification in advanced GC<sup>[40,43-45]</sup>. Herein, as in some related previous studies, MLR was shown to be a better prognostic factor than the other clinicopathological parameters examined; however, no consensus has been made on the optimal categorization of MLR, as each study has used different standardization. In the current study, the GC + SLNM patients were categorized according to the number of harvested lymph nodes, and the data indicated that 5-year survival rates were associated with SLNM per lymph node harvested. Specifically, younger patients with  $MLR \geq 0.06$  and 0.06-0.04 had lower survival than older patients.

As discussed above, young adults may be more likely to present for diagnosis at an advanced disease stage. In the absence of an effective predictive marker, surveillance endoscopy of patients with positive family histories seems to be the only way to detect early stage GC. Such patients should also be educated on the signs and symptoms of GC, and more attention should be paid to younger patients with upper gastrointestinal symptoms, to improve their rate of early diagnosis. Multivariate analyses have indicated that younger patients undergoing curative resection have longer survival<sup>[46]</sup>. As D2 lymphadenectomy leads to the examination of more nodes, and improves prognostic accuracy in patients with or without MLNs<sup>[47]</sup>, wider use of D2 lymphadenectomy may be essential for patients with GC, especially those

of younger age.

In conclusion, among the GC + SLNM patients examined in this study, younger patients tended to have shorter survival than their older counterparts. In particular, younger patients with the highest MLR had the worst prognosis. Thus, the field should strive towards improving earlier detection rates for GC patients to help improve prognosis of these patients. For younger patients, who may be at greater risk of disease-related mortality but at less risk of surgery-related morbidities, D2 lymphadenectomy may be considered because it allows sampling of many more lymph nodes.

## COMMENTS

### Background

The current data on age-related differential prognoses for gastric cancer (GC) is inconsistent. This study was designed to investigate the potential age-related differences in survival of GC patients with solitary lymph node metastasis (SLNM) and to determine the clinical efficacy of metastatic lymph node ratio (MLR) as a more sensitive predictor of GC recurrence than traditional histopathological parameters.

### Research frontiers

Among the patients with GC + SLNM examined in this study, the younger patients ( $\leq 50$ -years-old) experienced greater mortality and shorter survival times than the older patients. In particular, the younger patients with the highest MLR had the worst prognosis.

### Innovations and breakthroughs

Overall and 5-year survival rates of patients with GC and SLNM are lower in patients  $\leq 50$ -years-old. Thus, efforts should be made to diagnose these cases earlier, and more sensitive intraoperative prognostic methods, such as calculating the MLR, should be applied. The younger age of these patients translates to their general better health and good candidacy for uncomplicated recovery from surgery, thus allowing for the realization of benefit from the more accurate surgical prognosis methods.

### Applications

Strategies to improve earlier detection of GC should be devised and implemented, especially targeting the younger patient population. To achieve more accurate prognosis in this patient population, methods that allow for sampling of more lymph nodes, such as D2 lymphadenectomy, should be applied.

### Terminology

Gastric carcinoma is usually detected at a later disease stage in younger individuals, and this fact may have caused a shorter survival time. It is generally accepted that GC patients with SLNM have a worse survival rate than those without SLNM. MLR is considered a simpler and more effective method for prognosing patients with GC who undergo curative or radical resections. D2 lymphadenectomy is considered when sampling many more lymph nodes.

### Peer review

The authors thoroughly explained the problem of prognostic value of examined lymph node count in GC cases with SLNM, with respect to patient age. They have described, in detail, their data based on clinicopathological parameters and sufficiently discussed the implications of their findings in relation to the collective body of literature on this topic. The study showed that there is an age-related component to cumulative and 5-year survival of these cases, with younger age ( $\leq 50$ -years-old) and higher MLR status being predictive of less and shorter survival.

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## P115 promotes growth of gastric cancer through interaction with macrophage migration inhibitory factor

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### Abstract

**AIM:** To investigate the role of P115 in the proliferation of gastric cancer cells and the mechanism involved.

**METHODS:** The RNA and protein level of P115 and macrophage migration inhibitory factor (MIF) in gastric cancer and normal gastric tissue/cells were measured and the effect of P115 on cell proliferation was assessed. The role of P115 in cell cycle checkpoints was investigated and the related proteins and signaling pathways, such as cyclin D1, Mcm2, p53, PCNA as well as the MAPK signaling pathway were determined. The interaction between P115 and MIF and the effect of P115 on MIF secretion were examined. The data were analyzed *via* one-way ANOVA comparisons between groups and  $P < 0.05$  was considered significant.

**RESULTS:** P115 and MIF were both specifically expressed in gastric cancer tissues compared with normal gastric mucosa (both  $P < 0.01$ ). The mRNA and protein levels of P115 and MIF in gastric cancer cell lines MKN-28 and BGC-823 were higher than in the human gastric epithelial cell line GES-1 (both  $P < 0.01$ ).

In MKN-28 and BGC-823 cell lines, P115 promoted cell proliferation and G<sub>0</sub>-G<sub>1</sub> to S phase transition. In addition, several cell cycle-related regulators, including cyclin D1, Mcm2, PCNA, pERK1/2 and p53 were up-regulated by P115. Furthermore, the interaction between P115 and MIF was confirmed by co-immunoprecipitation assay. ELISA showed that P115 stimulated the secretion of MIF into the culture supernatant ( $P < 0.01$ ) and the compensative expression of MIF in cells was observed by Western blotting.

**CONCLUSION:** P115 promotes proliferation of gastric cancer cells through an interaction with MIF.

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**Key words:** Gastric cancer; P115; Migration inhibitory factor; Proliferation; Protein interaction

**Core tip:** Gastric cancer is one of the most common cancers. P115 is a tether protein which plays a key role in cell proliferation through combination with binding partners, including migration inhibitory factor (MIF). The present study showed that P115 and MIF were specifically expressed in gastric cancer tissues and cells. P115 promoted cell proliferation and G<sub>0</sub>-G<sub>1</sub> to S phase transition. Cell cycle regulators, including cyclin D1, Mcm2, PCNA, pERK1/2 and p53 were up-regulated by P115. P115 interacted with MIF and stimulated the secretion of MIF into the culture supernatant. In summary, P115 promotes proliferation of gastric cancer cells through an interaction with MIF.

Li XJ, Luo Y, Yi YF. P115 promotes growth of gastric cancer through interaction with macrophage migration inhibitory factor. *World J Gastroenterol* 2013; 19(46): 8619-8629 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8619.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8619>

## INTRODUCTION

Gastric cancer is one of the most common cancers worldwide with a significant impact on human health<sup>[1]</sup>. Despite significant developments in the diagnosis and treatment of gastric cancer, the prognosis remains poor. Extensive surgery combined with chemotherapy is the most common therapy choice in the early stages of gastric cancer<sup>[2]</sup>, while additional treatment options, such as gene therapy are desperately needed. With significant advances in genomics and proteomics, the discovery of a novel oncogene for therapeutic intervention remains a future challenge.

Cancer growth is a highly complex process involving alterations in gene expression and the interaction of many proteins. Golgi-vesicular transport protein P115 is a tether protein that plays an important role in many signal pathways required for cell proliferation<sup>[3]</sup> and has been extensively studied<sup>[4-6]</sup>. Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be described and extensively studied<sup>[7]</sup>. More recently, MIF has been reported to be overexpressed in a number of cancers, including esophageal squamous cell carcinoma<sup>[8]</sup>, glioblastoma<sup>[9]</sup>, neuroblastoma<sup>[10]</sup>, colonic cancer<sup>[11]</sup> and colorectal cancer<sup>[12]</sup>. The ability of MIF to promote tumor progression has been demonstrated and MIF has been shown to be a potential target for anti-cancer therapy. Hudson *et al.*<sup>[13]</sup> and Jung *et al.*<sup>[14]</sup> reported that MIF antagonized the activity of p53, which led to cancer progression. It was shown that the binding partner of MIF was JAB1/CSN5<sup>[15]</sup> which is known to be involved in the differentiation and morphogenesis of cells<sup>[16]</sup>. Furthermore, it is well known that upon binding to one of its receptors-CD74, MIF can increase the phosphorylation of Akt, ERK, MAPK and Stat3 which are all necessary for tumor proliferation.

Recently, a yeast two-hybrid interaction was examined to identify the intracellular proteins which might bind to MIF and mediate its secretion, and it was shown that P115 was a binding partner of MIF<sup>[17]</sup>. Previous research in our laboratory also demonstrated the same result. The objective of the present study was to evaluate the expression, the function in cell proliferation and the biological mechanism of P115 in gastric cancer.

## MATERIALS AND METHODS

### Cell culture

Human gastric cancer cell lines BGC-823 and MKN-28 were obtained from the American Type Culture Collection (Manassas, VA, United States). The human gastric epithelial cell line GES-1 was obtained from the cell bank of the Fourth Military Medical University. The cells were cultured in RPMI 1640 medium (Gibco, Maryland, United States) supplied with 10% FBS (Gibco, Maryland, United States), 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in humidified 5% CO<sub>2</sub>.

### Immunohistochemistry

Thirty gastric cancer and 30 normal gastric mucosa speci-

mens were obtained from the Department of Pathology, the First Affiliated Hospital, Chongqing Medical University from September 2008 to November 2009. Normal gastric mucosa specimens were obtained from normal tissues adjacent to the cancer tissue, and were pathologically confirmed as non-cancerous. The procedure was approved by the Ethics Committee. Samples were incubated with anti-P115 and anti-MIF rabbit polyclonal antibody (Cell Signaling Technology, United States) at 4 °C overnight, and then incubated with biotinylated goat anti-rabbit antibody (Santa Cruz Biotechnology, TX, United States) at room temperature for 15 min. DAB substrate was then used in the chromogenic reaction.

### Construction of plasmids and transfection

The pCD-shRNA was reconstructed from pGPU6/GFP/Neo. Four shRNAs targeting P115 and shNC were designed as shown in Table 1. ShRNAs were ligated into the *Bam*H I and *Bbs* I-digested pGPU6/GFP/Neo vector. The P115 expressing plasmid, pEGFP-N1-P115, was obtained from Jikai Company (Shanghai, China). Cells were seeded in 6-well plates and were transfected with 2 µg plasmids after reaching 70%-80% confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, United States) following the manufacturer's instructions.

### Reverse transcription-polymerase chain reaction and quantitative real-time polymerase chain reaction

RT-PCR was carried out using the AccessQuick™ One-Step reverse transcription-polymerase chain reaction [RT-PCR kit (Promega Co., Madison, United States)] according to the manufacturer's protocol. The oligonucleotide primers used were as follows: P115 sense: 5'-AACCTGGTGGCTGAACGGCAAG-3', P115 antisense: 5'-AGAAGCTTCACACCAGGCCAGC-3'. MIF sense: 5'-CGGGTTCTCTCCGAGCTCACC3', MIF antisense: 5'-TGATGTAGACCCTGTCCGGGCTGA-3'. β-actin sense: 5'-GACCCAGATCATGTTTGTAGACC-3', β-actin antisense: 5'-GCCAGGATAGAGCCACCAAT-3'. Total RNA was reverse transcribed to synthesize cDNA at 45 °C for 45 min. PCR was performed in a single reaction volume of 25 µL. The schedule consisted of incubation for 5 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min, then incubation for 10 min at 72 °C. The PCR products were subjected to 1.5% agarose gel electrophoresis. Quantitative real-time RT-PCR was performed using specific sense and antisense primers in a 25 µL reaction volume containing 12.5 µL of Absolute™ QPCR SYBR Green mix (Invitrogen), 0.25 pmol of each primer, and 0.5 µg of mRNA. Oligonucleotide primers were as follows: P115 sense: 5'-GGAGGGGAACAGTGATGGAG-3', P115 antisense: 5'-CAAAGCTGCTGCAATAACCC-3'. β-actin sense: 5'-CGGGAAATCGTGCGTGAC-3', β-actin antisense: 5'-TGGAAGGTGGACAGCGAGG-3'. The number of amplification cycles was 35, and the reaction were performed for 3 min at 50 °C, 20 s at 95 °C, and 30 s at 60 °C, with an initial step at 95 °C for 3 min.



Table 1 Sequences of shRNA

shRNA name	Target site	Sequences
P115-shRNA1	1117 bp S	5'-CACCGCAGCTTTGTACTATCCTAATTTCAGAGA ATTAGGATAGTACAAAGCTGCTTTTTTG-3'
	A	5'-GATCCAAAAAAGCAGCTTTGTACTATCCTAAT TCTCTGAAATTAGGATAGTACAAAGCTGC-3'
P115-shRNA2	1318 bp S	5'-CACCGCGCTGTGCTGTTCTCTATTGTTCAAGAGA CAATAGAGAACAGCACAGCGCTTTTTTG-3'
	A	5'-GATCCAAAAAAGCGCTGTGCTGTTCTCTATTG TCTCTGAACAATAGAGAACAGCACAGCGC-3'
P115-shRNA3	1578 bp S	5'-CACCGCAACCTCCAGTTTCTTACTCAAGAGA GTAAAGAAACTGGAGGGTTGCTTTTTTG-3'
	A	5'-GATCCAAAAAAGCAACCTCCAGTTTCTTTAC TCTCTGAAGTAAAGAACTGGAGGGTTC-3'
P115-shRNA4	1777 bp S	5'-CACCGCAGTTGGTCCAAGGCTTATGTTCAAGAGACATAAGCCTTGGACCAACTGCTTTTTTG-3'
	A	5'-GATCCAAAAAAGCAGTTGGTCCAAGGCTTATG TCTCTGAACATAAGCCTTGGACCAACTGC-3'
NC-shRNA	- S	5'-CACCGTTCTCCGAACGTGTACGTTTCAAGAGA ACGTGACACGTTCCGAGAACTTTTTTG-3'
	A	5'-GATCCAAAAAAGTCTCCGAACGTGTACACGT TCTCTGAAACGTGACACGTTCCGAGAAC-3'

### Western blotting analysis

Cells were lysed in 100  $\mu$ L RIPA lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 1% NP-40, 150 mmol/L NaCl, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 1 mmol/L NaF) at 4 °C for 30 min. Cell debris was removed by centrifugation at  $12000 \times g$  for 20 min at 4 °C. Protein concentrations were determined by the Bradford assay. An equal amount of lysate (40  $\mu$ g) was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Bedford, United States). The membranes were blocked with 5% nonfat milk at room temperature for 1 h and then incubated for 2 h with primary antibodies. The membranes were then incubated for 1 h with an appropriate horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, TX, United States). Antibodies to P115, MIF, cyclin D1, Mcm2, PCNA, p53 and  $\beta$ -actin were obtained from Cell Signaling Technology (MA, United States). Electrochemiluminescence was performed according to the manufacturer's instructions using a Bio-Rad imaging system. Quantity One software was used to quantify the density of bands.

### Cell proliferation assay

Cells were seeded in 96-well plates at a density of 2000 cells/well and allowed to proliferate for 24 h, 48 h and 72 h. Cell proliferation ability was assessed by MTT assay. Briefly, MTT (5 mg/mL) was added to each well and the plate was incubated for a further 4 h before removal of the media. DMSO was then added to each well to solubilize the formazan crystals. The absorbance was read at a wavelength of 595 nm using a microtiter plate reader. All experiments were carried out in triplicate.

### Flow cytometric analysis of cell cycle distribution

Flow cytometric analysis was performed as previously described<sup>[18]</sup>. Forty-eight hours after transfection, cells

were harvested and fixed with 75% ethanol at -20 °C overnight. Cells were stained with propidium iodide (25 mg/mL) and RNaseA (200 mg/mL) at 37 °C for 30 min. The DNA content was analyzed using a FACScan flow cytometer (Beckman Coulter, Germany).

### Co-immunoprecipitation of P115 and MIF

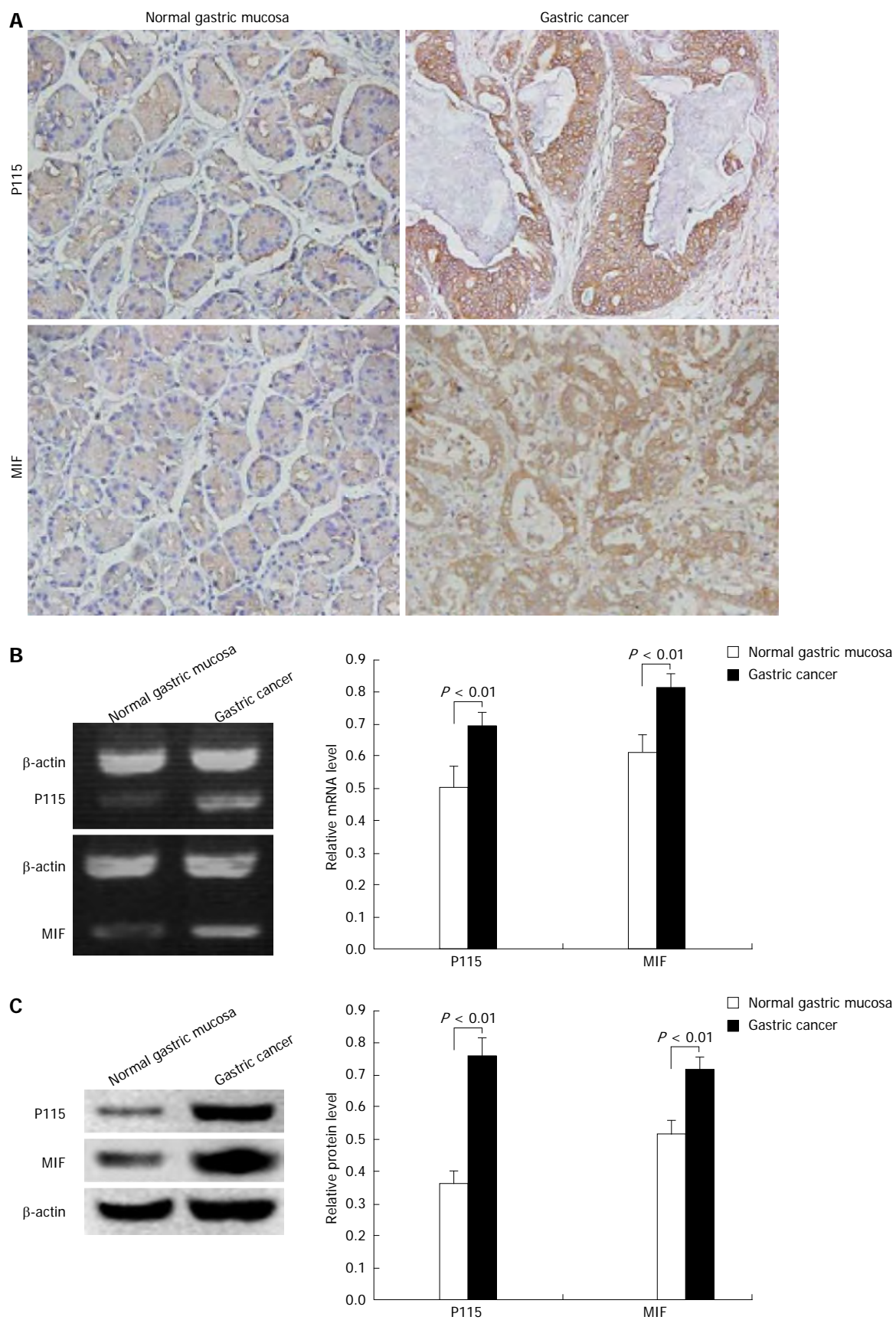
Cells were lysed in lysis buffer at 4 °C for 30 min. Cell debris was removed by centrifugation at  $14000 \times g$  for 5 min at 4 °C. To remove non-specific binding, protein G sepharose beads containing mouse IgG were added to 200  $\mu$ L protein and shaken slowly for 2 h at 4 °C. The sample was then centrifuged at  $2500 \times g$  for 5 min at 4 °C and the supernatant was carefully removed for immunoprecipitation. 1  $\mu$ g MIF antibody was incubated with the supernatant overnight and 42  $\mu$ L protein G Sepharose beads were then added. The mixture was incubated for 3 h at 4 °C on a tube roller to precipitate protein complexes. The beads were obtained by centrifugation at  $1000 \times g$  for 60 s and washed twice with PBS. Finally, 20  $\mu$ L loading buffer was added for SDS-polyacrylamide gel electrophoresis to assess P115.

### ELISA assay

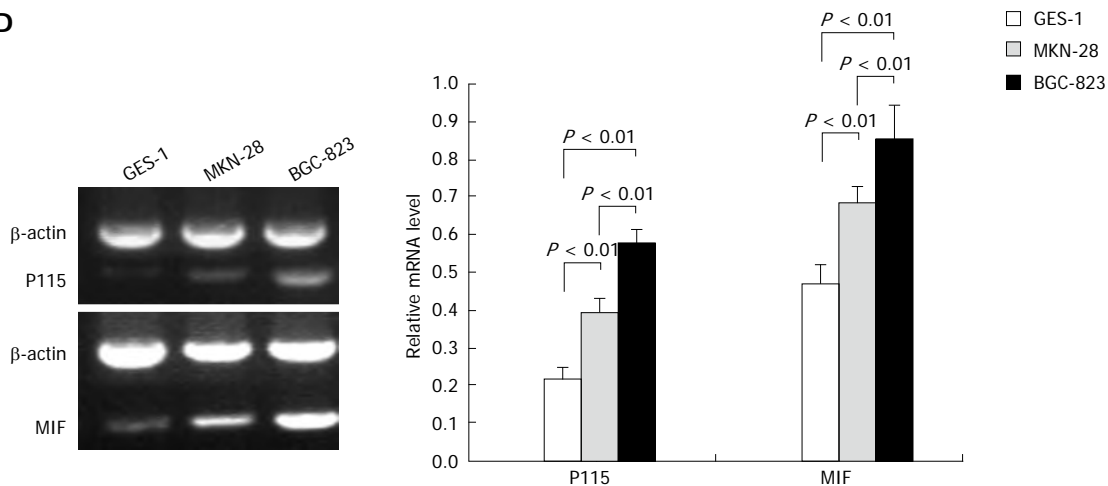
MIF level in the culture supernatant was determined by ELISA according to the manufacturer's recommendations. A polyclonal anti-MIF antibody was used as the capture antibody, and absorbance was measured at 450 nm in a microplate reader. The concentration of MIF in each sample was obtained by comparing absorbance values against the standard curve using r-MIF. Each experiment was performed in triplicate.

### Statistical analysis

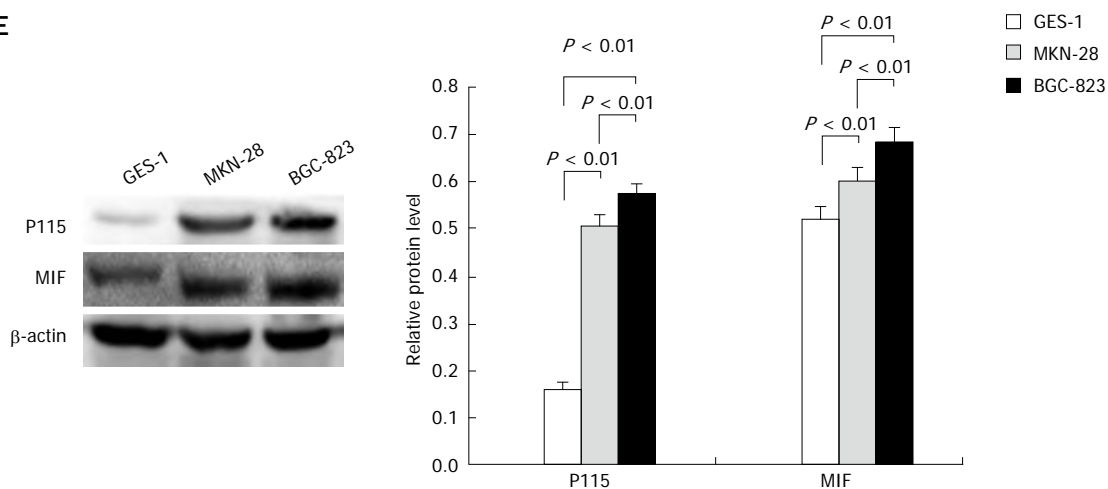
The data were expressed as mean  $\pm$  SD of three independent experiments. The data were analyzed *via* one-way ANOVA comparisons between different groups with



D



E



**Figure 1 P115 and macrophage migration inhibitory factor were specifically expressed in gastric cancer.** A: Immunohistochemistry showed that in gastric cancer tissue, P115 was expressed in Golgi and cytoplasm near the nucleus, and macrophage migration inhibitory factor (MIF) was expressed in cytoplasm and sparsely in membrane. In normal gastric mucosa tissue, P115 and MIF were negatively expressed (DAB stained,  $\times 200$ ). Real-time reverse transcription-polymerase chain reaction (RT-PCR) (B) and Western blotting (C) showed that mRNA and protein levels of P115 and MIF in gastric cancer tissue were higher than those in normal gastric mucosa tissue. RT-PCR (D) and Western blotting (E) showed that mRNA and protein levels of P115 and MIF in MKN-28 and BGC-823 cells were higher than those in the normal gastric mucosa epithelial cell line GES-1.  $\beta$ -actin was used as a loading control for RT-PCR and Western blotting. Data are mean  $\pm$  SD of three experiments.

significance value set at  $P < 0.05$ .

## RESULTS

### P115 and MIF were specifically expressed in gastric cancer tissues

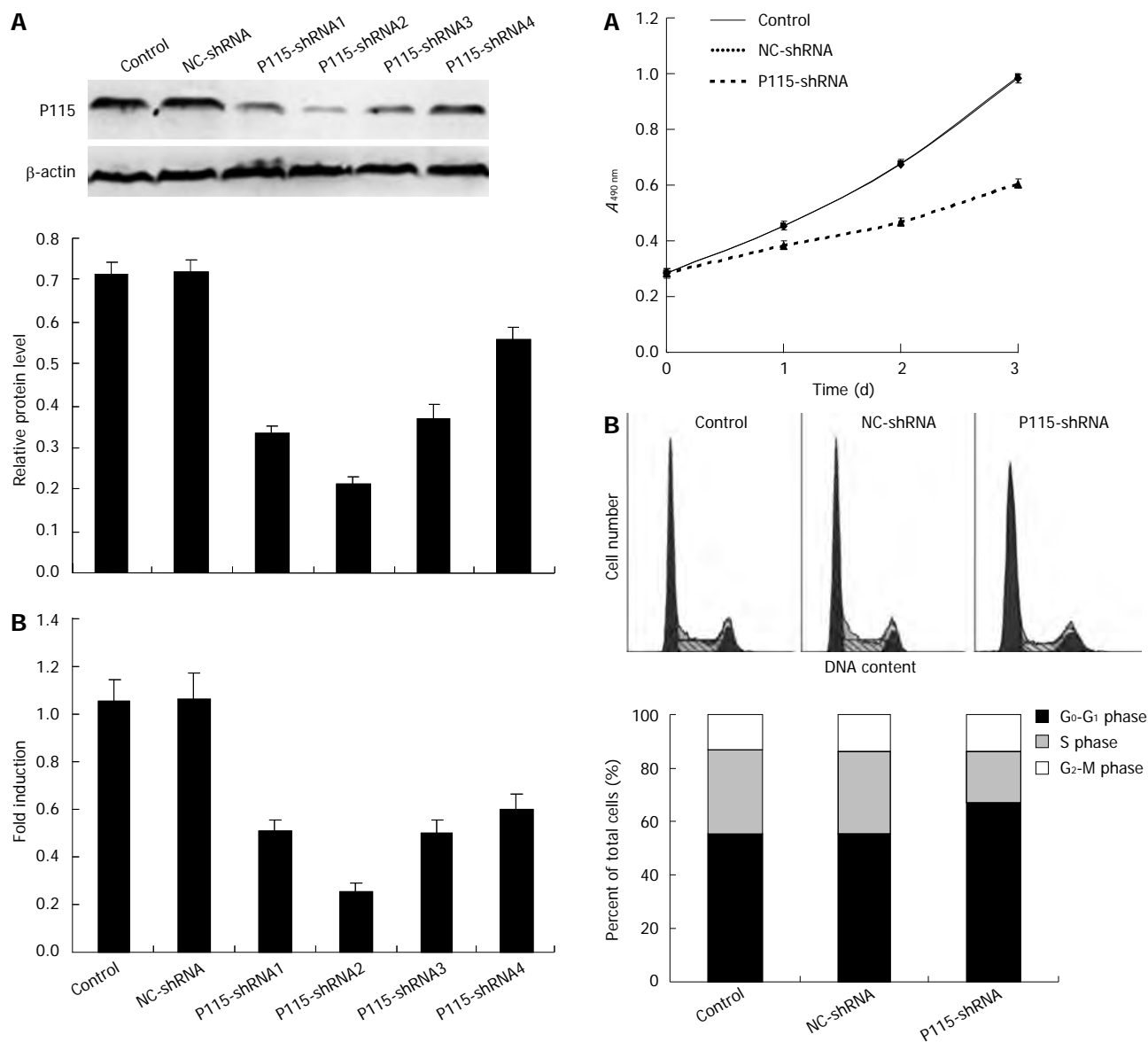
To examine whether P115 and MIF were specifically expressed in gastric cancer, the protein levels of P115 and MIF in human gastric tissue were first measured by immunohistochemistry (Figure 1A). It was shown that P115 was expressed in Golgi and cytoplasm near the nucleus, there were 12 positive samples (40.0%) in normal gastric mucosa and 22 positive samples (73.3%) in gastric cancer with 63.6% showing a strong positive rate (14 cases). MIF was expressed in cytoplasm and sparsely in membrane, there were 14 positive samples (46.7%) in normal gastric mucosa and 24 positive samples (80%) in gastric cancer with 66.7% showing a strong positive rate (16 cases).

Furthermore, the tissue homogenates of normal gas-

tric mucosa and gastric cancer were lysed to measure P115 and MIF levels. Semi-quantitative RT-PCR analysis showed that P115 and MIF mRNA in gastric cancer ( $0.694 \pm 0.046$  and  $0.814 \pm 0.040$ , respectively,  $n = 3$ ) were 1.377 and 1.326 times that in normal mucosa ( $0.504 \pm 0.046$  and  $0.614 \pm 0.054$ , respectively,  $n = 3$ ) (Figure 1B, both  $P < 0.01$ ). As shown in the semi-quantitative analysis of Western blotting results (Figure 1C), compared with normal gastric mucosa, the expression of P115 and MIF increased by 2.085- and 1.391-fold in gastric cancer ( $0.759 \pm 0.058$  vs  $0.364 \pm 0.037$ ;  $0.715 \pm 0.040$  vs  $0.514 \pm 0.044$ , respectively,  $n = 3$ ; both  $P < 0.01$ ).

### P115 and MIF were specifically expressed in gastric cancer cell lines

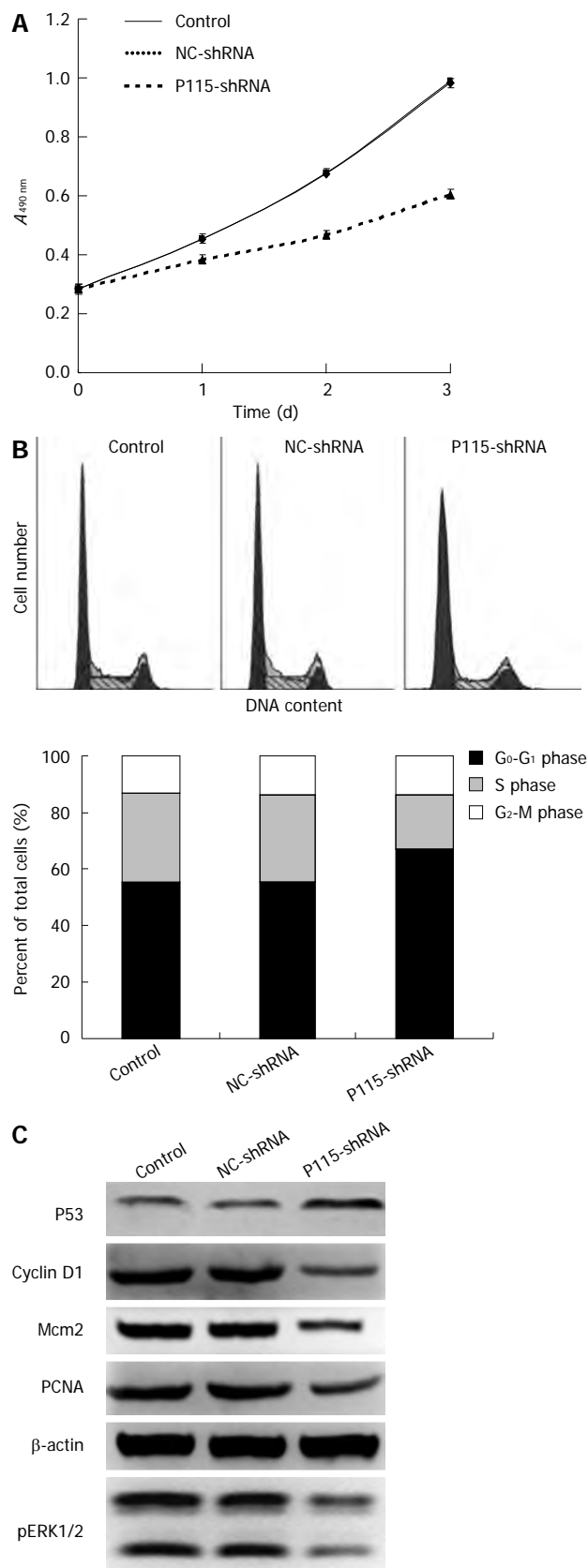
P115 and MIF mRNA in different cell lines are shown in Figure 1D. P115 mRNA in the MKN-28 ( $0.391 \pm 0.042$ ,  $n = 3$ ) and BGC-823 ( $0.513 \pm 0.038$ ,  $n = 3$ ) cell lines was 1.836- and 2.408-fold that in the normal gastric



**Figure 2** P115-shRNA plasmids reduced expression of P115 in BGC-823 cells. Cells were transfected with 2  $\mu$ g P115-shRNA for 36 h and P115-shRNA2 was found to have the best silencing efficacy measured by Western blotting (A) and real-time polymerase chain reaction (PCR) (B).  $\beta$ -actin was used as a loading control for Western blotting and glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for real-time PCR. Data are mean  $\pm$  SD of three experiments.

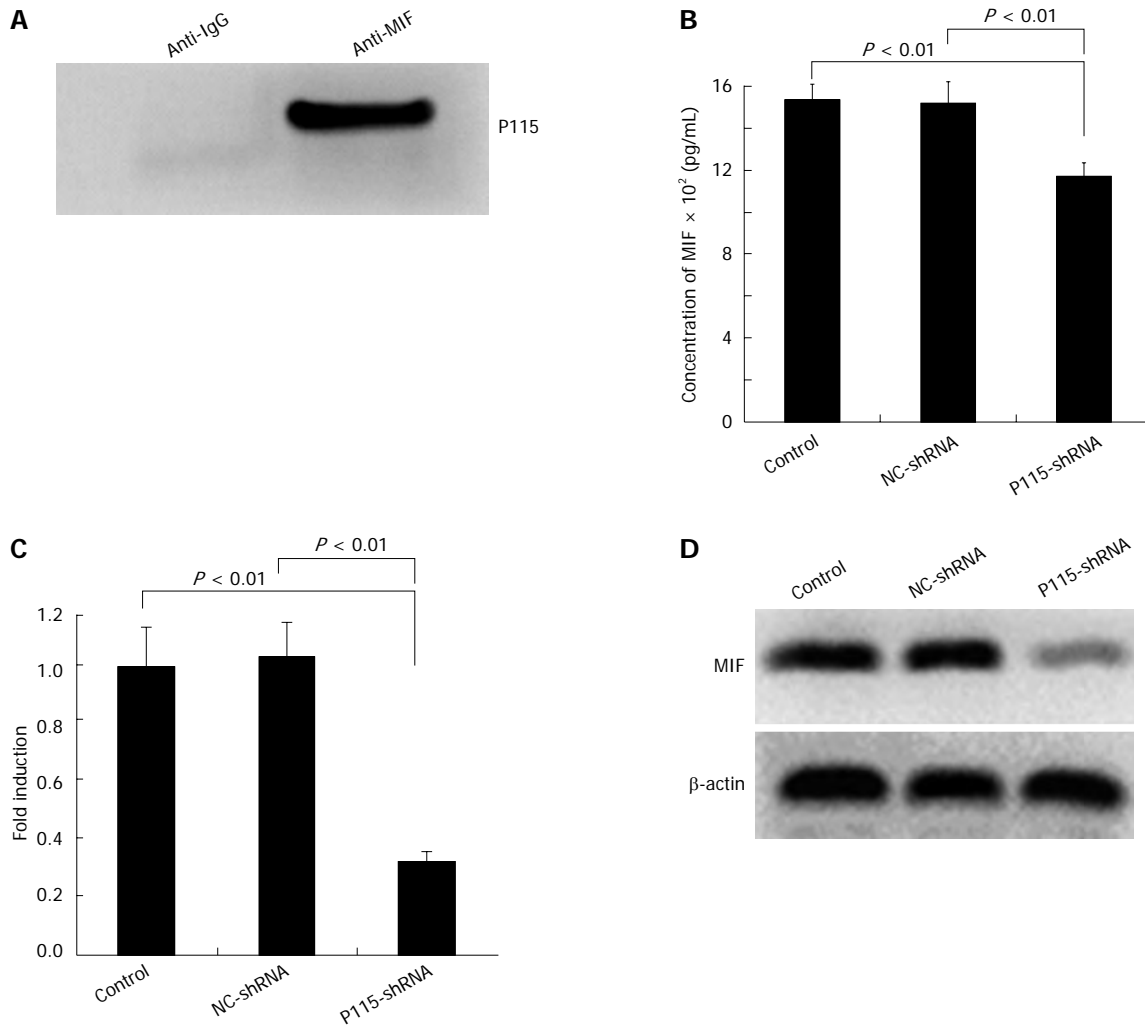
mucosa epithelial cell line GES-1 ( $0.213 \pm 0.036$ ,  $n = 3$ ), respectively (both  $P < 0.01$ ). Moreover, in the poorly differentiated cell line, BGC-823, it was 1.312 times that in MKN-28 cells ( $P < 0.01$ ). Correspondingly, MIF mRNA in MKN-28 ( $0.683 \pm 0.046$ ,  $n = 3$ ) and BGC-823 ( $0.895 \pm 0.104$ ,  $n = 3$ ) cells was 1.453 and 1.904 times that in GES-1 ( $0.470 \pm 0.052$ ,  $n = 3$ ) and BGC-823 cells was 1.310 times that in MKN-28 cells (all  $P < 0.01$ ).

Similar to the results of RT-PCR, the protein levels of P115 and MIF were markedly higher in MKN-28 and BGC-823 cells than in GES-1 cells (Figure 1E), and were higher in BGC-823 cells. Semi-quantitative analysis showed that the expression of P115 in MKN-28 ( $0.507 \pm 0.020$ ,  $n = 3$ ) and BGC-823 ( $0.547 \pm 0.015$ ,  $n = 3$ ) cells was 3.229- and 3.484-fold that in GES-1 cells ( $0.157$



**Figure 3** P115-shRNA inhibited cell proliferation and G<sub>0</sub>-G<sub>1</sub> to S phase transition. A: After transfection with 2  $\mu$ g P115-shRNA for 24, 48 and 72 h, the proliferation rate of BGC-823 cells was inhibited as detected by MTT assay; B: BGC-823 cells were transfected with 2  $\mu$ g P115-shRNA for 48 h. The cell cycle was then measured by flow cytometry (B) and cell cycle regulators were measured by Western blotting (C).  $\beta$ -actin was used as a control for sample loading.





**Figure 4** P115-shRNA inhibited the secretion and expression of macrophage migration inhibitory factor (MIF) in culture supernatant and cells. A: Macrophage migration inhibitory factor (MIF) in BGC cells was extracted through protein precipitation with a multiple clone antibody, and P115 was detected in the protein complex by Western blotting; B: 48 h after transfection with 2  $\mu$ g P115-shRNA, the secretion of MIF into the supernatant was inhibited as measured by ELISA. MIF in cells was reduced as measured by real-time PCR (C) and Western blotting (D).  $\beta$ -actin was used as a loading control for Western blotting and glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for PCR. Data are mean  $\pm$  SD of three experiments.

$\pm 0.010$ ,  $n = 3$ ), and in BGC-823 it was 1.079-fold that in MKN-28 cells (all  $P < 0.01$ ). The expression of MIF in MKN-28 ( $0.601 \pm 0.017$ ,  $n = 3$ ) and BGC-823 ( $0.687 \pm 0.015$ ,  $n = 3$ ) cells was 1.154- and 1.319-fold that in GES-1 cells ( $0.521 \pm 0.020$ ,  $n = 3$ ), and in BGC-823 it was 1.143-fold that in MKN-28 cells (all  $P < 0.01$ ).

#### P115-shRNA inhibited cell proliferation

To explore the biological function of P115, P115-shRNA plasmids expressing siRNA were constructed. First, the silencing efficiencies of 4 different P115-shRNAs were tested using Western blotting (Figure 2A) and real-time PCR (Figure 2B), which showed that the level of P115 was down-regulated most by 2  $\mu$ g P115-shRNA2 plasmids (protein:  $0.259 \pm 0.034$ ,  $n = 3$ ; mRNA:  $0.211 \pm 0.010$ ,  $n = 3$ ) after 36 h transfection in BGC-823 cells (expression of P115 was relatively high) compared with control cells (protein:  $0.727 \pm 0.018$ ,  $n = 3$ ; mRNA:  $1.041 \pm 0.086$ ,  $n = 3$ ) and NC-shRNA (protein:  $0.735 \pm 0.010$ ,  $n = 3$ ; mRNA:  $1.054 \pm 0.094$ ,  $n = 3$ ), with silencing effi-

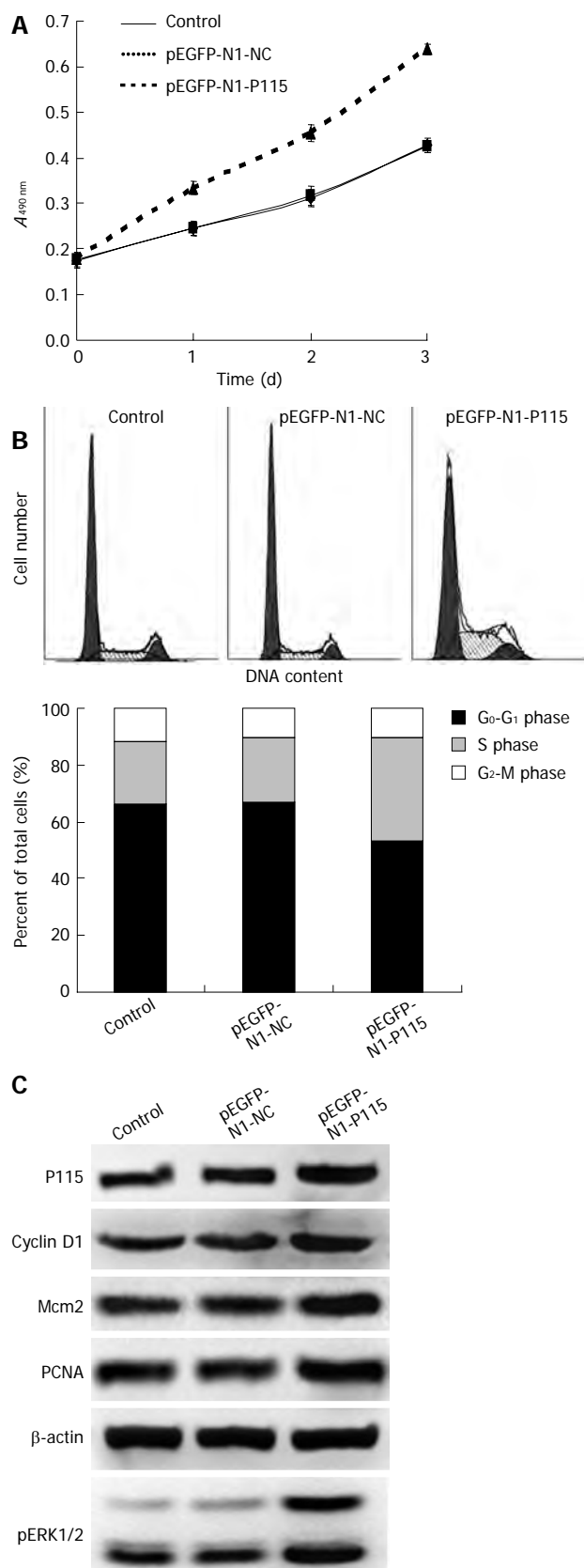
cacy up to 76.8%. Therefore, P115-shRNA2 was selected for subsequent study. The proliferation rate of BGC-823 cells was then determined by MTT assay 24 h, 48 h and 72 h after transfection, and showed that the growth rate of P115-shRNA treated BGC-823 cells was obviously decreased (Figure 3A) compared with NC-shRNA.

#### P115-shRNA inhibited G<sub>0</sub>-G<sub>1</sub> to S phase transition

The role of P115 in the cell cycle checkpoints was investigated (Figure 3B). FACS analysis revealed that P115-shRNA resulted in an 11.3% and 11.18% increase in cell number at G<sub>0</sub>-G<sub>1</sub> phase compared with control and NC-shRNA in BGC-823 cells.

#### P115-shRNA inhibited expression of cyclin D1, Mcm2, PCNA, pERK1/2 and p53

As P115-shRNA caused cell cycle arrest, it was supposed that P115 could lead to a change in G<sub>0</sub>-G<sub>1</sub> phase-related proteins and signaling pathways, such as cyclin D1, Mcm2, p53, PCNA as well as the MAPK signaling



**Figure 5** P115 promoted cell proliferation and G<sub>0</sub>-G<sub>1</sub> to S phase transition. (A) After transfection with 2  $\mu$ g pEGFP-N1-P115 for 24, 48 and 72 h, the proliferation rate of MKN-28 cells was increased as detected by MTT assay. (B) MKN-28 cells were transfected with 2  $\mu$ g pEGFP-N1-P115 for 48 h. The cell cycle was then measured by flow cytometry (B) and cell cycle regulators were measured by Western blotting (C).  $\beta$ -actin was used as a control for sample loading. Data are mean  $\pm$  SD of three experiments.

pathway. Therefore, the above proteins and phosphorylation of ERK1/2 were assessed. It was shown that cyclin D1, Mcm2, PCNA and pERK1/2 were significantly decreased by P115-shRNA in BGC-823 cells, which explained the effect of P115 on cell cycle phase. In addition, p53 was up-regulated by P115-shRNA (Figure 3C).

#### Interaction between P115 and MIF was detected by co-immunoprecipitation assay

MIF in BGC-823 cells was extracted through protein precipitation with a multiple clone antibody and the protein complex was detected by Western blotting. As shown in Figure 4A, P115 was detected in the protein complex, indicating that there was an interaction between MIF and P115 protein.

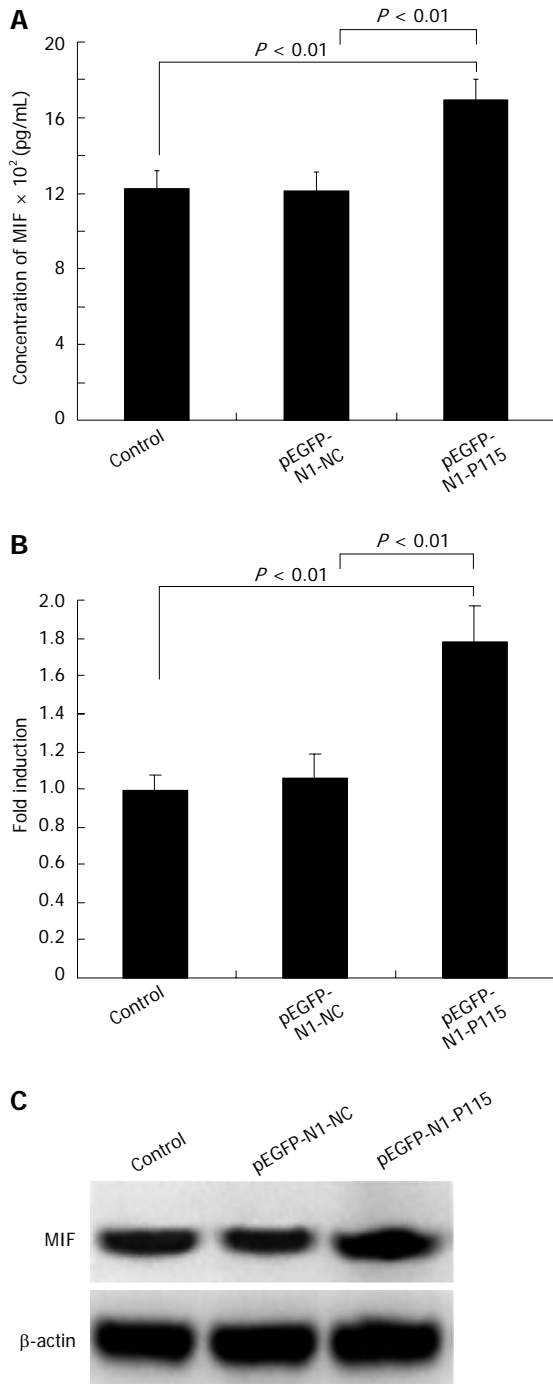
#### P115-shRNA inhibited expression of MIF in cells and secretion of MIF into supernatant

Considering that MIF is a secretory protein, the level of MIF in the culture supernatant was assessed by ELISA. This assay showed that the secreted concentration of MIF in the culture supernatant in P115-shRNA treated BGC-823 cells was markedly reduced ( $1173.67 \pm 63.47$  pg/mL,  $n = 3$ ) compared with control ( $1535.62 \pm 77.25$  pg/mL,  $n = 3$ ) and NC-shRNA treated cells ( $1517.69 \pm 102.51$  pg/mL,  $n = 3$ ), this difference was statistically significant ( $P < 0.01$ , Figure 4B). In addition, MIF mRNA and protein in cells were also detected. As shown in Figure 4C, P115-shRNA decreased the level of MIF mRNA in BGC-823 cells, and Western blotting showed the same trend as real-time PCR, in that P115-shRNA decreased the expression of MIF (Figure 4D).

#### pEGFP-N1-P115 promoted cell proliferation, G<sub>0</sub>-G<sub>1</sub> to S phase transition, expression of G<sub>0</sub>-G<sub>1</sub> phase-related proteins and secretion of MIF into supernatant

The role of P115 in gastric cancer cells was assessed from another point of view. It was shown that after transfection with 2  $\mu$ g pEGFP-N1-P115 for 24, 48 and 72 h, the proliferation rate of MKN-28 cells (expression of P115 was relatively low) was markedly increased (Figure 5A) and the transition of G<sub>0</sub>-G<sub>1</sub> phase to S phase in MKN-28 cells was accelerated by 13.71% and 13.9%, respectively, compared with control and pEGFP-N1-NC (Figure 5B), suggesting that stimulation of cell growth by P115 was associated with the distribution of cell cycle phase. Correspondingly, cyclin D1, Mcm2, PCNA and pERK1/2 were significantly increased by pEGFP-N1-P115 in MKN-28 cells (Figure 5C).

ELISA showed that the secreted concentration of MIF in the culture supernatant in pEGFP-N1-P115 treated MKN-28 cells was markedly increased ( $1696.38 \pm 107.95$  pg/mL,  $n = 3$ ) compared with control ( $1227.64 \pm 90.58$  pg/mL,  $n = 3$ ) and pEGFP-N1-NC treated cells ( $1208.63 \pm 101.78$  pg/mL,  $n = 3$ ), and the difference was statistically significant ( $P < 0.01$ , Figure 6A). As shown in Figure 6B, MIF mRNA in pEGFP-N1-P115 treated cells was increased and Western blotting also indicated that



**Figure 6** P115 promoted the secretion and expression of MIF in culture supernatant and cells. (A) 48 h after transfection with 2  $\mu$ g pEGFP-N1-P115, the secretion of migration inhibitory factor (MIF) into the supernatant was measured by ELISA. MIF in cells was increased by pEGFP-N1-P115 as measured by real-time polymerase chain reaction (PCR) (B) and Western blotting (C).  $\beta$ -actin was used as a loading control for Western blotting and glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for PCR. Data are mean  $\pm$  SD of three experiments.

pEGFP-N1-P115 increased the expression of MIF (Figure 6C).

## DISCUSSION

The complicated molecular mechanisms of carcinogen-

esis and the interaction of multiple oncogenes in gastric cancer challenge our ability to identify novel and rational molecular therapeutic targets. The present study demonstrates that P115 may be a potential tumor biomarker and therapeutic target which is overexpressed in human gastric cancer. Interaction with MIF may be involved in its molecular mechanism.

P115 has been demonstrated to be involved in intra-Golgi transport<sup>[6]</sup> and can bind to the Golgi-associated proteins, GM130<sup>[19]</sup> and giantin<sup>[20]</sup>, which both play an important role in mitosis, that is, P115 is essential for biogenesis of the Golgi apparatus<sup>[21,22]</sup>. MIF is a secretory protein which plays an important upstream role in the regulation of diverse cellular responses<sup>[23-25]</sup>. The role of MIF has been emphasized by the finding that high expression of MIF is associated with the incidence or the severity of oncologic diseases<sup>[26-28]</sup>. The data from this study showed that overexpression of P115 significantly enhanced the secretion of MIF, which indicated that P115 might be one of the stimuli inducing MIF secretion through direct interaction. Merk *et al.*<sup>[29]</sup> reported that MIF was co-secreted with P115, indicating that P115 had a specific role in MIF export, which is consistent with our results. MIF lacks a signal sequence and is secreted by an unconventional route for protein export. Stimuli induce the rapid release of MIF from preformed and cytoplasmic pools, which is followed by an upregulation of MIF mRNA expression and a replenishment of intracellular protein content<sup>[30,31]</sup>. Therefore, the protein and mRNA expression of MIF in cells was detected. As expected, when P115 was overexpressed or silenced, MIF protein and mRNA in cells were also enhanced or reduced compensatively.

The biological mechanism of MIF on tumor growth includes the induction of growth-related protein expression and inhibition of apoptosis-related protein expression<sup>[32]</sup>. Jung *et al.*<sup>[14]</sup> demonstrated that MIF interacted with p53 *in vivo* and directly promoted tumorigenesis by inhibiting p53 accumulation. Our data demonstrated that P115 knockdown enhanced the expression of p53, which was considered a result of MIF reduction. It is known that p53 is a classic tumor suppressor gene that can promote cell cycle arrest and apoptosis in response to DNA damage. Absence or down-regulation of p53 can interfere with these important checkpoints for maintaining genetic stability and allows cells to survive and proliferate. This may explain our results where knockdown of P115 led to the inhibition of cell proliferation and apoptosis (results not shown).

To further explore the molecular mechanism of P115 influencing cell growth, key proteins involved in the G<sub>0</sub>-G<sub>1</sub> phase relevant signaling pathway were determined. It was reported that the ERK1/2 pathway was necessary for transcriptional induction of cyclin D1 which promoted progression from G<sub>1</sub> to S phase<sup>[33]</sup>. In addition, Mcm2 and PCNA are both important proteins for initiation of DNA synthesis<sup>[34]</sup>. As shown in our results, cyclin D1, Mcm2 and PCNA, as well as pERK1/2 were markedly reduced by P115-shRNA, which was consistent with

G<sub>0</sub>-G<sub>1</sub> arrest. Researchers have reported that recombinant MIF can activate the ERK-MAP kinase pathway, and subsequently increase cell proliferation rate in fibroblasts and a colon cancer cell line<sup>[35]</sup>. Therefore, it is concluded that MIF is the key factor in the biological function of P115 in cell proliferation.

In conclusion, our study demonstrates that P115 is overexpressed in gastric cancer tissue and cells. Knock-down of P115 blocks cell proliferation *in vitro*, and the mechanism involves P115 stimulating the secretion of MIF directly by interacting with MIF, subsequently, leading to progression of cell cycle through relevant proteins. Although additional functional studies are required, P115 as well as the interaction between P115 and MIF may be a potential therapeutic target for the treatment of gastric cancer.

## COMMENTS

### Background

Cancer growth is a highly complex process involving alterations in gene expression and the interaction of many proteins. Golgi-vesicular transport protein P115 is a tether protein that plays an important role in many signal pathways required for cell proliferation. Some studies have reported the function of P115 in intra-Golgi transport, but there are few studies on the role of P115 in cancer cell proliferation and on its biological mechanism.

### Research frontiers

The ability of migration inhibitory factor (MIF) to promote tumor progression has been demonstrated. Recently, a yeast two-hybrid interaction was examined to identify the intracellular proteins which might bind to MIF and mediate its secretion, and it was shown that P115 was a binding partner of MIF.

### Innovations and breakthroughs

This study showed that over-expression of P115 could significantly enhance the secretion of MIF, which indicated that P115 might be one of the stimuli inducing MIF secretion through a direct interaction. Through interaction with MIF, P115 promoted progression from G<sub>1</sub> to S phase and then influenced the growth of cancer cells. Correspondingly, cyclin D1, Mcm2 and PCNA, as well as pERK1/2 were markedly reduced by P115-shRNA, being consistent with G<sub>0</sub>-G<sub>1</sub> arrest.

### Applications

The study results suggest that P115 as well as the interaction of P115 and MIF may be a potential therapeutic target for treatment of gastric cancer.

### Terminology

Protein-protein interaction: the majority of proteins in living systems function due to interaction with each other in stable or dynamic protein complexes. P115 is a tether protein which has been demonstrated to be involved in intra-Golgi transport. MIF is a secretory protein which plays an important role in regulation of diverse cellular responses.

### Peer review

This is a good descriptive study in which authors explore the expression and role of two important proteins in gastric cancer biology. The results are significant and suggest that P115 promote cells proliferation through interaction with MIF and provide a potential therapeutic target for treatment of gastric cancer.

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## Clinicopathological and biological significance of *cripto* overexpression in human colon cancer

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### Abstract

**AIM:** To assess the clinicopathological and biological significance of *cripto* in human colorectal cancer.

**METHODS:** Real-time reverse-transcription polymerase chain reaction (PCR) was used to examine *cripto* mRNA levels in primary colon cancer and normal colon tissues as well as normal and metastatic lymph nodes from colon cancers. Human colon cancer LS-174T cells were transfected with *cripto* small interfering RNA (siRNA), and mRNA and protein levels were evaluated using real-time PCR and western blot analysis, respectively. The growth of cancer cells was evaluated using the MTT assay and colony formation in soft agar. Invasion was examined using a Transwell assay, and the expressions of matrix metalloproteinase (MMP)-7 and MMP-9 were determined using western blot assay.

**RESULTS:** *Cripto* was significantly overexpressed in

primary colon cancer and metastatic lymph nodes. Silencing *cripto* gene expression with *cripto* siRNA resulted in a significant decrease in colony formation in soft agar in the colon cancer cell line LS-174T. *Cripto* siRNA treatment decreased the migration and invasion capabilities of the colon cancer cell line LS-174T *in vitro*. Furthermore, *cripto* siRNA treatment inhibited the expression of matrix MMP-7 and MMP-9.

**CONCLUSION:** The results provide evidence that *cripto* siRNA could be an effective approach for the inhibition of cancer cell invasion and migration and thus has potential for use in devising novel preventive and therapeutic strategies for colon cancer metastasis.

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**Key words:** Colon cancer; Invasion; Metastasis; *Cripto*; Small interfering RNA; Matrix metalloproteinases

**Core tip:** To assess the clinicopathologic and biological significance of *cripto* as a novel target for colon cancer gene therapy, pathological and *in vitro* studies were carried out. *Cripto* was significantly overexpressed in primary colon cancer and metastatic lymph nodes. *In vitro* studies found that *cripto* siRNA resulted in a significant reduction in colony formation in soft agar and in the migration and invasion abilities of colon cancer cells. Furthermore, *cripto* siRNA led to an inhibition of MMP-7 and MMP-9. These results suggest that the *cripto* gene be useful for devising novel preventive and therapeutic strategies for colon cancer.

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## INTRODUCTION

The *cripto* gene is a member of the epidermal growth factor-CFC family of signaling proteins first cloned from the human teratocarcinoma cell line NTERA2<sup>[1]</sup>. Cripto is overexpressed in most malignant solid tumors, including colon, breast, lung, ovarian, and pancreatic cancers<sup>[2-7]</sup>. In contrast, *cripto* is generally absent from or found at low levels in normal tissues. *In vitro*, *cripto* exhibits many of the properties of an oncogene, including transformation of immortalized cells, induction of cell migration, and stimulation of branching morphogenesis<sup>[8]</sup>. These findings suggest that *cripto* serves an important function in carcinogenesis and in the development of some tumors.

Recent reports show that *cripto* overexpression may be closely related to invasion and metastasis in some human cancers<sup>[9,10]</sup>. Ertoy *et al.*<sup>[9]</sup> evaluated *cripto* expression in matched sets of non-neoplastic cervical epithelium, primary cervical carcinoma, and metastatic tumors in the lymph nodes using immunopositivity staining. Strong *cripto* immunopositivity was found to be significantly correlated with tumor size and lymphovascular space involvement ( $P < 0.05$ ). More importantly, the level of *cripto* expression increased in metastatic lymph nodes compared with their primary tumors. Despite the clear association between *cripto* overexpression and human breast cancer, the clinicopathological and biological significance of *cripto* overexpression in human colon cancer remains undiscovered.

Colorectal cancer is the third most common malignant neoplasm worldwide<sup>[10]</sup> and the second leading cause of death attributed to cancer<sup>[11]</sup>. Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis remains poor. Enhanced understanding of the signaling mechanisms that regulate the metastasis of colon cancer may provide important insights with which to establish improved therapeutic strategies.

In this study, we demonstrate that *cripto* is highly overexpressed in primary and metastatic colon cancer tissues. In addition, we demonstrate that RNA interference (RNAi) *cripto* gene expression decreases the proliferation, migration, and invasion capability of colon cancer cell lines *in vitro*. To define the mechanisms underlying *cripto* invasion inhibition, we investigate the effect of *cripto* small interfering RNA (siRNA) transfection on the expression levels of mRNA and proteins of matrix metalloproteinase (MMP)-7 and MMP-9. Based on the results of this study, we conclude that *cripto* is a potential novel target of gene therapy for colon cancer metastasis.

## MATERIALS AND METHODS

### Tissue specimens and treatment

Thirty-nine paired samples of colon cancer and distant normal colon tissue were obtained from 39 inpatients who had undergone surgery from 2009 to 2010 in the Affiliated People's Hospital of Jiangsu University. Eighteen metastasized lymph nodes were obtained from patients

**Table 1 Clinicopathologic characteristics of colon cancer patients *n* (%)**

Characteristic	No. of patients
Sex	
Male	21 (53.8)
Female	18 (46.2)
Age (yr), mean (range)	62 (35–81)
≤ 65	20 (51.3)
> 65	19 (48.7)
Dukes staging	
A + B	12 (30.8)
C + D	27 (69.2)
Tumor differentiation	
Well	0
Moderate	29 (74.4)
Poor	10 (25.6)

undergoing surgical therapy for the treatment of colon cancer. Tumor histotype and grade of differentiation were defined according to WHO criteria. Clinical and pathological stages were defined according to Dukes Staging criteria. Inpatients did not receive any chemotherapy or radiotherapy prior to surgery. Eleven normal lymph nodes without evidence of cancer were obtained from patients undergoing carotid endarterectomy. None of these patients had any history or clinical evidence of cancer.

To facilitate real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis, the specimens were identified and bisected. One portion was processed for real-time RT-PCR, whereas the other portion was sent for routine pathology analysis. All specimens were immediately snap-frozen in liquid nitrogen to prevent RNA degradation. The specimens were then stored at -70 °C until total RNA processing could be performed. This study was conducted with approval from the Medical Ethical Committee, and all patients provided written informed consent to participate in the study (Table 1).

### Cell lines

The human colon cancer cell lines LS-174T and GEO were obtained from the Institute of Cell Biology, Shanghai, China. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C under a 5%CO<sub>2</sub> atmosphere. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were plated in 96-well plates at a density of  $2 \times 10^3$  cells/well. For real-time RT-PCR, cells were seeded in 6-well plates at a density of  $1 \times 10^5$  cells/well.

### RNA isolation and complementary DNA synthesis

Total cellular RNA was isolated from colon cancer cell lines, normal lymph nodes, and lymph nodes from primary colon cancer patients using Trizol. Final RNA pellets were dissolved in 20 µL of diethyl pyrocarbonate-treated water. RNA yield was determined by spectroscopy. For complementary DNA (cDNA) synthesis, 5 µg of total RNA was transcribed with cDNA transcription reagents using 0.5 µg of oligo(dT)18 primer for subsequent quantitative, RT-PCR.

**Table 2** Primers of *cripto*, matrix metalloproteinase-7 and matrix metalloproteinase-9

Genes	Forward(5'-3')	Reverse(5'-3')
Cripto	CAATTCGGCCCTCGGCTCTC	TTCAGGCAGCAGGTTCTGTTC
MMP-7	AACTCCCCTCGTCATAGAAAT	GATACGATCCTGTAGGTGAC
MMP-9	CGGAGTGAGTTGAACACAG	GTCCCACTGGGGATTATAC

MMP: Matrix metalloproteinase.

**Real-time RT-PCR**

Real-time RT-PCR analyses were performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, CA, United States). Primers and TaqMan probes were designed using Primer Express<sup>TM</sup> 1.0 (Applied Biosystems) software, and probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3' end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine). Real-time PCR was conducted in a total volume of 50  $\mu$ L with 1  $\times$  TaqMan Master Mix (Applied Biosystems) and primers. Thermal cycling parameters included one cycle at 95  $^{\circ}$ C for 3 min, 45 cycles involving denaturation at 95  $^{\circ}$ C for 30 s, annealing at 52  $^{\circ}$ C for 45 s, and extension at 72  $^{\circ}$ C for 45 s, followed by a final extension at 72  $^{\circ}$ C for 10 min. The relative amount of cDNA in each sample was calculated by dividing the CT value with the corresponding value of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All reactions were performed in triplicate. The data were normalized to the internal control gene, *GAPDH*, to control for RNA preparation. Real time RT-PCR results were analyzed using Q-Gen software<sup>[12]</sup>, which expresses data as mean normalized expression (MNE). MNE is directly proportional to the amount of RNA of the target gene (*cripto* and *MMPs*) relative to the amount of RNA of *GAPDH*.

**Primer design**

Primers for *cripto*, *MMP-7*, and *MMP-9* were designed using Perkin-Elmer Primer Express software. The primer sequences are presented in Table 2.

**Cripto siRNA sequence**

Cripto siRNAs corresponding to *cripto* mRNA with dTdT on 3'-overhangs were designed and chemically synthesized according to the recommendation of the manufacturer (Dharmacon Research, United States). Six siRNAs targeting the coding sequence of *cripto* mRNA (S1-S6) were used in the current experiment. Information on the *cripto* siRNAs is provided in Table 3. The scrambled siRNA served as a control and its sequences were 5'-UUCUCCGAACGUGUCACGUTdTdT-3' and 5'-ACGUGACACGUUCGGAGAATdTdT-3'.

**In vitro transfection**

Transfection of siRNA was performed using a commercial reagent, oligofecamine (Invitrogen, United States), in 6-well plates following the manufacturer's instructions. Briefly, the day before transfection, confluent layers of

cells were trypsinized, counted, and resuspended. Thereafter,  $1 \times 10^5$  of cells were plated into each well of the 6-well plates, such that approximately 70% confluence could be achieved the next day at the time of transfection. Oligofecamine was diluted in serum-free RPMI 1640 and mixed with siRNA at a 1:2 ratio (4  $\mu$ L of 20  $\mu$ mol/L of siRNA formulated with 8  $\mu$ L of oligofecamine). The cells were then incubated for another 48 h. Cell numbers were determined using a hemocytometer before subsequent assays.

**MTT assay**

Cells plated in 96-well plates were grown in their respective media for 48 h after the addition of siRNA. At each time point, cells were checked visually for growth and proliferation. MTT (Sigma) was then added to the wells, and the cells were incubated at 37  $^{\circ}$ C for 4 h. MTT solubilization solution (10% Triton X-100 in acidic isopropanol, 0.1 mol/L HCl) was added to the wells, and the cells were incubated overnight. Colorimetric measurements were performed using a microplate reader (Molecular Devices) at 560 nm, and the background at 650 nm was subtracted.

**Anchorage-independent growth assay**

For the anchorage-independent growth experiments, LS-174T cells ( $1 \times 10^4$  cells/well) were seeded in 0.3% FBS supplemented with complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar-medium base layer in 24-well plates. After 15 d, the colonies were stained with nitroblue tetrazolium, and colonies larger than 50  $\mu$ m were acquired using a micro-Scopeman camera system (Moritex Europe Ltd.) and analyzed with Image-Pro Plus (Media Cybernetics) software.

**In vitro invasion/migration assay**

Transwell migration and invasion assays were performed using LS-174T cells cultured in 12-well plates containing either 8  $\mu$ m pore Biocoat<sup>®</sup> control inserts (migration assays) or Matrigel-coated inserts (invasion assays) according to the manufacturer's instructions (Becton Dickinson, Bedford, MA, United States). The membranes were rehydrated with warm serum-free Dulbecco's modified Eagle's medium (1.0 mL/chamber) for 2 h. The upper chamber was filled with  $1 \times 10^5$  cells in L-15 medium containing 5% FBS. The lower chamber was filled with L-15 medium containing 25% FBS as a chemoattractant. After the chambers were incubated for 24 h at 37  $^{\circ}$ C under a 5% CO<sub>2</sub> atmosphere, the non-invading cells were removed from the upper surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were fixed and stained with hematoxylin and eosin.

The number of cells that penetrated the filter was counted by a technician blinded to the experimental settings in four microscopic fields of each filter under  $\times 20$  magnification. The percentage of invasion was expressed as the ratio of the mean cell number from the invasion chamber to the mean cell number from the control chamber according to the manufacturer's recommenda-



Table 3 *Cripto* siRNA sequence

siRNA	Sense (5' → 3')	Antisense (5' → 3')	MW	Position
S1	UUCGGCCUCGGUCUCCCCATT	UGGGAAGACCGAGGCCGAATT	13347.2	175-195
S2	CAGAACCUGCUGCCUGAAUTT	AUUCAGGCAGCAGGUUCUGTT	13317.2	236-256
S3	CUGUGAGCACGAUGUGCGCTT	GCGCACAUCGUGCUCACAGTT	13347.2	314-334
S4	GAGAACUGUGGGUCUGUGCTT	GCACAGACCCACAGUUCUCTT	13332.2	336-356
S5	UGCUGGCACGGUCAGCUCCTT	GGAGCUGACCGUGCCAGCATT	13362.2	396-416
S6	CUACCACCGUCUGCACGUATT	UACGUGCAGACGGUGGUAGTT	13332.2	495-515

tion. The percentage of migration was expressed as the ratio of the mean cell number in control inserts containing S1 siRNA transfected cells to mean cell numbers in control inserts containing untreated cells (untreated cells were given a value of 100%).

#### Western blot assay for MMP-7 and MMP-9

Seventy-two hours after transfection, cells were washed twice in PBS, and total protein was extracted in 150 mmol/L NaCl, 50 mmol/L Tris·HCl (pH 7.5), 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 10 mg/mL leupeptin, 1% aprotinin, and 2 mmol/L PMSF. Ten micrograms of protein sample was loaded onto a 10% SDS-PAGE and electroblotted onto a PVDF nylon membrane (Millipore, Bedford). Membranes were blocked in 0.05% Tween 20 (v/v) PBS containing 5% skimmed milk and then incubated with MMP-7, MMP-9, and  $\beta$ -actin antibodies (Santa Cruz Biotechnology). Membranes were then incubated with a HRP-linked goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Finally, the membrane was reacted with DAB reagent and washed with PBS once protein bands had appeared.

#### Statistical analysis

Statistical analyses included the independent *t*-test and analysis of variance. Statistical analyses were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, United States).

## RESULTS

#### *Cripto* is highly expressed in primary colon cancer

To determine whether or not *cripto* was expressed in human colon cancer, real-time RT-PCR was conducted on 39 paired samples to determine *cripto* mRNA expression levels in clinical tissues. The results showed that *cripto* expression in primary colon cancer samples was significantly higher (mean expression in cancer tissue was more than 16-fold higher;  $P < 0.001$ ) than that in normal tissues.

#### *Cripto* is significantly overexpressed in lymph nodes containing metastatic colon cancer

Real-time RT-PCR analysis was performed on 11 normal lymph nodes and 18 lymph nodes containing metastatic colon cancer. The results indicated that *cripto* expression in metastatic lymph nodes was significantly higher (mean

expression in cancer tissue was approximately 150-fold higher;  $P < 0.001$ ) than that in normal lymph nodes.

#### Screening of *cripto* siRNA

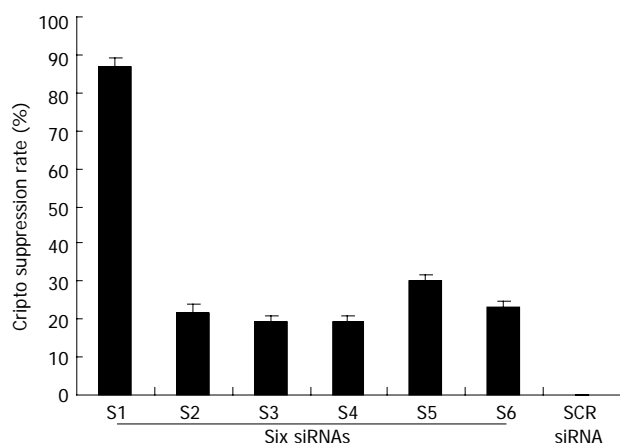
The capability of siRNA to inhibit *cripto* expression was quantified by real-time RT-PCR analysis 48 h following siRNA exposure. S1-S6 (targeting the coding sequence of *cripto* mRNA) showed various suppressant effects on *cripto* mRNA expression in LS-174T cells which express high levels of *cripto* mRNA<sup>[13,14]</sup>. Among them, S1, which targets nucleotides 175-195, exhibited the strongest effect. At a concentration of 100 nmol/L, S1 reduced the *cripto* mRNA level by 89% 48 h after the start of transfection (Figure 1). In contrast, the control-scrambled siRNA treatment showed no effect on *cripto* mRNA levels, thus supporting the specificity of *cripto* siRNA. To characterize the potency of S1 further, the dose and time dependency of its effects on *cripto* mRNA in LS-174T cells were examined, and results indicated that S1 downregulated the *cripto* mRNA level in a dose-dependent manner (Figure 2).

#### Effects of *cripto* siRNA on proliferation in LS-174T cells in vitro

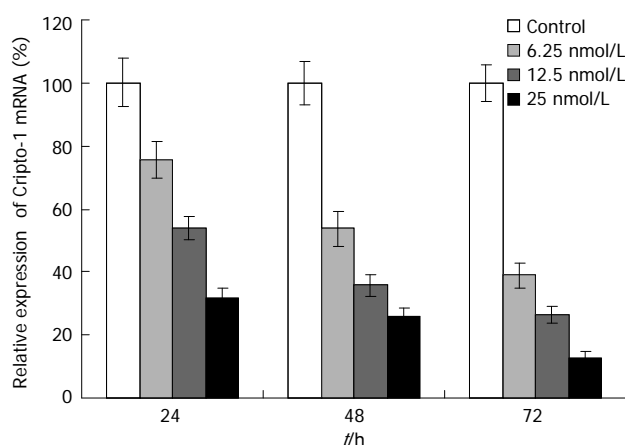
We then evaluated the biological effects of *cripto* suppression on colon cancer LS-174T cells using different types of assays. The MTT assay showed that cellular proliferation in the monolayer culture was unaffected by either siRNA (Figure 3A). However, colony formation in soft agar was strongly inhibited by treatment with *cripto* siRNA but not by control siRNA (Figure 3B). Figure 3B shows that treatment with *cripto* siRNA induced significant anchorage-independent growth inhibition in a dose-dependent manner (Figure 3).

#### Downregulation of *cripto* decreased cancer cell invasion and migration capabilities of colon cancer cell lines in vitro

Colony formation in soft agar is a property closely associated with malignancy. Given the known role of *cripto* siRNA in the downregulation of anchorage-independent growth of LS-174T cells, we attempted to determine whether or not the *cripto* gene contributes to cell invasion and migration in colon cancer. Cell migration and invasion studies were performed using Matrigel matrix assays. Tumor cells require both migration and invasion properties to invade through the Matrigel matrix. Two independent experiments were performed. The results showed that S1 treatment, but not scrambled siRNA treatment,



**Figure 1** Suppression of *cripto* mRNA by six siRNA transfection in LS-174T cells. The colon cancer cell line LS-174T was transfected with 100 nmol/L of six siRNA after 48-h incubation. Real-time polymerase chain reaction quantification of the relative amount of *cripto* transcript was performed using the *cripto* primers and probe set as described in "Materials and Methods," using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. All data are presented as the mean  $\pm$  SD of three independent experiments; the level of *cripto* mRNA relative to GAPDH in untreated cells maintained under identical experimental conditions was taken as 100%.

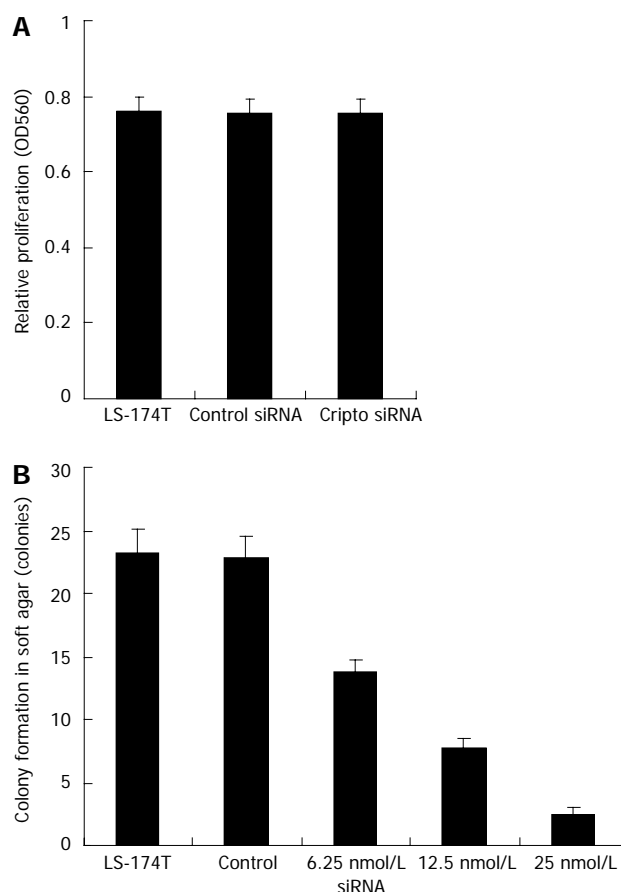


**Figure 2** Effects of S1 siRNA on *cripto* mRNA of colon cancer LS-174T cells. The LS-174T colon cancer cell line was transfected with different dose of S1 siRNA for 24, 48, 72 h. *Cripto* mRNA was evaluated by real-time reverse transcription-polymerase chain reaction as described in "Materials and Methods," using GAPDH as internal standard. All data are presented as the mean  $\pm$  SD of three independent experiments; the level of *cripto* mRNA relative to GAPDH in untreated cells maintained under identical experimental conditions was taken as 100%.

resulted in a significant low level of migration and invasion potential of LS-174T cells (Figure 4).

#### Effects of *cripto* siRNA on MMP-7 and MMP-9 expression in colon cancer cells

To explore whether or not the invasiveness of transfected cells was associated with MMP induction, real-time PCR and western blot assays were conducted to detect alterations in the expression level of MMP-7 and MMP-9. As illustrated in Figure 5, *cripto* suppression resulted in decreases in both mRNA and MMP-7 and MMP-9 protein levels compared with those in control cells. Another



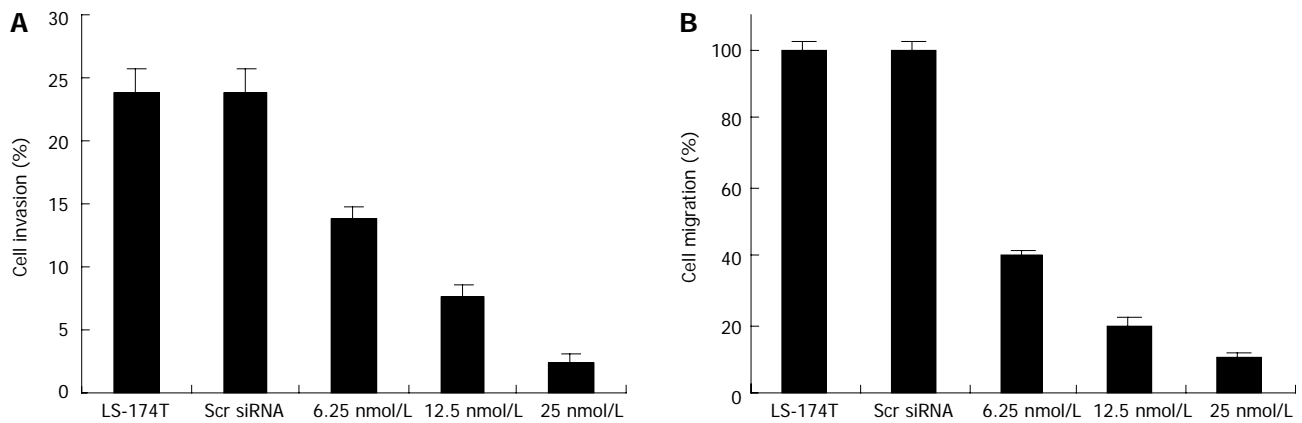
**Figure 3** Effects of S1 siRNA on cell proliferation and anchorage-independent growth of colon cancer LS-174T cells. A: S1 siRNA treatment decreases cell proliferation. Cells plated in 96-well plates were grown in their respective media for 48 h after the addition of siRNA. Cell proliferation was examined by MTT assay; B: S1 siRNA treatment reduces anchorage-independent growth of LS-174T cells. The anchorage-independent growth was evaluated by colony formation in soft agar.

experiment on transfection with *cripto* siRNA in the colon cancer cell line GEO also exhibited invasion inhibition and downregulation of MMP-7 and MMP-9 expression (data not shown). These results indicate that *cripto* suppression by RNAi could inhibit invasion and migration capabilities by reducing MMP-7 and MMP-9 expression in human colon cancer cells.

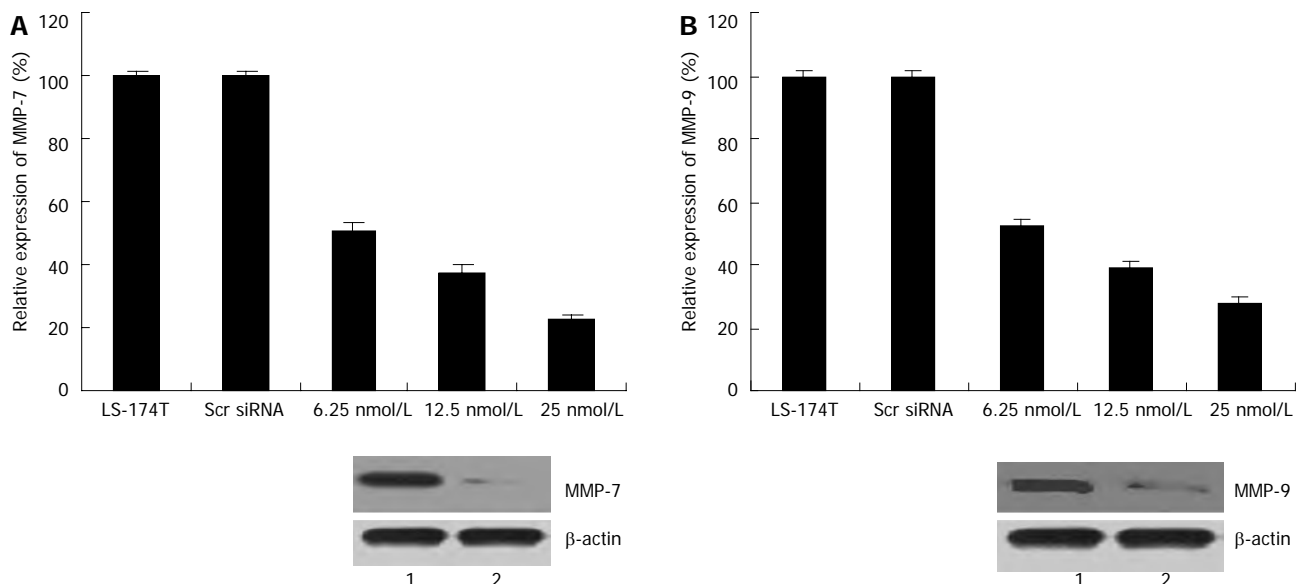
## DISCUSSION

The primary modalities for colon cancer therapy are surgery, radiotherapy, and chemotherapy. One of the main limitations of current treatment modalities is that systemic therapies for metastatic disease are not curative. To improve the choice of therapeutic strategy, the mechanism of invasion and metastasis of colon cancer must be clarified.

The *cripto* gene is known to be overexpressed in numerous solid cancers, and its overexpression appears to be associated with enhanced proliferation and malignant potential<sup>[1,2,5]</sup>. We found that *cripto* was significantly overexpressed in primary and metastatic colon cancer tissues through real-time RT-PCR. To determine whether or not



**Figure 4** Effects of S1 siRNA on migration and invasion of colon cancer LS-174T cells. The LS-174T colon cancer cell line was used in this experiment. Cell migration was assessed in BioCoat control cell culture chambers. Transwell migration and invasion assays were performed using LS-174T cells cultured in 12-well plates containing either Matrigel-coated inserts (invasion assays) or 8  $\mu$ m pore BioCoat® control inserts (migration assays), according to the manufacturer's instructions. Control, scrambled siRNA-, and S1 siRNA-treated cells were added to control and Matrigel chambers. A: The percentage of invasion was expressed as the ratio of mean cell number from invasion chamber to mean cell number from control chamber according to the manufacturer's recommendation; B: The percentage of migration was expressed as the ratio of mean cell number in control inserts containing siRNA-treated cells to mean cell number in control inserts containing untreated control cells. Control cells were given a value of 100%.



**Figure 5** Effects of S1 treatment on matrix metalloproteinase-7 and -9 expression of colon cancer line LS-174T. After cancer cells were treated with S1 siRNA for different times, cells were harvested, and total RNA and proteins were extracted. Real-time reverse transcription-polymerase chain reaction and western blotting were performed to detect mRNA and protein levels, respectively, of matrix metalloproteinase (MMP)-7 and -9. A: Expression level of mRNA and protein of MMP-7 (2 h); B: Expression level of mRNA and protein of MMP-9 (72 h). 1: Scr siRNA; 2: S1(25 nmol/L).

*cripto* was a potential target for colon cancer gene therapy, the colon cancer cell line LS-174T was treated with *cripto* siRNA, and anchorage-independent growth and the capacity for invasion and migration were determined using different assays. The results showed that cancer cells transfected with *cripto* siRNA inhibited the anchorage-independent growth, invasive capacity, and migration capability of colon cancer cells. Finally, our results provide mechanistic insight into the function of *cripto* in the regulation of invasion and migration through the suppression of MMP-7 and MMP-9 expression, which suggests that *cripto* may serve as a novel tumor marker for colon cancer metastasis.

The mechanisms by which *cripto* regulates invasive potential and migration capability remain unclear. Normanno *et al.*<sup>[15]</sup> recently showed that *cripto* overexpression enhanced invasion capability by inhibiting anoikis in breast cancer. The process of metastasis is complex, occurring in a series of steps, including cell invasion and degradation of the basement membranes and stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis.<sup>[16-19]</sup> The MMPs comprise a family of related enzymes that degrade the extracellular matrix and are considered to be important factors in facilitating tumor invasion. Among

the MMPs, MMP-7 and MMP-9 have been considered important factors in facilitating invasion and metastases in human colon cancer<sup>[20-24]</sup>. We further investigated whether or not *cripto*-induced invasion of LS-174T cells is mediated through MMP-7 and MMP-9. Real-time RT-PCR and Western blot analysis were performed to detect MMP expression in the colon cancer cell line LS-174T. Cripto siRNA-transfected cells showed significantly low levels of mRNA and MMP-7 and MMP-9 proteins. Data from these results show that the downregulation of *cripto* expression after siRNA treatment decreases MMP expression. To the best of our knowledge, this study is the first to report on the primary mechanism responsible for the decrease in invasion potential observed after *cripto* siRNA treatment.

Based on our studies, we speculate that transfection with *cripto* siRNA decreases invasion and metastasis in human colon cancer through MMP downregulation. Thus, targeting *cripto* for molecular intervention may be an attractive therapeutic strategy for colon cancer. Many studies have proven that RNAi technology siRNA can be used successfully for gene silencing *in vivo*<sup>[25,26]</sup>. Thus, the application of RNAi mediated by siRNA to knock down *cripto* expression in colon cancer may prove to be a valuable strategy for patients with advanced colon cancer.

## COMMENTS

### Background

The *cripto* gene is always overexpressed in most malignant solid tumors, including colon, breast, lung, ovarian, and pancreatic cancers. Recent reports showed that *cripto* overexpression may very well be closely related to invasion and metastasis in a few of human cancers, including cervical carcinoma and breast cancer, but the clinicopathologic and biological significance of *cripto* overexpression in human colon cancer remains unclear.

### Research frontiers

To explore the clinicopathologic and biological significance of *cripto* as a novel target for colon cancer gene therapy, pathological and *in vitro* studies were carried out using RNA interference (RNAi). The results suggest that the *cripto* gene may be useful for devising novel preventive and therapeutic strategies for colon cancer.

### Innovations and breakthroughs

The results showed that RNAi *cripto* can decrease cell migration and invasion by inhibiting matrix metalloproteinases.

### Applications

The results here suggested that the detection of *cripto* is helpful in understanding the development of colorectal carcinoma, and that *cripto* siRNA could be an effective approach for the inhibition of invasion and migration of human colon cancer.

### Terminology

RNAi's main function is to adjust and shut down gene expression and regulate all kinds of activities of cells. Small interfering RNA (siRNA) is generated within the cell in the process of RNAi, which consists of fragments of about 21-25 nuclear acids of the double-stranded RNA molecule.

### Peer review

The biological significance of *cripto* in the occurrence and development of colorectal carcinoma *in vivo*, *in vitro* were investigated. The results showed that *cripto* was significantly overexpressed in primary colon cancer and metastatic lymph nodes. Furthermore, the molecular mechanism of invasion and the regulation of *cripto* gene expression in colon cancer cells were explored by RNA interference technology. The study found, MMP-7 and -9 gene is involved in the regulation of *cripto* invasion of colorectal cancer cells. There is important innovation and scientific significance of the study.

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## Randomized trial in malignant biliary obstruction: Plastic vs partially covered metal stents

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tially covered self-expandable metal stent (pcSEMS) to plastic stent (PS) in patients treated for malignant, infrahilar biliary obstruction.

**METHODS:** Multicenter prospective randomized clinical trial with treatment allocation to a pcWallstent® (SEMS) or a 10 French PS. Palliative patients aged  $\geq 18$ , for infrahilar malignant biliary obstruction and a Karnofsky performance scale index  $> 60\%$  from 6 participating North American university centers. Primary endpoint was time to stent failure, with secondary outcomes of death, adverse events, Karnofsky performance score and short-form-36 scale administered on a three-monthly basis for up to 2 years. Survival analyses were performed for stent failure and death, with Cox proportional hazards regression models to determine significant predictive characteristics.

**RESULTS:** Eighty-five patients were accrued over 37 mo, 42 were randomized to the SEMS group and 83 patients were available for analyses. Time to stent failure was  $385.3 \pm 52.5$  d in the SEMS and  $153.3 \pm 19.8$  d in the PS group,  $P = 0.006$ . Time to death did not differ between groups ( $192.3 \pm 23.4$  d for SEMS vs  $211.5 \pm 28.0$  d for PS,  $P = 0.70$ ). The only significant predictor was treatment allocation, relating to the time to stent failure ( $P = 0.01$ ). Amongst other measured outcomes, only cholangitis differed, being more common in the PS group ( $4.9\%$  vs  $24.5\%$ ,  $P = 0.029$ ). The small number of patients in follow-up limits longitudinal assessments of performance and quality of life. From an initially planned 120 patients, only 85 patients were recruited.

**CONCLUSION:** Partially covered SEMS result in a longer duration till stent failure without increased complication rates, yet without accompanying measurable benefits in survival, performance, or quality of life.

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### Abstract

**AIM:** To compare efficacy and complications of par-

**Key words:** Randomized; Biliary; Obstruction; Stent; Plastic; Metal; Palliative; Common bile duct

**Core tip:** This randomized trial is one of very few comparing partially covered self-expandable metal stent (SEMS) to 10 French plastic stent (PS) in the temporary palliation of malignant biliary obstruction. In 85 patients, time to stent failure was significantly longer ( $385.3 \pm 52.5$  d) in SEMS vs PS ( $153.3 \pm 19.8$  d),  $P = 0.006$ . Time to death did not differ ( $192.3 \pm 23.4$  d for SEMS vs  $211.5 \pm 28.0$  d for PS,  $P = 0.70$ ). Amongst other measured outcomes, only cholangitis differed and was more common in PS (4.9% vs 24.5%,  $P = 0.029$ ).

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## INTRODUCTION

Malignant obstructive jaundice is associated with many symptoms that negatively impact quality of life such as anorexia, pruritus and malabsorption<sup>[1-3]</sup>. Endoscopic retrograde cholangio-pancreaticography (ERCP) with placement of a biliary stent is the procedure of choice for palliation of infrahilar common bile duct (CBD) malignant biliary obstruction<sup>[4]</sup>.

Both plastic and self-expandable metallic stents can palliate malignant biliary obstruction, and although randomized trial data have shown uncovered metallic stents to remain patent for longer periods compared to plastic stents<sup>[3]</sup>, the latter remain widely utilized<sup>[3,5,6]</sup> at least in part due to their lower upfront costs. The more recently introduced covered self-expandable metallic stents remain poorly studied in a randomized clinical trial setting, and may be associated with added complications such as pancreatitis and cholecystitis, as well as stent migration. This holds true for both fully covered and partially covered stents<sup>[7-15]</sup>.

The primary aim of our study was thus to compare the stent patency's of a partially covered metal stent and a commonly used plastic stent in a randomized controlled trial for patients with low to mid-CBD malignant biliary obstruction. We additionally sought to better characterize the safety of the partially covered metal stent and attempted to identify clinical variables that would allow clinicians to choose a metallic or plastic biliary stent.

## MATERIALS AND METHODS

### Study design and randomization

The study was a randomized clinical trial. Randomization

was performed using sealed envelopes in which patients were allocated in a 1:1 proportion to either a partially covered Wallstent<sup>®</sup> Endoscopic Biliary Endoprosthesis with Permalume<sup>™</sup> covering comprised of two components: the implantable metallic stent and the Unistep<sup>™</sup> Plus Delivery System, (Boston Scientific, Natick, MA, United States) (self-expandable metal stent, SEMS), or a 10 French (Fr) Amsterdam-type polyethylene plastic stent (PS) biliary stent. The sealed envelopes were opened only at the time of intent of stent insertion in the ERCP suite after confirmation that all selection criteria had been fulfilled. The allocation sequence was performed centrally and patient enrolment and participant assignment was carried out by a third party not directly involved with the patient's care or the measurement of outcomes. Neither patient, treating team, or the evaluators of outcomes were blinded to treatment allocation due to the nature of the intervention and follow-up care required. Each investigator received written approval for the study from his respective Institutional Review Boards prior to study initiation and patient enrollment. The trial did not require prior registration as it was started before 2004 and both stents are FDA approved.

### Study population

Inclusion criteria were age 18 or older, and the provision of a signed written voluntary informed consent form approved by the Institutional Review Boards at participating centers. All patients demonstrated laboratory, imaging and/or histological evidence of malignant biliary obstruction. The cause of obstruction could be any intrinsic or extrinsic malignancy extending no more proximal than 1cm below the common hepatic ductal bifurcation. A Karnofsky performance scale was applied. A Karnofsky score  $> 60\%$  is a validated measure of patient function, previously used in Pancreatico-biliary cancer patients<sup>[16]</sup>. Patients were required to have anticipated life expectancy that would allow for completion of full follow-up. Exclusion criteria were jaundice related to intrahepatic cholestasis or obstruction, or a prior attempt at a curative surgical resection for the biliary obstructing lesion. There were 6 participating North American university centers (Fletcher Allen Health Care at the University of Vermont, McGill University Health Centre, St. Elizabeth's Medical Center, Dartmouth Hitchcock Medical Center, Duke University Medical Center, and Thomas Jefferson University).

### Outcome measures

The primary endpoint of the trial was the time to occurrence of stent failure as defined by the appearance of one or more cholestatic symptoms accompanied by a 50% increase in bilirubin from the lowest post-stent insertion value recorded prior to this follow-up event, and/or cholangitis (defined as the new onset of pain, fever, and jaundice) whether associated with a stent replacement or not. Repeat ERCP for stent replacement or suspected obstruction using a stent of any type was con-

sidered to represent a stent failure. Secondary outcomes were death, cholestatic symptoms, laboratory data, technical success (defined as the successful delivery and deployment of the initial stent to the desired location in the biliary tree) measured at the time of the procedure, the presence of adverse events, and the Karnofsky performance score. We also administered the short form (SF)-36 general quality of life measurement scale, which is a questionnaire measuring patient's perceptions about functional health and well-being previously administered to bilio-pancreatic cancer patients<sup>[1]</sup>.

### Interventions and follow-up management

ERCP was performed by experienced endoscopists; stents were placed with or without prior dilatation or sphincterotomy after sealed-envelope randomization to stent type was done after confirmation of obstruction meeting inclusion criteria. The patient then received either the SEMS or the PS. The length of each type of stent was determined by the biliary anatomy and left to the discretion of the endoscopist as part of the medical effectiveness philosophy of the trial thereby enhancing generalizability of the results. A cholangiogram was performed to document stent patency and position. No prophylactic antibiotics were used. Each patient had one- and three-month follow-up, followed by quarterly scheduled follow-up sessions up to 2 years following stent insertion.

Failed plastic stents were replaced with covered metal stents, while failed covered metal stents were replaced with either one or more plastic or metal stent(s) inserted through the metal stent. The decision for the choice of stent type following stent failure was left to the discretion of the endoscopist and recorded.

### Data collection

Dedicated, standardized electronic case report forms were completed by trained research assistants and downloaded into a web-based remote data entry repository. Internal validity of recorded data and missing data quality checks were performed centrally by trained research personnel. At baseline, investigation variables included any significant medical history, the tumor type, stage, and location, the date of diagnosis, the length and maximum diameter of stricture, and administration of any prior anticancer treatment, as well as the Karnofsky Score. Variables assessed at baseline and at periodic follow-up visits (months 1, 3, 6, 9, 12, and if the patient survived, 3-monthly up to month 24) following the index procedure included a cholestatic symptom assessment, the use of any adjuvant treatment such as radiation and/or chemotherapy, laboratory test results (chemistry, hematology), and the Karnofsky index. All adverse events were recorded, including the occurrences of cholangitis, pancreatitis, and cholecystitis using standardized definitions<sup>[17]</sup>.

### Sample size calculation

The planned enrollment was 120 patients. Sample size

predictions were calculated using a model of binomial proportions and independent samples. Assuming a 25% improvement in stent patency duration with expandable metallic stenting and using a 1-sided type I error rate of 5% and a type II error rate of 20%, approximately 60 patients were thought to be needed in each group.

### Statistical analysis

Amongst descriptive variables, continuous variables are reported as means and standard deviations as well as medians where appropriate, and categorical variables as proportions. Inferential testing was carried out using *t*-tests for continuous and  $\chi^2$  for categorical variables. Karnofsky scores and quality of life scores were assessed for both intra- and between-group differences comparing baseline values to the last visit on record at the 1-mo, the 3-mo visit, and the 6-mo visits; both within and across groups. We used a *t*-test with either the pooled or Satterthwaite method, depending on the results of the equality of variances test at each follow-up period.

Survival analyses were performed for both stent failure and patient survival using both intention-to-treat (ITT) and per protocol (PP) analyses. In the first group, only subjects who had at least a 50% drop in bilirubin at 1 mo were included. In the second, all subjects were included as originally randomized. Kaplan-Meier curves were created for the SEMS and PS groups, and compared with a log-rank test. Cox proportional hazards regression models were also used to determine if significant covariates were associated with either time-to-stent failure or time-to-death. The proportional hazards assumption was tested with the use of a Kolmogorov-Smirnov Supremum test. The following covariates were included for these analyses in addition to stent randomization group: obstruction (for prediction of mortality), tumor type, known metastatic cancer, chemotherapy or radiation therapy, and the baseline Karnofsky score. Covariates that were associated with the outcome with a *P*-value of 0.15 or less in a univariate model were entered into a multivariable model. There was no planned interim analysis.

## RESULTS

### Patient population

A total of 85 patients were accrued over 37 mo. The study was closed prior to completion of enrollment of the estimated 120 patients due to a marked slowing of patient accrual (trial fatigue). Of the 85 patients included, 42 were randomized to the SEMS group, and 43 to the PS group. Three patients had evaluable baseline patient data but were excluded from further analyses because of inclusion protocol violations (2 were never stented, and one received a metal covered stent when in fact randomized to plastic stent). Of the 82 patients with analyzable outcomes data, 41 received a SEMS and 41 a PS; the CONSORT diagram is shown in Figure 1. Population characteristics at baseline for both groups are shown in Table 1.



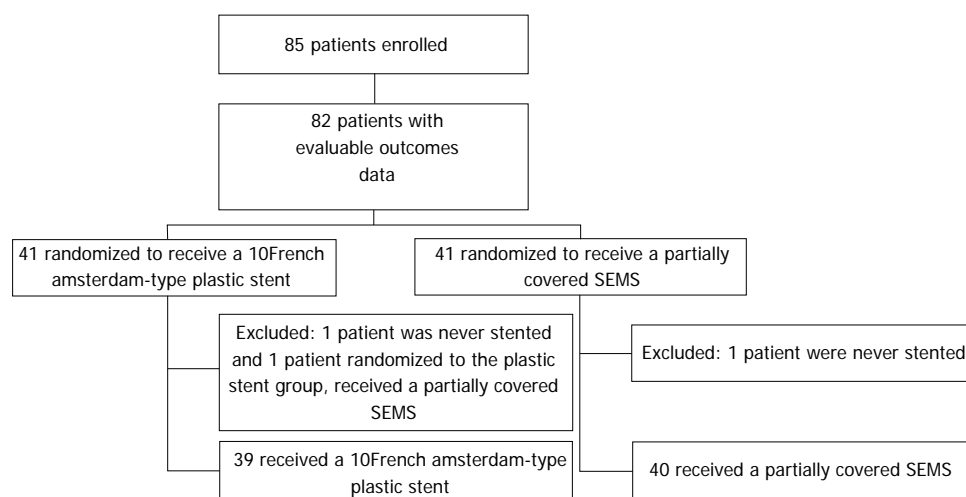


Figure 1 Consolidated standards of reporting trials diagram. SEMS: Self-expandable metal stent.

Twenty-nine point eight percent were inpatients; amongst these, the mean hospital stays related to the procedure were  $2.5 \pm 1.6$  d in the SEMS group, and  $4.9 \pm 4.7$  d in the PS group.

The mean length of Wallstents used was  $61.4 \pm 11.2$  mm (median: 60 mm, range: 40–80 mm); 95.1% patients received a 10Fr diameter and 4.9% an 8F diameter. In the PC group, the stent length was  $76.0 \pm 18.2$  mm (median: 70, range: 50–120 mm); all patients had a 10Fr diameter.

### Primary outcome results

In ITT analysis, the time to stent failure was  $385.3 \pm 52.5$  d in the SEMS and  $153.3 \pm 19.8$  d in the PS group ( $P = 0.006$ ) (Figure 2A). Corresponding results were  $396.5 \pm 56.8$  d and  $164.3 \pm 24.1$  d, respectively ( $P = 0.025$ ) using a PP approach. After adjustment for possible confounding variables, in ITT analysis, the only independent significant predictor of a failed stent was the stent group allocation (HR = 0.29, 95%CI: 0.12–0.75,  $P = 0.011$ ); similar findings were noted with the PP analysis (HR = 0.22, 95%CI: 0.06–0.80,  $P = 0.013$ )

### Secondary outcome

**Procedural outcomes:** No differences in intra-procedural events were noted. Overall, 69.4% of all stent insertions were carried out in an out-patient setting. Optimal stent insertion and positioning was noted in 95.3% of patient with in the SEMS and 97.4% of patients in the PS groups, respectively. The length of the SEMS used was  $61.4 \pm 11.2$  mm (median: 60 mm, range: 40–80 mm) and a diameter of  $59.64 \pm 11.2$  mm (median: 60 mm, range: 40–80 mm); 95.1% patients received a 10Fr diameter and 4.9% a 8F diameter. In the PC group, the stent length was  $76.0 \pm 18.2$  mm (median: 70 mm, range: 50–120 mm); All patients had a 10Fr diameter. Sphincterotomy was carried out prior to stent insertion in 18.8% of cases, and balloon dilatation in 3.5%. The distal end of the stent was positioned outside the CBD into the duodenum in

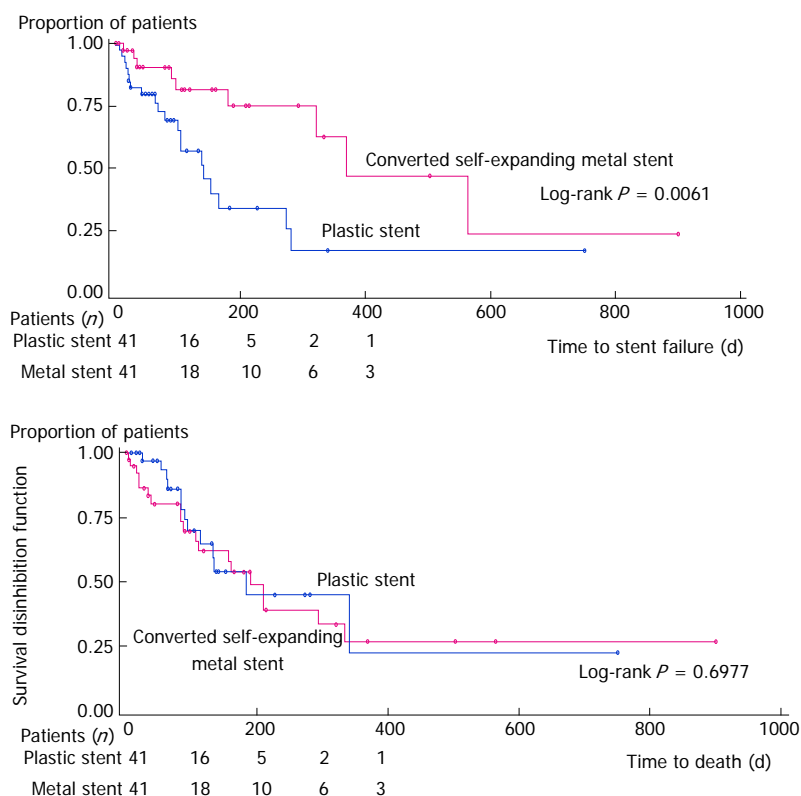
93.0%. Some form of tissue sampling was carried out at the time of ERCP in 56.0% of patients.

**Time to death:** The time to death did not differ between both groups:  $192.3 \pm 23.4$  d for SEMS *vs*  $211.5 \pm 28.0$  d for PS ( $P = 0.70$ ) using an ITT approach (Figure 2B). Similar conclusions were reached using the PP approach  $248.5 \pm 26.8$  d *vs*  $251.3 \pm 32.5$  d, respectively ( $P = 0.66$ ). After adjustment for possible confounding variables, no significant predictor of time to death was found in ITT or PP analysis.

**Additional secondary outcomes:** Complications including the development of pancreatitis, cholangitis, and cholecystitis are (2.4% *vs* 2.4%,  $P = 1.0000$ ; 4.9% *vs* 24.5%,  $P = 0.029$ ; 4.8% *vs* 0.0%,  $P = 0.4741$ ). Only cholangitis differed, with a greater frequency in the PS group.

The percentage reduction in bilirubin value from baseline to the 1-mo visit was no different in SEMS than in the PS group [74.0%, (95%CI: 60.0–87.9) *vs* 63.7% (95%CI: 45.5–81.9), respectively,  $P = 0.37$ ]. No statistical differences in Karnofsky performance scores were noted between the two treatment groups when comparing the differences in scores for the last, 1, 3, and 6 mo visits compared to baseline. In the pre-planned paired analysis to assess intra-group differences, patients receiving the SEMS, showed significant improvements noted at 6 mo and at the last available visit ( $P = 0.015$ , and  $P = 0.022$ , respectively). There were also significant improvements noted for patients in the PS group compared to baseline both at 1 mo and at the last available date of follow-up ( $P = 0.045$ , and  $P = 0.0014$ , respectively; full data available upon request).

Overall, 29.4% of patients had one or more cholestatic symptoms at follow-up, 24.3% for the SEMS group and 33.3% for the PS group ( $P = 0.213$ ). Additional symptom reporting showed no difference between both groups with regards to individual cholestatic symptoms (data not shown).



**Figure 2** Survival analysis. A: Time to stent failure for partially covered self-expanding metal stent and plastic stent patient groups; B: Time to death for partially covered self-expanding metal stent and plastic stent patient groups.

**Quality of life-SF-36 measures:** Seventy-four patients answered the quality of life questionnaires over a total of 174 visits during a 12-mo follow-up (31 patients answered only once to the SF-36 questionnaire, 17 answered to two questionnaire and 13 responded to 3 questionnaires). Among these, 38 had received a SEMS and 36 a PS. At baseline, patients in the SEMS group exhibited lower means than those in the PS group for all 8 summary scores, indicating worse quality of life parameters; the differences however were not statistically significant except for physical functioning (46.4 *vs* 63.9,  $P = 0.008$ ). The SEMS group scores improved gradually such that, by 9 mo, most were arithmetically greater than scores from the PS group although without significant differences. There remained, however, only a very small number of patients able to complete the questionnaires in follow-up (9 patients at 9 mo, and 5 at 12 mo). In paired analysis, statistical significant improvements were noted amongst SEMS patients in physical functioning (6 mo *vs* baseline), and vitality (1 mo *vs* baseline). Significant bettering of quality of life was noted amongst PS patients at 1 month *vs* baseline for bodily pain, social functioning, and mental health, as well as in vitality for the 9 mo *vs* baseline comparison (full quality of life scores are available upon request).

## DISCUSSION

Stenting for malignant biliary obstruction remains prin-

cipally a palliative procedure<sup>[6,7]</sup>; temporary stenting until the time of exploratory or potentially curative surgery is performed (with the advent of useful adjuvant treatment methods), although the efficacy of this approach remains unproven and may in fact be harmful<sup>[18-20]</sup>. RCT data have suggested the superiority of uncovered metal over plastic biliary stenting<sup>3</sup> owing to the larger internal luminal diameter, thus preventing premature blockage from bacterial biofilm encrustation and sludge formation<sup>[21]</sup>. Indeed, a Cochrane meta-analysis of 5 trials by Moss *et al*<sup>[7]</sup> concluded that uncovered metal stents had a lower risk of recurrent biliary obstruction than plastic stents (RR = 0.52, 95%CI: 0.39-0.69), with no difference in technical or therapeutic success, complications or 30-d mortality. An additional trial performed since, also confirmed the superiority of uncovered SEMS over Tannenbaum plastic stents<sup>[22]</sup>, a plastic stent variant without side holes that may contribute to prolonged plastic stent patency.

Although these trials assessed uncovered metal biliary stents, these conclusions were largely presumed to be generalizable to (partially and completely) covered metal stents, and probably is the reason for a paucity of studies examining this latter comparison. This assumption, however, can be questioned and is of contemporary significance for two reasons: (1) plastic biliary stents remain very commonly inserted as initial method of stenting in the face of increasing use of covered and uncovered metal stents for non hilar biliary obstruction<sup>[6]</sup>; and (2)

**Table 1 Patient characteristics at baseline**

Characteristic		Partially covered SEMS (n = 42)	10-French polyethylene plastic stent (n = 43)	P value
Gender (male)		51.2%	50.0%	0.9119
Age (yr)		70.8 ± 12.9	73.3 ± 10.7	0.3896
Co-morbidities:	Cardiovascular	53.7%	47.5%	0.5676
	Respiratory	22.0%	20.0%	0.8209
	Neurologic	19.5%	22.5%	0.7343
	GI, liver, biliary	75.6%	77.5%	0.8362
	Renal, urinary	15.0%	30.0%	0.0982
	Musculoskeletal	25.0%	35.0%	0.3148
	Endocrine	47.5%	30.0%	0.0976
Cholestatic symptoms	Jaundice	97.5%	100.0%	0.2968
	Clay-colored stools	85.4%	97.5%	0.0453
	Abdominal pain	36.6%	52.5%	0.1404
Abdominal pain	Abdominal pain	53.7%	40.0%	0.2056
Pruritus		51.2%	50.0%	0.9119
Dark urine		75.6%	75.0%	0.9489
Fever	Fever	9.8%	5.0%	0.3972
Constitutional symptoms	Weight Loss	73.2%	47.5%	0.0155
	Anorexia	51.2%	50.0%	0.9119
	Papilla	2.78%	7.9%	0.2951
Obstruction location	Distal common bile duct	72.2%	47.4%	0.0198
	Mid common bile duct	22.2%	39.5%	0.0845
	Proximal common bile duct	2.8%	5.3%	0.5595
Type of primary tumor	Ampullary carcinoma	2.6%	7.5%	0.3036
	Cholangiocarcinoma	0.0%	5.0%	0.1422
	Gallbladder adenocarcinoma	2.6%	2.5%	0.9767
	Metastatic Cancer	10.3%	7.5%	0.6501
	Pancreatic adenocarcinoma	69.2%	67.5%	0.8662
	Other	0.0%	2.5%	0.3024
	Unknown	15.4%	7.5%	0.2519
Metastat. cancer prim. location	Colon	28.6%	0.0%	0.0002
	Lung	28.6%	20.0%	0.355
	Other	42.9%	80.0%	0.0004
Tumor stage	T1	4.0%	19.2%	0.0292
	T2	32.0%	11.5%	0.0217
	T3	16.0%	42.3%	0.0077
	T4	48.0%	26.9%	0.0443
Nodes	N0	36.4%	61.9%	0.0187
	N1	63.6%	38.1%	0.0187
Metastatic tumor	M0	29.2%	36.0%	0.5038
	M1	70.8%	64.0%	0.5038
Chemotherapy or radiation		11.1%	8.82%	0.7255
Laboratory data	Alkaline phosphatase (IU/L)	630.5 ± 347.7	532.7 ± 331.4	0.1486
	Bilirubin (mg/dL)	9.56 ± 6.99	11.33 ± 7.82	0.3082
	Hematocrit	43.97% ± 50.36%	37.06% ± 5.95%	0.3280
	Hemoglobin (g/dL)	12.01 ± 1.63	12.39 ± 2.09	0.3305
	INR	1.17 ± 0.19	1.25 ± 0.36	0.5922
	AST (IU/L)	168.64 ± 98.34	191.26 ± 149.25	0.6687
Karnosky performance scores	ALT (IU/L)	240.03 ± 178.35	265.03 ± 240.48	0.8926
		81.8 ± 10.8	82.0 ± 12.03	0.9151

SEMS: Self-expandable metal stent; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alkaline phosphatase.

covered metal stents are reported to exhibit greater rates of migration, and perhaps other complications (such as cholecystitis and pancreatitis) compared to uncovered metal stents<sup>[5,11,23]</sup>. Both these realizations justify the aims of the current trial.

Only two randomized controlled trials have compared plastic to covered metal stents. In the multicenter trial by Isayama *et al.*<sup>[24]</sup>, investigators compared a covered metal biliary stent to a rarely used type of plastic stent with a double lumen (found to be superior to polyethylene stents with regards to stent patency<sup>[25]</sup>) in patients

with lower biliary malignant obstruction attributable to pancreatic head cancer. In the Isayama multicentric trial<sup>[24]</sup>, the cumulative stent patency was significantly greater in the covered metal stent group: the respective mean and median stent patency durations were 285 and 419 d, *vs* 202 and 133 d observed for the plastic stent group patients respectively ( $P = 0.0072$ ). Interestingly, the covered metal stent group experienced more frequent cholecystitis (4 *vs* 0), pancreatitis (1 *vs* 0), and migration (5 *vs* 1), although these differences did not, at least taken separately, achieve statistical significance.

These results validate the findings of the current trial that noted, using life-table analysis, that the time to stent failure was  $385.3 \pm 52.5$  d in the SEMS, and  $153.3 \pm 19.8$  d in the PS group ( $P = 0.006$ ). Times to stent occlusion were all shorter, although the between-group differences remained, in the only other randomized trial assessing plastic *vs* covered metal stents by Soderlund *et al*<sup>[26]</sup>. In that study, 22 of 51 plastic stent and 9 of 49 covered metal stent group patients ( $P = 0.009$ ) developed stent failure after medians of 1.1 and 3.5 mo ( $P = 0.007$ ), with median patency times of 1.8 mo *vs* 3.6 mo ( $P = 0.002$ ), respectively.

Even though insertion of a plastic stent is favored in patients with an estimated short survival, such as those with large tumors (over 30 mm), liver metastases, younger age, or adenocarcinoma histology<sup>[27-29]</sup>, summary RCT data have shown that infrahilar biliary stenting, either pre-operatively or as sole palliation, does not improve mortality<sup>[3,18,19]</sup>. Interestingly, however, an observational trial and an as yet unpublished additional meta-analysis have suggested improvement in survival using expandable metal stent technologies, yet remain unconfirmed<sup>[30,31]</sup>. Furthermore, while other such comparisons have failed to demonstrate such a benefit<sup>5</sup>, two trials have recently suggested a patient survival benefit with the percutaneous insertion of covered rather than uncovered metal stents for infrahilar biliary obstruction, due to pancreatic cancers<sup>[15]</sup> and cholangiocarcinomas<sup>[32]</sup>.

Despite difficulty in accrual leading to early termination of the study including 85 patients and not the projected 130 patients, the strengths of the current trial include the multi-institutional participation, the medical effectiveness design, and the adopted ITT analytical approach that all increase the generalizability of results. The *a priori* standardized definitions and independent measurements of outcomes all strengthen the validity, minimizing the chance of bias. Life table analysis and multivariable adjustment further ensure the clinical relevance of the findings. Other than improved stent patency, only the outcome of cholangitis differed amongst both groups, occurring more frequently in the plastic stent group. Cholecystitis was a rare outcome, as was pancreatitis, and the trial was not powered to demonstrate any differences in these less frequent endpoints. No differences in procedural outcomes were noted. The between-group quality of life comparisons were limited by the small number of survivors, yet the pre-post stenting analyses within each group using paired analyses confirm what few trials have shown: that a number of quality of life domains improve following successful biliary drainage<sup>[1,33,34]</sup>, and that such benefits were observed both with metal and plastic stents. Post-stent insertion improvements in functional status using Karnovsky scores were also noted at 1 mo and extended to the last available patient visit recorded.

These results, taken as a whole, suggest that the observed benefits of the SEMS studied over the common type of PS used as comparator are attributable to the

prolonged stent patency. The resulting decreased rates of cholangitis outweigh any possible risks attributable to increased stent migration, pancreatitis or cholecystitis. Further characterization of optimal patient groups may relate to issues such as cystic duct involvement that predicts cholecystitis<sup>[23,35]</sup>.

Perhaps just as relevant as efficacy findings are cost-effectiveness issues that are being analyzed separately as part of the current trial. Indeed, past cost-effectiveness modeling have suggested that the presence of distant metastases, especially if numerous, is associated with shorter survival time in patients with pancreatic cancer, and that a metallic stent should not be used in this type of patients<sup>[27,36,37]</sup>. Rather, SEMS should be reserved for patients expected to live for at least 6 mo<sup>[38,39]</sup>. Nonetheless, as mentioned earlier, plastic stents remain commonly inserted, at least in part due to their lower upfront costs compared to their metal expandable equivalents.

In conclusion, the present study confirms that insertion of a partially covered SEMS for patients with infrahilar biliary obstructing tumors results in a longer duration until stent failure as compared to a commonly used plastic stent (in this case, an Amsterdam-type polyethylene stent) without increased complication rates. There were no measurable benefits in survival, performance, or quality of life. Additional trials and meta-analytical evaluation are required to more confidently assess these important additional patient outcomes.

## COMMENTS

### Background

Despite existing evidence in the literature favoring metal over plastic stenting in distal biliary obstruction palliation, few data exist assessing partially covered metal stents, especially in a contemporary setting.

### Research frontiers

Plastic stent palliation remains widespread, while partially covered metal biliary stents appear to migrate more frequently than their uncovered counterparts, but have gained popularity.

### Innovations and breakthroughs

There have been 2 previously published studies in the literature that have compared partially covered metal to plastic biliary stenting with limited generalizability, suggesting the superiority of the metal stent alternative.

### Applications

The current article validates the conclusion that partially covered metal stents result in a significantly longer time to stent patency, without a prolonged survival time in patients undergoing palliation for distal biliary malignant obstruction. Additional and summary data are required to confirm the robustness of the latter finding, while the advent of completely covered metal stents require further assessments, owing to the theoretical benefit of decreased stent in growth versus the possible increased risk of stent migration.

### Peer review

The findings of this randomized trial appear to confirm that of two previous studies in the literature and is limited by the small sample size, even though a significant difference in the primary outcome measure was noted. The achieved statistical power limits the interpretation of other endpoints.

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## ***Clostridium difficile*-associated disease: Adherence with current guidelines at a tertiary medical center**

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### **Abstract**

**AIM:** To assess adherence with the the Society for Healthcare Epidemiology of America (SHEA)/ the Infectious Diseases Society of America (IDSA) guidelines for management of *Clostridium difficile* (*C. difficile*)-associated disease (CDAD) at a tertiary medical center.

**METHODS:** All positive *C. difficile* stool toxin assays in adults between May 2010 and May 2011 at the University of Maryland Medical Center were identified. CDAD episodes were classified as guideline adherent or non-adherent and these two groups were compared to determine demographic and clinical factors predictive of adherence. Logistic regression analysis was performed to assess the effect of multiple predictors on guideline adherence.

**RESULTS:** 320 positive *C. difficile* stool tests were identified in 290 patients. Stratified by disease severity

criteria set forth by the SHEA/IDSA guidelines, 42.2% of cases were mild-moderate, 48.1% severe, and 9.7% severe-complicated. Full adherence with the guidelines was observed in only 43.4% of cases. Adherence was 65.9% for mild-moderate CDAD, which was significantly better than in severe cases (25.3%) or severe-complicated cases (35.5%) ( $P < 0.001$ ). There was no difference in demographics, hospitalization, ICU exposure, recurrence or 30-d mortality between adherent and non-adherent groups. A multivariate model revealed significantly decreased adherence for severe or severe-complicated episodes (OR = 0.18, 95%CI: 0.11-0.30) and recurrent episodes (OR = 0.46, 95%CI: 0.23-0.95).

**CONCLUSION:** Overall adherence with the SHEA/IDSA guidelines for management of CDAD at a tertiary medical center was poor; this was most pronounced in severe, severe-complicated and recurrent cases. Educational interventions aimed at improving guideline adherence are warranted.

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**Key words:** *Clostridium difficile*; Metronidazole; Vancomycin; Adherence to the Infectious Diseases Society of America Guidelines; Hospital Acquired Infections

**Core tip:** This study assesses a tertiary care medical center's adherence with updated guidelines on the management of *Clostridium difficile* (*C. difficile*)-associated diseases in adults. We found that overall adherence is poor, especially in patients with severe disease. Factors associated with poor adherence and limitations of current guidelines are identified. Our data suggests that educational interventions aimed at improving *C. difficile* guideline adherence are warranted.

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guidelines at a tertiary medical center. *World J Gastroenterol* 2013; 19(46): 8647-8651 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i46/8647.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8647>

## INTRODUCTION

*Clostridium difficile* (*C. difficile*) is the major infectious cause of nosocomial diarrhea and can cause prolonged hospital stays, renal failure, toxic megacolon, and death<sup>[1-3]</sup>. In 1995, the Society for Healthcare Epidemiology of America (SHEA) published a clinical position paper on *C. difficile*-associated disease (CDAD)<sup>[4]</sup>. Based on data from small, randomized, controlled studies showing no outcome-difference when comparing metronidazole and vancomycin, the 1995 position paper considered them equally effective; however, it stated, “metronidazole may be preferred to reduce the risk of vancomycin resistance among other organisms in hospitals”.

Updated clinical practice guidelines for the management of CDAD in adults were published in 2010 by SHEA and the Infectious Diseases Society of America (IDSA)<sup>[5]</sup>. The 15-year interval between the two sets of recommendations was marked by dramatic changes in CDAD epidemiology and outcomes, with increases in prevalence, severity, and therapy resistance; emergence of hypervirulent strains may have contributed to these trends<sup>[6-8]</sup>. Additionally, new data suggested vancomycin might be superior for CDAD treatment in some cases. Zar *et al*<sup>[9]</sup> prospective, randomized, comparative efficacy study of metronidazole *vs* vancomycin demonstrated superiority of vancomycin for the treatment of severe CDAD. These results influenced the 2010 SHEA/IDSA guidelines that recommended vancomycin as first-line treatment for severe CDAD, while maintaining a recommendation for metronidazole in mild-moderate cases. These guidelines recommend treating an initial recurrence in the same manner as the initial episode, and a second recurrence with vancomycin in a tapering and/or pulsed regimen<sup>[5]</sup>.

The 2010 SHEA /IDSA recommendations promote significant clinical practice changes. Since adherence to the guidelines may affect patient outcomes and infection control, we sought to determine adherence with the updated SHEA/IDSA CDAD guidelines at a tertiary care medical center.

## MATERIALS AND METHODS

The Institutional Review Board of the University of Maryland Baltimore approved this study and waived the requirement for informed consent. All positive *C. difficile* stool tests (Quick Check A/B Toxin Assay; Wampole Laboratories, Princeton, New Jersey) in adults between May 2010 and May 2011 at the University of Maryland Medical Center were retrospectively identified. Medical charts were reviewed for demographics, clinical informa-

tion, and adherence to CDAD guidelines.

Classifications defined in the updated 2010 SHEA/IDSA guidelines were used. These guidelines define mild-moderate CDAD as the presence of a white blood cell count  $\leq 15000/\text{mm}^3$  and a serum creatinine level  $\leq 1.5$  times the premorbid level. Conversely, severe CDAD is defined by the presence of a white blood cell count  $\geq 15000/\text{mm}^3$  or a serum creatinine level  $\geq 1.5$  times the premorbid level. Severe-complicated CDAD is defined by the presence of hypotension, shock, ileus, or megacolon. According to the guidelines, the correct treatment for mild-moderate CDAD is metronidazole 500 mg orally three times per day for 10-14 d. For treatment of severe CDAD, recommended treatment is oral vancomycin 125 mg four times per day for 10-14 d. For severe-complicated CDAD, the recommended treatment is oral vancomycin 500 mg four times per day in addition to intravenous metronidazole 500 mg every eight hours. If complete ileus exists, then rectal administration of vancomycin should be considered. Patients with a first recurrence are recommended to receive the same treatment as per their initial episode. For a second recurrence, vancomycin in a tapered and/or pulsed regimen is recommended.

Specific data collected included age, gender, disease severity as defined by the 2010 SHEA/IDSA guidelines, location of treatment (stratified into outpatient, hospital ward or intensive care unit), non-CDAD antibiotic treatment during the month preceding diagnosis, presence of immunosuppression, if the episode was a recurrence, 30 d mortality, and agent selection and dosage of CDAD treatment. CDAD episodes were classified as guideline adherent if treatment provided was with the correct agent(s) at the correct dosage(s). If one of these parameters was not in accordance with the guidelines, then the treatment regimen was deemed non-adherent. Partial adherence was defined as the patient receiving the correct antibiotic, but at the wrong dose. Patients stratified into adherent and non-adherent groups were compared to determine demographics and clinical factors predictive of guideline adherence. Logistic regression analysis was performed to assess the effect of multiple predictors on guideline adherence (SAS, version 9.2).

## RESULTS

About 320 positive *C. difficile* stool tests were identified in 290 patients (average age 57.6 years, 43.1% female). Of the cases, 95.9% were in hospitalized patients and 15.6% were identified as a recurrence. Stratified by disease severity criteria set forth by the SHEA/IDSA guidelines, 42.2% of cases were mild-moderate, 48.1% severe, and 9.7% severe-complicated. Most (80.6%) of the severe-complicated cases met this criterion due to hypotension or shock. Full adherence with the guidelines was observed in 43.4% of cases; 65.9% for mild-moderate, which was significantly better than in severe (25.3%) and severe-complicated cases (35.5%) ( $P < 0.001$ ) (Figure 1). Of the severe CDAD cases, 55.3% were managed incor-



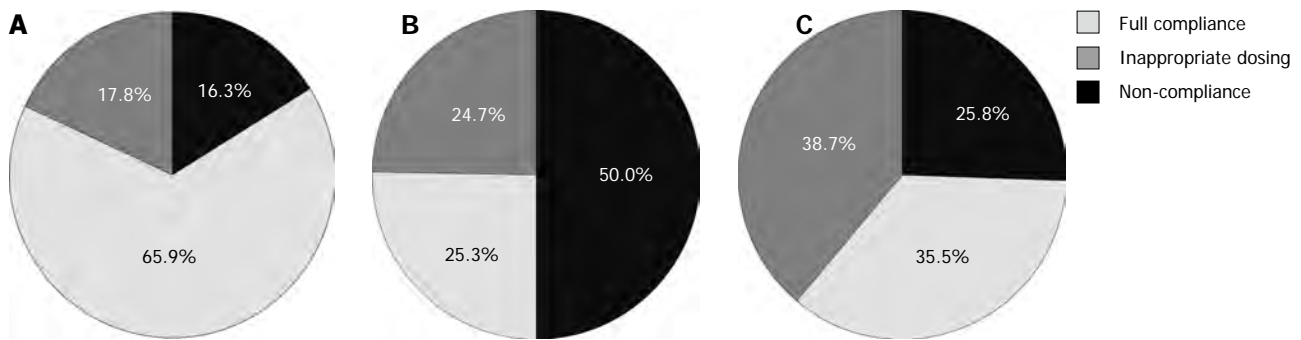


Figure 1 Rates of adherence with the 2010 the Society for Healthcare Epidemiology of America/the Infectious Diseases Society of America guidelines stratified by severity of *Clostridium difficile*-associated disease: (A) Mild-moderate, (B) Severe, and (C) Severe-complicated. Compliance was significantly better in mild-moderate vs severe or severe-complicated disease,  $P < 0.001$ .

**Table 1 Comparison of demographics, disease severity, and other clinical factors between guideline adherent and guideline non-adherent groups  $n$  (%)**

	Guideline Compliant, $n = 139$	Guideline Non-compliant, $n = 181$	Unadjusted $P$ value	Adjusted $P$ value
Demographics				
Mean $\pm$ SD, yr	56.8 $\pm$ 14.1	59.4 $\pm$ 16.2	0.13	
Female	61 (44.2)	77 (55.8)	0.81	
Disease severity				
Mild-moderate	89 (65.9)	46 (34.1)	$< 0.001^1$	$< 0.001^1$
Severe	39 (25.3)	115 (74.7)		
Severe-complicated	11 (35.5)	20 (64.5)		
Severe + severe-complicated	50 (27.0)	135 (73.0)		
Other factors				
Hospitalized	133 (43.3)	174 (56.7)	0.84	
ICU	60 (40.0)	90 (60.0)	0.24	
Prior antibiotics ( $< 30$ d)	88 (39.1)	137 (60.9)	0.02	0.08
Recurrence	17 (34.0)	33 (66.0)	0.14	0.04
Immunosuppressed	57 (51.8)	53 (49.2)	0.03	0.49
30-d mortality	15 (41.7)	21 (58.3)	0.82	

<sup>1</sup>On unadjusted analysis, mild-moderate disease is compared to both severe and severe complicated disease. On adjusted analysis, mild-moderate disease is compared to the combination of severe and severe-complicated disease.

rectly with metronidazole. Partial adherence, where the correct drug was given at the incorrect dose, occurred in 17.8% of mild-moderate, 24.7% of severe, and 38.7% of severe-complicated cases (Figure 1).

On bivariate analysis (Table 1), factors significantly associated with adherence included disease severity, immunosuppression (IS), and documented receipt of antibiotics in the preceding 30 d. There was no difference in age, gender, hospitalization, ICU exposure, recurrence or 30-d mortality between adherent and non-adherent groups. IS patients were classified as mild-moderate more often than non-IS patients (60.0% vs 32.9%,  $P < 0.001$ ). A multivariate model controlling for disease severity, prior antibiotics, IS, and recurrence status revealed significantly decreased adherence for severe/severe-complicated episodes (OR = 0.18, 95%CI: 0.11-0.30) and recurrent episodes (OR = 0.46, 95%CI: 0.23-0.95) but no significant

difference for prior antibiotics or IS status.

## DISCUSSION

Our results reveal poor overall adherence with the 2010 SHEA/IDSA guidelines for management of CDAD at a tertiary care academic medical center. Guideline adherence is worst in severe, severe-complicated, and recurrent CDAD. Our data suggests a lack of familiarity with current guidelines, as most providers continue to treat all initial episodes of CDAD with metronidazole, which was suggested as preferable by the 1995 SHEA clinical position paper on CDAD management. In fact, over half of our severe CDAD population, which should be treated with vancomycin, was incorrectly treated with metronidazole. This also explains the significantly improved adherence observed in mild-moderate patients whose treatment was not changed by the updated guidelines. We considered other possible causes of guideline non-adherence, such as the high cost of vancomycin and concern for vancomycin-resistance in other organisms, which has been shown to be significant in other nosocomial settings<sup>[10,11]</sup>. While the cost of branded oral vancomycin is approximately fifty-fold higher than oral metronidazole, our pharmacy routinely administers the generic intravenous formulation orally, which reduces the cost-difference dramatically<sup>[12]</sup>, and makes cost concerns negligible. This finding also suggests an increased need for more intensive antibiotic stewardship, as not all incidences of non-adherence are likely due to knowledge. Antibiotic stewardship has been proposed as an effective method of increasing compliance at medical centers<sup>[13-15]</sup>. The exact impact of concerns over vancomycin resistance in other organisms on prescribing practices at our institution is unknown. We suspect this impact is small, as research on vancomycin use for CDAD has been conflicting with regards to rates of colonization and infection with resistant organisms<sup>[16-18]</sup>.

Partial adherence with the guidelines, where the correct drug was chosen but an incorrect dosage was administered, occurred frequently as noted in Figure 1. The dosage of vancomycin chosen was often higher than recommended by the guidelines. While this is a form of

non-adherence, it may be appropriate, as patients given the recommended dosage (125 mg four times daily) can have low fecal levels during the first day of treatment<sup>[19]</sup>. Additionally, one would expect a higher dose to be equally effective and, given poor systemic absorption, little difference in side effects.

Interestingly, we found clusters of cases in specific wards of the hospital, such as the Trauma Unit or the Cancer Center, suggesting nosocomial spread of the infection, which is a major source of morbidity, mortality, and cost from the disease<sup>[20-22]</sup>. This finding highlights the need for improved hand hygiene and infection control measures such as contact precautions.

Two patient populations expose limitations of applying the SHEA/IDSA guidelines: immunosuppressed patients and patients with end-stage renal disease. As noted in our results, immunosuppressed patients were significantly more likely to have their disease severity classified as mild-moderate. This may reflect an inability to mount a severe-defining white blood cell count. The frequent incidence of neutropenia among cancer patients in particular suggests a limitation of applying the guidelines to this group. Similarly, the end-stage renal disease population, in whom serum creatinine fluctuations cannot be used as indicators of disease severity, present a problem with applying the SHEA/IDSA guidelines.

The present work has limitations. Our study was retrospective, took place in a single tertiary medical center, and 95.9% of CDAD cases were in hospitalized patients. Therefore, our results may not be applicable to community hospitals or outpatients. Additionally, our study was not powered to detect differences in 30-d mortality. Our institution was also using a toxin assay for diagnosis at the time of data collection, which is less sensitive than PCR<sup>[23,24]</sup>. Furthermore, the study by Zar *et al*<sup>[9]</sup> that was used to develop the treatment guidelines has had questions raised with regards to its methodology<sup>[25,26]</sup>. Further studies that directly survey providers about their treatment practices and measure the effects of guideline adherence on mortality and morbidity factors such as length of stay and complications of CDAD are warranted.

In conclusion, our results suggest that many providers are unfamiliar with the 2010 SHEA/IDSA *C. difficile* guidelines. Educational interventions and antibiotic stewardship may prove beneficial to improve adherence, and potentially patient outcomes.

## COMMENTS

### Background

*Clostridium difficile* (*C. difficile*) remains a major cause of nosocomial diarrhea, resulting in prolonged hospital stays, renal failure, toxic megacolon, and death. This also contributes to a significant economic burden on medical facilities across the globe. While the number of nosocomial infections increases, the correct treatment for this disease is of paramount importance.

### Research frontiers

In 1995, the Society for Healthcare Epidemiology of America (SHEA) published guidelines that suggested that Metronidazole is the preferred agent for treatment of *C. difficile*. Recently, updated clinical practice guidelines for the management of *C. difficile* colitis have been published by SHEA and the Infectious Diseases So-

ciety of America (IDSA) suggesting that oral Vancomycin be preferred in cases of severe and severe-complicated disease, but adherence to these new guidelines is unclear at this time. In this study, the authors observe compliance to the new 2010 guidelines at a tertiary medical center.

### Innovations and breakthroughs

Despite advances in health care sanitation technique, *Clostridium* infections continue to increase. In this study, the authors observed that compliance to the updated 2012 SHEA/IDSA guidelines is poor at the tertiary care hospital, suggesting a need for increased education and antibiotic stewardship for providers. The authors also identified specific areas that the guidelines fail to address clearly; end-stage renal disease patients and patients who are significantly immunosuppressed.

### Applications

By recognizing poor compliance at our tertiary care facility, steps can be made to increase education and antibiotic stewardship at other facilities. In addition, the study suggests the guidelines should be updated to include the aforementioned patient populations with specific guidelines pertaining to their management.

### Terminology

Compliance in the study is defined as using the proper dosage (both strength and frequency) in the proper duration for a specific *C. difficile*-associated diarrhea (CDAD) infection. The guidelines define mild to moderate CDAD as the presence of a white blood cell count  $\leq 15000/\text{mm}^3$  and a serum creatinine level  $\leq 1.5$  times the premorbid level. Conversely, severe CDAD is defined by the presence of a white blood cell count  $\geq 15000/\text{mm}^3$  or a serum creatinine level  $\geq 1.5$  times the premorbid level. Severe-complicated CDAD is defined by the presence of hypotension, shock, ileus, or megacolon.

### Peer review

The authors report the results of a study conducted to assess adherence with the SHEA/IDSA guidelines for management of CDAD at a tertiary medical center. The study is well-designed, includes sufficient number of patients and the paper is well written. Despite the fact that the study is single-centered, and includes only hospitalized patients which reduces its generalizability (as mentioned by the authors), the results are considerable. This is a worthy study and appears of high clinical interest.

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## Assessment of the diagnostic performance and interobserver variability of endocytoscopy in Barrett's esophagus: A pilot *ex-vivo* study

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### Abstract

**AIM:** To investigate a classification of endocytoscopy (ECS) images in Barrett's esophagus (BE) and evaluate its diagnostic performance and interobserver variability.

**METHODS:** ECS was applied to surveillance endoscopic mucosal resection (EMR) specimens of BE *ex-vivo*. The mucosal surface of specimen was stained with 1% methylene blue and surveyed with a catheter-type endocytoscope. We selected still images that were most representative of the endoscopically suspect lesion and matched with the final histopathological diagnosis to accomplish accurate correlation. The diagnostic performance and inter-observer variability of the new classification scheme were assessed in a blinded fashion by

physicians with expertise in both BE and ECS and inexperienced physicians with no prior exposure to ECS.

**RESULTS:** Three staff physicians and 22 gastroenterology fellows classified eight randomly assigned unknown still ECS pictures (two images per each classification) into one of four histopathologic categories as follows: (1) BEC1-squamous epithelium; (2) BEC2-BE without dysplasia; (3) BEC3-BE with dysplasia; and (4) BEC4-esophageal adenocarcinoma (EAC) in BE. Accuracy of diagnosis in staff physicians and clinical fellows were, respectively, 100% and 99.4% for BEC1, 95.8% and 83.0% for BEC2, 91.7% and 83.0% for BEC3, and 95.8% and 98.3% for BEC4. Interobserver agreement of the faculty physicians and fellows in classifying each category were 0.932 and 0.897, respectively.

**CONCLUSION:** This is the first study to investigate classification system of ECS in BE. This *ex-vivo* pilot study demonstrated acceptable diagnostic accuracy and excellent interobserver agreement.

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**Key words:** Endocytoscopy; Barrett's esophagus; Dysplasia; Esophageal adenocarcinoma; Interobserver agreement

**Core tip:** The current gold standard for surveillance of esophageal adenocarcinoma in Barrett's esophagus (BE) is endoscopic random biopsy and pathological diagnosis. Endocytoscopy (ECS) has the potential to provide a virtual histological diagnosis *in vivo* and in real-time. However, a major issue relates to that interpretation of cellular and nuclear images may be subject to similar interobserver variability associated with conventional histopathological diagnosis, and there have been no



reliable classification systems for the endocytoscopic diagnosis. We presented the first study to investigate classification system of ECS in BE. This *ex-vivo* pilot study demonstrated acceptable diagnostic accuracy and excellent interobserver agreement.

Tomizawa Y, Iyer PG, Wongkeesong LM, Buttar NS, Lutzke LS, Wu TT, Wang KK. Assessment of the diagnostic performance and interobserver variability of endocytoscopy in Barrett's esophagus: A pilot *ex-vivo* study. *World J Gastroenterol* 2013; 19(46): 8652-8658 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8652.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8652>

## INTRODUCTION

Recent advances in endoscopic imaging may lead to improved detection and facilitate therapy of dysplasia and esophageal adenocarcinoma (EAC) in Barrett's esophagus (BE)<sup>[1-5]</sup>. Histology is regarded as the gold standard for diagnosis of dysplasia and EAC<sup>[6,7]</sup>, but sampling error and interobserver variability among gastrointestinal pathologists have been well described<sup>[8-11]</sup>.

Endocytoscopy (ECS) is a probe-based technique which captures ultra-high magnified images of the epithelial surface, with the capability to discriminate cellular and subcellular features. ECS, thus, has the potential to provide a virtual histological diagnosis *in vivo* and in real-time. ECS has been investigated throughout the gastrointestinal tract for the identification of lesions in the esophagus<sup>[12-16]</sup>, small intestine<sup>[17]</sup>, and colon<sup>[18-21]</sup>. However, a major issue relates to the fact that interpretation of cellular and nuclear images may be subject to similar interobserver variability associated with conventional histopathological diagnosis<sup>[22,23]</sup>. To date, there have been no reliable classification systems for the endocytoscopic diagnosis of BE and Barrett's EAC. Accurate diagnosis based on a simple and reproducible classification system is warranted before ECS can be implemented into clinical practice.

Our aim was to develop simplified scheme for the classification of endocytoscopic images in BE and to evaluate its diagnostic performance and interobserver variability among experienced and inexperienced users of ECS in an *ex-vivo* setting.

## MATERIALS AND METHODS

### Patients and tissue specimens

ECS was performed *ex-vivo* on endoscopic mucosal resection (EMR) specimens obtained from patients undergoing endoscopic surveillance of BE at our institution. All EMR procedures were performed by a single endoscopist using the cap technique (EMR Kit; Olympus America, Center Valley, PA). Lesions targeted for EMR were endoscopically suspect areas, such as nodules or polyps, or dysplastic/neoplastic-appearing mucosa, such as irregular,

friable, ulcerated, or villous-appearing mucosa, as seen under high-definition white-light imaging and narrow band imaging<sup>[24]</sup>. The study was approved by the Institutional Review Board and a written informed consent was obtained from all patients.

### Endocytoscopy procedure

As soon as retrieved from the patient, the mucosal surface of each EMR specimen was immediately rinsed with 3-5 mL of 20% N-acetylcysteine to remove excess mucus, followed by the application of 1-1.5 mL of 1% methylene blue solution as contrast agent. *Ex-vivo* ECS imaging was performed using a flexible, catheter-type endocytoscope (XEC120, Olympus Medical Systems Co., Tokyo, Japan) which provides 1100 × magnification at a 120 μm × 120 μm field of view. The stained surface of each specimen was surveyed with the endocytoscope, and the area most representative of the endoscopically suspect lesion was identified. ECS imaging of the lesion was videotaped for approximately one minute.

### Histopathology assessment

Histopathological assessment of the EMR specimens was performed according to the protocol in our BE unit, as previously published<sup>[25]</sup>. Patients had their pathological diagnosis of EMR specimens confirmed by at least two experienced gastrointestinal pathologists with expertise in Barrett-associated neoplasia.

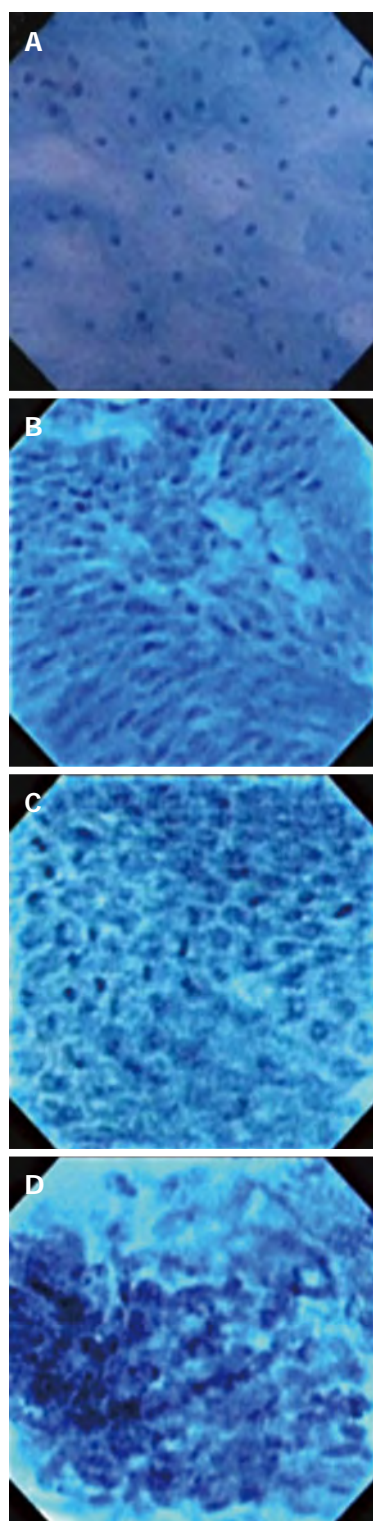
### ECS image analysis and classification

Following histopathological diagnoses of EMR specimens, the corresponding ECS videos and images were reviewed by investigators uninvolved in the subsequent blinded image assessment. To accomplish accurate correlation of endocytoscopic images with histological findings, we selected snapshots that were most representative of the histopathological findings for each specimen and matched final histopathological findings with their respective ECS images. With consideration to the previously proposed esophageal endocytoscopic atypia classification by Inoue *et al.*<sup>[26]</sup>, we classified the endocytoscopic images as follows (BEC; Barrett's EndoCytoscopy): (1) BEC1 - squamous epithelium; (2) BEC2-BE without dysplasia; (3) BEC3 - BE with dysplasia; and (4) BEC4-BE with EAC.

This classification scheme is based on the interpretation of ECS features (Figure 1): BEC1 images consist of cytoplasm-rich, rhomboid cells in a regular pattern; BEC2 images consist of increased cell numbers and different-sized nuclei/cells; BEC3 images consist of increased nucleus-cytoplasm ratio with dense chromatin and prominent nuclear fission; BEC4 images consist of cells of various sizes that are irregularly arranged, with blurred and enlarged nuclei. Two representative endocytoscopic images for each classification were selected for analysis of diagnostic performance and interobserver variability.

### Diagnostic performance and interobserver variability

The diagnostic performance and inter-observer variabil-



**Figure 1 Classification of endocytoscopic images of Barrett's esophagus.**

A: Squamous epithelium (BEC 1) Cytoplasm-rich, rhomboid cells in a regular pattern; B: Barrett's esophagus without dysplasia (BEC 2) Increased cell number and different-sized nuclei/cells; C: Barrett's esophagus with dysplasia (BEC 3) Increased nucleus-cytoplasm ratio, and dense chromatin and nuclear fission are prominent; D: Esophageal adenocarcinoma (BEC 4) Cells of various sizes, irregularly arranged, with blurred and enlarged nuclei (magnification  $\times 1125$ ).

ity of the new classification scheme were assessed in a blinded fashion. Experienced physicians with expertise

in both BE and ECS were provided with a brief 5-min presentation on the new classification, as shown in Figure 1. Inexperienced physicians consisted of clinical fellows ( $n = 22$ ) in the Division of Gastroenterology and Hepatology with no prior exposure to ECS. They were provided with a 30 min presentation on ECS imaging and the new classification scheme. During the training session, fellows were presented with two non-study sets of pictures representative of each BEC classification for learning purposes. They were given the opportunity to ask questions and review the criteria. The training session and image classification by experienced and inexperienced physicians were conducted separately.

Immediately following the training session, participants were shown the randomly assigned unknown ECS pictures and asked to classify each image as: (1) BEC1; (2) BEC2; (3) BEC3; and (4) BEC4. The participants were blinded to patient history, endoscopic findings, and histopathological diagnoses. During the image classification session, the participants were not allowed to review previously seen images or to change their answers.

### Statistical analysis

Data analysis was performed using the SPSS (Chicago, Illinois, United States) statistical software program. Classification accuracy, sensitivity, specificity, and positive and negative predictive values were calculated to assess diagnostic performance. Interobserver agreement was determined using intraclass correlation coefficient (ICC), which assesses agreement beyond chance among investigators. ICC was derived from a 2-way random effects model because both people effects and measures effects were random. An ICC of 0.4-0.75 indicates fair to good reliability, whereas an ICC greater than 0.75 shows excellent reliability.

## RESULTS

A total of 20 patients were included in this study: squamous epithelium ( $n = 2$ ), BE without dysplasia ( $n = 6$ ), BE with dysplasia ( $n = 6$ ), and BE with EAC ( $n = 6$ ). A total of eight representative endocytoscopic images (two images per each classification) from different patients were utilized for this study. The overall classification accuracy for each category among experienced ( $n = 3$ ) and inexperienced ( $n = 22$ ) physicians were 100% and 99.4% for BEC1, 95.8% and 83.0% for BEC2, 91.7% and 83.0% for BEC3, and 95.8% and 98.3% for BEC4, respectively. If we combined BEC2 and BEC3 as diagnosis of BE, the classification accuracy would be 95.8%, even in ECS naive observers. The sensitivities, specificities, positive predictive values and negative predictive values for each category are shown in Table 1.

The interobserver agreements for the experienced and inexperienced physicians in classifying each category were 0.932 and 0.897, respectively. When a dichotomized category (BEC 1 and 2 *vs* BEC 3 and 4) was used, interobserver agreements for the experienced and inexpe-

**Table 1** Diagnostic performance of endocytoscopy in Barrett's esophagus

	Classification	Sensitivity	Specificity	PPV	NPV
Experienced physicians (staff physician)	BEC 1	1.000%	1.000%	1.000%	1.000%
	BEC 2	1.000%	0.944%	0.857%	1.000%
	BEC 3	0.833%	0.944%	0.833%	0.944%
	BEC 4	0.833%	1.000%	1.000%	0.947%
Inexperienced physicians (clinical fellow)	BEC 1	0.977%	1.000%	1.000%	0.992%
	BEC 2	0.636%	0.894%	0.667%	0.881%
	BEC 3	0.705%	0.871%	0.646%	0.898%
	BEC 4	0.955%	0.992%	0.977%	0.985%
Both experienced and inexperienced (physicians staff physician and clinical fellow)	BEC 1	0.980%	1.000%	1.000%	0.993%
	BEC 2	0.680%	0.900%	0.694%	0.894%
	BEC 3	0.720%	0.880%	0.667%	0.904%
	BEC 4	0.940%	0.993%	0.979%	0.980%

PPV: Positive predictive value; NPV: Negative predictive value.

**Table 2** Interobserver agreement of endocytoscopy in Barrett's esophagus

Classification	Staff physician (95%CI)	GI fellow (95%CI)
BEC 1-4	0.932 (0.794-0.985)	0.897 (0.784-0.973)
BEC 1 and 2 vs 3 and 4	0.851 (0.593-0.965)	0.581 (0.358-0.856)

GI: Gastrointestinal.

rienced physicians in classifying into this category were 0.851 and 0.581, respectively (Table 2).

## DISCUSSION

Barrett's esophagus (BE) is a well-established precursor of esophageal adenocarcinoma (EAC) whose incidence is rising in Western countries. Patients with BE are therefore advised to undergo periodic surveillance to detect dysplastic mucosa and pre-cancerous lesions at an early stage at a time where intervention can be curative. The current gold standard for surveillance is periodic endoscopic random biopsy within the BE segment and pathological diagnosis. Due to inherent limitations of the gold standard, there have been considerable interests in advanced endoscopic imaging techniques to enhance dysplasia and EAC detection. Dysplasia can be patchy in distribution within the BE segment and sampling error can occur with random biopsy techniques. Novel imaging technologies that can reliably detect dysplasia or EAC in real-time would facilitate targeted biopsy and, hence, a reduction in sampling error.

Endocytoscopy (ECS) can provide real-time virtual histological images during endoscopic observation and potentially identify areas that harbor dysplastic or cancerous cells. However, interpretation of ECS images may be subject to similar interobserver variability associated with conventional histopathological diagnosis. A first step is to standardize ECS image criteria for accurate tissue diagnosis. To date, no studies have been conducted on the development and use of a classification system for endocytoscopic images in BE. In this study, we proposed a classification scheme based on ECS cellular and archi-

tectural features for categorizing Barrett's tissue, with the aim that the classification remains simple and easy to learn and adopt.

Overall, we had an acceptable classification accuracy for each Barrett tissue category when using our classification system and high accuracy was obtained for the differentiation of BE without dysplasia from dysplastic tissue among experienced observers. Although the number of percentage of accuracy among staff physicians appears low in BE with dysplasia, it is obvious that the small number of denominator is the reason. Our group of inexperienced observers (clinical fellows) classified squamous epithelium and EAC with high accuracy of 99.4% and 98.3%, respectively, and BE with and without dysplasia with acceptable accuracy of 83.0%. These results suggest our classification scheme is reliable and easy to learn. Interobserver agreement regarding both experienced and inexperienced groups was interpreted as excellent (ICC = 0.932 and 0.897, respectively). In classifying into two dichotomized category (BEC 1 and 2 vs BEC 3 and 4), interobserver agreement for the experienced physicians was still interpreted as excellent (ICC = 0.851) and interobserver agreement for the fellows showed good reliability (ICC = 0.581).

In this study, all the misdiagnoses of BE with dysplasia (BEC 3) were answered as non-dysplasia (BEC 2). It may imply that our criteria are similar to those of histological diagnosis. Misdiagnosis for non-dysplastic BE occurred in the inexperienced group, and all the misdiagnoses were answered as dysplasia. These facts probably reflect some of the dilemma that exists with pathological interpretation of non-dysplastic and dysplastic BE.

The reported diagnostic accuracy and interobserver agreement of ECS images should be interpreted with caution. In this structured pilot *ex-vivo* study, we intended to show a "classic" unambiguous image for each category and, thus, selected representative images. In BE, the microscopic epithelial changes that represent transition from metaplasia to dysplasia to cancer occur on a continuum. We did not assess how our classification performs near the margins of the transitions. The use of the representative images may maximize diagnostic



accuracy and interobserver agreement and minimize the correlation of the study findings with what will be observed during real-time use *in vivo*. We did not evaluate using “real-time” images correlated with biopsy results using our classification. Further study is warranted in real scanning to validate our classification. An additional study limitation is related to the *ex-vivo* nature of this preliminary study. The performance of *in-vivo* catheter-type ECS is clearly operator dependent. Challenges that lie ahead are the difficulty of maintaining a long, thin, flexible catheter onto the esophageal surface in stable position and in focus. During *in-vivo* observations, gastrointestinal motility may hinder collection of interpretable images. In one *in-vivo* study of ECS in BE<sup>[27]</sup>, 76% of ECS images were recognized as poor quality. The study also had a high false-positive rate of 43%, resulting in both a sensitivity and positive predictive value of 42% in all image sequences. Conversely, an *in-vivo* feasibility ECS study for esophageal cancer conducted in Japan reported that clear and interpretable images were obtained in all cases, with the positive predictive value and the false-positive rate for esophageal malignancy being 94% and 6.3%, respectively<sup>[26]</sup>. Another *in-vivo* feasibility study also presented high quality images for interpretation<sup>[12]</sup>. Expertise in handling the ECS device could explain the difference in image quality obtained, and we believe the technical aspects can be overcome as already reported in the previous two *in-vivo* studies. A new endoscope-type ECS (XGIF-Q260EC1 and XCF-Q260EC1; Olympus) has recently been introduced and been reported to obtain more sensitive, ultra-magnified images<sup>[28-30]</sup>. The new ECS enables easy switch from a conventional endoscopic view to ultra-magnification endocytoscopic view by the press of a button at the top of the endoscope. The new device could reduce the technical burden of maintaining an ECS probe on a moving surface.

Our classification system does not differentiate between low grade (LGD) and high grade (HGD) dysplasia. It is well known the reproducibility of histopathological interpretation of LGD and HGD even among skilled pathologists is a challenge<sup>[10]</sup>. We still do not have definitive consensus about the management of LGD in BE, and management is individualized. It is clear that surveillance of any dysplastic lesions in BE segment is of importance given the established dysplasia-carcinoma sequence in EAC. In this study, we aimed to assess the potential of ECS in enhancing surveillance of dysplastic lesions in BE. We therefore proposed the two distinct criteria of dysplastic BE *vs* non-dysplastic BE instead of LGD and HGD.

In conclusion, we proposed a simple diagnostic classification system for ECS in BE. In this pilot *ex-vivo* study, acceptable accuracies regarding the diagnosis of squamous epithelium, non-dysplastic BE, dysplastic BE, and EAC were demonstrated. Interobserver agreement in classifying each category was interpreted as excellent, even among observers inexperienced in ECS. The applicability of the proposed classification scheme in the *in-vivo* setting remains to be seen.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Barrett's esophagus is a well-established precursor of esophageal adenocarcinoma, therefore it is very important to detect dysplastic pre-cancerous lesions at an early stage at a time where intervention can be curative. The current gold standard of endoscopic random biopsy has inherent limitations. There have been considerable interests in advanced endoscopic imaging techniques to enhance dysplasia detection.

### Research frontiers

Novel imaging technologies that can reliably detect dysplasia or early esophageal cancer would facilitate targeted biopsy and, hence, a reduction in sampling error. Endocytoscopy can provide real-time virtual histological images during endoscopic observation and potentially identify areas that harbor dysplastic or cancerous cells and facilitate targeted biopsy.

### Innovations and breakthroughs

A major issue relates to the fact that interpretation of cellular and nuclear images by endocytoscopy may be subject to similar interobserver variability associated with conventional histopathological diagnosis. To date, there have been no reliable classification systems for the endocytoscopic diagnosis of Barrett's esophagus and esophageal adenocarcinoma. In this study, the authors proposed a classification scheme based on endocytoscopy cellular and architectural features for categorizing Barrett's tissue, with the aim that the classification remains simple and easy to adopt. This is the first study to investigate classification system of endocytoscopy in Barrett's esophagus. In this study, the diagnostic performance and inter-observer variability of the new classification scheme were assessed in a blinded fashion by physicians with expertise in both Barrett's esophagus and endocytoscopy and inexperienced physicians with no prior exposure to endocytoscopy. Overall, the authors had an acceptable classification accuracy for each Barrett tissue category when using our classification system, and high accuracy was obtained for the differentiation of Barrett's esophagus without dysplasia from dysplastic tissue among experienced observers. Interobserver agreement in classifying each category was interpreted as excellent among both experienced and inexperienced observers.

### Applications

The results of this structured pilot *ex-vivo* study suggest that our classification scheme is reliable and easy to learn.

### Peer review

The study to investigate classification system of endocytoscopy in Barrett's esophagus is very interesting. This *ex-vivo* pilot study demonstrated acceptable diagnostic accuracy and excellent interobserver agreement.

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## Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis

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onset ulcerative colitis (UC) in an 18-mo-old affected child.

**METHODS:** We analysed the interleukin-10 (*IL10*) receptor genes at the DNA and RNA level in the proband and his relatives. Beta catenin and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) receptors were analysed in the proteins extracted from peripheral blood cells of the proband, his relatives and familial adenomatous polyposis (FAP) and PTEN hamartoma tumor syndrome (PHTS) patients. Samples were also collected from the proband's inflamed colorectal mucosa and compared to healthy and tumour mucosa collected from a FAP patient and patients affected by sporadic colorectal cancer (CRC). Finally, we examined mesalazine and azathioprine effects on primary fibroblasts stabilised from UC and FAP patients.

**RESULTS:** Our patient was a compound heterozygote for the *IL10RB* E47K polymorphism, inherited from his father, and for a novel point mutation within the *IL10RA* promoter (the -413G->T), inherited from his mother. Beta catenin and tumour necrosis factor  $\alpha$  receptors-I (TNFR1) protein were both over-expressed in peripheral blood cells of the proband's relatives more than the proband. However, TNFR2 was over-expressed only in the proband. Finally, both TNF $\alpha$ -receptors were shown to be under-expressed in the inflamed colon mucosa and colorectal cancer tissue compared to healthy colon mucosa. Consistent with this observation, mesalazine and azathioprine induced, in primary fibroblasts, *IL10RB* and TNFR2 over-expression and TNFR1 and TNF $\alpha$  under-expression. We suggest that  $\beta$ -catenin and TNFR1 protein expression in peripheral blood cells could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients with early-onset UC.

**CONCLUSION:** A synergistic effect of several variant alleles of the *IL10* receptor genes, inherited in a Mende-

### Abstract

**AIM:** To investigated the molecular cause of very early-

lian manner, is involved in UC onset in this young child.

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**Key words:** Inflammatory bowel disease; Ulcerative colitis; Interleukin 10 receptors; Tumour necrosis factor  $\alpha$  receptors; Beta catenin

**Core tip:** We identified a novel point mutation within the interleukin-10 (*IL10*) receptor genes promoter (the -413G->T), associated with mRNA under-expression. We propose that this mutation has a synergistic effect with other variant alleles of *IL10* receptor genes in very-early ulcerative colitis (UC) onset in this young child.  $\beta$ -catenin and tumour necrosis factor  $\alpha$  receptors-I (TNFRI) protein were both over-expressed in peripheral blood cells of proband relatives, whereas TNFRII was over-expressed only in the proband. We suggest that  $\beta$ -catenin and TNFRI protein expression could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients with early-onset UC.

Galatola M, Miele E, Strisciuglio C, Paparo L, Rega D, Delrio P, Duraturo F, Martinelli M, Rossi GB, Staiano A, Izzo P, De Rosa M. Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis. *World J Gastroenterol* 2013; 19(46): 8659-8670 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i46/8659.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8659>

## INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host<sup>[1]</sup>. Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinicopathological subtypes of IBD, common in developed countries, affecting the quality of life of approximately 1.4 million individuals in the United States and 2.2 million people in Europe<sup>[2-4]</sup>.

Accumulating data suggest that these disorders result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host<sup>[5]</sup>. Active IBD is defined as an infiltration of the lamina propria by innate immune cells (neutrophils, macrophages, dendritic and natural killer T cells) and adaptive immune cells (B and T cells). Increased numbers and activation of these cells in the intestinal mucosa enhance local levels of tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and several pro-inflammatory interleukins (IL)<sup>[5-8]</sup>.

Genome-wide association studies (GWAS) have been successful in IBD, identifying 99 non-overlapping genetic risk loci, including 28 that are shared between CD and UC<sup>[9,10]</sup>. Analyses of the genes and genetic loci implicated in IBD show several pathways that are cru-

cial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species generation, autophagy, adaptive immunity regulation, endoplasmic reticulum stress and metabolic pathways associated with cellular homeostasis. Early studies have suggested the existence of both protective and predisposing alleles<sup>[11]</sup>. Again, many genetic changes might affect genetic regions other than coding regions, indicating that allele-specific gene-expression changes contribute to the disease risk<sup>[12]</sup>.

The relative importance of each individual pathway in the pathogenesis of IBD has not been determined. There is enthusiasm for a model in which mucosal inflammation results from defective activity of Treg cells. In this model, effector T cells that react to the microbial flora or other GI antigens are kept in check by a population of regulatory cells; defects in these cells lead to GI inflammation. IL10 production by Treg cells appears to be required for suppression of colitis<sup>[13]</sup>.

A recent study has demonstrated that IBD with an early onset can be monogenic. Mutations in *IL10* or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children<sup>[14]</sup>.

*IL10R* consists of two  $\alpha$  (*IL10RA*) and two beta (*IL10RB*) molecules. *IL10RA* and *IL10RB* genes have been mapped on chromosomes 11q23.3 and 21q22, respectively, and many single-nucleotide polymorphisms (SNPs) have been identified<sup>[15]</sup>. Recently, Moran *et al*<sup>[16]</sup> identified *IL10R*s polymorphisms that confer risk for developing very early-onset IBD. Each novel, nonsynonymous SNP was identified only in the heterozygous state, and none of the resulting amino acid changes were predicted to be deleterious by SIFT or Polyphen.

The aims of this work were to clarify the molecular basis of UC in an 18-mo-old affected child. To this aim, we investigated the pathogenetic mechanisms of IL10 pathway alteration in the onset of UC in the proband, and we clarified the molecular changes associated with them. Moreover, we propose  $\beta$ -catenin and tumour necrosis factor  $\alpha$  receptors- I (TNFRI) as molecular biomarkers of subclinical disease among apparently healthy family members of the index case. Finally, we have investigated the effect of mesalazine and azathioprine, the main pharmacological therapy used for IBD treatment, on the expression of IL10 receptors, TNF $\alpha$  and TNF $\alpha$  receptors.

## MATERIALS AND METHODS

### Patients

The proband, exhibiting UC, was referred by paediatric gastroenterologists to the laboratory for genetic analysis. He was admitted to the hospital for bloody diarrhoea, asthenia, fever and a severe anaemia (haemoglobin 3.7 g/dL). He underwent upper and lower GI endoscopy. The upper GI endoscopy did not reveal any macroscopic and/or microscopic sign of disease. Ileocolo-



noscopy showed a severe ulcerative pancolitis, (E4-S1) according to the Paris classification<sup>[17]</sup>. The colonoscopic grade of inflammation was characterised by the presence of marked erythema, absent vascular pattern, friability erosions, associated with spontaneous bleeding and ulcerations, suggesting a grade 3 according to the Mayo endoscopic score<sup>[18]</sup>. A severe grade of inflammation was confirmed histologically by the diffuse presence of a large number of neutrophilic leukocytes (> 50/HPF) with crypt abscesses and significant acute inflammation with ulcerations in lamina propria. The presence of granulomas was excluded at any colonic levels, as well as at level of the distal ileum.

The child was treated with blood transfusions, antibiotics and steroid therapy without improvement. A rescue therapy with cyclosporine followed by mesalazine and azathioprine was then started. His following clinical history was characterised by relapsing-remitting symptoms and by the lack of response to drugs. The proband's mother referred episodes of bloody diarrhoea, but she refused colonoscopy.

Blood samples from proband and healthy family members were collected at the same hospital as the patient. Normal colorectal mucosa and colorectal cancer tissues were sampled from patients with FAP or sporadic colon cancer operated on the "Istituto Nazionale dei Tumori" in Naples.

Samples from all subjects who participated in the study were collected after being granted authorisation from the "Comitato etico per le attività Biomediche - Carlo Romano" of the University of Naples Federico II, with protocol number 120/10. Such authorisation is given only once the study has received ethical approval, and participants' informed and written consent has been obtained.

### Molecular analysis of *IL10RA* and *IL10RB* messenger

**Reverse transcription polymerase chain reaction of *IL10RA* and *IL10RB* of full length coding regions:** Total RNA was extracted from 3 mL of peripheral blood cells of the UC patient and his healthy family members, using Trizol reagent (Invitrogen, Life Technologies, CA), cDNA was synthesised and 1 µL of the cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) as previously described<sup>[19]</sup>, using the following pairs of oligonucleotides: *IL10RA*-5'UTR-FP/*IL10RA*-3'UTR-RP; *IL10RB*-5'UTR-FP/*IL10RB*-3'UTR-RP. Two fragments of 2023 bp and 1197 bp, respectively, were produced. The PCR products were analysed on a 1% agarose gel in a tris-acetic acid (TAE)-EDTA standard buffer, and visualised by ethidium bromide staining (Table 1).

**Sequence analysis of *IL10RA* and *IL10RB* mRNA:** Sequence analysis of *IL10RA* and *IL10RB* full length coding regions was performed on amplified fragments from the cDNA of the proband and his healthy family members, using the following primer pairs, localised

inside these regions: *IL10RA*-5'UTRb-FP; *IL10RA*-3'UTRb-RP; *IL10RA*-3cFP; *IL10RA*-4cRP; *IL10RA*-6cFP; *IL10RA*-7cRP; *IL10RB*-5'UTRb-FP; *IL10RB*-3'UTRb-RP; *IL10RB*-4cFP; *IL10RB*-5cRP (Table 1). The analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of *IL10RA* and *IL10RB* mRNA sequences [GenBank Accession numbers: NM\_001558.3 and NM\_000628.3, respectively]; all oligonucleotides were obtained with primer-BLAST Software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

**Real time RT-PCR quantification analysis:** Real time PCR quantification analysis was performed for *IL10RA* and *IL10RB* messengers. The relative expression was calculated with the comparative Ct method. Patient numbering corresponds to that adopted in Figure 1A. Three millilitres of peripheral blood cells from the UC patient, his healthy family members and 8 healthy subjects were pelleted after erythrocyte lysis and resuspended in Trizol reagent. The mean value across all of the healthy samples (H1-8) was used as a calibrator to measure the relative expression. *IL10RA* and *IL10RB* mRNA quantification was carried out by amplifying fragments spanning the junctions between exons 3-4, for *IL10RA* messenger and exons 4-5 for *IL10RB* messenger, compared to the glucuronidase transcript fragment, using the oligonucleotides described above: *IL10RA*-3cFP/*IL10RA*-4cRP; *IL10RB*-4cFP/*IL10RB*-5cRP (Table 1). The quantitative real time assays were performed using the iCycler iQ Real Time Detection System BIO-RAD as previously described<sup>[19]</sup>.

### Molecular analysis of *IL10RA* gene

**Genomic PCR and sequencing:** Genomic DNA was extracted from 3 mL of peripheral blood cells of UC patient, using Nucleon BACC2 Kit (Amersham Biosciences). Genomic PCR and sequencing of all exons was performed for *IL10RA* gene, using oligonucleotides complementary to intronic neighbouring boundary regions of each exon, described in Table 1. The GenBank Accession number of *IL10RA* genomic sequence is: (NC\_000011.9/gi:224589802). Mutational analysis of *IL10RA* promoter region, from bp -2159 to bp +1, was performed by PCR and sequencing. This region was amplified into three overlapping fragments of 788, 782 and 788 bp in molecular weight, respectively, using the following primer pairs: *IL10RA*p1-FP/*IL10RA*p1-RP; *IL10RA*p2-FP/*IL10RA*p2-RP; *IL10RA*p3-FP/*IL10RA*p3-RP (Table 1).

**Amplification refractory mutation-PCR of the -413G->T *IL10*-RA promoter mutation:** We set up an amplification refractory mutation-PCR (ARMS-PCR) reaction to analyse 200 DNA extracted from blood samples of control subjects apparently healthy, for the -413G->T promoter mutation identified in the UC proband and his mother.

**Table 1** Oligonucleotide sequences

RT-PCR of IL10RA and IL10RB of full length coding regions		
IL10RA-5'UTR-FP:	GTCCAGCCCCAAGGGTAG	[NM_001558.3; start: + 5]
IL10RA-3'UTR-RP:	CACCCACATACCCTGCACTA	[NM_001558.3; start: + 2027]
IL10RB-5'UTR-FP:	GTCGTGTGCTTGGAGGAAG	[NM_000628.3; start: + 57]
IL10RB-3'UTR-RP:	GTGGCTAAGTCCAGGGTCTG	[NM_000628.3; start: + 1223]
Sequence analysis of IL10RA and IL10RB messenger/real time RT-PCR quantification analysis		
IL10RA-5'UTRb-FP:	TCAGACGCTCATGGGACA	[NM_001558.3; start: + 132]
IL10RA-3'UTRb-RP:	CCCAGTGGACTTGCAGAAA	[NM_001558.3; start: + 1938]
IL10RA-3cFP:	AACCTGGACCGTCACCAACAC	[NM_001558.3; start: + 405]
IL10RA-4cRP:	AATCTTCCCGAGGATGAAGC	[NM_001558.3; start: + 506]
IL10RA-6cFP:	AGTACCCAGTGTCTTGCTC	[NM_001558.3; start: + 871]
IL10RA-7cRP:	CAAAAAGGCCTCCTCATCAA	[NM_001558.3; start: + 983]
IL10RB-5'UTRb-FP:	CATGGCGTGGAGCCCT	[NM_000628.3; start: + 99]
IL10RB-3'UTRb-RP:	GATGGTCTTGGCCCTTGTT	[NM_000628.3; start: + 1177]
IL10RB-4cFP:	GTGCAATACTGAAAAACGGT	[NM_000628.3; start: + 565]
IL10RB-5cRP:	CCCTCGAACTTGAACACAATAA	[NM_000628.3; start: + 678]
Genomic PCR and sequencing		
IL10RAp1-FP:	GCGGTTTGAGGCTCAGC	[NC_000011.9; start: + 117856447]
IL10RAp1-RP:	CAAGACGGAGGCTGAGGA	[NC_000011.9; start: + 117857234]
IL10RAp2-FP:	CTAGCAGGGGAAGAGCAGC	[NC_000011.9; start: + 117855574]
IL10RAp2-RP:	AACCTTCGTCTCCAGGTTT	[NC_000011.9; start: + 117856355]
IL10RAp3-FP:	TGAGCCAAGTGACACAGAGG	[NC_000011.9; start: + 117855023]
IL10RAp3-RP:	TTGAACATATACCCTGCTGAAGAG	[NC_000011.9; start: + 117855810]
IL10RA-1FP:	CTGTCACTCCACGCCAA	[NC_000011.9; start: + 17857104]
IL10RA-1RP:	TCTCCACTGGATGGAGAACTTTA	[NC_000011.9; start: + 117857327]
IL10RA-2FP:	TTGGTAAAATTGGGGTCATCA	[NC_000011.9; start: + 117859029]
IL10RA-2RP:	GCCCTCAGGCACTCACTTC	[NC_000011.9; start: + 117859328]
IL10RA-3FP:	AAGCTCGTTTCCAGTGCCTA	[NC_000011.9; start: + 117860120]
IL10RA-3RP:	GGCAGACATGGTGAGCTATG	[NC_000011.9; start: + 117860439]
IL10RA-4FP:	ACAAACCTGTGGCCAAGTTT	[NC_000011.9; start: + 117863822]
IL10RA-4RP:	CACACAAGGGTGCTTCCAG	[NC_000011.9; start: + 117864202]
IL10RA-5FP:	ATCACCTCTAAAGGCCACC	[NC_000011.9; start: + 117864629]
IL10RA-5RP:	GGATGCAGAGCTATGTGAAGC	[NC_000011.9; start: + 117864993]
IL10RA-6FP:	TTTCATGGGACCAGAGTCCT	[NC_000011.9; start: + 117866223]
IL10RA-6RP:	CTGGCTGGAGGAAAAGAG	[NC_000011.9; start: + 117864993]
IL10RA-7.1FP:	GCTCTCTCTGGGCCT	[NC_000011.9; start: + 117869338]
IL10RA-7.1RP:	CGGCCCTCAGAGTTTGA	[NC_000011.9; start: + 117869854]
IL10RA-7.2FP:	ACCTGGGAGCAACAGGTG	[NC_000011.9; start: + 117869775]
IL10RA-7.2RP:	CGTGCTAACTTCTGCC	[NC_000011.9; start: + 117870445]
ARMS PCR of the -413G->T IL10-RA promoter mutation		
IL10RA-ARMS-FP-N:	CCGGCACGCCAGGCAAAAAGCGGCTCGGTCTG	[NC_000011.9; start: + 117856738]
IL10RA-ARMS-FP-M:	CCGGCACGCCAGGCAAAAAGCGGCTCGGTCT	[NC_000011.9; start: + 117856738]
IL10RA-ARMS-RP:	GCCTCCAGTGCCTTCGGATCAA	[NC_000011.9; start: + 117856897]
Gene copy number quantification of IL10RA gene		
IL10RA-4cFP:	TCCTCGGGAAGATTACGTA	[NM_001558.3; start: + 493]
IL10RA-4c2RP:	TGCGAATGGCAATTCATAC	[NM_001558.3; start: + 594]
IL10RA-7cFP:	ACTGAAGAGCCCCAGTTCCT	[NM_001558.3; start: + 1065]
IL10RA-7c2RP:	GCTGTCGTGCTATTGCTGC	[NM_001558.3; start: + 1187]

RT-PCR: Reverse transcription polymerase chain reaction; IL10: Interleukin-10.

This ARMS reaction was performed with following oligonucleotide primers: *IL10RA*-ARMS-FP-N; *IL10RA*-ARMS-FP-M; *IL10RA*-ARMS-R (Table 1).

#### Gene copy number quantification of *IL10RA* gene:

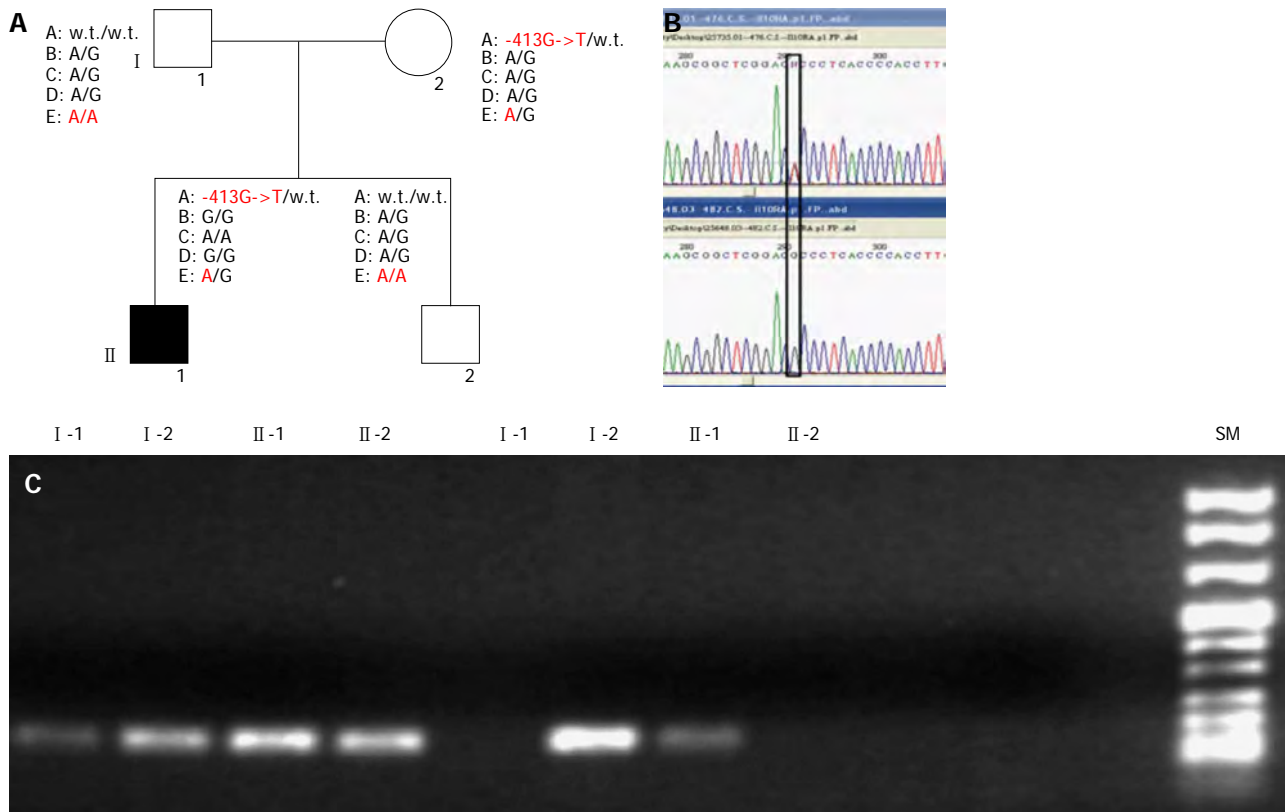
For the genomic quantification of *IL10RA* gene, specific amplified fragments were compared to a fragment of the exon 15 of *MUTYH* gene. For *IL10RA* specific quantification, two short fragments, one inside exon 4 and the other inside exon 7, were amplified, using the following primer pairs: *IL10RA*-4cFP/*IL10RA*-4c2RP; *IL10RA*-7cFP/*IL10RA*-7c2RP (Table 1). Patient numbering corresponds to that adopted in Figure 1A.

#### In silico analysis

In silico analysis of the -413G->T point mutation was performed using the Patch 1.0 software. Patch is a pattern-based program for predicting transcription factor binding sites (TFBS) in DNA sequences. It uses the set of binding sites from TRANSFAC® Public 6.0 and is free online available at the web site: <http://www.biobase-international.com/>.

#### *β*-catenin, TNFRI and TNFRII protein analysis in peripheral blood cells of UC patients

**Western blotting assay of *β*-catenin, TNFRI and TNFRII proteins:** Total protein was extracted from 3 mL



**Figure 1** Molecular characterisation of variant alleles within *interleukin-10* receptor genes in the inflammatory bowel diseases family members. A: Pedigree of the inflammatory bowel diseases (IBD) family and genomic single-nucleotide polymorphisms identified: interleukin-10 (*IL10*) RA: -413G->T (A); *IL10RA*-rs.: 2256111 Esone 4 c.549A->G (p.153Ala->Ala) (B); *IL10RA*-rs.:2229113 Esone 7 c.1051A->G (p.351Arg->Gly) (C); *IL10RA*-rs.:9610 3'UTR c.2543G->A (D); *IL10RB*-rs.: 2834167 Esone 1 c.139G->A (p.47 Lys ->Glu) (E); B: Sequence analysis of *IL10RA* promoter region. Sequence analysis was performed on amplified fragments from gDNA of the patients. Reported here are the electropherogram around the identified mutation -413G->T. The specific mutated nucleotide is shown within the black box; C: Gel-electrophoresis of the amplification refractory mutation-polymerase chain reaction performed for the -413G->T *IL10RA* promoter mutations. Patient numbering corresponds to that adopted in the shown above pedigree.

of peripheral blood cells (approximately  $5-7 \times 10^3$ /mL cells) using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions. Concentrations were determined and Western blotting assay was performed as previously described<sup>[19]</sup>. The primary antibody against amino-terminal  $\beta$ -catenin was from Cell Signaling Technology (Beverly, MA). Primary antibodies against TNFRI and TNFRII were from R&D System (R and D System, Minneapolis). The antibody against actin was from Santa Cruz (Santa Cruz, CA). H1-5 and H6-10 are mixes of healthy subjects. PHTS and FAP are two patients affected by PTEN hamartoma tumour syndrome and adenomatous polyposis coli syndrome, respectively. I -1, I -2, II -1 and II -2 are UC family members as reported in Figure 1A.

#### Real time PCR quantification analysis of *COX2* mRNA:

Real time PCR quantification analysis was performed for *COX2* messengers. Relative expression was calculated with the comparative Ct method and normalised against the Ct of Glucuronidase (GUS) mRNA. The quantitative RNA real time assays were performed as described before. To better normalise the healthy values, we used three blood mixes as controls, each containing five samples collected from healthy subjects, for a total of fifteen controls. H1-5,

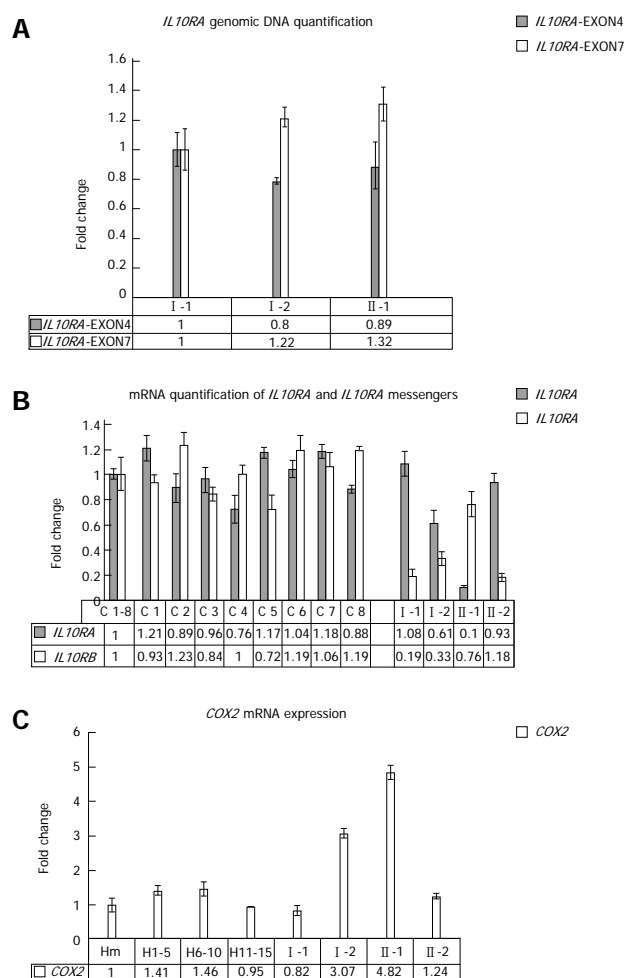
H6-10, H11-15 are mixes of healthy subjects. Hm is the mean value among all healthy samples used as calibrator to measure the relative expression. Patient numbering corresponds to that adopted in Figure 1A.

#### $\beta$ -catenin, TNFRI and TNFRII proteins expression in colorectal mucosa

**Western blotting assay of  $\beta$ -catenin, TNFRI and TNFRII proteins:** Total protein was extracted from the injured colorectal mucosa of the IBD proband and from healthy and tumour mucosa collected from patients affected by FAP and sporadic colorectal cancer using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions. Western blotting analysis of  $\beta$ -catenin (amino-terminal antigen), TNFRI and TNFRII was performed as previously described.

#### Incubation with mesalazine and azathioprine of established colon fibroblast culture:

**established colon fibroblast culture:** Samples of colorectal mucosa from IBD proband and one FAP patient were washed three times in PBS containing 300 U/mL penicillin, 300  $\mu$ g/mL streptomycin, and 2.5  $\mu$ g/mL amphotericin B (all from Gibco BRL, Karlsruhe, Germany), finely minced with scissors (tissue pieces of approximately 30 mm<sup>3</sup>) and digested in 2 mL 0.1% collagenase II (Boehringer Man-



**Figure 2** Real time polymerase chain reaction analysis of interleukin-10 receptors and *COX2* performed on peripheral blood cells. A: Copy number quantification of interleukin-10 (*IL10*) gene. Real time polymerase chain reaction (PCR) quantification analysis was performed for *IL10RA*. *IL10RA*-exon4: Amplified fragment at the boundaries of exon 4 and IVS4 of the gene; *IL10RA*-exon7: Amplified fragment at the boundaries of exon 7 and IVS7 of the gene; Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. B: Real time PCR quantification analysis of *IL10RA* and *IL10RB* mRNA. Real time RT-PCR quantification analysis was performed for *IL10RA* and *IL10RB* mRNA. C1-8: Mean value between all healthy samples used as calibrator to measure the relative expression; C1 to C8: Healthy subjects. Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. C: Real Time PCR quantification analysis of *COX2* messenger. H1-5, H6-10, H11-15: Mixes of healthy subjects; Hm: Mean value between all healthy samples used as calibrator to measure the relative expression; Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A.

nheim, Mannheim, Germany) in DMEM-15% FBS for 2 h at 37 °C, 5% CO<sub>2</sub>. The cell suspension was then collected by centrifugation, washed twice with serum-free DMEM medium, and subsequently cultured for 7 d in DMEM-15% FBS/CHANG C medium (1:1), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (all from Gibco BRL, Karlsruhe, Germany). Primary fibroblasts from IBD and FAP patients were stabilised, cultured on plates, and incubated with mesalazine (30 mmol/L) and azathioprine (30 mmol/L) for 12 h, alternatively. A combination of real time PCR of *IL10* receptors and Western blotting analysis of TNFα and TNFα receptors were performed as previously described.

## RESULTS

### Variant alleles of the *IL10* receptor genes act in a synergistic manner in the onset of UC

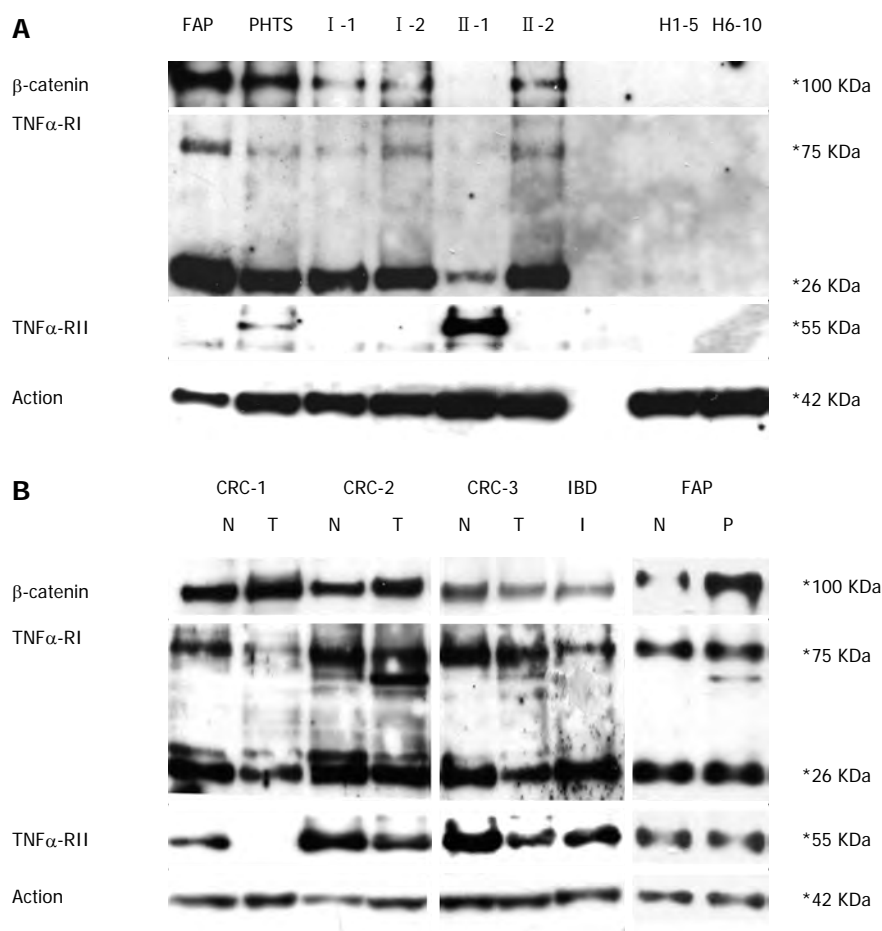
Molecular screening of *IL10RA* and *IL10RB*, performed on the proband and his relatives, revealed the presence of multiple SNPs in the patient, inherited from his parents, as shown in Figure 1A.

Specifically, the proband was heterozygous for the *IL10RB* E47K polymorphism (rs2834167, A/G genotype), inherited from his father, described to be associated with a low level of specific mRNA expression (to the A allele). As shown in Figure 1, he was also carrier of an *IL10RA* promoter point mutation (the -413G->T point mutation), inherited from his mother and not previously described in literature. In silico analysis of this mutation, performed using the Patch 1.0 software, shows that it alters a binding site for the Sp1 transcription factor. This genomic variant represents a specific mutation of this IBD family because it was not identified in 200 healthy subjects. The proband's father and his brother were both homozygous for *IL10RB* E47K polymorphism (rs rs:2834167 A/A genotype; 47K/K), whereas his mother was heterozygous A/G. Only the proband and his mother were carriers of the -413G->T point mutation identified in the promoter region of the *IL10RA* gene. For the following SNPs of *IL10RA*, the rs2256111, localised in the exon 4 (c.549A->G; p.153Ala->Ala), the rs:2229113, localised in the exon 7 (c.1051A->G; p.351Arg->Gly) and the rs:9610, localised in the 3'UTR (c.2543G->A), the proband was homozygous G/G, G/G and A/A, respectively. These three polymorphisms were A/G heterozygous in all other family members (Figure 1A). Using DNA real-time PCR for gene dosage of *IL10RA* gene, we ruled out the presence of intragenic or whole gene deletion (Figure 2A).

### *IL10* receptor variants are associated with mRNA under-expression

Associated with these genomic variants, we observed a under-expression of *IL10RA* and *IL10RB* mRNA in the proband compared to the average values of 8 healthy subjects, which segregates with each specific variant among the family members. In fact, as revealed by mRNA real-time quantification of both mRNAs of *IL10* receptors shown in Figure 2B, only the proband and his mother, carriers of the -413G->T promoter point mutation, showed a decrease in *IL10RA* mRNA. In contrast, the proband's father and his brother, both homozygous A/A for the *IL10RB* E47K polymorphism, show very low levels of *IL10RB* mRNA expression (fold change of approximately 0.19 and 0.18, respectively), whereas the proband and his mother, who were heterozygous A/G for this polymorphism, showed approximately 50% mRNA expression of the *IL10RB* compared to the mean value across eight healthy samples used as a calibrator (fold change of approximately 0.5 and 0.7 for the proband's mother and the proband himself, respectively). Furthermore, only the proband and his mother showed





**Figure 3**  $\beta$ -catenin, tumour necrosis factor  $\alpha$  receptors-I and II protein expression performed on peripheral blood cells and colon mucosa. **A:** Western blotting assay of  $\beta$ -catenin tumour necrosis factor  $\alpha$  receptors-I (TNFRI) and TNFRII performed on protein extracts from peripheral blood cells. Familial adenomatous polyposis (FAP): Patient affected by adenomatous polyposis coli; PHTS: Patient affected by PTEN hamartoma tumour syndrome; I-1, I-2, II-1, II-2: Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. H1-5, H6-10: mixes of healthy subjects; **B:** Western blotting assay of  $\beta$ -catenin TNFRI and TNFRII performed on protein extracts from colon mucosa. FAP: Patient affected by adenomatous polyposis coli; colorectal cancer (CRC)1, CRC2, CRC3: Patients affected by sporadic colorectal mucosa; inflammatory bowel diseases (IBD): Affected proband; N: Healthy colon mucosa; T: Colon tumour; P: Colon polyp; I: Inflamed colon mucosa.

COX2 overexpression, analysed in peripheral blood cells (Figure 2C).

#### Alteration of WNT/ $\beta$ -catenin pathway and TNF $\alpha$ receptors expression in the UC patient

As shown in Figure 3A,  $\beta$ -catenin and TNFRI protein were both over-expressed in the peripheral blood cells of the proband's relatives more than the proband. In contrast, TNFRII was over-expressed only in the proband. None of these proteins were detectable in healthy controls. When investigated in colon mucosa, both TNF $\alpha$  receptors were observed to be under-expressed in the inflamed colon mucosa and colorectal cancer compared to healthy colon mucosa. In the FAP patient, normal colon mucosa and polyps express TNF $\alpha$  receptors at the same level. Furthermore, as expected,  $\beta$ -catenin expression is much higher in the polyp than in normal mucosa. (Figure 3B)

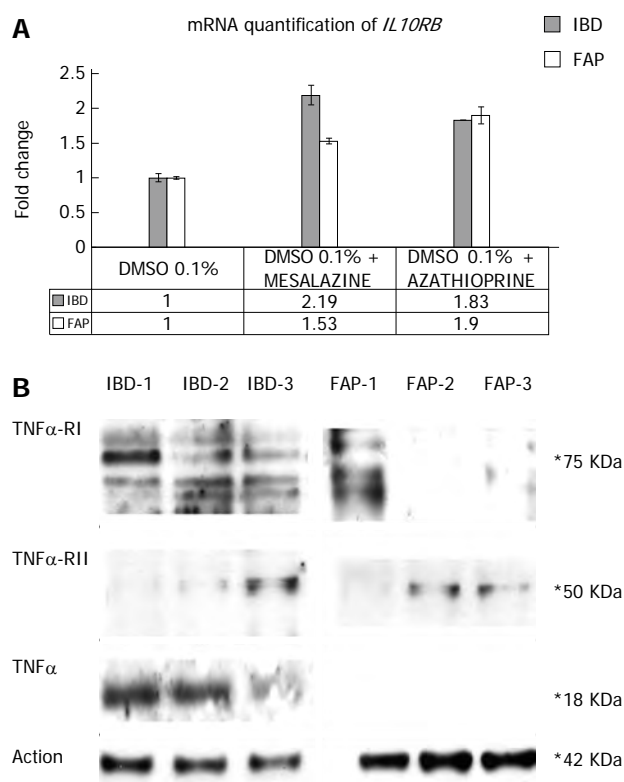
#### Effects of mesalazine and azathioprine on primary fibroblasts

Finally, we show that after incubation with mesalazine

and azathioprine of primary fibroblasts of the proband and of a FAP patient, drugs induce *IL10RB* mRNA and TNFRII protein over-expression, whereas TNFRI protein was under-expressed. A decrease of TNF $\alpha$  expression was also observed after incubation with azathioprine but not with mesalazine only in the IBD patient. Fibroblasts isolated from an FAP patient did not show any signal for TNF $\alpha$  hybridisation in our experimental conditions (Figure 4).

## DISCUSSION

A recent study demonstrated that mutations in *IL10* or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children<sup>[20,21]</sup>. In another approach to determining the genetic basis for these disorders, Moran *et al*<sup>[16]</sup> identified risk SNPs for very early onset IBD. Two SNPs, rs2228054 and rs2228055, were frequently found in the heterozygous state among IBD patients and inherited as a haplotype. The authors propose that the conferred risk may be due to one or both SNPs. Alternatively, the increased



**Figure 4** Effects of mesalazine and azathioprine on inflammatory bowel diseases and familial adenomatous polyposis primary fibroblasts. **A:** Real time polymerase chain reaction (PCR) quantification analysis of interleukin-10 (*IL10*) mRNA; Real time RT-PCR quantification analysis was performed for *IL10RB* mRNA on primary fibroblasts extracted from an inflammatory bowel diseases (IBD) and a familial adenomatous polyposis (FAP) patient and incubated with mesalazine and azathioprine; **B:** Western blotting assay of tumour necrosis factor  $\alpha$  receptors-I (TNFRI) and TNFRII and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) performed on protein extracts from primary fibroblasts of an IBD and of a FAP patient. IBD-1: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO only; IBD-2: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO and mesalazine; IBD-3: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO and azathioprine; FAP-1: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO only; FAP-2: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO and mesalazine; FAP-3: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO and azathioprine.

risk may reside in a regulatory region (*e.g.*, promoter) in linkage disequilibrium with these SNPs and suggest that this risk haplotype exerts a mild phenotype in the general population resulting in disease only in the presence of other genetic variants or environmental triggers<sup>[16]</sup>.

As suggested by Moran *et al.*<sup>[16]</sup> and also described for other human diseases<sup>[22]</sup>, our results confirm that early-onset IBD could be attributed to a synergistic effect of several variant alleles of the genes encoding *IL10* receptors. These variants, alone, could only give rise to a sub-clinical manifestation of the disease. In fact, the proband's father and his brother, both carriers of homozygous A/A polymorphism E47K for the *IL10RB* gene but without the -413G->T promoter mutation in the *IL10RA* gene, were apparently not affected. The proband's mother shows a genotype very similar to the proband. In fact, they are both heterozygous for the E47K *IL10RB*

gene polymorphism and for the -413G->T promoter mutation in the *IL10RA* gene. They show different mRNA expression for the *IL10RA* gene and quantitative real-time PCR revealed a 0.1 and 0.6-fold change for the *IL10RA* mRNA in the proband and his mother, respectively. This different gene expression could be due to other intragenic SNPs in the *IL10RA* gene whose alleles are different, such as, the rs.:2256111, localised in exon 4 (c.549A->G; p.153Ala->Ala), the rs.:2229113, localised in exon 7 (c.1051A->G; p.351Arg->Gly) and the rs.:9610, localised in the 3'UTR (c.2543G->A), that were homozygous G/G, G/G and A/A in the proband but A/G heterozygous in all other family members. However, we cannot rule out other gene expression regulatory mechanisms. Possibly due to the different *IL10RA* mRNA expression, the proband's mother has not developed the disease. However, she referred to an episode of rectal bleeding and shows increased levels of *COX2* mRNA expression in peripheral blood cells.

In a recent study, 66 early onset IBD patients were analysed. The authors identified 16 patients with loss-of-function mutations in the *IL10* or *IL10R* genes. A variety of mutations were discovered. Most patients were born from consanguineous parents and they carried homozygous biallelic mutations (point mutations or deletions). However, some patients also presented compound heterozygous mutations. Genotype/phenotype correlations were not clearly observed. In fact, siblings sharing the same homozygous *IL10RB* mutation showed a remarkably distinct level of disease severity, suggesting that the phenotypic manifestation is dependent on other intrinsic or extrinsic factors that remain presently unknown<sup>[21,23]</sup>.

Non-coding single nucleotide polymorphisms (SNPs) can be associated with qualitative and quantitative changes. Furthermore, genetic changes may affect transcription-factor-binding sequences, locus accessibility, translational efficiency and trans-regulators such as noncoding RNAs and microRNAs<sup>[12]</sup>. Cis- or trans-expression quantitative trait loci are detected for approximately half of the IBD risk regions, indicating that allele-specific gene-expression changes contribute to disease risk<sup>[24]</sup>.

Unexpectedly, we observed  $\beta$ -catenin and TNFRI protein over-expression in the peripheral blood cells of the proband's apparently healthy relatives more than in the proband himself. FAP and PHTS patients, but not healthy subjects, also expressed this protein, as previously described<sup>[19]</sup>. Therefore, we suggest that these proteins could represent a good candidate for molecular markers of sub-clinical disease in relatives of patients with UC. Previous studies showed that faecal calprotectin concentration in patients with CD and relatives differed significantly from controls, suggesting that there is a high prevalence of subclinical disease in first-degree relatives of these patients. This result conforms to an additive inheritance pattern in which the genetic basis for this abnormality may represent a risk factor for CD and UC<sup>[25,26]</sup>.

Because no therapeutic approach was successful in patients who are carriers of IL10 pathway alterations, we investigated the effect of mesalazine and azathioprine on the expression of IL10 receptors, TNF $\alpha$  and TNF $\alpha$  receptors. In agreement with our hypothesis, we found TNFRI under-expression and TNFRII and *IL10RB* over-expression in primary fibroblasts incubated with mesalazine and azathioprine, in both the UC and FAP patients. In the UC patient only, azathioprine, but not mesalazine, induces a TNF $\alpha$  decrease.

These observations could suggest that these drugs are only able to partially restore IL10 pathway function in UC, by activation of *IL10RB*, but not *IL10RA*, transcription. On the other hand, under-expression of TNFRI and over-expression of TNFRII could increase the risk of colorectal cancer-associated colitis in UC patients. As described by Chang *et al.*<sup>[27]</sup>, TNFRI has tumour suppressor activity in the context of colitis-associated cancer, and the role of TNFRII in cell proliferation is well known.

Current therapeutic strategies for paediatric IBD include the use of exclusive enteral nutrition, corticosteroids, mesalamine, sulfasalazine, immunomodulators (azathioprine, 6-mercaptopurine, methotrexate) and anti-TNF $\alpha$ -antibodies<sup>[22,28]</sup>. Aminosalicylates are the undisputed first-line option for treating and maintaining remission in UC<sup>[29]</sup>. However, the role that these drugs may play in the management of Crohn's disease has been controversial. Thiopurine drugs, azathioprine and mercaptopurine, have been shown to be effective in inducing and maintaining remission in IBD<sup>[30]</sup>. Most epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC<sup>[14,31]</sup>, although some studies failed to show this, as described by Velayos *et al.*<sup>[32]</sup>.

TNF signals *via* two cell surface receptors, TNFRI and TNFRII, resulted in several, sometimes opposing, cellular responses that vary by context and cell nature<sup>[33,34]</sup>. In the colonic mucosa, TNF is involved in both cell survival and cell death<sup>[35]</sup>. Additionally, increased levels of TNF have been found in the setting of cancers, including those of the pancreas, skin, and ovaries<sup>[36]</sup>. With specific regard to colon carcinogenesis, TNF activity has been shown both to promote and to protect from neoplastic transformation<sup>[37-39]</sup> and there are case studies of development of cancer in other organ systems (lymphatic and skin) following the use of anti-TNF for IBD or rheumatological disease<sup>[40]</sup>. For this reason, we investigated protein expression of TNF receptors in colon mucosa of the UC patient compared to that of normal and cancer colon mucosa from patients affected by FAP and sporadic colorectal cancer. In agreement with the hypothesis suggested by Chang *et al.*<sup>[27]</sup> about the tumour suppressor activity of TNFRI in the context of colitis-associated carcinogenesis, we found not only a decrease in the expression of TNFRI but also of TNFRII in colorectal cancer when compared to normal colon mucosa for each patient. The expression of TNF receptor proteins in colon mucosa of our UC patient was at an

intermediate level between that observed in colorectal tumour tissue and normal mucosa of CRC patients.

In conclusion, our results, in agreement with data from recently published literature<sup>[5,16,22]</sup>, indicate that early-onset UC could be caused by a synergistic effect of more variant alleles of the *IL10* receptors gene, resulting in alteration of the IL10 pathway. In our opinion, a dosage model of nonallelic non-complementation fits well with this case, whereby mutations in two different genes can behave as alleles of the same locus by causing or exacerbating the same phenotype. However, we cannot exclude, as described for others syndromes, that different mechanisms, such as alternative splicing mechanisms<sup>[41,42]</sup> or allelic variants of modifier genes, could contribute to the observed phenotypic variability<sup>[22]</sup>.

In addition, we suggest that the expression of  $\beta$ -catenin and TNFRI protein could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients. Recent findings suggest that chronic inflammation in IL10-/- mice increased P- $\beta$ -catenin552 expression. Moreover, TNFRI exerts its tumour suppressor activity by modulating activation of  $\beta$ -catenin and controlling epithelial proliferation<sup>[43]</sup>. It clearly appears that classical therapeutic approaches do not seem adequate for IBD patients who are carriers of IL10 pathway alterations because under-expression of TNFRI signalling would confer increased risk of developing colitis associated-carcinoma. Allogenic hematopoietic stem cell transplantation could represent a causal therapeutic approach for IL10R-deficient patients, useful for the treatment of the intractable ulcerating enterocolitis of the infant, as recently suggested<sup>[14,15,20-22]</sup>.

## COMMENTS

### Background

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host. Mutations in interleukin-10 (*IL10*) or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children.

### Research frontiers

Increased numbers and activation of immune cells in the intestinal mucosa enhance local levels of tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and several proinflammatory IL. Recent work has demonstrated that IBD with an early onset can be monogenic and *IL10* polymorphisms have been associated with IBD in genome-wide association studies. The aims of this work were to clarify the molecular basis of disease in this young child, shedding light on a synergistic effect of *IL10RA* and *IL10RB* polymorphisms. The authors also assessed the possible presence and inheritance of subclinical intestinal inflammation in apparently healthy relatives of this patient with ulcerative colitis (UC).

### Innovations and breakthroughs

Recent studies have shown that loss-of-function mutations in *IL10RA*, *IL10RB* and *IL10* genes, in immunodeficient patients, are associated with severe, infantile-onset IBD. In particular, literature reports have highlighted the role of *IL10RA* polymorphisms in the risk for developing very early onset UC. This is the first study reporting that *IL10RA* polymorphisms could have synergistic effect with those of *IL10RB*. The authors propose that these risk polymorphisms exert a mild phenotype in the general population resulting in disease only in the presence of other genetic variants in the *IL10RA* or *IL10RB*. Furthermore, these observations would suggest an inherited abnormality of beta catenin and TNFRI in the proband's relatives.



## Applications

This work expands the understanding of the complex inheritance pattern of very early onset ulcerative colitis. It seems possible that the subclinical phenotypic manifestations identified in the first-degree relatives of the proband represents the consequence of inherited defects of *IL10R* genes, which then represent one of the risk factors for the disease. This study could contribute to identifying at-risk families for very early onset UC allowing clinicians to perform genetic tests and appropriate care.

## Terminology

IL10 is an anti-inflammatory cytokine secreted by a variety of cell types and is critical for maintaining immune homeostasis in the gastrointestinal tract. IL10 activates downstream signalling by binding to IL10R, comprised of two  $\alpha$  subunits (encoded by *IL10RA*) and two beta subunits (encoded by *IL10RB*).

## Peer review

The authors investigated the molecular cause of very early-onset inflammatory bowel disease in an 18-mo-old child as well as his relatives. They concluded that a synergistic effect of several variant alleles of the *IL10 receptor* genes, inherited in a Mendelian manner, is involved in IBD onset in this young child. This study supports a special enthusiasm about the potential power of genomics to define the aetiology and/or phenotype of diseases. When a single specific case or family is studied, the discovery of new functional polymorphisms and the functional consequences of these mutations deserves attention even if the functional characterisation and the real pathogenic contribution of susceptible genes are hard to assess in complex disorders such as IBD.

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## Dietary-suppression of hepatic lipogenic enzyme expression in intact male transgenic mice

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### Abstract

**AIM:** To study, in intact male transgenic mice, the effects of three diets based on olive oil and olive oil diet supplemented with lovastatin and orlistat on hepatic lipogenic enzymes expression, considered markers of cell proliferation.

**METHODS:** Forty  $Apc^{Min/+}$  mice were randomly divided into 4 groups and fed for 10 wk: olive oil (OO) group,  $n = 10$  animals received a diet with olive oil 12%; olive oil plus lovastatin (LOVA) group,  $n = 10$  animals received the same diet with olive oil supplemented with lovastatin 5 mg/kg; olive oil plus orlistat (OR) group,  $n = 10$  animals fed the diet with olive oil supplemented with orlistat 50 mg/kg and SD group,  $n = 10$  animals fed a standard diet. The activity of lipogenic enzymes and their gene expression were evaluated by radiomet-

ric and real-time reverse transcription-polymerase chain reaction assay, respectively.

**RESULTS:** After 10 wk of dietary treatment, the body weight was no different among animal groups ( $21.3 \pm 3.1$  g for standard group,  $22.1 \pm 3.6$  g for OO group,  $22.0 \pm 3.2$  g for LOVA group and  $20.7 \pm 3.4$  g for OR group, data expressed as mean  $\pm$  SD), observing a generalized well-being in all animals. All the dietary managed treated groups presented significantly reduced hepatic levels of fatty acid synthase, farnesyl pyrophosphate synthase and 3-hydroxyl-3-methyl-glutaryl CoA reductase activity and gene expression when compared with the mice fed the standard diet. To evaluate cell proliferation in the liver of treated mice, the levels of cyclin E mRNA have been measured, demonstrating a significant reduction of *cyclin E* gene expression in all treated groups. Evidence of reduced hepatic cell proliferation was present overall in OO group mice.

**CONCLUSION:** We confirm the role of lipogenic enzymes as markers of cell proliferation, suggesting that appropriate dietary management alone or with drugs can be a feasible approach to counteract hepatic cell proliferation in mice.

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**Key words:** Lipogenic enzymes; Liver; Markers of cell proliferation; Transgenic mice; Dietary treatment

**Core tip:** The olive oil diet significantly reduces the enzymatic activities, as well as the expression of hepatic cell cycle related genes. The addition of drugs as lovastatin and orlistat to olive oil diet more down-regulated the studied lipogenic enzymes, demonstrating that the inhibition of these enzymes with natural components of diet could have a potential benefit in association with canonical chemical substances to counteract hepatic

cell proliferation in mice.

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## INTRODUCTION

Several alterations of lipid metabolism are often found in tumors, where neoplastic lipogenesis is essential for cancer cell survival<sup>[1]</sup>. Cancer cells esterify fatty acids predominantly to phospholipids, essential component of cell membranes. The main pathway through which proliferating cells gain lipids for membrane synthesis is the endogenous mevalonate pathway<sup>[2,3]</sup>. Increased synthesis of mevalonate and mevalonate derived isoprenoids supports increased cell proliferation through the activation of growth-regulatory proteins and oncoproteins and by promoting DNA synthesis<sup>[4,5]</sup>.

Endogenous fatty acid synthesis is dependent on the activity of fatty acid synthase (FAS). This enzyme is over-expressed in many types of malignancies, including prostate, breast, lung and colon cancer<sup>[6-8]</sup>. The tumor environment contains regions of poor oxygenation and high acidity, and FAS over-expression could confer a selective growth advantage upon these unfavorable conditions<sup>[9]</sup>.

It is known that hydroxyl-3-methyl-glutaryl CoA reductase (HMGCoAR) activity is up-regulated severalfold in colon tumors and not regulated by feedback inhibition from cholesterol compared with normal mucosa<sup>[2,10,11]</sup>. Alterations in biosynthetic processes of the mevalonate pathway and in the levels of enzyme products participating in this biochemical system may contribute to the cell growth advantage acquired during carcinogenic process and to the development of malignancy.

In cancer, high levels of mevalonate-derived metabolites, such as isoprenoid compounds have been demonstrated<sup>[5,12,13]</sup>. Several HMGCoA metabolites, such as farnesyl pyrophosphate (FPP) and geranyl pyrophosphate are implicated in oncogene activation and tumorigenesis<sup>[14]</sup>. FPP, produced by activity of FPP synthase, is the substrate for the farnesylation of a wide number of proteins implicated as potential growth regulators. FPP synthase gene is over-expressed in different human tumors as well as an upregulation of FPP synthase has been detected in about 85% of hepatocellular carcinoma<sup>[15]</sup>. An elevated FPP synthase expression has also been observed in rat prostate tumor cell lines<sup>[16]</sup>.

Previously, we demonstrated an high FPP synthase activity in human colorectal cancer<sup>[17]</sup>.

The regulation of lipogenic enzymes abundance in cancer cells is complex and occurs at the transcriptional or posttranscriptional levels. Several studies show that

blockade of these enzymes can attenuate the growth and survival of tumor cells<sup>[1,18,19]</sup>, being potential target for cancer therapy.

Moreover, promotion and progression of carcinogenesis are susceptible to nutritional interventions aimed at counteracting cancer development<sup>[20]</sup>. In this respect, olive oil consumption has been demonstrated to reduce the incidence of aberrant crypt foci in azoxymethane-treated rats<sup>[21]</sup>. Furthermore, olive oil is able to down-regulate the expression of cyclooxygenase-2 and BCL-2 proteins that plays a crucial role in colorectal carcinogenesis<sup>[22]</sup>. Olive oil healthy effects can be attributed not only to the higher relationship between unsaturated and saturated fatty acids, but also to the antioxidant property of its phenolic compounds, as oleuropein and hydroxytyrosol (HT). As antioxidants, polyphenols may protect cell constituents against oxidative damage and act as highly effective chemopreventive agents<sup>[23,24]</sup>.

Olive oil polyphenols are quickly absorbed by intestine, but the biotransformation of absorbed HT should take place mostly in the liver. Taking into account this point, the aim of the present study was to test in the  $Apc^{Min/+}$  mouse model three diets based on olive oil and olive oil diet supplemented with lovastatin and orlistat, known agents with antitumor activity and inhibitors of HMGCoAR and FAS, respectively. Since high serum concentration of the lipid and liver steatosis have been observed in  $Apc^{Min/+}$  mice<sup>[25,26]</sup>, this experimental model was selected to evaluate a putative hepatic dietary-induced down-regulation of lipogenic enzymes.

## MATERIALS AND METHODS

### Animals and experimental study design

Five-week-old C57BL/6J mice with an heterozygote mutation for the *Apc* gene ( $Apc^{Min/+}$ ) were obtained from Charles River (Calco, CO, Italy). The mice were maintained in the animal care facility at our Institute. They were kept in temperature, air- and light-controlled conditions and received food and water *ad libitum*. Animals did not receive any surgical or hormonal manipulation but were kept anatomically and physiologically intact. All animals received care in compliance to the "Guide for the Care and Use of Laboratory Animals". The procedures related to animal use have been communicated to the Italian Ministry of Health and approved.

Forty  $Apc^{Min/+}$  mice were randomly divided into 4 groups and fed for 10 wk: olive oil (OO) group,  $n = 10$  animals received a diet with olive oil 12% (12.5% protein, 12% oils and fats, 3% fibers); lovastatin (LOVA) group,  $n = 10$  animals received the same diet with olive oil supplemented with lovastatin 5 mg/kg; orlistat (OR) group,  $n = 10$  animals fed the diet with olive oil supplemented with orlistat 50 mg/kg and SD group,  $n = 10$  animals fed a standard diet (18.5 % protein, 5% oils and fats, 4.2% fibers). Any diet was provided in pellets by Mucedola Srl, Settimo Milanese, Italy. Body weight and food intake were measured every 3 d.



After 10 wk of dietary treatment, the animals were killed by cervical dislocation. The liver from each animal was immediately excised and washed with phosphate buffered saline. Samples of fresh liver tissue were rapidly frozen and stored at -80 °C and the counterpart specimens were fixed in 10% buffered formalin to assess histological analysis.

### FAS activity assay

FAS activity was determined on frozen liver samples. After tissue homogenization and centrifugation, an aliquot of supernatant (50 µL) was pre-incubated with 100 mmol/L potassium phosphate buffer, pH = 7 for 15 min at 37 °C. Subsequently, 20 µL of reaction mix (2.5 mmol/L NADPH, 1.25 mmol/L acetyl-CoA, 1.25 mmol/L malonyl-CoA and 0.02 mmol/L 2-<sup>14</sup>C-malonyl-CoA (52 mCi/mmol, Amersham Biosciences, United Kingdom) were added and samples were incubated for 10 min at 37 °C. Reactions were stopped by the addition of 500 µL 1 mol/L HCl/methanol (6:4, v:v); fatty acids were extracted with 1 mL of petroleum ether and incorporation of 2-<sup>14</sup>C-malonyl-CoA was analyzed by scintillation counting. FAS activity was expressed as picomoles of incorporated 2-<sup>14</sup>C-malonyl-CoA per minute per milligram of total proteins (pmol/min/mg prot).

### Preparation of microsomal fraction

Frozen hepatic tissue specimens were placed in cold homogenization buffer containing 0.3 mol/L sucrose, 10 mmol/L EDTA (pH = 7.4) and 1 mmol/L 2-β-mercaptoethanol. Each homogenate was centrifuged at 900 × *g* for 5 min at 4 °C; the supernatant was further centrifuged at 8700 *g* for 10 min; the pellet was discarded and the supernatant was centrifuged at 10000 *g* for 10 min to obtain microsomal fraction. Each pellet was resuspended in 0.2 mL ice-cold buffer containing 20 mmol/L imidazol (pH = 7.4), 5 mmol/L dithiothreitol.

### FPP synthase activity assay

FPP synthase assay was carried out with some modifications of the procedure of Krisans *et al.*<sup>[13]</sup> and described by Gupta *et al.*<sup>[27]</sup>. Briefly, FPP synthase was assayed in 150 µL containing 25 mmol/L Hepes, pH = 7, 2 mmol/L MgCl<sub>2</sub>, 1 mmol/L dithiothreitol, 5 mmol/L KF, 1% noctyl-β-glucopyranoside, 3.3 µmol/L [4-<sup>14</sup>C] IPP (18 Ci/mmol), 3 µmol/L unlabeled IPP and 20 µmol/L geranyl diphosphate. Reactions were started by adding 40 µL of microsomal fraction containing 100 µg of total protein and incubated for 45 min at 37 °C. Reactions were stopped by the addition of 150 µL 2.5 mol/L HCl in 80% ethanol containing 100 µg/mL farnesol as a carrier. The samples were hydrolyzed for 30 min at 37 °C to convert the FPP to farnesol and neutralized by the addition of 150 µL of 10% NaOH. The reaction product (farnesol) was extracted into 1 mL of *N*-hexane and an aliquot (200 µL) of the organic phase was used for radioactivity counting. One unit of enzyme activity is defined as the amount of enzyme required to synthesize

**Table 1 Sequences of amplification primers**

Gene		Primer
FPP synthase	Sense	5'-AAAATGTGGCACTGACATCCAGG-3'
	Antisense	5'-GGGTGCTGCGTACTGTTCATG-3'
FAS	Sense	5'-GATCCTGGAACGAGAACACGA-3'
	Antisense	5'-GAGACGTGTCACTCCTGGACTTG-3'
HMGCoAR	Sense	5'-GCTTGAGCATCCTGACATAC-3'
	Antisense	5'-GAACCATAGTCCCACGTCT-3'
Cyclin E	Sense	5'-GTCTTCGCAGATCGCAGA-3'
	Antisense	5'-GAGACCTTCTGCGACTCCA-3'
β-actin	Sense	5'-GCCTCTGGTCGTACCACTGGC-3'
	Antisense	5'-AGGGAGGAAGAGGATGCGCA-3'

FPP: Farnesyl pyrophosphate; FAS: Fatty acid synthase; HMGCoAR: 3-hydroxy-3-methyl-glutaryl CoA reductase.

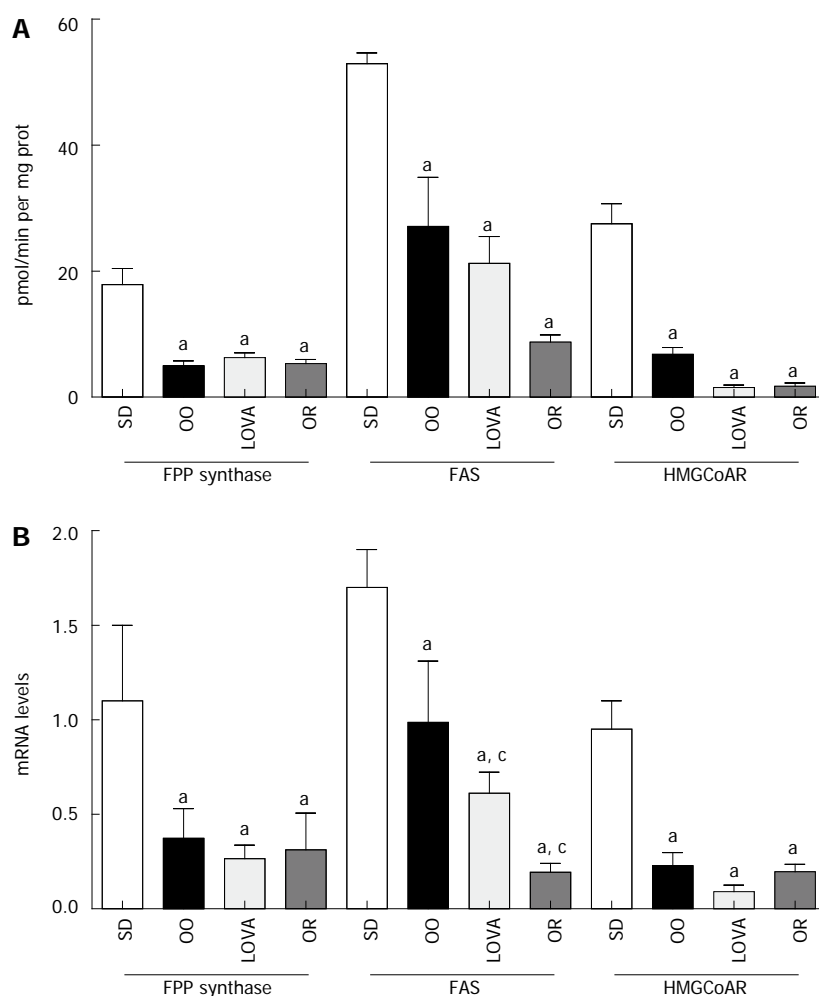
one pmol of FPP per min. Parallel samples were assayed to evaluate the total and the nonspecific radioactivity. In all experiments, enzyme assays were carried out in duplicate. The coefficient percentages of intra- and inter-assay variation were 3% and 4%, respectively.

### HMGCoAR activity assay

HMGCoAR activity was measured by radiochemical assay using *DL*-3-hydroxy-3-methyl-[3-<sup>14</sup>C]-glutaryl-coenzyme A (<sup>14</sup>C-HMGCoA) as substrate. Briefly, 50 µL of microsomal fraction containing about 50 µg of total protein were pre-incubation for 10 min at 37 °C with 50 µL of cofactors solution containing 1 mol/L potassium-phosphate buffer (pH = 7.4), 100 mmol/L EDTA (pH = 7.4), 50 mmol/L dithiothreitol, 200 mmol/L glucose-6-phosphate, 25 mmol/L NADP and 0.5 U of glucose-6-phosphate dehydrogenase. Reactions were started by adding 10 µL of <sup>14</sup>C-HMGCoA (specific activity 5.0 mCi/mmol) in each sample followed by an incubation for 20 min at 37 °C. Reactions were stopped by the addition of 20 µL of KOH 33% and incubated for 45 min to consent the hydrolysis of substrate. The subsequent addition of 50 µL of 5 mol/L HCl and the incubation for 60 min at 37 °C were performed to convert the formed [<sup>14</sup>C]-mevalonic acid in [<sup>14</sup>C]-mevalonolactone. The reaction product (<sup>14</sup>C-mevalonolactone) was extracted into 300 µL of *N*-hexane and an aliquot (200 µL) of the organic phase was used for radioactivity counting. HMGCoAR activity was expressed as picomoles of [<sup>14</sup>C]-mevalonate formed per minute per milligram of microsomal proteins (pmol/min/mg prot). In all experiments, enzyme assays were carried out in duplicate.

### Gene expression analysis

Total RNA from samples of liver tissue was isolated with TRI-Reagent (Mol. Res. Centre Inc. Cincinnati, O, United States), following the manufacturer's instruction. Briefly, the tissue was homogenized in 0.25 mL of cold 0.9% NaCl; then, 0.75 mL of TRI-Reagent and 0.2 mL of chloroform were added to the homogenate. The samples were vigorously shaken and centrifuged and the RNA present in the aqueous phase was precipitated with 0.5 mL of isopropanol. The RNA pellet was washed



**Figure 1** Farnesyl pyrophosphate synthase, fatty acid synthase and 3-hydroxyl-3-methyl-glutaryl CoA reductase activity, mRNA levels in the four groups of treatment. A: Activity levels in the four groups of treatment. <sup>a</sup> $P < 0.05$  vs SD group (one-way analysis of variance and Tukey's Multiple Comparison Test). Data are expressed as mean  $\pm$  SE; B: mRNA levels in the four groups of treatment. <sup>a</sup> $P < 0.05$  vs SD group (one-way analysis of variance and Tukey's Multiple Comparison Test); <sup>c</sup> $P < 0.05$  vs OO group (Tukey's Multiple Comparison Test). Data expressed as  $n$  molecules mRNA target gene/ $n$  molecules mRNA  $\beta$ -actin. SD: Standard diet; OO: Olive oil; LOVA: Olive oil plus lovastatin; OR: Olive oil plus orlistat; FPP: Farnesyl pyrophosphate; FAS: Fatty acid synthase; HMGCoAR: 3-hydroxyl-3-methyl-glutaryl CoA reductase.

once with 1 mL of 75% ethanol, dried, resuspended in sterile water and quantified by UV absorbance. 2  $\mu$ g of total RNA were used for the reverse transcription reaction performed in 20  $\mu$ L of final volume at 41  $^{\circ}$ C for 60 min, using 30 pmol of antisense primer (Table 1) for analyses of the FAS, FPP synthase, HMGCoAR, cyclin E and the  $\beta$ -actin gene.  $\beta$ -actin gene was utilized as reference gene. Real-time PCRs were performed in 25  $\mu$ L of final volume containing 2  $\mu$ L of cDNA, master mix with SYBR Green (iQ SYBR Green Supermix Bio-Rad, Milan, Italy) and sense and antisense primers for the *FAS*, *FPP* synthase, *HMGCoAR*, *cyclin E* and the  *$\beta$ -actin* gene (Table 1).

Real-time polymerase chain reaction (PCR) was carried out in an CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) using the following protocol: 45 cycles at 95  $^{\circ}$ C for 3 min, 95  $^{\circ}$ C for 10 s, 55  $^{\circ}$ C for 30 s followed by a melting curve step at 65  $^{\circ}$ C–95  $^{\circ}$ C with a heating rate of 0.5  $^{\circ}$ C per cycle for 80 cycles. The PCR products were quantified by external calibration curves, one for each tested gene, obtained with serial dilutions of

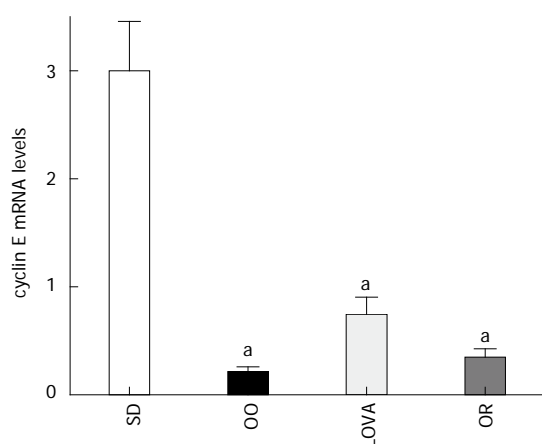
known copy number of molecules ( $10^2$ – $10^7$  molecules). All expression data were normalized by dividing the target amount by the amount of  $\beta$ -actin used as internal control for each sample. The specificity of the PCR product was confirmed by gel electrophoresis.

### Statistical analysis

The significance of the differences among experimental groups was evaluated with one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test. Differences were considered significant at a 5% probability level.

## RESULTS

After 10 wk of dietary treatment, the body weight (in grams) was no different among animal groups ( $21.3 \pm 3.1$  g for standard group,  $22.1 \pm 3.6$  g for OO group,  $22.0 \pm 3.2$  g for LOVA group and  $20.7 \pm 3.4$  g for OR group, data expressed as mean  $\pm$  SD), observing a generalized well-being in all animals.



**Figure 2** Cyclin E mRNA levels (mean  $\pm$  SE) in the four groups of treatment. Data expressed as  $n$  molecules mRNA cyclin E gene/ $n$  molecules mRNA  $\beta$ -actin. <sup>a</sup> $P < 0.05$  vs SD group (one-way analysis of variance and Tukey's Multiple Comparison Test). SD: Standard diet; OO: Olive oil; LOVA: Olive oil plus lovastatin; OR: Olive oil plus orlistat.

All the dietary managed treated groups presented significantly reduced levels of hepatic lipogenic enzymes activity when compared with the mice fed the standard diet, being such reduction particularly marked in LOVA group for HMGCoAR and in OR group for FAS (one-way analysis of variance and Tukey's Multiple Comparison Test,  $P < 0.05$ ) (Figure 1A).

The levels of FPP synthase, FAS and HMGCoAR mRNA in liver tissue followed the same behavior of protein activities (Figure 1B). Differences statistically significant were observed between the three groups of treatment and the mice group treated with standard diet for all enzymes studied (one-way analysis of variance and Tukey's Multiple Comparison Test,  $P < 0.05$ ). For FAS mRNA levels, a significant reduction was also detected between LOVA group and OR group compared to OO group (Figure 1B).

To evaluate cell proliferation in the liver of treated mice, the levels of cyclin E mRNA have been measured, demonstrating a significant reduction of *cyclin E* gene expression in all treated groups. Evidence of reduced hepatic cell proliferation was present overall in OO group mice (Figure 2).

## DISCUSSION

Our data support appropriate dietary management as a feasible approach to counteract hepatic cell proliferation in mice, targeted to the selective down regulation of FAS, FPP synthase and HMGCoAR considered biomarkers of cell proliferation.

Understanding the distribution of roles within a biochemical pathway is clearly important and it provides a rationale for selecting a particular reaction step suitable for therapeutic intervention.

Statins having biochemical effects on cholesterol synthesis, are considered as potential anti-tumor agents<sup>[28]</sup>, inhibiting tumor cell growth by restricting either chole-

sterol availability or cholesterol synthesis<sup>[28,29]</sup>. Previously, we have demonstrated that the combined treatment with eicosapentaenoic acid (EPA) and lovastatin enhanced the regulatory effect on gene expression of HMGCoAR and low density lipoprotein receptor in HepG2 cell line<sup>[30]</sup>. Moreover, we detected a synergistic effect in the inhibition of cancer cell proliferation obtained by combination of EPA and Lovastatin, demonstrating an inhibition at the lower doses with respect to the substances used separately.

On the other hand, orlistat, as an anti-obesity drug, is a novel and selective FAS inhibitor in tumors<sup>[1]</sup>. Moreover, FAS inhibition by orlistat reduces proliferation and promotes apoptosis in prostate, breast and gastric cancer cell lines<sup>[31,32]</sup>.

In our previous study<sup>[33]</sup>, we showed a down-regulation of FAS observed after HT treatment in SW620 cell line, suggesting that FAS might be a molecular target for anti-proliferative activity of olive oil polyphenols in a metabolically defined subset of colon cancer.

In this study the reduction of *cyclin E* gene expression in mice liver by these compounds demonstrates their ability to inhibit cell proliferation. This finding supports the role of lipogenic enzymes as markers of cell growth.

The olive oil diet significantly reduces the enzymatic activities, as well as the expression of hepatic cell cycle related genes. The addition of drugs as lovastatin and orlistat to olive oil diet more down-regulated the studied lipogenic enzymes, demonstrating that the inhibition of these enzymes with natural components of diet could have a potential benefit in association with canonical chemical substances to counteract hepatic cell proliferation in mice.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Several alterations of lipid metabolism are often found in tumors, where neoplastic lipogenesis is essential for cancer cell survival. Increased synthesis of mevalonate and mevalonate derived isoprenoids supports increased cell proliferation through the activation of growth-regulatory proteins and oncoproteins and by promoting DNA synthesis. Promotion and progression of carcinogenesis are susceptible to nutritional interventions aimed at counteracting cancer development. In this respect, olive oil consumption has been demonstrated to reduce the incidence of colorectal cancer. Olive oil healthy effects can be attributed not only to the higher relationship between unsaturated and saturated fatty acids, but also to the antioxidant property of its phenolic compounds, as oleuropein and hydroxytyrosol.

### Research frontiers

Alterations in biosynthetic processes of the mevalonate pathway and in the levels of enzyme products participating in this biochemical system may contribute to the cell growth advantage acquired during carcinogenic process and to the development of malignancy. The blockade of these enzymes can attenuate the growth and survival of tumor cells, being potential target for cancer therapy. Their paper demonstrates that the inhibition of lipogenic enzymes with natural components of diet could have a potential benefit in association with canonical chemical substances to counteract hepatic cell proliferation in mice.

# Innovations and breakthroughs

The study supports the role of lipogenic enzymes as markers of cell growth. Reported data on possible benefits of olive oil to counteract hepatic cell proliferation in mice, highlight the importance and the innovation that an appropriate dietary treatment can be useful in cancer prevention.

# Applications

Further studies will be designed to translate the findings in clinical practice. Olive oil through its ability to suppress the lipogenic enzymes may provide well-tolerated novel therapies, particularly in metabolic disorders-related tumors, as gastrointestinal cancers.

# Terminology

Lipogenic enzymes are involved in lipid metabolism. The lipogenesis is essential for cell membrane synthesis. The main pathway through which proliferating cells gain lipids for membrane synthesis is the endogenous mevalonate pathway, where the 3-hydroxyl-3-methyl-glutaryl CoA reductase (HMGCoAR) is the key enzyme. Among HMGCoAR metabolites, farnesyl pyrophosphate (FPP), produced by activity of FPP synthase, is the substrate for the farnesylation of a wide number of proteins considered potential growth regulators. Moreover, endogenous fatty acid synthesis is dependent on the activity of fatty acid synthase (FAS). FAS is a multi-enzyme protein containing domains for acyl-carrier peptide and the seven different catalytic activities required for the conversion of acetyl-CoA and malonyl-CoA to palmitate. Expression of FAS is linked to specific functions such as the conversion and storage of energy in the liver and in adipose tissue, and is possibly involved in the regulation of food intake.

# Peer review

The aim of this research is to study the effects of three diets based on olive oil and olive oil diet supplemented with lovastatin and orlistat on hepatic lipogenic enzymes expression in Apc<sup>Min/+</sup> mice which are divided randomly into 4 groups of 10 animals per group that were fed for 10 wk: Concentration of olive oil used was 12%; lovastatin 5 mg/kg; orlistat 50 mg/kg and SD group that was fed a standard diet. The activity of lipogenic enzymes and their gene expression were evaluated by radiometric and real-time reverse transcription-polymerase chain reaction assay. Results show that all the dietary managed treated groups significantly reduced hepatic levels of fatty acid synthase, farnesyl pyrophosphate synthase and 3-hydroxyl-3-methyl-glutaryl CoA reductase activity and gene expression when compared with the mice fed the standard diet. The data are the potential interest and they confirm the role of lipogenic enzymes as markers of cell proliferation, suggesting that appropriate dietary management alone or with drugs can be a feasible approach to counteract hepatic cell proliferation in mice. The conclusions indicate that the observed effects could serve as markers for hepatic cell proliferation.

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## Alanine aminotransferase normalization at week 8 predicts viral response during hepatitis C treatment

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### Abstract

**AIM:** To investigate alanine aminotransferase (ALT) and sustained virological response (SVR) in chronic hepatitis C (CHC) during peginterferon-ribavirin treatment.

**METHODS:** One hundred and fifty-one genotype 1 CHC patients underwent treatment for 48 wk with peginterferon and ribavirin, and were retrospectively divided into two groups as having a rapid virological response (RVR) (Group 1,  $n = 52$ ) and not having an RVR (Group 2,  $n = 99$ ). We also subdivided each group into two according to the initial ALT level being high (Group 1h and Group 2h) or normal (Group 1n and Group 2n). HCV RNA and ALT levels were measured at baseline; at 4, 12, 24 and 48 wk during the treatment period; and at 24 wk follow-up. ALT levels were also obtained at 8 wk. According to the results of ALT, patients were enrolled in either the follow-up abnormal or follow-up normalized ALT groups at each interval. Patients with high and normal ALT levels were compared for each interval in terms of SVR.

**RESULTS:** The SVR rates were 83% vs 40% ( $P = 0.000$ ), 82% vs 84% ( $P = 0.830$ ), and 37% vs 44% ( $P = 0.466$ ) when comparing Group 1 with 2, 1h with

1n, and 2h with 2n, respectively. In Group 2h, the SVR rates were 34% vs 40% ( $P = 0.701$ ), 11% vs 52% ( $P = 0.004$ ), 12% vs 50% ( $P = 0.007$ ), 7% vs 50% ( $P = 0.003$ ), 6% vs 53% ( $P = 0.001$ ), and 0% vs 64% ( $P = 0.000$ ) when comparing patients with high and normalized ALT levels at week 4, 8, 12, 24, 48 and 72, respectively. The multiple logistic regression analysis revealed that RVR (OR = 7.05; 95%CI: 3.1-16.05,  $P = 0.000$ ), complete early virological response (cEVR) (OR = 17.55; 95%CI: 6.32-48.76,  $P = 0.000$ ), normalization of ALT at 8 wk (OR = 3.04; 95%CI: 1.31-7.06,  $P = 0.008$ ), and at 12 wk (OR = 4.21; 95%CI: 1.65-10.76,  $P = 0.002$ ) were identified as independent significant predictive factors for SVR.

**CONCLUSION:** Normalization of ALT at 8 wk may predict viral response during peginterferon-ribavirin treatment in genotype-1 CHC patients especially without RVR.

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**Key words:** Chronic hepatitis C; Genotype-1; Alanine aminotransferase; Rapid virological response; Sustained virological response; Interferon; Ribavirin

**Core tip:** Rapid virological response (RVR) has been acknowledged as a powerful on-treatment predictor of sustained virological response (SVR) in the treatment of chronic hepatitis C (CHC). However, RVR rates are relatively low and a new predictor is needed for CHC patients; especially those without RVR. In this context, on-treatment alanine aminotransferase (ALT) changes may be a new predictor for SVR. In this study, we found that ALT normalization at the 8 wk may be an important on-treatment predictor for CHC.

Dogan UB, Akin MS, Yalaki S. Alanine aminotransferase normalization at week 8 predicts viral response during hepatitis C

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## INTRODUCTION

The sustained virological response (SVR) after combined peginterferon and ribavirin treatment in chronic hepatitis C (CHC) patients is heterogeneous<sup>[1]</sup>. Thus, for the treatment of CHC, clinicians would like to establish predictive factors for SVR<sup>[2]</sup>. Pretreatment predictive viral factors are hepatitis C virus (HCV) genotype and serum HCV RNA levels at baseline, and many host factors including age, sex, weight, race, liver fibrosis, insulin resistance and recently acknowledged presence of interleukin-28 polymorphism<sup>[1,3,4]</sup>.

Once treatment is initiated, rapid virological response (RVR) is acknowledged as a powerful on-treatment predictor of SVR<sup>[5]</sup>. However, RVR rates are relatively low and a new predictor is needed for CHC patients; especially those without RVR. In this context, on-treatment alanine aminotransferase (ALT) changes may be a new predictor for SVR. There are few data evaluating the relationship between on-treatment ALT changes and SVR during combination treatment with peginterferon and ribavirin in patients with CHC.

The purpose of this study was to investigate the relationship between on-treatment ALT changes and SVR in genotype 1 CHC patients during peginterferon-ribavirin treatment.

## MATERIALS AND METHODS

### Patients

Medical records of patients with CHC, who were treated between 2008 and 2012 at the Adana Numune Training and Research Hospital, Turkey, were retrospectively reviewed. Eligible patients had chronic HCV genotype 1 infection with compensated liver disease and a detectable plasma HCV RNA level, and had not been previously treated for hepatitis C. Patients who were on treatment or had withdrawn because of adverse events, or were lost during follow-up were excluded from the study. Patients were also excluded if they had co-infection with hepatitis B or HIV, any other cause of liver disease such as alcohol abuse or autoimmune hepatitis, morbid obesity (Body Mass Index > 40), poorly controlled diabetes mellitus (glycated hemoglobin value > 8.5%), severe depression or a severe psychiatric disorder, or active substance abuse. Finally, 151 patients who were followed up for at least 6 mo after completion of treatment were included in the study. Most patients had undergone liver biopsy within 6 mo before screening. The liver histology was graded by the histological activity index according to the criteria of Ishak *et al.*<sup>[6]</sup>, which comprise two major components namely Histological Activity Index and fibrosis. The study was approved by our Institutional Review Board

and was conducted in accordance with provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

### Study design

We first categorized 151 patients into two main groups. Group 1 included 52 patients with RVR, and Group 2 included 99 patients without RVR. Each group was then subdivided into two according to the initial ALT level: Group 1h, patients who had initial abnormal ALT levels with RVR; Group 1n, patients who had initial normal ALT levels with RVR; Group 2h, patients who had initial abnormal ALT levels without RVR; and Group 2n, patients who had initial normal ALT levels without RVR. ALT patterns were analyzed throughout the course of treatment and follow-up period.

### Treatment with peg-interferon plus ribavirin

Patients with genotype 1 infection were administered peginterferon  $\alpha$ -2a at a dose of 180  $\mu$ g/wk or peginterferon  $\alpha$ -2b at the standard dose of 1.5  $\mu$ g/kg per week; both in combination with oral ribavirin at a dose of 1000-1200 mg/d, according to body weight (< 75 kg, 1000 mg/d;  $\geq$  75 kg, 1200 mg/d). Patients underwent treatment for 48 wk and were followed-up for 24 wk.

### Laboratory assessment

Patients were followed up by blood sample analysis and measurement of biochemical variables. Blood samples were tested for complete blood counts, serum ALT levels, HCV genotype (baseline only) and serum HCV RNA. Serum ALT levels were obtained from all patients at baseline and at weeks 4, 8, 12, 24 and 48 of combined peg-interferon and ribavirin treatment, and 24 wk after completing therapy. According to the results of ALT, patients were included in either the follow-up abnormal or follow-up normalized ALT groups at each interval. Patients with high and normal ALT levels were compared at weeks 4, 8, 12, 24, and 48 of treatment; and follow-up week 24 in terms of SVR. The upper normal limit for serum ALT was 40 IU/L in our laboratory.

### Efficacy assessments

HCV RNA levels were measured with the use of the Cobas TaqMan assay (Roche Diagnostics, Milan, Italy), which has a lower limit of quantitation of 20 IU/mL. Real-time polymerase chain reaction with Rotor Gene Q (Qiagen, Milan, Italy) was used for genotype determination. Measurements were obtained at screening visits (baseline); weeks 4, 12, 24 and 48 during the treatment period; and 24 wk follow-up. The primary endpoint of efficacy was SVR (undetectable serum HCV RNA levels at 24 wk after completing treatment). RVR was defined as undetectable serum HCV RNA level at the end of 4 wk. Patients with detectable HCV RNA at week 4 (no RVR) who had undetectable HCV RNA at week 12 were said to have a complete early virological response (cEVR). End of treatment response (ETR) was defined

**Table 1** Comparison of baseline characteristics and virological responses in patients with and without rapid virological response *n* (%)

	Patients with RVR (Group 1, <i>n</i> = 52)	Patients without RVR (Group 2, <i>n</i> = 99)	<i>P</i> <sup>1</sup> value
Age (yr)	55.9 ± 12.2	57.9 ± 11.1	0.312
Male	24 (46.2)	51 (51.5)	0.534
Initial ALT (IU/L)	87.3 ± 109.8	52.2 ± 40.4	0.005
Initial abnormal ALT level	33 (63.5)	49 (49.5)	0.103
Initial HCV RNA (log <sub>10</sub> IU/mL)	5.4 ± 1.1	6.1 ± 0.8	0.000
cEVR	52 (100)	58 (59)	0.000
ETR	48 (92)	56 (57)	0.000
SVR	43 (83)	40 (40)	0.000
ISHAK score, mean ± SD			
Biopsy of receipt	31 (59.6)	63 (63.6)	0.631
HAI	8.9 ± 3.8	8.2 ± 2.8	0.356
Fibrosis score	2.9 ± 1.3	2.7 ± 1.4	0.681

<sup>1</sup>Student's *t* test. RVR: Rapid virological response; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; EVR: Early virological response; ETR: End of treatment response; SVR: Sustained virological response; HAI: Histological activity index.

as the undetectable serum HCV RNA level at the end of treatment.

### Statistical analysis

Data management and statistical analyses were performed with SPSS for Windows Release 18.0.0 (SPSS Inc., Chicago, IL, United States). Results are expressed as the mean ± SD. Student's *t* test or analysis of variance was used to assess the significance of SVR rates. Univariate analysis and multiple logistic regression analysis were used to identify predictive factors for sustained response. In the multiple logistic regression analysis, we determined the strength of the influence of possible variables (RVR, cEVR, normalization of ALT at 8 and 12 wk) for sustained response. *P* < 0.05 was considered as statistically significant.

## RESULTS

### Characteristics and viral responses of the study patients according to RVR

RVR was achieved in 52 (34.4%) patients and cEVR was achieved in 110 (72.9%) patients. The remaining 41 patients who did not achieve a cEVR at week 12 had undetectable HCV RNA at 24 wk. Comparison of baseline characteristics and virological responses in patients with and without RVR are summarized in Table 1. The initial ALT level was higher in patients with RVR than patients without RVR, although there was no significant difference in the number of patients with initial abnormal ALT level between the groups. Initial HCV RNA (log<sub>10</sub> IU/mL) was significantly lower and the SVR rate was significantly higher in patients with RVR compared to patients without RVR. The overall SVR rate was 55%.

### Characteristics and viral responses according to initial ALT levels in patients with and without RVR

Baseline characteristics and virological responses according to the initial ALT level in patients with and without RVR were similar (Table 2).

### During treatment

Schematic diagrams showing patient group flow according to initial ALT level and subsequent pattern of changes in patients with and without RVR are shown in Figure 1.

### Viral responses in patients with initial normal ALT levels during treatment

Patients who had normal initial ALT levels showed nearly sustained normal ALT levels during treatment. Only one patient in Group 1n (Figure 1A) and two patients in Group 2n (Figure 1B) had variable ALT abnormality during treatment. SVR rates were 84% and 44% in Group 1n and 2n, respectively.

### Viral responses according to ALT normalization during treatment

Comparison of SVR rates in patients with high and normalized ALT levels at weeks 4, 8, 12, 24, 48 and 72 in the initial abnormal ALT level groups with and without RVR are summarized in Table 3 and illustrated in Figure 2. At 8 wk, normalization of ALT became significant in terms of SVR in both groups.

### Analysis of factors that predicted SVR to combination therapy

We performed univariate analysis using the  $\chi^2$  test to investigate the association of SVR with various factors. In the multiple logistic regression for the strength of influence factors, RVR (OR = 7.05; 95%CI: 3.1-16.05, *P* = 0.000), cEVR (OR = 17.55; 95%CI: 6.32-48.76, *P* = 0.000), normalization of ALT at week 8 (OR = 3.04; 95%CI: 1.31-7.06, *P* = 0.008), and at week 12 (OR = 4.21; 95%CI: 1.65-10.76, *P* = 0.002) were identified as independent significant predictive factors for SVR.

## DISCUSSION

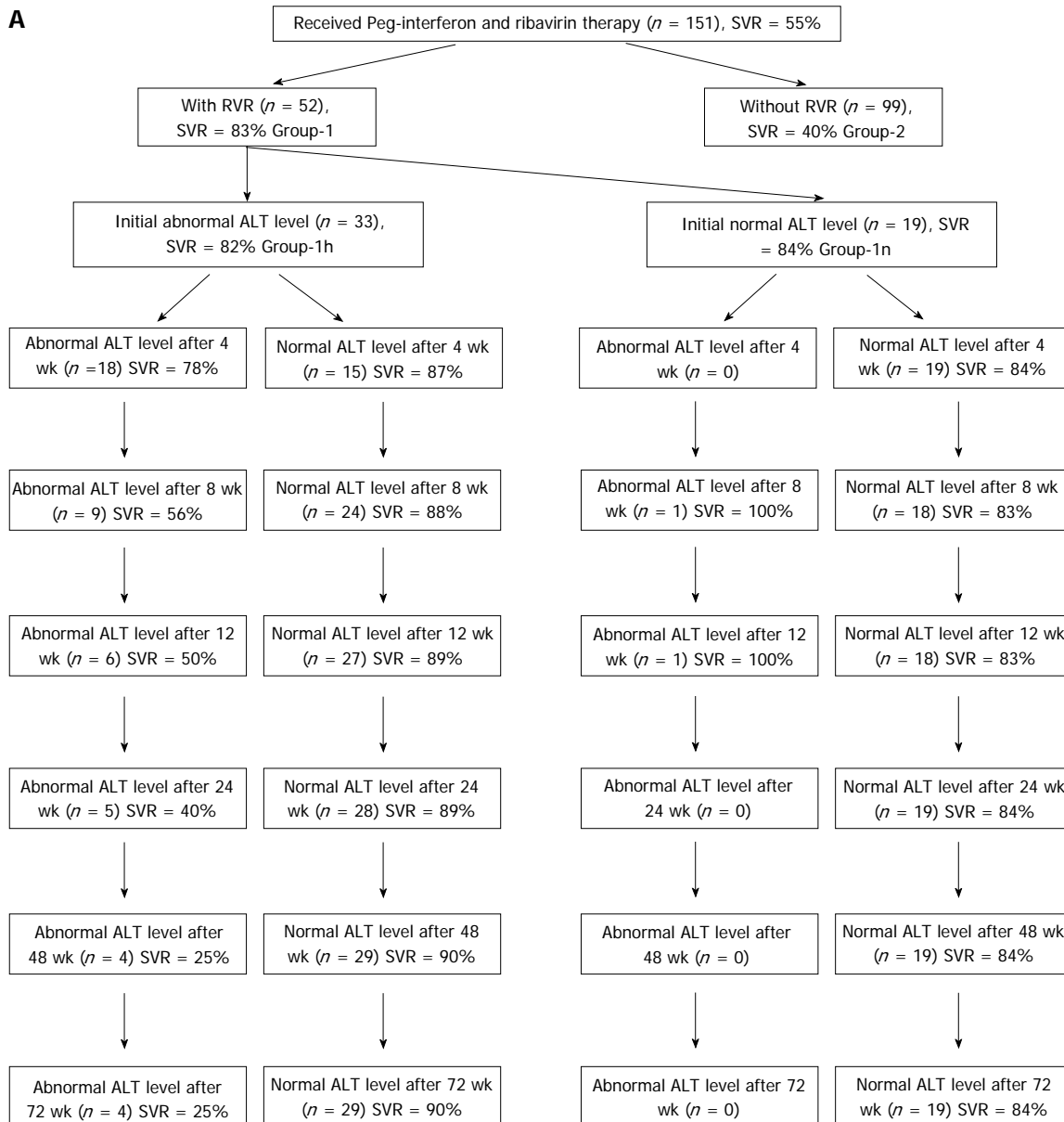
Treatment with pegylated interferon- $\alpha$  and ribavirin is a well-accepted standard of care for patients with CHC<sup>[2]</sup>. Although this approach appears to be highly effective for patients with HCV genotypes 2 or 3, who have a SVR of about 80%, the treatment algorithm is less effective for patients with HCV genotype 1, because these patients have SVR rates of just 40%-50%<sup>[7,8]</sup>. There are some pretreatment factors related to SVR. Clinicians need to know these factors for predicting SVR, to determine non-responders as early as possible in order to avoid prolonged treatment without benefit<sup>[2,9]</sup>. The viral factors are HCV genotype and serum HCV RNA levels at baseline and numerous host factors include age, sex, race, weight, liver fibrosis, and insulin resistance<sup>[1]</sup>. Recently, an interleukin-28 polymorphism has been acknowledged as

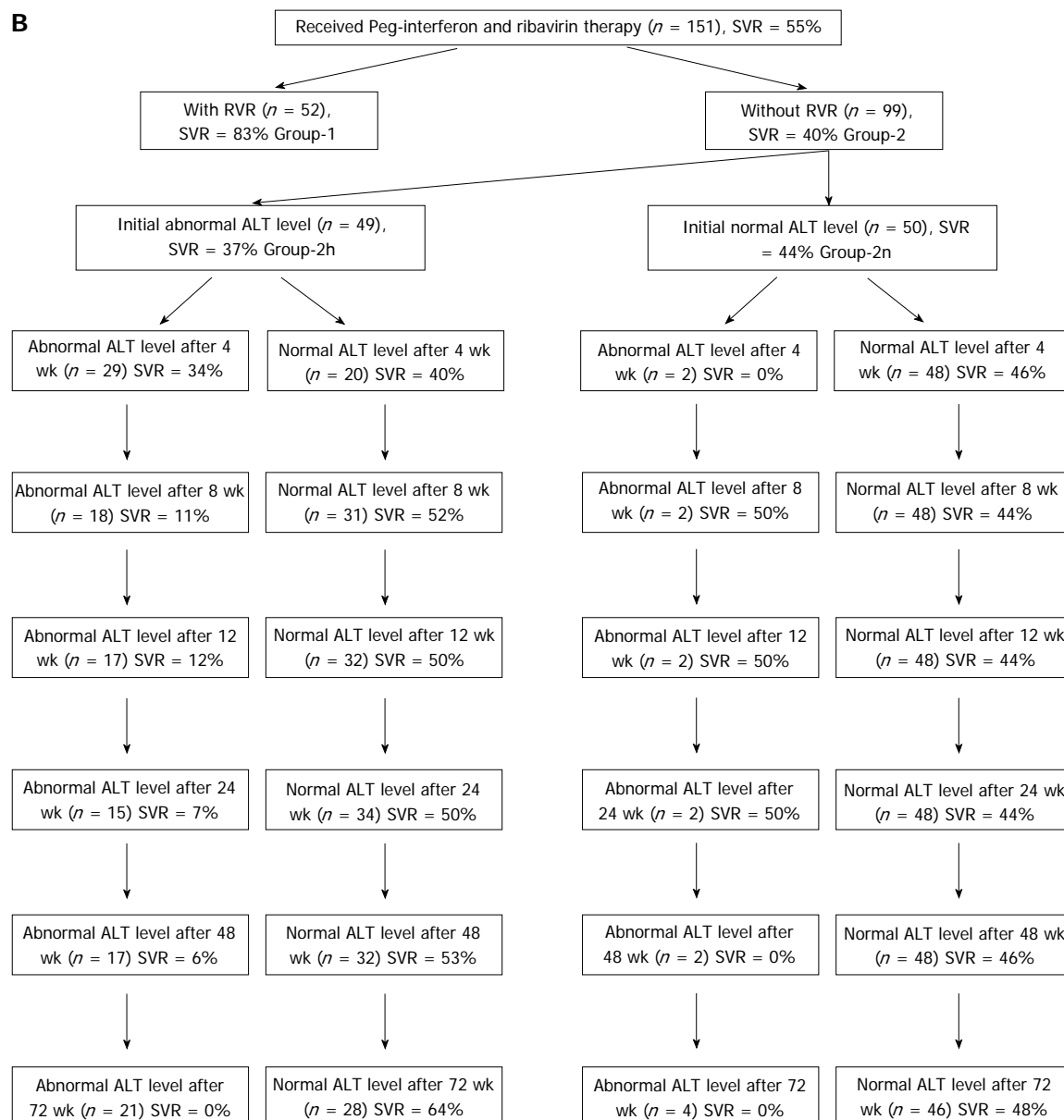


**Table 2** Comparison of baseline characteristics and virological responses according to the initial alanine aminotransferase level in patients with and without rapid virological response *n* (%)

	Patients with RVR (Group 1)		<i>P</i> <sup>1</sup> value	Patients without RVR (Group 2)		<i>P</i> <sup>1</sup> value
	Initial abnormal ALT level (Group 1h, <i>n</i> = 33)	Initial normal ALT level (Group 1n, <i>n</i> = 19)		Initial abnormal ALT level (Group 2h, <i>n</i> = 49)	Initial normal ALT level (Group 2n, <i>n</i> = 50)	
Age, yr	54.3 ± 13.8	58.6 ± 8.3	0.222	56.6 ± 12.6	59.2 ± 9.2	0.246
Male	18 (54.6)	6 (31.6)	0.114	26 (53.1)	25 (50.0)	0.763
Initial ALT (IU/ L)	122.4 ± 125.3	26.4 ± 8.2	0.002	78.1 ± 43.9	26.8 ± 7.4	0.000
HCV RNA (log10 IU/mL)	5.6 ± 0.9	5.1 ± 1.4	0.139	6.2 ± 0.9	6.1 ± 0.7	0.748
cEVR	33 (100)	19 (100)	NA	29 (59)	29 (58)	0.906
ETR	30 (91)	18 (95)	0.626	26 (53)	30 (60)	0.491
SVR	43 (82)	40 (84)	0.83	18 (37)	22 (44)	0.466
ISHAK Score, mean ± SD						
Biopsy of receipt	17 (51.5)	14 (73.7)	0.121	33 (67.4)	30 (60)	0.453
HAI	9.6 ± 3.7	8.0 ± 3.9	0.257	8.7 ± 2.6	7.7 ± 3.0	0.164
Fibrosis score	2.9 ± 1.2	2.9 ± 1.4	0.957	3.0 ± 1.4	2.4 ± 1.4	0.100

<sup>1</sup>Student's *t* test. RVR: Rapid virological response; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; EVR: Early virological response; ETR: End of treatment response; SVR: Sustained virological response; HAI: Histological activity index.





**Figure 1 Schematic diagram showing patient group flow according to initial alanine aminotransferase level and subsequent pattern of change in patients with and without rapid virological response.** A: Change in patients with rapid virological response (RVR); B: Change in patients without RVR. ALT: Alanine aminotransferase; SVR: Sustained virological response.

a powerful pretreatment predictor of SVR<sup>[3,4]</sup>.

Once treatment is initiated, the monitoring of viral responses such as RVR and early virological response (EVR) can further aid in predicting treatment response<sup>[5]</sup>. As for the response-guided approach, RVR is regarded as the most important predictor for SVR<sup>[10-12]</sup>. In a recent retrospective analysis of 1383 patients, it was shown that achieving RVR correlated with a high probability (86%-100%) of SVR to peginterferon-ribavirin combination therapy, regardless of genotype<sup>[13]</sup>. In another retrospective analysis, it was shown that the SVR rate was 42% in the absence of RVR at week 48<sup>[14]</sup>. Unfortunately, RVR rates are small and range from 7.4%-37%<sup>[15]</sup>. Also, there is a positive correlation between the magnitude of the decrease in HCV RNA at week 4 and the probability of

SVR<sup>[16]</sup>. We previously demonstrated that patients with a  $\geq 3$  log<sub>10</sub> drop in HCV RNA at week 4 have a high probability of achieving an SVR when treated with either peginterferon  $\alpha$ -2a-ribavirin or peginterferon  $\alpha$ -2b-ribavirin<sup>[17]</sup>. In addition, EVR is an important parameter for the decision to terminate or continue treatment because patients without EVR can hardly achieve SVR<sup>[18]</sup>. RVR seems to be the single important on-treatment factor for SVR. Consequently, there is a need for a new on-treatment predictor for SVR; especially in patients without RVR. In this context, on-treatment ALT changes may be a new predictor for SVR.

In general, a decreased pattern of ALT level is the accepted basic indicator of interferon therapeutic effect in CHC, and several studies have shown that delayed

**Table 3** Comparison of sustained virological response in patients with high and normalized alanine aminotransferase levels at week 4, 8, 12, 24, 48 and 72 in patients with and without rapid virological response

	Initial abnormal ALT level in patients with RVR (Group 1h, n = 33)		$P^2$ value	Initial abnormal ALT level in patients without RVR (Group 2h, n = 49)		$P^1$ value
	Follow-up abnormal ALT	Follow-up normalized ALT		Follow-up abnormal ALT	Follow-up normalized ALT	
After 4 wk						
No. of patients	18	15	0.525	29	20	0.701
SVR rate	78	87		34	40	
After 8 wk						
No. of patients	9	24	0.049	18	31	0.004
SVR rate	56	88		11	52	
After 12 wk						
No. of patients	6	27	0.028	17	32	0.007
SVR rate	50	89		12	50	
After 24 wk						
No. of patients	5	28	0.001	15	34	0.003
SVR rate	40	89		7	50	
After 48 wk						
No. of patients	4	29	0.006	17	32	0.001
SVR rate	25	90		6	53	
After 72 wk						
No. of patients	4	29	0.006	21	28	0.000
SVR rate	25	90		0	64	

<sup>1</sup>Student's *t* test; <sup>2</sup>Student's  $\chi^2$  test. RVR: Rapid virological response; ALT: Alanine aminotransferase; SVR: Sustained virological response.

normalization of ALT levels may indicate poor response to interferon therapy<sup>[9,19]</sup>, although the viral response was not always associated with biochemical response<sup>[6,20]</sup>.

Serum ALT, a surrogate marker of hepatocyte damage or death, decreases during antiviral treatment, and shows the lowest activity at the end of treatment<sup>[21]</sup>. The mechanism of decline of ALT level is not clear; however it can be explained by a reduction in infected cells, a non-cytolytic cure, or cell removal irrelevant of ALT dynamics. However, a decreased ALT level at the early phase of treatment is not related to apoptotic activity<sup>[22]</sup>. Theoretically, the rapid declines in ALT may reflect a rapid decrease of ongoing inflammation in the same manner as removal of the virus. The pattern of viral elimination shows a rapid decrease in the first month. Ribeiro *et al*<sup>[21]</sup> showed that the RVR significantly correlated with the decline in ALT levels at week 4 of treatment. A retrospective study of 111 patients with chronic HCV infection treated by conventional interferon and ribavirin also demonstrated that the larger decline in ALT level within the first 2 and 4 wk was a predictor of SVR<sup>[23]</sup>. These correlations suggest that ALT dynamics can be presented as a possibility to reflect rapid virological changes; especially in patients with elevated baseline ALT levels.

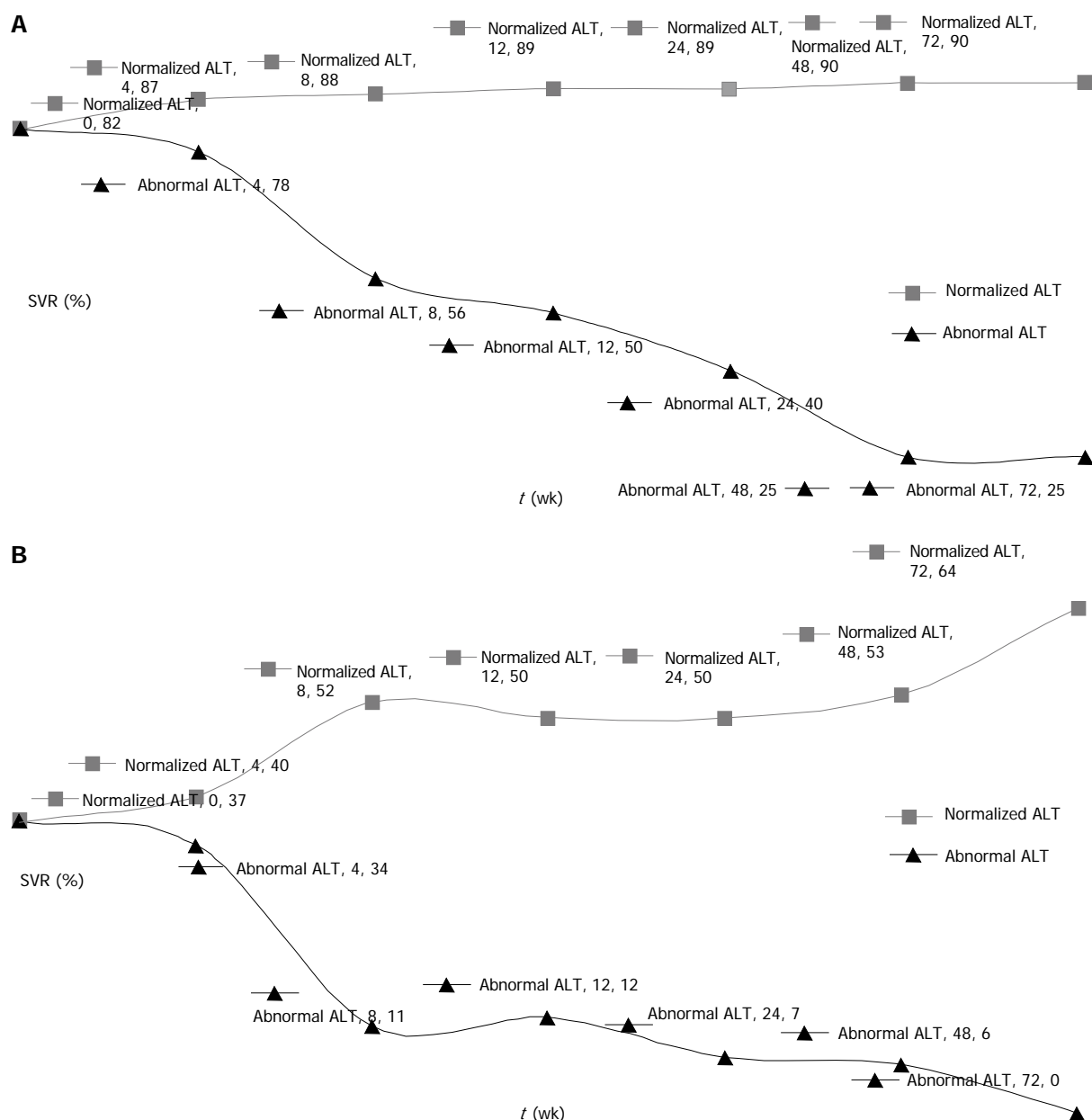
Kim *et al*<sup>[24]</sup> reported that, instead of RVR, the rapid normalization of serum ALT level after initiation of treatment may play an additional role in predicting SVR. They retrospectively analyzed changes in ALT levels between baseline and week 4 of treatment in 168 patients with chronic HCV infection. Rapid normalization of ALT within 1.5 times of the normal range after treatment was found to be significantly associated with improved SVR in patients with genotype I HCV infection (34.1% *vs* 20.0%,  $P = 0.01$ ) and non-genotype-1 infection (88.1%

*vs* 66.7%,  $P = 0.11$ ) who had initially high ALT levels. This result suggests that rapid normalization of ALT at week 4 of treatment could be used as a strategy for predicting SVR in patients with elevated baseline ALT levels; however, its use is limited because of the paucity of knowledge about RVR and the difficulty of application in normal ALT levels.

A recent report suggested that mild ALT elevations (peak ALT value  $1.5 \times$  baseline value) during treatment may reflect ongoing viral activity in non-responders, but a more significant rise may reflect a good virological response due to an immunomodulating effect of interferon<sup>[25]</sup>. However, it was difficult to use these data to analyze the reason for on-treatment ALT elevation and to elucidate the relationship between on-treatment ALT elevation and SVR; especially at week 4 of treatment.

In our study, patients with genotype 1 CHC were divided into two groups as those with or without RVR, because RVR is the most important on-treatment predictor of SVR. The SVR rate was also found to be high in patients with RVR (83% *vs* 40.0%,  $P < 0.001$ ) in our study. Each group was further subdivided into two according to the initial ALT level being high or normal. The SVR rates were similar between patients with high and normal ALT levels at baseline and at week 4 in patient with and without RVR. SVR rates were found to be significantly higher in patients with normalized ALT at week 8 and thereafter.

In the patient group with RVR, SVR starts at 82% at baseline in patients with initially abnormal ALT level. SVR declines in patients with continuing abnormal ALT levels and increases in patients with normalized ALT levels. However, this difference becomes significant, with 56% *vs* 88%, only after 8 wk treatment. Later, this difference increases but at a slower rate, reaching 25% *vs* 90%



**Figure 2** Sustained virological response rates in patients with follow-up abnormal and normalized alanine aminotransferase with and without rapid virological response. A: With rapid virological response (RVR); B: Without RVR. SVR: Sustained virological response; ALT: Alanine aminotransferase.

at week 48 (Figure 2A). However, it is difficult to comment on patients with continuing abnormal ALT levels because of the lower number of patients.

In the patient group without RVR, the decrease in SVR is larger in patients with continuing abnormal ALT levels. SVR starts at 37% at baseline in patients with initial abnormal ALT levels, and declines in patients with continuing abnormal ALT levels and increases in those with normalized ALT levels, as in patients with RVR. The difference in SVR levels in those groups becomes significant at 8 wk, reaching 11% *vs* 52%. The difference in SVR continues to increase slightly, reaching 6% *vs* 53% at week 48 (Figure 2B).

Although SVR was found to be significantly correlated with the decline of ALT level at week 4 of treatment in a few studies<sup>[21,23,24]</sup>, high levels of ALT may also reflect

a good virological response due to an immunomodulating effect of interferon<sup>[25]</sup>. Clinicians must also know the baseline ALT level in order to be able to predict SVR. Furthermore, RVR is already the most important predictor at week 4 of treatment and it is still unclear whether the use of serum ALT levels, instead of RVR, is helpful for predicting SVR in clinical practice. The main problem is to predict SVR in patients without RVR. In our study, SVR was found to be higher in patients with normalized ALT at week 4 of treatment; however, the difference was not significant at that stage (34% *vs* 40.0%,  $P = 0.701$ ). SVR rates continued to increase and became significant at 8 wk in non-RVR patients with normalized ALT. At week 12 of treatment and later, SVR rates were found to be higher in these patients; however, cEVR was already a more important criterion for SVR at this stage, compared



to the ALT (OR = 17.55 *vs* 4.21). Therefore determination of ALT levels at 8 wk would be better than at 4 and 12 wk.

In our opinion, if a patient with initial abnormal ALT without RVR still has abnormal ALT level at 8 wk, peginterferon-ribavirin treatment may be discontinued because SVR is expected to be only 11%.

In conclusion, normalization of ALT at the 8 wk may predict viral response during peginterferon-ribavirin treatment in patients with genotype 1 CHC; especially without RVR.

## COMMENTS

### Background

Rapid virological response (RVR) is acknowledged as a powerful on-treatment predictor of sustained virological response (SVR) during peginterferon-ribavirin treatment of chronic hepatitis C (CHC). However, RVR rates are relatively low and a new predictor is needed for CHC patients; especially those without RVR.

### Research frontiers

The authors investigated the relationship between on-treatment alanine aminotransferase (ALT) changes and SVR in patients with genotype 1 CHC during peginterferon-ribavirin treatment.

### Innovations and breakthroughs

The authors found that normalization of ALT at 8 wk may predict viral response during peginterferon-ribavirin treatment in patients with genotype 1 CHC; especially without RVR.

### Applications

If the patients with initial abnormal ALT without RVR still had abnormal ALT level at 8 wk, peginterferon-ribavirin treatment may be discontinued because SVR is expected to be only 11%.

### Peer review

This study investigated the relationship between on-treatment ALT changes and SVR in patients with genotype 1 CHC during peginterferon-ribavirin treatment, and demonstrated that on-treatment ALT changes may be a new predictor for SVR.

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## Metastatic type 1 gastric carcinoid: A real threat or just a myth?

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### Abstract

**AIM:** To describe disease characteristics and treatment modalities in a group of rare patients with metastatic gastric carcinoid type 1 (GCA1).

**METHODS:** Information on clinical, biochemical, radiological, histopathological findings, the extent of the disease, as well as the use of different therapeutic modalities and the long-term outcome were recorded. Patients' data were assessed at presentation, and thereafter at 6 to 12 monthly intervals both clinically and biochemically, but also endoscopically and histopathologically. Patients were evaluated for the presence of specific symptoms; the presence of autoimmune disorders and the presence of other gastrointestinal malignancies in other family members were also recorded. The evaluation of response to treatment was defined using established WHO criteria.

**RESULTS:** We studied twenty consecutive patients with a mean age of 55.1 years. The mean follow-up period was 83 mo. Twelve patients had regional lymph node metastases and 8 patients had liver metastases. The primary tumor mean diameter was  $20.13 \pm 10.83$  mm (mean  $\pm$  SD). The mean Ki-67 index was  $6.8\% \pm 11.2\%$ . All but one patient underwent endoscopic or surgical excision of the tumor. The disease was stable in all but 3 patients who had progressive liver disease. All patients remained alive during the follow-up period.

**CONCLUSION:** Metastatic GCA1 carries a good overall prognosis, being related to a tumor size of  $\geq 1$  cm, an elevated Ki-67 index and high serum gastrin levels.

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**Key words:** Metastatic gastric carcinoids; Gastrin; Chromogranin A; Somatostatin analogues; Stomach neuroendocrine tumor

**Core tip:** Metastatic gastric carcinoid type 1 (GCA1) are extremely rare and there is no data regarding their natural history, treatment and prognosis. Based on our study, metastatic GCA1 carries a good overall prognosis. Metastatic spread appears to be related to a tumor size of  $\geq 1$  cm, an elevated Ki-67 index, and to high serum gastrin levels. Endoscopic surveillance is extremely important for follow-up. Surgical resection should be performed only in patients in whom total tumor excision is expected. Although still controversial, somatostatin analogues could be considered as first line treatment to lower the elevated gastrin levels and suppress enterochromaffin like cell hyperplasia.

Grozinsky-Glasberg S, Thomas D, Strosberg JR, Pape UF, Felder S, Tsolakis AV, Alexandraki KI, Fraenkel M, Saiegh L, Reissman P, Kaltsas G, Gross DJ. Metastatic type 1 gastric carcinoid: A real threat or just a myth? *World J Gastroenterol* 2013; 19(46): 8687-8695 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8687.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8687>

## INTRODUCTION

Gastric carcinoids (GCAs) are neuroendocrine tumors (NETs) of the gastric mucosa originating from enterochromaffin like (ECL) cells<sup>[1]</sup>. GCAs arise either spontaneously or in response to a chronic hypergastrinemia state; due to their rarity (only 2% of all carcinoids and 8.7% of gastrointestinal carcinoids)<sup>[2,3]</sup>, the predictors of metastatic disease have not been systematically addressed.

GCAs are divided into three distinct types. Type 1 (GCA1) represents the majority (approximately 75%) and is associated with chronic atrophic gastritis and autoimmune destruction of parietal cells. Type 2 (GCA2) (approximately 5%-10%) occurs almost always in the context of Multiple Endocrine Neoplasia type 1 (MEN1). Both types 1 and 2 GCAs occur in the setting of elevated serum gastrin which exerts a trophic effect on gastric enterochromaffin-like (ECL) cells leading to neuroendocrine cell hyperplasia and multifocal polypoid carcinoid tumors. These tumors are well differentiated and carry an excellent overall prognosis. Type 3 GCAs (15%-25%) are not related to hypergastrinemia and follow an aggressive course<sup>[4-6]</sup>.

Type 1 GCAs are usually discovered during upper gastrointestinal tract (GIT) endoscopy performed for non specific symptoms (nausea, abdominal pain, dyspepsia)<sup>[7]</sup>, or during investigation of anemia<sup>[8-10]</sup>. In the past, type 1 GCA was frequently diagnosed in women in their 5<sup>th</sup> to 7<sup>th</sup> decades; however, with the more extensive use of endoscopy, the diagnosis occurs at a younger age<sup>[11]</sup>.

Traditionally, GCA1s are endoscopically removed<sup>[12,13]</sup>;

antrectomy could be considered to remove the source of excessive gastrin secretion<sup>[14]</sup>. Importantly, somatostatin analogues (SSAs) have been increasingly used in the treatment of patients with GCA1 or GCA2<sup>[15]</sup>, based on their capability to inhibit gastrin release, reduce the ECL cell hyperplasia<sup>[16-20]</sup>, and to substantially decrease tumor load<sup>[21-23]</sup>.

Metastatic GCA1 are extremely rare and little is known about their natural history, treatment and prognosis. We conducted a multicenter, retrospective analysis to describe disease characteristics and treatment modalities in a group of rare patients with metastatic GCA1.

## MATERIALS AND METHODS

Twenty consecutive patients with metastatic GCAs1 treated in five tertiary referral centers for at least 6 mo were studied. Information on clinical presentation, biochemical profile, imaging, histopathological findings and disease extent (using the TNM classification)<sup>[24]</sup> were recorded. The use of varying therapeutic modalities and the long-term outcome of these patients were also recorded. Patients' data were assessed at presentation, and thereafter at 6-12 monthly intervals both clinically and biochemically, but also endoscopically and histopathologically.

### Clinical assessment

Patients were evaluated for the presence of symptoms such as abdominal pain, nausea, vomiting and dyspepsia; the presence of autoimmune disorders associated with pernicious anemia and the presence of other gastrointestinal malignancies in other family members were also recorded.

### Biochemical evaluation

Pernicious anemia was defined as a low serum vitamin B<sub>12</sub> level (normal range 180-670 pmol/L) and at least one positive antibody against parietal cells, intrinsic factor or proton-pump antigen. Serum gastrin and chromogranin A (CgA) were measured after an overnight fast, and thereafter at regular intervals (3-6 mo) during the study period. Treatment with proton pump inhibitors (PPIs) was discontinued for at least 3 wk before blood samples were taken. Serum CgA and gastrin were measured using commercially available radioimmunoassay kits: CGA-RIACT, CISBIO International, France (normal reference range of 19.4-98.1 ng/mL), or Euro-Diagnostica, Malmö (upper normal limit 4 nmol/L) for CgA, and DiaSorin, Stillwater, Minnesota 55082-0285, United States (normal reference range of 40-108 mU/L) or EURO-Diagnostica, Malmö (upper normal limit 60 pmol/L) for gastrin, respectively.

### Imaging assessment

All patients underwent imaging assessment at diagnosis, including either <sup>111</sup>In-pentetreotide scintigraphy (Octreoscan) or (68)Gallium-DOTA-TATE/-TOC/-NOC



Table 1 Clinical and histopathological characteristics of the study patients

Patient No.	No. of lesions	Size, largest lesion (mm)	Ki-67%	CT before Tx	SRS/ <sup>68</sup> Ga before Tx	Distant mets	Gastrin (40-108 mU/L)	Surgery	Residual disease	SSA/monthly dosage (mg)	Outcome	F/U Period (mo)
							at Dx	Last				
1	multiple	15	2%	-	Uptake (stomach, LN)	no	1811	125	wedge resection	no	no	108
2	solitary	35	2%	Liver mets	Uptake (stomach, Liver)	liver	-	-	Billroth 2 + LN	liver	no	84
3	multiple	21	2%	Liver mets	Uptake (stomach, Liver)	liver	2204	325	none	liver	San LAR 30	36
4	multiple	55	20%	Stomach lesion	Uptake (stomach)	diaphragm	1800	-	Billroth 2 + LN	no	San LAR 30	24
5	multiple	25	1%	Stomach lesion	Uptake (stomach, LN)	no	1403	-	Billroth 2 + LN	no	no	18
6	multiple	-	15%	Hepato-gastric ligament LN	no data	no	-	-	ER	no	no	12
7	solitary	17	4%	LN	Uptake (stomach, LN)	no	700	600	wedge resection	no	no	132
8	multiple	15	1%	Liver mets and LN	Uptake (LN, Liver)	liver	407	190	ER, largest	liver	San LAR 30 + INF 50 mcg/wk	36
9	solitary	10	1%	Liver mets and LN	no data	liver	-	-	wedge resection	no	no	360
10	solitary	30	5%	Stomach lesion	no uptake	no	5130	43	Billroth 2 + LN	no	none	120
11	multiple	17	1%	-	no uptake	no	-	-	Billroth 2 + LN	no	none	168
12	multiple	14	5%	-	no uptake	no	-	-	Billroth 2 + LN	no	none	72
13	solitary	47	15%	-	Uptake (stomach, Liver)	liver	5470	45	ER	liver	none	120
14	multiple	30	2%	-	Uptake (LN, Liver)	liver	1336	335	ER, recurrent	liver	none	48
15	multiple	10	2%	No pathology	no uptake	no	3500	-	Billroth 2 + LN	no	none	48
16	multiple	30	2%	Stomach lesion	no data	no	-	-	Billroth 2 + LN	no	none	36
17	solitary	-	1%	Liver mets	Uptake (liver)	liver	1612	10	Billroth 2 + LN	liver	none	36
18	multiple	20	-	Stomach lesion, mesenteric and gastro-hepatic LN	no data	no	506	-	wedge resection	no	none	60
19	multiple	20	-	No pathology	no uptake	no	1600	-	Billroth 2 + LN	no	none	72
20	multiple	15	-	Liver mets	Uptake (stomach, Liver)	liver	458	336	ER	liver	San LAR 30	72

Nr. of lesions, solitary: one lesion seen on endoscopy, multiples  $\geq 2$  lesions seen on endoscopy; CT: Computerized tomography; SRS: Somatostatin receptor scintigraphy (Octreoscan); (<sup>68</sup>Ga: (<sup>68</sup>Ga) Gallium-DOTA-TATE/-TOC/-NOC PET; Tx: Treatment; SSAs: Somatostatin analogues; LN: Lymphadenopathy; mets: Metastases; SomA: Somatoline Autogel; SanLAR: Sandostatin LAR; INF: Interferon  $\alpha$ ; Billroth 2 + LN: Gastro-jejunostomy and lymph nodes dissection; wedge resection: wedge resection (triangular resection) of a part of the stomach; ER: Endoscopic resection; SD: Stable disease; PR: Partial response; CR: Complete response.

PET (17 patients), computerized tomography (CT) of the abdomen (13 patients), or both modalities (11 patients) (Table 1).

### Endoscopic and histopathological assessment

All patients underwent upper GI endoscopy and 6/20 also underwent endoscopic ultra-sonography (EUS). Upper GI endoscopy with multiple biopsies was performed in order to assess the lesions and map surrounding gastric mucosa for changes of atrophic gastritis; the “dominant” lesions were biopsied and removed if possible. EUS was performed to assess invasion of the muscularis propria, regional lymph node involvement and/or visible metastases. Histopathological diagnosis was performed using biopsies taken from both the tumors and the surrounding mucosa at diagnosis or periodically during the follow-up period, or, in case of tumor excision - from the surgical specimen. Sections were immunostained for chromogranin (CG), neuron specific enolase (NSE), synaptophysin (SYN), and the Ki-67 proliferative index using the MIB-1 antibody. The diagnosis of NETs was confirmed morphologically during endoscopy together with a positive immunocytochemical staining for NSE, SYN and/or CG.

**Table 2 Factors of significance in the suspicion of metastatic gastric carcinoid type 1 *n* (%)**

Characteristics	All GCA1 patients ( <i>n</i> = 254)	Metastatic GCA1 patients ( <i>n</i> = 20)	<i>P</i> value at diagnosis (metastatic <i>vs</i> all GCA1)
Age (yr), mean $\pm$ SD	58.5 $\pm$ 12.7	55.1 $\pm$ 12.8	0.050
Size of largest tumor (mm, mean $\pm$ SD)	7.9 $\pm$ 12.1	20.14 $\pm$ 11	< 0.001
Ki-67 (% , mean $\pm$ SD)	1.9 $\pm$ 2.4	6.8 $\pm$ 11.2	< 0.001
Symptomatic	112 (44)	18 (90)	< 0.001
Gastrin levels (mL/L, mean $\pm$ SD) at diagnosis	898 $\pm$ 418	2138.4 $\pm$ 1562	< 0.001

GCA1: Gastric carcinoid type 1.

**Table 3 Demographic and clinical characteristics of the patients included in the study *n* (%)**

Characteristics	All patients <i>n</i> = 20
Age (yr), mean $\pm$ SD	55.1 $\pm$ 12.8
Male:female, <i>n</i>	9:11
Caucasians	95%
Size of primary tumor (mm), mean $\pm$ SD	20.14 $\pm$ 11
Symptomatic	18 (90)
Atrophic gastritis	20 (100)
Other autoimmune diseases	2 (10)
Familial aggregation	3 (15)

### Evaluation of response to treatment

Disease response was defined using established WHO criteria<sup>[24]</sup>.

Patients were considered in remission if symptoms disappeared, gastrin and CgA levels were substantially reduced (> 50% reduction) or returned to normal range and if there was no evidence of residual disease following treatment. The study was approved by the local institutional ethical committees and informed consent was obtained from all patients.

### Statistical analysis

Results were expressed as mean  $\pm$  SD. Nonparametric ANOVA (Kruskal-Wallis one-way ANOVA) was used to assess and compare different parameters (such as the mean age at diagnosis, the size of the largest tumor, the Ki-67 *etc.*) at diagnosis (Table 2), or the levels of gastrin at diagnosis and following surgical treatment/at last visit (Table 1). Post hoc comparisons were made using Mann-Whitney *U* test. A *P* value of < 0.05 was considered significant.

## RESULTS

The clinical characteristics of all patients included in the study are shown in Table 3. The cohort included 9 men and 11 women with a mean age of 55.1 years. Whereas women are usually at higher risk for autoimmune atrophic gastritis, our cohort included patients of both genders, showing only a slight preponderance in the number of female patients. The mean duration of follow-up was 83 mo (range 12-360 mo). Other autoimmune diseases (*e.g.*, Hashimoto's thyroiditis, Sjögren's syndrome) were diagnosed in two patients (10%). In three patients (15%)

there was a first-degree relative with history of gastric (2 patients) or pancreatic (one patient) adenocarcinoma.

### Basal evaluation (at diagnosis)

At diagnosis gastroscopy revealed macroscopic gastric carcinoid tumors (described as “nodules”, “ulcers” or “polyps”) in all patients, with a mean diameter of 20.13  $\pm$  10.83 mm (mean  $\pm$  SD) (range 4-55 mm). The tumors were single in 6/20 patients (30%), and multiple (defined as  $\geq$  2 tumors seen on gastroscopy) in the remaining 14 (70%). ECL cell hyperplasia was observed in all patients. The mean Ki-67% proliferation index was 6.8%  $\pm$  11.2% (range 1%-20%). None of the patients included in the present series presented with ZES and the associated MEN1 syndrome or with characteristics of type 3 gastric carcinoids (Tables 1 and 4).

EUS was intended to be performed in all patients in order to reveal any residual and/or sub-mucosal tumors. Signs of aggressiveness or invasiveness at first biopsy were demonstrated in seven out of 12 patients with available data (58%) and included: ulceration of the primary lesion in two patients (17%); vascular invasion in two patients (17%); invasion of the muscularis mucosa and lamina propria in four patients (33%). Peri-gastric/gastro-hepatic ligament lymph node invasion was observed in 9 patients (45%) as demonstrated by CT scan and/or Octreoscan or (68)Ga-DOTATOC/NOC/TATE PET-CT; distant metastases were present at initial diagnosis in 9 patients (45%), and included liver metastases in eight and diaphragmatic metastases in one out of the 20 patients.

### Treatment

Ten out of the twenty patients (50%) underwent total gastrectomy or a Billroth 2 operation (gastro-jejunostomy) and lymph node dissection, another 4 patients (20%) underwent antrectomy and wedge resection, whereas endoscopic resection of the dominant lesion was performed in 5 patients (25%). One patient underwent only primary tumor biopsy (Table 1, patient No. 3).

Histopathological analysis following tumor resection demonstrated positive staining by immunohistochemistry (IHC) for neuroendocrine markers (chromogranin and synaptophysin) in all patients (100%), for vesicular monoamine transporter 2 (VMAT2) in two patients (10%), and for neuron specific enolase (NSE) in seven patients (35%). Ki-67 indices were available in 17 out of the 20 patients included; eleven tumors were defined as ENETS grade 1

**Table 4** Features associated with the diagnosis of gastric carcinoid type 1 in our patients

Patient No.	Vitamin B12 levels (n. 180-670 pmol/L)	APCA	Gastrin levels (n. 40-108 mU/L)	Prior use of PPIs	1 <sup>st</sup> gastroscopy (macroscopic)	Histo-pathology	<i>H. Pylori</i>
1	45	positive (1/20)	1811	no	multiple	CAG + IM	negative
2	165	positive	-	no	solitary	CAG + IM + NECH	-
3	333	positive (1:160)	2204	no	multiple	CAG + NECH	negative
4	186	positive (1:20)	1800	no	multiple	CAG + NECH	negative
5	104	positive (1:20)	1403	no	multiple	CAG + NECH	negative
6	122	positive (1:80)	-	no	multiple	CAG	-
7	121	-	700	no	solitary	CAG + IM + NECH	-
8	86	-	407	no	multiple	CAG + IM + NECH	-
9	50	-	-	no	solitary	CAG + NECH	-
10	-	positive (1:40)	5130	no	solitary	CAG	-
11	-	-	-	no	multiple	CAG	-
12	-	-	-	no	multiple	CAG	-
13	184	positive (1:160)	5470	no	solitary	CAG	-
14	121	positive	1336	no	multiple	CAG	-
15	215	-	3500	no	multiple	CAG	-
16	-	-	-	no	multiple	CAG + IM + NECH	-
17	345	-	1612	no	solitary	CAG	-
18	130	-	506	no	multiple	CAG	-
19	181	-	1600	no	multiple	CAG	negative
20	167	positive	458	no	multiple	CAG	-

APCA: Antiparietal cells antibodies; PPIs: Proton pump inhibitors; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; NECH: Neuroendocrine cells hyperplasia; *H. pylori*: *Helicobacter pylori*.

(Ki-67  $\leq$  2%) and six tumors as grade 2 (Ki-67 between 2%-20%). The final value for the mean Ki-67 proliferation index measured 4.8%, slightly lower than the Ki-67 value at first endoscopy (6.8%); interestingly, the Ki-67 was significantly higher in the liver/lymph node metastases than in the primary tumor in 4/20 patients.

Based on local team decision, five out of the 20 patients assessed were treated with somatostatin analogues (SSAs): in four patients Sandostatin LAR (Novartis, Basel, Switzerland) 30 mg/month, in one patient Somatuline Autogel (Ipsen, Paris) 90 mg/month, whereas in one patient pegylated interferon alpha was added to the SSA at a dosage of 50 micrograms per week, as anti-secretory and anti-proliferative therapy.

Treatment related adverse events were reported in only 3 patients and included diarrhea (one patient), fatigue (in the patient treated with interferon alpha) and gastrectomy-related dumping syndrome in one patient.

None of the patients received chemotherapy or peptide receptor radioligand therapy, to date.

### Laboratory and imaging assessment at diagnosis

Gastrin and CgA levels were elevated at diagnosis in all patients with available data (14/20 patients for gastrin, and 13/20 patients for CgA) and reached  $2138.4 \pm 1562$  mU/L for gastrin (normal range 40-108 mU/L) and  $507.6 \pm 403.7$  ng/mL for CgA (normal range 19.4-98.1 ng/mL), respectively. No clear correlation was found between initial gastrin and CgA serum levels and the number or size of the tumors.

High levels of anti-parietal cells antibodies were found in all patients in whom their titer was determined. The levels of vitamin B12 were low in all but six patients, with a mean value of  $162 \pm 87$  pmol/L (normal range

180-670 pmol/L) (Table 4).

Data on functional imaging -  $^{111}\text{In}$ -pentetreotide scintigraphy (Octreoscan) or (68)Ga-DOTATOC/NOC/TATE PET-CT (performed based on local availability) were available at diagnosis in 17/20 included patients: in 12 patients (71%) there was increased tracer uptake by the gastric lesions as well as by the perigastric metastatic lymph nodes and liver lesions. Twelve patients underwent (68)Ga-DOTATOC/NOC/TATE-PET-CT demonstrating an increased uptake by the tumor and metastases in 9 patients, and no pathological uptake in the remaining 3 patients. Five patients performed an Octreoscan, showing increased uptake by the tumor in 3, and no pathological uptake in 2.

Interestingly, in the five patients with no pathological uptake by either functional imaging method, the Ki-67 index of proliferation was  $\leq$  2% and the tumor size was  $> 1$  cm.

### Follow-up assessment and treatment outcome

All patients remained alive during the follow-up period. During follow-up after the first intervention, the disease was stable in all patients: in the subgroup who underwent total gastrectomy or Billroth 2 operation (gastro-jejunostomy) and lymph node dissection (10 patients, 50%), as well as in the subgroup of the 4 patients (20%) who underwent antrectomy and wedge resection, the disease did not progress or recur during follow-up. The same was observed in the other patients in the present series, including those who underwent repeated endoscopic resection of the largest lesions. In the seven patients with persistent liver disease, somatostatin analogue treatment was administered in three patients: in two Sandostatin LAR 30 mg/month alone, (inducing disease stabilization in

one patient and complete response in the other), whereas in the third patient pegylated interferon  $\alpha$  (PegIntron) at a dosage of 50 micrograms/week was added to Sandostatin LAR 30 mg/month, and induced partial response of the liver metastases. All patients tolerated treatment with SSAs well and none discontinued treatment during the follow-up period. Apart from a slight perturbation in the control of pre-treatment diabetes mellitus in one patient (Table 1, patient 3), there were no other adverse effects associated with somatostatin analogue treatment. Eighteen patients (90%) had symptoms attributed to the disease (such as abdominal pain, nausea, vomiting or dyspepsia) that improved in all following treatment.

Serum gastrin decreased progressively in all patients with available data, from  $2138.4 \pm 1562$  mI/L pre-treatment to  $223 \pm 193$  mI/L at the last visit (normal range 40-108 mI/L,  $P < 0.005$ ). The levels of serum CgA also significantly decreased, from  $507.6 \pm 403.7$  ng/mL to  $57 \pm 44.7$  ng/mL (mean  $\pm$  SD) (normal range 19.4-98.1 ng/mL,  $P < 0.005$ ).

## DISCUSSION

GCAs are rare neoplasms, accounting for about 1.25% of all malignancies<sup>[25]</sup>. Their incidence, however, is increasing, most probably as result of the widespread use of endoscopy and imaging. Despite the relatively indolent biological behaviour of GCA1 tumors, approximately 8%-23% have been reported as presenting with an aggressive clinical course, metastasizing to regional lymph nodes and rarely to the liver<sup>[7]</sup>.

The European Neuroendocrine Tumor Society (ENETS) consensus guidelines on GCA1 treatment are based on tumor size (less or more than 1 cm) and specify that, despite a preference for a conservative approach, based on endoscopic follow-up, lesion resection is recommended whenever possible<sup>[26]</sup>. Specifically, in patients with lesions of more than 1 cm, EUS should be performed to assess gastric wall and lymph nodal involvement before the decision about the type of excision (endoscopic mucosal resection, EMR, or subtotal gastrectomy/wide resection) is taken. Although biotherapy with somatostatin analogues (SSAs) is still a matter of debate according to the ENETS guidelines, we and others have recently demonstrated the beneficial effect of long acting SSAs monthly administration on inhibition of gastrin and CgA levels and of tumor progression, as shown from the regression of ECL-cell hyperplasia and tumor disappearance observed in the great majority of treated patients<sup>[21,27,28]</sup>. The combination of octreotide and  $\alpha$ -interferon has been also reported to be of value in a patient with metastatic disease to the liver<sup>[7]</sup>.

As the therapeutic modalities to inhibit tumor progression in metastatic GEP-NETs are still unsatisfactory, new approaches are under investigation. Recent preclinical data demonstrated possible beneficial effects of interferon-beta (IFN- $\beta$ ) in inhibiting cell proliferation and stimulating apoptosis in a PNET cell line model<sup>[29-31]</sup>.

Moreover, a new gastrin/CCK2 receptor antagonist molecule, YF476, appears to induce potent inhibition of ECL cell proliferation compared with dopamine agonists or dopamine/somatostatin chimera molecules, and to provide new insights for the therapy of hypergastrinemic gastric NETs associated with low acid states, such as in our patients<sup>[32]</sup>. Noteworthy, a recent phase II study demonstrated good tolerability for the multi receptor ligand SSA pasireotide (SOM230) in patients with GEP NETs refractory to available SSAs<sup>[33]</sup>.

In the present study we sought to define risk factors for increased malignant potential at the time of diagnosis in patients with GCA1. From a total of 254 consecutive patients with GCA1 followed and treated at 5 tertiary referral medical centers, we identified 20 patients with metastatic disease to locoregional lymph nodes or liver at presentation (7.9%). In our series, the patients with metastatic GCA1 were younger, had larger tumors, had a higher Ki-67 proliferation index, and presented with higher gastrin levels compared with the group of patients with non-metastatic GCA1 tumors (Table 2). These results are in accordance with a recent study published by Saund MS and coworkers<sup>[34]</sup>, demonstrating that in a group of 984 patients with localized GCA1, tumor size and depth predict lymph node metastasis; they recommended endoscopic resection for intraepithelial tumors  $< 2$  cm and perhaps tumors  $< 1$  cm invading into the lamina propria or submucosa.

In the present series, most of the patients with metastatic GCA1 were symptomatic, with presence of epigastric or abdominal pain, dyspepsia, bloating, nausea, loose stools or early satiety. A possible explanation for these symptoms may be the presence of atrophic gastritis together with achlorhydria in all patients with GCA1, as well as the increased levels of gastrin<sup>[35,36]</sup>.

Of note, there was a clear correlation between the size of the tumor at diagnosis and tumor metastatic spread in our study, as in all patients included the tumor size was  $\geq 1$  cm. Moreover, the mean Ki-67 index of proliferation in the metastatic GCA1 was significantly higher than in the localized tumors (Table 2), most probably due to an increased number of patients with grade 2 tumors in our series (6/20 patients, 30%) and indicating the utmost importance of performing immunohistochemical staining for this marker in all patients with GCA1. Findings of aggressiveness and/or invasiveness at diagnosis (*e.g.*, ulceration of the lesion, vascular invasion, muscularis propria or lamina propria invasion) are all predictive factors for an aggressive biological behaviour, in parallel with a tumor size of  $\geq 1$  cm. In this high risk group, EUS or cross-sectional imaging should be performed to assess the presence of lymph nodes/liver metastatic disease.

Regarding the imaging characteristics of metastatic GCA1, it appears from our study that no radiological parameters, tumor number or tumor uptake on somatostatin receptor scintigraphy could distinguish between local and metastatic tumors. All of the metastatic GCA1 patients accomplished tumor resection with a low compli-



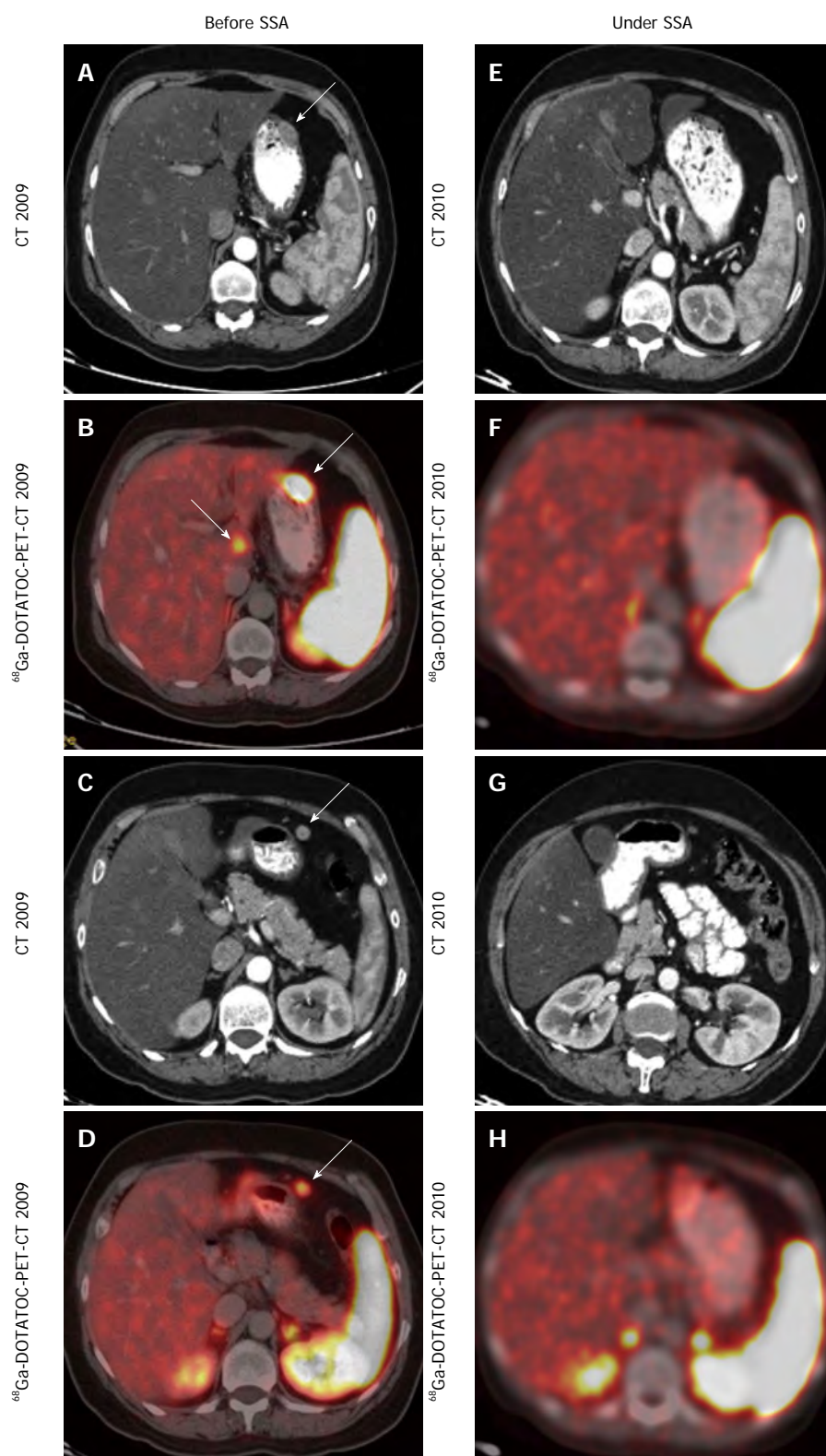


Figure 1 Computed tomography and  $^{68}\text{Ga}$ -DOTATOC-PET-Computed tomography images before and during treatment with somatostatin analogue (sandostatin LAR 30 mg/mo). Pathologic uptake in the gastric and hepatic lesion (A + B) adjacent lymphadenopathy and liver lesion (C + D), disappeared on follow up imaging (E - H).

cation rate, and with an excellent outcome. Following or in parallel with tumor resection, medical therapy was administered in five patients, based on clinical experience. Importantly, under treatment with SSAs, the disease

stabilized in 3 patients, in one patient the primary tumor, the metastatic lymph nodes and the liver metastases regressed and completely disappeared (Figure 1 and Table 1), whereas in another patient, pegylated interferon  $\alpha$

was added to the SSA and induced disease stabilization. In none of the twenty patients with metastatic GCA1 was disease progression observed over a mean follow-up period of 54 mo.

Based on the results of our study, metastatic GCA1 do exist, are extremely rare, and carry a good overall prognosis. Metastatic spread appears to be related to a tumor size of  $\geq 1$  cm, and therefore endoscopic ultrasound evaluation is recommended in such patients. Elevated Ki-67 index of tumor proliferation, as well as high serum gastrin levels, represent additional risk factors for metastatic disease. Endoscopic resection and/or subtotal gastrectomy are recommended by the ENETS guidelines in all patients with gastric carcinoids of  $\geq 1$  cm; however, in our personal opinion<sup>[21]</sup>, SSAs might be considered as possible treatment in order to lower the elevated gastrin levels, suppress ECL cell hyperplasia, and obviate the need for surgical excisions, particularly in patients with multiple or relapsing tumors, as well as in those with metastatic disease of the liver. Treatment with SSAs could be theoretically continued as long as gastrin/CGA levels are suppressed, in parallel with disease stabilization observed on regular endoscopic follow-up. However, this approach is still problematic by the lack of controlled trials, the high cost of these drugs as well as the limited accessibility to SSAs in some areas. Although the potential role of SSAs (“cold” SSAs, as monthly injections, or radioactive “hot” SSAs, PRRT) cannot be denied - it remains still controversial and it has to be confirmed in larger studies. Moreover, surgical procedures should be most probably performed only in patients in whom total tumor excision can be expected. Therefore, in these patients, endoscopic surveillance (as well as repeated oncological surveillance by imaging in metastatic cases) is the most important measure. Prospective multicenter randomised studies, including larger number of patients, would be optimal for definition of the best therapeutic approach, the duration of treatment and its efficacy in terms of long-term survival. However, due to the extreme rarity of this condition, the probability for such trials is remote, and therefore clinicians who manage these patients will most probably have to rely on personal experience and data from retrospective studies, such as ours.

## COMMENTS

### Background

Gastric carcinoids (GCAs) are rare neuroendocrine tumors (NETs) of the gastric mucosa originating from enterochromaffin like (ECL) cells. Type 1 (GCA1) represents the majority, and usually carries an excellent overall prognosis.

### Research frontiers

Metastatic GCA1 are extremely rare and little is known about their natural history, treatment and prognosis. The present study represents a multicenter, retrospective analysis aiming to describe disease characteristics and treatment modalities in a group of rare patients with metastatic GCA1.

### Innovations and breakthroughs

The authors demonstrated that the metastatic potential of GCA1 appears to be related to a tumor size of  $\geq 1$  cm, an elevated Ki-67 index and high serum gastrin levels. Endoscopic ultrasound is recommended in patients with these risk factors. Somatostatin analogues may be used, particularly in patients with

multiple relapsing tumors, and with metastatic disease. Surgical procedures should be performed only in patients in whom total tumor excision is expected.

### Applications

By understanding the potential malignant behavior of these rare tumors, this study may represent a future strategy for therapeutic intervention in patients with metastatic GCA1.

### Peer review

This is a useful multicenter, retrospective analysis of a rare disease and provides helpful information on risk factors, tumor characteristics, treatment procedures and prognosis in a wide and rare group of patients with metastatic GCA1.

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## Risk factors to predict severe postoperative pancreatic fistula following gastrectomy for gastric cancer

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### Abstract

**AIM:** To allow the identification of high-risk postoperative pancreatic fistula (POPF) patients with special reference to the International Study Group on Pancreatic Fistula (ISGPF) classification.

**METHODS:** Between 1997 and 2010, 1341 consecutive patients underwent gastrectomy for gastric cancer at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine, Japan. Based on the preoperative diagnosis, total or distal gastrectomy and sufficient lymphadenectomy was performed, mainly according to the Japanese guidelines for the treatment of gastric cancer. Of these, 35 patients (2.6%) were diagnosed with Grade B or C POPF according to the ISGPF classification and were treated intensively. The hospital records of these patients were reviewed retrospectively.

**RESULTS:** Of 35 patients with severe POPF, 17 (49%) and 18 (51%) patients were classified as Grade B and C POPF, respectively. From several clinical factors, the

severity of POPF according to the ISGPF classification was significantly correlated with the duration of intensive POPF treatments ( $P = 0.035$ ). Regarding the clinical factors to distinguish extremely severe POPF, older patients ( $P = 0.035$ , 65 years  $\leq$  vs  $<$  65 years old) and those with lower lymphocyte counts at the diagnosis of POPF ( $P = 0.007$ ,  $< 1400/\text{mm}^3$  vs  $1400/\text{mm}^3 \leq$ ) were significantly correlated with Grade C POPF, and a low lymphocyte count was an independent risk factor by multivariate analysis [ $P = 0.045$ , OR = 10.45 (95%CI: 1.050-104.1)].

**CONCLUSION:** Caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

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**Key words:** Pancreatic fistula; International Study Group on Pancreatic Fistula classification; Gastric cancer; Gastrectomy; Complication

**Core tip:** Although several possible risk factors associated with the occurrence of postoperative pancreatic fistula (POPF) have been reported, there have been no generally accepted risk factors to predict POPF changing into extremely severe POPF. In this study, we demonstrated that older patients ( $P = 0.035$ ) and those with lower lymphocyte counts at the diagnosis of POPF ( $P = 0.007$ ) were significantly associated with extremely severe International Study Group on Pancreatic Fistula grade C POPF, and a low lymphocyte count was identified as an independent risk factor by multivariate analysis ( $P = 0.045$ , OR = 10.45).

Komatsu S, Ichikawa D, Kashimoto K, Kubota T, Okamoto K, Konishi H, Shiozaki A, Fujiwara H, Otsuji E. Risk factors to predict severe postoperative pancreatic fistula following



gastrectomy for gastric cancer. *World J Gastroenterol* 2013; 19(46): 8696-8702 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8696.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8696>

## INTRODUCTION

Recent advances in less invasive treatment techniques and the perioperative management of gastric cancer have decreased the mortality and morbidity rates associated with this disease<sup>[1,2]</sup>. However, postoperative pancreatic fistula (POPF) is still a major complication following gastrectomy. Once POPF develops, it sometimes contributes to lethal complications, such as abdominal abscesses, secondary anastomotic leakage, and intra-abdominal hemorrhage.

Many surgeons have previously reported several possible risk factors for the occurrence of POPF. It has been reported that the incidence of POPF associated with surgical procedures is higher following radical or extended lymphadenectomy<sup>[3,4]</sup>, splenectomy, or pancreaticosplenectomy<sup>[5-7]</sup>. Host-related factors on POPF have also been clarified, in which the occurrence of POPF has been significantly correlated with a higher body mass index (BMI) and visceral fat area (VFA), being male, hyperlipidemia, and comorbidities<sup>[8-11]</sup>.

Thus, to decrease the incidence of POPF, several clinical studies have been performed with the aims of avoiding unnecessary surgery and standardizing surgical procedures<sup>[4,12]</sup>. Moreover, in order to lower the risk of tissue damage and make surgical procedures easier, surgical devices such as ultrasonic activated coagulating scalpels have been developed and most surgeons are currently cautious of the risk factors associated with POPF. However, to date, after patients develop POPF, there are no generally accepted risk factors to predict the change to severe POPF in these patients. Indeed, indicators that provide an objective description of the patient's condition at specific points in the disease process of POPF are useful to improve understanding of the complications that may be encountered.

In this study, we confirmed that the severity of POPF according to the International Study Group on Pancreatic Fistula (ISGPF) classification was correlated with the duration of intensive POPF treatments. Furthermore, we clarified an independent risk factor to predict the worst outcome of POPF treatment with special reference to the ISGPF classification.

## MATERIALS AND METHODS

### *Patients and surgical procedures*

Between 1997 and 2010, 1341 consecutive patients underwent gastrectomy for gastric cancer at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine. Of these, 35 patients (2.6%) were diagnosed with severe POPF and were treated intensively.

Patients underwent preoperative assessments including gastric endoscopy, computed tomography (CT) scans, and laboratory tests. Based on the preoperative diagnosis, total or distal gastrectomy and sufficient lymphadenectomy was performed, mainly according to the Japanese guidelines for the treatment of gastric cancer<sup>[13]</sup>. Patients with T1 and N0 tumors underwent D1, D1 +  $\alpha$ , or D1 +  $\beta$  lymphadenectomy. Patients with T2 or more advanced tumors and those with N1 or more advanced tumors underwent D2 lymphadenectomy. Briefly, D1 lymphadenectomy indicated dissection of the perigastric lymph nodes (nodal stations No. 1, 2, 3, 4, 5 and 6) and D1 +  $\alpha$  lymphadenectomy indicated dissection of the perigastric lymph nodes and nodes at the base of the left gastric artery (No. 7). D1 +  $\beta$  lymphadenectomy indicated dissection of the perigastric lymph nodes and stations No. 7, 8a (anterosuperior group of the common hepatic artery), and 9 (celiac artery) lymph nodes. In the D2 dissection, the perigastric lymph nodes and all second-tier lymph nodes were completely retrieved. Depending on the location of the tumor, lymphadenectomy was added along the distal side of the splenic artery (No. 11d) and at the splenic hilum (No. 10), together with splenectomy or splenectomy with distal pancreatectomy<sup>[14]</sup>.

### *Definition of POPF using the ISGPF classification*

POPF was retrospectively defined according to the ISGPF definition<sup>[15]</sup>: output via an operatively placed drain (or a subsequently placed percutaneous drain) of any measurable volume of drain fluid on or after postoperative day 3, with an amylase content more than 3 times higher than the upper normal serum value. We comprehensively diagnosed POPF according to not only drain amylase (D-AMY) levels, but also changes in the properties of the drain, clinical findings, laboratory data, and imaging findings such as ultrasonography (US) or CT scans. Patients who had no drains or whose drains were removed that developed postoperative fever ( $< 38^{\circ}\text{C}$ ), leukocytosis, and peripancreatic fluid collection detected on US or CT scans were also diagnosed with POPF.

POPF was graded according to the ISGPF criteria as follows: grade A had no clinical impact and required no treatment; grade B required a change in management or adjustment in the clinical pathway; and grade C required a major change in clinical management or deviation from the normal clinical pathway and required aggressive clinical intervention. Patients requiring only repositioning of their drains belonged to Grade B POPF. Patients were classified as grade C POPF if US and CT findings showed peripancreatic fluid collection and drains needed to be placed interventionally in order to improve severe clinical data and conditions. In this study, severe POPF were regarded as a clinically significant pancreatic fistula corresponding to grade B and C POPF.

### *Treatment strategy for POPF following gastrectomy*

Patients with POPF, which is diagnosed by high D-AMY level and have no abnormal physical finding and labo-

**Table 1** Characteristics of 35 patients with postoperative pancreatic fistula following gastrectomy *n* (%)

Sex	Male	30 (86)
	Female	5 (14)
Age (yr)	mean + SD	67.3 ± 9.5
BMI (kg/m <sup>2</sup> )	mean + SD	22.1 ± 3.2
pT-stage	T1	3 (9)
	T2	11 (31)
	T3	15 (43)
	T4	6 (17)
pN-stage	N0	10 (29)
	N1	3 (9)
	N2	9 (26)
	N3	13 (36)
pStage	I	6 (17)
	II	3 (9)
	III	18 (51)
	IV	8 (23)
Gastrectomy	Distal	10 (29)
	Total or others <sup>1</sup>	25 (71)
Splenectomy	Presence	21 (60)
	Absence	14 (40)
Pancreaticosplenectomy	Presence	12 (34)
	Absence	23 (66)
Lymphadenectomy	< D2	8 (23)
	D2 ≤	27 (77)
Resection status	R0	10 (29)
	R1	15 (42)
	R2	10 (29)
ISGPF classification	Grade B	17 (49)
	Grade C	18 (51)

<sup>1</sup>Others; proximal gastrectomy and remnant gastrectomy. ISGPF: International Study Group on Pancreatic Fistula; BMI: Body mass index.

ratory data, could be followed without any treatments. The abdominal drainage tube is normally removed after the D-AMY level has been reduced to a level lower than three times the serum AMY level. Patients with POPF, which is diagnosed by high D-AMY level and have abnormal findings such as fever, abdominal pain and other laboratory data, start to undergo intensive POPF treatments. After emergency CT examination, if the drainage tube position is good, antibiotics, octreotide acetate and total parenteral nutrition should be started. If the fluid drainage tube position is not satisfactory, an additional or alternative drainage tube can be placed into the abnormal fluid cavity by percutaneous CT or ultrasonography-guided technique. Moreover, bacterial infection of drainage fluid and/or the suspicion of it was detected following these POPF treatments. The drainage tube would be changed into an irrigation type drainage tube. Then, continuous irrigation and drainage with saline would be performed. If these series of conservative POPF treatments were not effective, open drainage and debridement for POPF abscess by laparotomy would be performed and the irrigation type drainage tube and an enteral feeding tube would be placed. After that, comprehensive POPF treatments consist of continuous irrigation drainage with saline, antibiotics, octreotide acetate and enteral nutrition.

## Statistical analysis

The  $\chi^2$  test and Fisher's exact probability test were performed for categorical variables, while the Student's *t* test and Mann-Whitney *U* test for unpaired data of continuous variables were performed to compare clinicopathological characteristics between the two groups. Multivariate stepwise logistic regression analysis was performed to identify the independent risk factors associated with Grade C POPF. Multivariate odds ratio are presented with 95%CI. In all of these analyses, *P* values less than 0.05 were considered significant.

## RESULTS

### Clinicopathological characteristics of patients with severe POPF

Table 1 shows the characteristics of 35 patients with severe POPF. The mean patient age was 67.3 years and the male:female ratio was 6:1. More than 80% of patients were male and the incidence of patients with pT3-T4, pStage III-IV, and D2 or more lymphadenectomy was high. Of 35 patients with severe POPF, 17 (49%) and 18 (51%) patients were classified as grade B and grade C POPF, respectively. The median intensive treatment period of POPF was 20 d. Twenty nine patients were diagnosed with POPF by their D-AMY levels and were retrospectively judged to meet the ISGPF criteria. The remaining 6 patients were diagnosed with POPF by their clinical condition, laboratory data, and CT findings because POPF was detected after drain removal.

### Comparison of clinicopathologic factors and ISGPF classification in patients with severe POPF between short and long duration intensive treatments

No current standard definition of POPF reflects the duration of intensive treatments according to the severity of POPF following gastrectomy for gastric cancer. Therefore, we compared possible clinicopathologic factors and ISGPF classification between short (< 20 d) and long (≥ 20 d) duration intensive treatments (Table 2). The cut off value of each continuous clinical data was decided by a ROC curve. As a result, the severity of POPF according to the ISGPF classification was significantly correlated with the duration of intensive POPF treatments (*P* = 0.035). There were no significant differences between both groups for other clinicopathologic factors, although POPF after pancreaticosplenectomy was associated with long duration intensive POPF treatments (*P* = 0.149). Therefore, we confirmed that the ISGPF classification reflects the duration of intensive treatments according to the severity of POPF and is a reliable classification of POPF following gastrectomy for gastric cancer.

### Comparison of clinical factors between Grade B and C POPF according to the ISGPF classification

We compared several clinical factors between grade B

**Table 2** Comparison of clinicopathologic factors and International Study Group on Pancreatic Fistula classification in patients with severe postoperative pancreatic fistula between short and long duration of intensive treatments *n* (%)

Variables		Intensive treatment periods for POPF		<sup>1</sup> <i>P</i> value
		< 20 d	20 d ≤	
Sex	Male	10 (83)	20 (87)	0.827
	Female	2 (17)	3 (13)	
Age (yr)	< 65	5 (42)	7 (30)	0.772
	65 ≤	7 (58)	16 (70)	
BMI (kg/m <sup>2</sup> )	< 21	5 (42)	6 (27)	0.636
	21 ≤	7 (58)	16 (73)	
pT-stage	T1 T2	3 (25)	3 (13)	0.391
	T3 T4	9 (75)	20 (87)	
pN-stage	N1 N2	5 (42)	16 (70)	0.217
	N3	7 (58)	7 (30)	
pStage	I II	4 (33)	5 (22)	0.736
	III IV	8 (67)	18 (78)	
Splenectomy	Presence	7 (58)	14 (61)	0.827
	Absence	5 (42)	9 (39)	
Pancreatico-splenectomy	Presence	2 (17)	10 (43)	0.149
	Absence	10 (83)	13 (57)	
Lymphadenectomy	< D2	2 (17)	6 (26)	0.685
	D2 ≤	10 (83)	17 (74)	
Resection status	R0	5 (42)	5 (22)	0.728
	R1	4 (33)	11 (48)	
	R2	3 (25)	7 (30)	
Blood loss (g)	< 1000	3 (25)	9 (39)	0.476
	1000 ≤	9 (75)	14 (61)	
Operation time (min)	< 330	6 (50)	11 (48)	0.815
	330 ≤	6 (50)	12 (52)	
Preoperative Hb (g/dL)	< 10	5 (42)	3 (13)	0.091
	10 ≤	7 (58)	20 (87)	
Preoperative Alb (g/dL)	≤ 3.5	3 (30)	6 (29)	1.000
	3.5 <	7 (70)	15 (71)	
<sup>2</sup> Lymphocyte counts (/mm <sup>3</sup> )	< 850	5 (42)	18 (78)	0.073
	850 ≤	7 (58)	5 (22)	
ISGPF classification	grade B	9 (75)	8 (35)	0.035 <sup>3</sup>
	grade C	3 (25)	15 (65)	

<sup>1</sup>*P* values were derived from the  $\chi^2$  or Fisher's exact test and were considered significant at  $< 0.05$ . <sup>2</sup>Lymphocyte counts at the diagnosis of POPF;

<sup>3</sup>Significant values. ISGPF: International Study Group on Pancreatic Fistula; POPF: Postoperative pancreatic fistula; BMI: Body mass index.

and C POPF according to the ISGPF classification in order to detect the predictive factors of extremely severe POPF (Table 3). As a result, older patients ( $P = 0.035$ ,  $\leq 65$  years old *vs*  $> 65$  years old) and those with low lymphocyte counts at the diagnosis of POPF ( $P = 0.007$ ,  $< 1400/\text{mm}^3$  *vs*  $\geq 1400/\text{mm}^3$ ) were significantly associated with Grade C POPF. The cut-off value of  $1400/\text{mm}^3$  is calculated by the ROC curve to distinguish between grade B and C POPF (Figure 1). The incidence of other clinical factors, which were presented in Table 3 and others such as underlying disease, methods of reconstruction, HbA1c, postoperative Hb, Alb, preoperative serum total protein, total cholesterol, triglyceride, %LVC and FEV 1.0% *etc.*, did not significantly differ between both groups (data not shown). Furthermore, logistic regression analysis revealed that a low lymphocyte count was an independent risk factor by multivariate analysis [ $P = 0.045$ ,

OR = 10.45 (95%CI: 1.050-104.1)] (Table 4).

## DISCUSSION

Until recently, there has been no universally recognized definition of POPF following gastrectomy for gastric cancer. Accordingly, different definitions of POPF have been reported, which has resulted in highly variable rates of POPF, ranging from 5.8% to 49.7%<sup>[7,10,16-20]</sup>. Therefore, it is impossible to accurately evaluate the incidence and severity of POPF. Obama *et al*<sup>[21]</sup> were the first to utilize the ISGPF classification, which was formulated as an objective definition of POPF following pancreatic surgery in 2005<sup>[15]</sup>, to evaluate the feasibility of laparoscopic gastrectomy with radical lymphadenectomy for gastric cancer. The incidence of ISGPF grade B or C including both open and laparoscopic gastrectomy was 5.1% (12/233)<sup>[21]</sup>. Miki *et al*<sup>[22]</sup> also reported using the ISGPF classification that a high content of drain AMY on 1POD could be used to predict severe POPF. The incidence of ISGPF grade B or C following total gastrectomy with D2 lymphadenectomy was 22.1% (23/104). Ji-ang *et al*<sup>[11]</sup> recently reported that severe POPF, defined as ISGPF grade B or C, was associated with being male and a high BMI in patients undergoing laparoscopic gastrectomy for gastric cancer. The incidence of ISGPF grade B or C following laparoscopic distal gastrectomy for early gastric cancer was 4.2% (34/798). Miyai *et al*<sup>[23]</sup> advocated that simple predictive scoring system might be useful for many clinicians to assess the risk of POPF after laparoscopic gastrectomy (LAG). The incidence of ISGPF grade B or C following LAG was 3.9% (11/277). These reports clarified the significance of using the same definition of POPF and detecting the risk factors of POPF using the ISGPF classification. However, it remains unclear whether the ISGPF classification following pancreatic surgery can be applied to POPF following gastrectomy to reflect the extent of the severity of POPF and treatment outcomes.

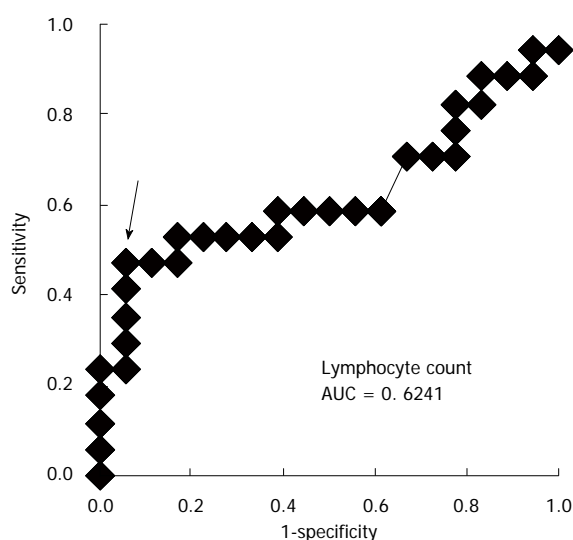
In order to elucidate whether the ISGPF classification can be translated into a clinically useful definition for POPF following gastrectomy, we showed that differences in the severity of POPF defined by the ISGPF classification indeed reflected intensive treatment periods. As a result, we confirmed that the ISGPF classification was a reliable classification that was significantly correlated with the duration of intensive treatments in patients with severe POPF following gastrectomy for gastric cancer (Table 1). These results contribute to universal recognition of the ISGPF classification as one of the candidate definitions of POPF following gastrectomy for gastric cancer.

Although several possible risk factors associated with the occurrence of POPF have been reported, there have been no generally accepted risk factors to predict extremely severe POPF, which requires several intensive treatments. During intensive treatments, indicators that provide an objective description of the patient's condi-

**Table 3** Comparison of clinical factors between grade B and C postoperative pancreatic fistula according to International Study Group on Pancreatic Fistula classification *n* (%)

Variables		Total	ISGPF classification		<sup>1</sup> <i>P</i> value
			Grade B ( <i>n</i> = 17)	Grade C ( <i>n</i> = 18)	
Sex	Male	30	14 (82)	16 (89)	0.944
	Female	5	3 (18)	2 (11)	
Age (yr)	< 65	12	9 (53)	3 (17)	0.035 <sup>3</sup>
	65 ≤	23	8 (47)	15 (83)	
BMI (kg/m <sup>2</sup> )	< 21	13	9 (53)	4 (24)	0.158
	21 ≤	21	8 (47)	13 (76)	
pT-stage	T1 T2	6	4 (24)	2 (11)	0.402
	T3 T4	29	13 (76)	16 (89)	
pN-stage	N1 N2	21	9 (53)	12 (67)	0.629
	N3	14	8 (47)	6 (33)	
pStage	I II	9	5 (29)	4 (22)	0.921
	III IV	26	12 (71)	14 (78)	
Splenectomy	Presence	21	11 (65)	10 (56)	0.836
	Absence	14	6 (35)	8 (44)	
Pancreaticosplenectomy	Presence	12	4 (24)	8 (44)	0.344
	Absence	23	13 (76)	10 (56)	
Lymphadenectomy	< D2	8	3 (18)	5 (28)	0.691
	D2 ≤	27	14 (82)	13 (72)	
Resection status	R0	10	4 (24)	6 (33)	0.892
	R1	15	7 (41)	8 (45)	
	R2	10	6 (35)	4 (22)	
Blood loss (g)	< 1000	23	11 (65)	12 (67)	0.815
	1000 ≤	12	6 (35)	6 (33)	
Operation time (min)	< 330	19	10 (59)	9 (50)	0.854
	330 ≤	16	7 (41)	9 (50)	
Preoperative Hb (g/dL)	<10	8	5 (29)	3 (17)	0.443
	10 ≤	27	12 (71)	15 (83)	
Preoperative Alb (g/dL)	≤ 3.5	9	4 (27)	5 (29)	0.825
	3.5 <	23	11 (74)	12 (71)	
<sup>2</sup> Lymphocyte counts (/mm <sup>3</sup> )	< 1400	26	9 (53)	17 (94)	0.007 <sup>3</sup>
	1400 ≤	9	8 (47)	1 (6)	

<sup>1</sup>*P* values were derived from the  $\chi^2$  or Fisher's exact test and were considered significant at < 0.05; <sup>2</sup>Lymphocyte counts at the diagnosis of postoperative pancreatic fistula; <sup>3</sup>Significant values. ISGPF: International Study Group on Pancreatic Fistula; BMI: Body mass index.



**Figure 1** The cut-off value of 1400/mm<sup>3</sup> is calculated by the receiver operating characteristic-curve to distinguish between grade B and C postoperative pancreatic fistula. Arrow is the point of cut-off value.

tion at the diagnosis of POPF are useful for understanding the complications that may be encountered. In this

**Table 4** Results of multivariable logistic regression; risk factor for extremely severe postoperative pancreatic fistula

Covariate	OR	95% Confidence limit	<i>P</i> value
Lymphocyte counts (/mm <sup>3</sup> ) < 1400 vs 1400 ≤	10.45	1.05-104.1	0.045
Age (yr) 65 ≤ vs < 65	3.39	0.602-1.886	0.166

study, we demonstrated that older patients (*P* = 0.035) and those with lower lymphocyte counts at the diagnosis of POPF (*P* = 0.007) were significantly associated with extremely severe grade C POPF, and a low lymphocyte count was identified as an independent risk factor by multivariate analysis (*P* = 0.045, OR = 10.45) (Table 2).

At first, we hypothesized that there were some differences among the previously reported risk factors associated with the occurrence of POPF between grade B and C in patients with severe POPF. However, contrary to our expectations, there was no correlation with the previously reported factors associated with the occurrence of POPF such as radical or extended lymphadenectomy<sup>[3,4]</sup>, splenectomy or pancreaticosplenectomy<sup>[5-7]</sup>, a higher BMI



and VFA, being male, hyperlipidemia, and comorbidities<sup>[8-11]</sup>. In this study, blood lymphocyte counts at the diagnosis of POPF were the only independent risk factor to predict the severity of POPF patients. This result implies that factors associated with the occurrence of POPF may not affect the severity of severe POPF by changing it from grade B to C POPF.

The reason why a low lymphocyte count was the only independent risk factor to predict extremely severe POPF remains unclear. One possible reason is that a change in the severity of POPF from grade B to C POPF may be associated with host-related immunity. Hogan *et al.*<sup>[24]</sup> suggested that a perioperative reduction in circulating lymphocyte levels was an independent predictive factor for wound complications following excisional breast cancer surgery. As discussed in their report, which resulted in selective antibiotic prophylaxis being required for these immune-compromised patients, POPF patients with lower lymphocyte counts at diagnosis may require more intensive treatments to avoid POPF developing into extremely severe POPF, such as grade C POPF. Another possible reason was that a low lymphocyte count may be caused by a delay in the diagnosis of severe POPF and this data may reflect a pre-septic state<sup>[25,26]</sup>. Patients with POPF, which have only abnormal D-AMY data, can be followed without any treatment. These patients mainly resulted in grade A POPF; however, some of these patients may later develop severe POPF. Indeed, in our study, severe POPF patients with grade B started intensive treatments after an average of 5.7 d. In contrast, extremely severe POPF patients with grade C started these treatments after an average of 10.3 d (data not shown). Severe POPF sometimes presents no clinical symptoms such as high grade fever and abdominal pain until some time later. However, surgeons should bear in mind that some POPF patients may develop extremely severe POPF. Therefore, a lower lymphocyte count could provide an objective description and is useful for understanding the complications that may be encountered.

In conclusion, we confirmed that the ISGPF classification of POPF following gastrectomy was a reliable classification that correlated with the duration of intensive treatments in patients with severe POPF. Furthermore, we clarified an independent risk factor to predict the worst outcome of POPF treatment with special reference to the ISGPF classification. Therefore, caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

## COMMENTS

### Background

Despite recent advances in less invasive treatment techniques and the perioperative management of gastric cancer, postoperative pancreatic fistula (POPF) is still a major complication following gastrectomy. Once POPF develops, it sometimes contributes to lethal complications, such as abdominal abscesses, secondary anastomotic leakage, and intra-abdominal hemorrhage. However, to date, after patients develop POPF, there are no generally accepted risk factors

to predict these patients to change severe POPF.

### Research frontiers

Indicators that provide an objective description of the patient's condition at specific points in the disease process of POPF are useful to improve understanding of the complications that may be encountered. In this study, the authors confirmed that the severity of POPF according to the International Study Group on Pancreatic Fistula (ISGPF) classification was correlated with the duration of intensive POPF treatments. Furthermore, they clarified an independent risk factor to predict the worst outcome of POPF treatment with special reference to the ISGPF classification.

### Innovations and breakthroughs

Between 1997 and 2010, 1341 consecutive patients underwent gastrectomy for gastric cancer. Of these, 35 patients (2.6%) were diagnosed with Grade B or C POPF according to the ISGPF classification and were treated intensively. The severity of POPF according to the ISGPF classification was significantly correlated with the duration of intensive POPF treatments ( $P = 0.035$ ). Regarding the clinical factors to distinguish extremely severe POPF, older patients ( $P = 0.035$ ,  $\geq 65$  years old vs  $< 65$  years old) and those with lower lymphocyte counts at the diagnosis of POPF ( $P = 0.007$ ,  $< 1400/\text{mm}^3$  vs  $\geq 1400/\text{mm}^3$ ) were significantly correlated with Grade C POPF, and a low lymphocyte count was an independent risk factor by multivariate analysis [ $P = 0.045$ , OR = 10.45 (95%CI: 1.050-104.1)].

### Applications

The ISGPF classification of POPF following gastrectomy was a reliable classification that correlated with the duration of intensive treatments in patients with severe POPF. Caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

### Terminology

POPF: POPF following gastrectomy for gastric cancer is still a major complication following gastrectomy. ISGPF: grade A had no clinical impact and required no treatment; grade B required a change in management or adjustment in the clinical pathway; and grade C required a major change in clinical management or deviation from the normal clinical pathway and required aggressive clinical intervention.

### Peer review

This is a good descriptive study showing that the ISGPF classification was reliable in patients with POPF following gastrectomy for gastric cancer. Caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

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## Long-term follow up of endoscopic resection for type 3 gastric NET

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prove the usefulness of endoscopic resection in type 3 gastric neuroendocrine tumors (NETs).

**METHODS:** Of the 119 type 3 gastric NETs diagnosed from January 1996 to September 2011, 50 patients treated with endoscopic resection were enrolled in this study. For endoscopic resection, endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) was used. Therapeutic efficacy, complications, and follow-up results were evaluated retrospectively.

**RESULTS:** EMR was performed in 41 cases and ESD in 9 cases. Pathologically complete resection was performed in 40 cases (80.0%) and incomplete resection specimens were observed in 10 cases (7 vs 3 patients in the EMR vs ESD group,  $P = 0.249$ ). Upon analysis of the incomplete resection group, lateral or vertical margin invasion was found in six cases (14.6%) in the EMR group and in one case in the ESD group (11.1%). Lymphovascular invasions were observed in two cases (22.2%) in the ESD group and in one case (2.4%) in the EMR group ( $P = 0.080$ ). During the follow-up period (43.73; 13-60 mo), there was no evidence of tumor recurrence in either the pathologically complete resection group or the incomplete resection group. No recurrence was reported during follow-up. In addition, no mortality was reported in either the complete resection group or the incomplete resection group for the duration of the follow-up period.

**CONCLUSION:** Less than 2 cm sized confined submucosal layer type 3 gastric NET with no evidence of lymphovascular invasion, endoscopic treatment could be considered at initial treatment.

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**Key words:** Stomach; Neuroendocrine tumor; Endoscopic resection; Treatment; Carcinoid

### Abstract

**AIM:** To clarify the short and long-term results and to

**Core tip:** Endoscopic treatment was suitable for tumors measuring approximately 20 mm or smaller in size, with no lymph node or distant metastasis and limited to the submucosal layer of type 3 gastric neuroendocrine tumors (NETs), similar to endoscopic treatment guidelines applied to other gastrointestinal NETs.

Kwon YH, Jeon SW, Kim GH, Kim JI, Chung IK, Jee SR, Kim HU, Seo GS, Baik GH, Choi KD, Moon JS. Long-term follow up of endoscopic resection for type 3 gastric NET. *World J Gastroenterol* 2013; 19(46): 8703-8708 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8703.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8703>

## INTRODUCTION

Neuroendocrine tumors (NETs) are slow-growing malignancies with distinct biological and clinical characteristics. Although these tumors have long been a source of clinical and pathologic interest, their fundamental biology still eludes precise delineation<sup>[1]</sup>. Despite the relative rarity of gastric NETs, their diagnosis is increasing due to the recent widespread use of diagnostic endoscopy<sup>[2-4]</sup>. Yearly age-adjusted incidence is approximately 0.2 per population of 100000.

Enterochromaffin-like (ECL) cells, the main endocrine cell types in type 1 and type 2 gastric NETs, are highly susceptible to gastrin trophic stimuli. Under circumstances that cause hypergastrinemia, such as chronic atrophic gastritis (CAG) in pernicious anemia (type 1) or gastrin-producing neoplasms in Zollinger-Ellison syndrome (ZES)/multiple endocrine neoplasia (MEN) 1 (type 2), multiple ECL cell carcinoids occur in the oxyntic corpus and fundus mucosa of the stomach<sup>[5,6]</sup>. Type 1 and 2 gastric NETs are usually considered benign, with a low risk of malignancy. However, type 3 gastric NETs are composed of different endocrine cells, which grow sporadically, irrespective of gastrin, in an otherwise normal mucosa. Most of these tumors show lymphoinvasion, angioinvasion, and deep wall invasion at the time of diagnosis, and they often present with metastases, which are found in 50%-70% of well-differentiated, and in up to 100% of poorly differentiated tumors<sup>[6-9]</sup>. As a worse overall mortality of type 3 gastric NETs, aggressive surgery is considered the initial therapeutic approach, generally. Many reports on the efficacy of endoscopic treatment for gastric NETs have been published<sup>[10-13]</sup>. However, few studies have reported on endoscopic treatment of type 3 gastric NETs.

In this study, we will conduct a retrospective review of the outcomes and long-term prognosis of endoscopic treatment on type 3 gastric NETs. In addition, we demonstrate the efficacy of endoscopic treatment on type 3 gastric NETs.

## MATERIALS AND METHODS

### Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by University Hospital Kyungpook Trust (KNUMC\_12-1005). All patients provided informed written consent for this study.

### Patients

After receiving appropriate Institutional Review Board approval, members of the Korean college of Helicobacter and Upper Gastrointestinal Research retrospectively enrolled patients who were diagnosed with histologically proven gastric NETs from 10 hospitals between January 1996 and September 2011. Based on endoscopic findings, all gastric NETs were classified according to the Paris endoscopic classification<sup>[14]</sup>. Abdominal computed tomography (CT) scans were available for diagnosis of lymph node involvement or other organ metastasis. These patients were then analyzed with respect to their presenting signs and symptoms, associated disease, tumor characteristics (number, size, site, and the presence of metastasis), and outcome. From the 225 gastric NETs, we reviewed patients' plasma gastrin levels and other associated diseases, such as ZES and multiple endocrine neoplasia (MEN) type 1, to diagnose type 3 gastric NETs. The exact criteria used to decide between endoscopic or surgical treatment was dependent on the tumor size, tumor shape (combined ulceration or depressed lesions), or evidence of adjacent lymph node metastasis.

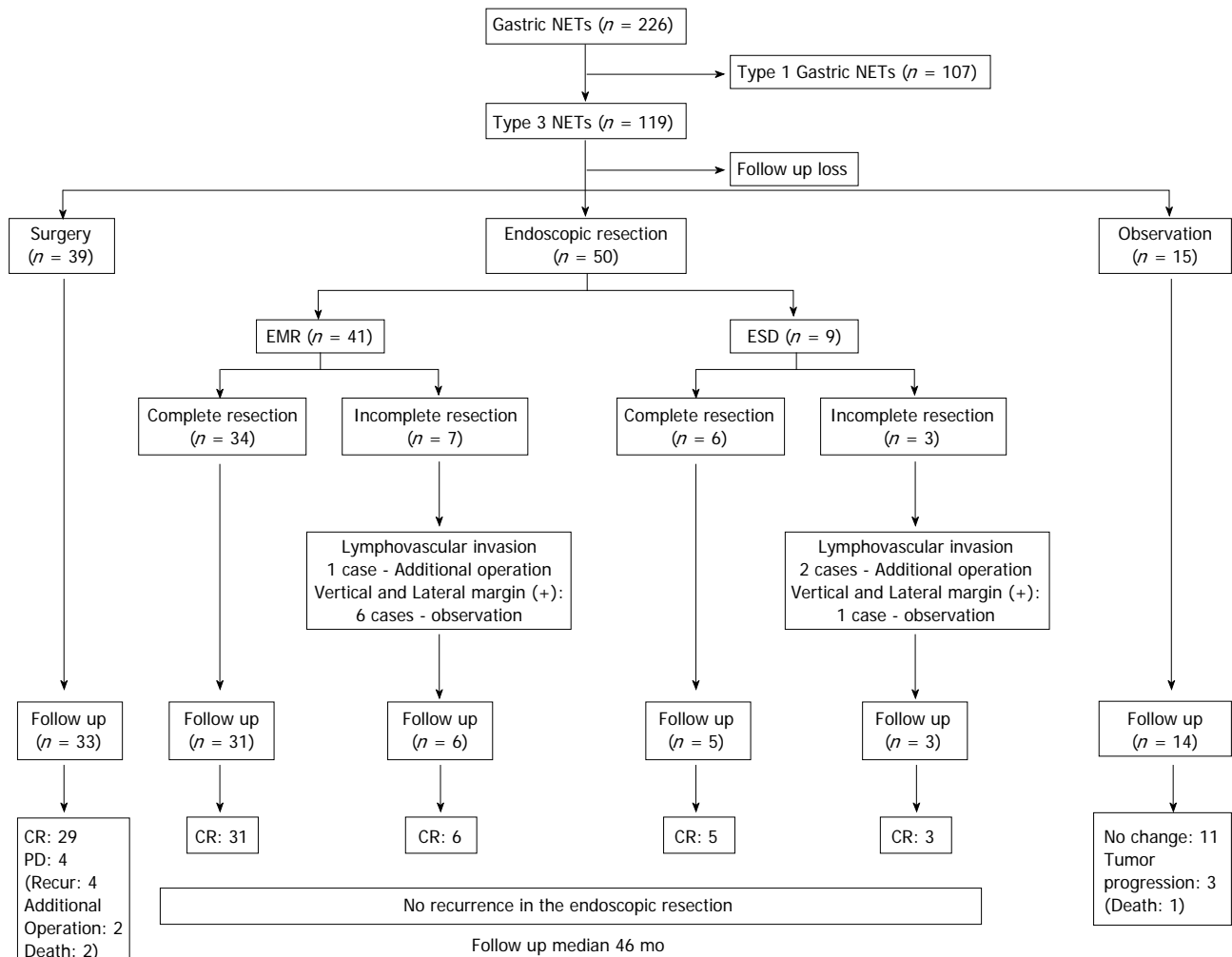
### Histopathologic findings and TNM stage of gastric NETs

Resection specimens processed by formalin fixation were serially sectioned at 2 mm intervals, and tumor involvement to the lateral and vertical margins was assessed. In addition, histopathological type, tumor size, depth of invasion, and lymphovascular invasion were evaluated microscopically. Pathologically complete resection was defined according to the following findings: (1) en bloc resection; (2) the tumor was a well-differentiated neuroendocrine tumor (classical-type carcinoid) according to World Health Organization (WHO) classification<sup>[15]</sup>; (3) tumor invasion was limited to the submucosal layer; (4) no lateral and vertical margin involvement; and (5) no lymphovascular invasion.

### Endoscopic findings and endoscopic mucosal resection and endoscopic submucosal dissection procedures

We evaluated tumor characteristics, such as the measured size, number, and location of tumors. Tumor size was estimated using biopsy forceps (FB 21K-1; Olympus Medical Systems Co, Tokyo, Japan), which was approximately 6 mm in length when opened. Tumor location was reported according to the longitudinal axis (fundus, cardia, body, or antrum). All lesions were imaged with adjacent anatomical structures to ensure that the exact location of





**Figure 1** Flow chart of type 3 gastric neuroendocrine tumors. Of all type 3 gastric neuroendocrine tumors (NETs) ( $n = 119$ ), 39 patients were treated with surgery, 50 patients were treated using an endoscopic method, and 15 patients were followed up only by observation. In the endoscopic treatment group, 41 patients were treated with endoscopic mucosal resection (EMR), and nine patients were treated with endoscopic submucosal dissection (ESD). Upon analysis of the resected specimens, histologically incomplete resections were found in seven cases in the EMR group and three cases in the ESD group, and lymphovascular invasion was found in one case in the EMR group and two cases in the ESD group. All cases of lymphovascular invasion were treated with an additional operation. During the median follow-up duration (46 mo), there was no recurrence of gastric NETs in the endoscopic resection group.

the tumor was recorded and proved histologically by endoscopic biopsy. Endoscopic ultrasonography was used for measuring the depth of invasion of gastric NETs. Endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) was performed after obtaining informed consent. Submucosal injection of saline mixed with epinephrine was performed to elevate tumor tissues from the underlying muscularis propria. Next, EMR, using a hood and snare, or submucosal dissection was applied for removal of the lesion.

#### Follow-up after endoscopic resection

The follow-up program consisted of endoscopic examinations at three, six, and twelve-month intervals, and CT scans and blood tests were performed at 12-month intervals. Follow-up endoscopy was performed depending on the follow-up program, and for histological examinations of NETs recurrence, biopsies were performed at iatrogenic ulcer scar lesions that had undergone endoscopic treatment. CT examination findings were normal in all

patients at the end of follow up.

#### Statistical analysis

All continuous variable data are presented as the mean  $\pm$  SE. Statistical significance was calculated using unpaired Student's *t* test. To assess the difference between two procedures, univariate analysis was performed using Student's *t*-test. Statistical significance was set at 0.05. All analyses were performed using SPSS version 18.0 (SPSS Inc., United States).

## RESULTS

#### Patient baseline characteristics

Overall, in the 226 cases of gastric NETs, 119 cases (52.4%) were diagnosed as type 3 gastric NETs. Of the 119 patients, 50 patients (42.0%) received endoscopic interventions for the treatment of type 3 gastric NET lesions (Figure 1). The average age of the patients was 58.6 (25-85) years. Twenty-eight (56.0%) patients were

**Table 1 Patient characteristics of 51 gastric endocrine tumors who underwent endoscopic resection *n* (%)**

Male:female	28:22
Mean age, yr	58.6 ± 12.2
Associated symptoms	
Abdominal discomfort	14 (28.0)
Body weight loss	1 (2.0)
Diarrhea	1 (2.0)
Other symptom	1 (2.0)
Associated disease	
Diabetes mellitus	5 (10.0)
Thyroid disease	1 (2.0)
Combined other malignancy	2 (4.0)
Number of tumors	
1	48 (96.0)
≥ 2	2 (4.0)
Tumor location	
Antrum	4 (8.0)
Body	38 (76.0)
Fundus or cardia	8 (16.0)
Tumor size	
≤ 10 mm	33 (66.0)
> 10 mm	17 (34.0)
EUS invasion depth	
Mucosa and submucosa	49 (98.0)
MP	1 (2.0)
Treatment methods	
EMR	41 (82.0)
ESD	9 (18.0)

GET: Gastric endocrine tumor; MP: Muscularis propria; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

male and 22 (44.0%) patients were female. Asymptomatic patients were the most common, and abdominal discomfort was the second most common presenting symptom (28.0%) in patients who had type 3 gastric NETs. Upon analysis of the associated underlying disease, five patients (10.0%) had diabetes mellitus (DM), one patient (2.0%) had thyroid disease and early gastric cancer (EGC), and two patients (4.0%) had other combined malignancies (Table 1).

### Tumor characteristic and metastasis

Based on the endoscopic findings, superficial elevated type (type IIa) and solitary lesions (96%) were most prevalent. Upon analysis of the location of the type 3 gastric NETs, 38 lesions (76.0%) were found on the body. Based on the EUS evaluation, there were 49 cases (98.0%) of confined tumors in the mucosal or submucosal layer, and one tumor (2.0%) was suspicious of invasion into the muscularis propria (MP) layer. No lymphatic invasion or other organ metastasis findings was observed in the imaging study (Table 1).

### Treatment modality and results

Of the 50 patients who had been treated with endoscopic intervention, 41 patients (82.0%) were treated by EMR and 9 patients (18.0%) were treated by ESD. The mean tumor size of the gastric NETs was 10.2 ± 6.3 mm, and compared with the mean tumor size, no significant difference was observed between the two groups (9.3 mm

**Table 2 Treatment outcomes after endoscopic treatment of gastric endocrine tumors *n* (%)**

	EMR ( <i>n</i> = 41)	ESD ( <i>n</i> = 9)	<i>P</i> value
Mean resection size (range, mm)	9.3 ± 5.6	14.2 ± 7.8	0.055
Tumor size > 10 mm	11 (26.8)	6 (66.7)	0.031
Pathologically complete resection	35 (85.4)	6 (66.7)	0.249
Lymphovascular invasion	1 (2.4)	2 (22.2)	0.080
Additional operation	1 (2.4)	2 (22.2)	0.080

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

vs 14.2 mm in the EMR vs ESD group, *P* = 0.055). All tumors were determined as pathologically well-differentiated neuroendocrine tumors. Upon analysis of the resected specimens, 11 tumors and six tumors in the EMR and ESD groups, respectively, were gastric NETs measuring 10 mm or more in size (*P* = 0.031); pathologically complete resections were achieved in 40 cases (80.0%), and incomplete resection specimens were seen in 10 cases (7 vs 3 patients in the EMR vs ESD group, *P* = 0.249). Lateral or vertical margin invasion was found in six cases (14.6%) in the EMR group and in one case in the ESD group (11.1%). Lymphovascular invasions were observed in two cases (22.2%) in the ESD group and in one case (2.4%) in the EMR group (*P* = 0.080) (Table 2).

The mean tumor size of a complete resection was 9.6 (2-32) mm, and the size for an incomplete resection was 12.4 (3-20) mm (*P* = 0.011). The mean tumor size of lymphovascular invasion cases was larger than that of the no lymphovascular invasion group, however, there was no significant difference (*P* = 0.416) (Table 3). All cases of with a lymphovascular invasion tumor underwent an additional operation, while other incomplete resection cases were followed up by observation (Figure 1). There were no complications after the endoscopic treatment procedures.

### Follow-up

Of the 50 patients who underwent endoscopic treatment, five patients (10.0%) were lost to follow-up, and 45 patients (90%) were included in the follow-up. The median follow-up duration was 46 (13-60) mo. No evidence of tumor recurrence was found upon endoscopic and histological examinations in both groups. There was also no evidence of recurrence during follow-up imaging studies. In addition, no mortality was reported in either the complete resection group or the incomplete resection group during the follow-up duration. If 5 years was used as a cut-off point, 20 patients showed a disease-free state during this period.

## DISCUSSION

Carcinoids were first described by Oberndorfer in 1907 to describe a group of tumors of the gastrointestinal tract that had a relatively indolent course and were con-

**Table 3 Analysis of resected tumors**

	Tumor size	P value
Complete resection (range, mm)		
Yes (n = 40)	9.6 ± 6.3	0.011
No (n = 10)	12.4 ± 6.1	
Lymphovascular invasion (range, mm)		
Yes (n = 3)	16.3 ± 4.2	0.416
No (n = 47)	9.8 ± 6.2	

sidered to be intermediate between adenomas and carcinomas in terms of malignancy potential. Currently, these tumors are also known by the modern term of gastric NETs, which include a subset of tumors demonstrating features of neuroendocrine differentiation<sup>[15]</sup>. Surgery has been the most common treatment of gastric NETs; however, these tumors often receive suboptimal management, and some patients still undergo inappropriate surgery. As the diagnosis of gastric NETs is increasing with the widespread use of screening diagnostic endoscopy, treatment using the endoscopic method is becoming a matter of concern. In type 1 gastric NETs, endoscopic polypectomy or endoscopic mucosal resection is small (< 1 cm) and few (< 3-5 cm) in number<sup>[16]</sup> because the clinical behavior of these tumors is usually indolent. Most are grade 1 tumors with TNM stage I disease and no mortality during prolonged follow-up<sup>[17]</sup>. Type 3 gastric NETs represent 15%-25% of NETs and are not related to hypergastrinemia and ECL hyperplasia. The lesions are typically solitary, larger than 1-2 cm, ulcerated, and deeply invasive. The lesions are usually located in the gastric fundus and body, but may also occur in the antrum; they are also more frequent in males<sup>[6,18-20]</sup> and are characterized by a far more aggressive course. Type 3 gastric NETs present with lymph node and distant metastases in more than 50% of cases. Therefore, partial or total gastrectomy with local lymph node resection is considered an acceptable treatment<sup>[21,22]</sup> in the absence of visceral metastases. Additionally, systemic chemotherapy is also considered appropriate if surgery is not feasible, even if, thus far, the results are not very encouraging<sup>[23]</sup>. Only small (< 10 mm), well differentiated (G1) type 3 gastric NETs may be treated non-operatively by endoscopic resection. Because of the generally favorable tumor biology, surgery and/or local ablation should be considered even in metastatic gastric NETs<sup>[3]</sup>. Recently, Saund *et al*<sup>[24]</sup> reported that tumor size and depth can predict lymph node metastasis for gastric NETs and that endoscopic resection may be appropriate for intraepithelial (IE) tumors <2 cm and perhaps tumors < 1 cm invading into the lamina propria or submucosa. In our present study, complete pathological resections were achieved in 80.4% of patients (85.4% in the EMR group *vs* 66.7% in the ESD group). Better results for the pathological complete resection rate for treatment have usually been reported with the ESD technique. However, in the current study, the EMR group showed a more preferable complete resection rate compared with the ESD group. We pre-

sumed that tumor size is a contributing factor. Based on analysis of resected tumor size, the mean tumor size of the ESD group was larger than that of the EMR group ( $P = 0.055$ ), and the pathologically complete resection ratio showed no significant difference in both modality groups ( $P = 0.249$ ). Even in cases with tumor sizes greater than 10 mm (14 cases), which were confined to the submucosal layer and no lymphovascular invasion, endoscopic treatment showed no recurrence during the follow-up duration. Considering these factors, the ESD technique was useful for large type 3 gastric NETs. The long-term results of the endoscopic treatment only group ( $n = 43$ ) showed no recurrence or mortality. Therefore, we could conclude that endoscopic treatment was suitable for tumors measuring approximately 20 mm or smaller in size, with no lymph node or distant metastasis and limited to the submucosal layer of type 3 gastric NETs, similar to endoscopic treatment guidelines applied to other gastrointestinal NETs.

Our study has some limitations. First, this study is a retrospective analysis of clinical records. However, the data are believed to be reliable because all patients with type 3 gastric NETs treated using the endoscopic method at 10 institutions between January 1996 and September 2011 were included. The second limitation is that this study has a possible selection bias because it was not randomized. However, we consider the selection bias to be minimal because the patient characteristics and the median tumor sizes of patients with type 3 gastric NETs were not different. Third, the outcome of the endoscopic resection and selection of methods for endoscopic resection were different for each institution. However, each operator had sufficient skill to perform the endoscopic procedure, and the modality of endoscopic treatment was generally accepted for the treatment of gastric NETs. The final limitation is that we enrolled patients according to the WHO 2000 system for NET classification, due to retrospective study design. Therefore, we could not evaluate tumor histology on the basis of proliferative activity (Ki-67 index, mitotic rate) in which gastric NETs are graded as G1, G2, or G3.

In a conclusion, if the tumor is confined in the submucosal layer, there is no evidence of lymphovascular invasion, and the tumor size is smaller than 2 cm, endoscopic treatment could be applied for the initial treatment of type 3 gastric NETs.

## COMMENTS

### Background

Lots of controversies still exist about the optimal treatment of gastric neuroendocrine tumors (NETs). Type 3 gastric NETs are known as more aggressive disease course compared with type 1 gastric NETs. So, management of type 3 gastric NETs are comparable to that used for gastric adenocarcinomas, which includes partial or total gastrectomy with extended lymph node resection. However, in the case of small sized tumor, endoscopic resection is applied for initial treatment, nowadays.

### Research frontiers

To evaluate of the long-term results and to prove the usefulness of endoscopic resection in type 3 gastric NETs.

## Innovations and breakthroughs

Endoscopic treatment was suitable for tumors measuring approximately 20 mm or smaller in size, with no lymph node or distant metastasis and limited to the submucosal layer of type 3 gastric NETs.

## Applications

This present study suggest that the tumor size, the depth of invasion and evidence of lymphovascular invasion must be considered before performing endoscopic treatment for type 3 gastric NETs.

## Peer review

This study described the efficacy of endoscopic resection for the type 3 gastric NETs which size is less than 2 cm, confined submucosal layer, and no evidence of lymphovascular invasion.

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## Pattern and distribution of colonic diverticulosis: Analysis of 2877 barium enemas in Thailand

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### Abstract

**AIM:** To determine the pattern and distribution of colonic diverticulosis in Thai adults.

**METHODS:** A review of the computerized radiology database for double contrast barium enema (DCBE) in Thai adults was performed at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Incomplete studies and DCBE examinations performed in non-Thai individuals were excluded. The pattern and distribution of colonic diverticulosis detected during DCBE studies from June 2009 to October 2011 were determined. The occurrence of solitary cecal diverticulum, rectal diverticulum and giant diverticulum were reported. Factors influencing the presence of colonic diverticulosis were evaluated.

**RESULTS:** A total of 2877 suitable DCBE examinations were retrospectively reviewed. The mean age of patients was  $59.8 \pm 14.7$  years. Of these patients,

1778 (61.8%) were female and 700 (24.3%) were asymptomatic. Colonic diverticulosis was identified in 820 patients (28.5%). Right-sided diverticulosis (641 cases; 22.3%) was more frequently reported than left-sided diverticulosis (383 cases; 13.3%). Pancolonic diverticulosis was found in 98 cases (3.4%). The occurrence of solitary cecal diverticulum, rectal diverticulum and giant diverticulum were 1.5% (42 cases), 0.4% (12 cases), and 0.03% (1 case), respectively. There was no significant difference in the overall occurrence of colonic diverticulosis between male and female patients (28.3% vs 28.6%,  $P = 0.85$ ). DCBE examinations performed in patients with some gastrointestinal symptoms revealed the frequent occurrence of colonic diverticulosis compared with those performed in asymptomatic individuals (29.5% vs 25.3%,  $P = 0.03$ ). Change in bowel habit was strongly associated with the presence of diverticulosis (a relative risk of 1.39;  $P = 0.005$ ). The presence of diverticulosis was not correlated with age in symptomatic patients or asymptomatic individuals ( $P > 0.05$ ).

**CONCLUSION:** Colonic diverticulosis was identified in 28.5% of DCBE examinations in Thai adults. There was no association between the presence of diverticulosis and gender or age.

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**Key words:** Colonic diverticulosis; Diverticular disease; Barium enema; Pattern; Thailand; Cecal diverticulum; Rectal diverticulum; Giant diverticulum

**Core tip:** Based on this study in the largest university hospital in Thailand, colonic diverticulosis was identified in 28.5% of double contrast barium enemas performed in Thai adults. The incidence of colonic diverticulosis in the present study was markedly higher than that previously reported from hospital-based data of colonic diverticulosis in Thailand in 1980. This study also demonstrated that there was no significant association be-

tween the presence of diverticulosis and gender or age. However, colonic diverticulosis was more commonly reported in patients with some gastrointestinal symptoms, especially those with a change in bowel habit.

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## INTRODUCTION

Colonic diverticulosis is a common gastrointestinal condition in which the large intestine contains outpouchings of the mucosa and submucosa through a weak area of the colon<sup>[1]</sup>. However, the actual prevalence of colonic diverticulosis is difficult to determine because most people with colonic diverticula are asymptomatic<sup>[2]</sup>. Double contrast barium enema (DCBE) is regarded as the investigation of choice for demonstrating the presence and extent of colonic diverticulosis<sup>[3,4]</sup>. It is evident that the prevalence and pattern of colonic diverticulosis differ among ethnic groups and lifestyles<sup>[5,6]</sup>; left-sided diverticulosis is most common in Western and developed countries, while right-sided diverticulosis is more prevalent in Asian and developing countries<sup>[4,7,8]</sup>.

Although some data on colonic diverticulosis from Asian countries are available<sup>[6,9,10]</sup>, information on colonic diverticulosis in the region of Southeast Asia is limited and outdated<sup>[11]</sup>. As the characteristics of colonic diverticulosis have changed with time<sup>[12,13]</sup>, this study aimed to determine the pattern and distribution of colonic diverticulosis in Thai adults in recent years.

## MATERIALS AND METHODS

After obtaining approval from our Institutional Review Board (SIRB 634/2554), a review of the computerized radiology database for DCBE in Thai adults (defined as individuals aged  $\geq 18$  years) was performed at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. All findings of colorectal lesions detected at DCBE from June 2009 to October 2011 were analyzed. Incomplete studies, e.g. patients who were unable to hold barium or DCBE performed in patients with colonic obstruction, were excluded. Barium studies in non-Thai individuals were also excluded. Written informed consent was given by all patients before undergoing fluoroscopic DCBE. The detailed techniques and interpretation of standard fluoroscopic DCBE performed in our institute were previously reported<sup>[14]</sup>. Briefly, DCBE demonstrates a diverticulum as a barium-filled outpouching of the colon which is joined to the colonic wall by a neck. The DCBE findings were interpreted and reported by a staff gastrointestinal radiologist.

Patients' characteristics, indication for DCBE, and anatomical distribution of colonic diverticula were analyzed. In this study, the colon was divided into 3 parts: the right-sided colon (the cecum, the ascending colon, and the hepatic flexure of the colon), the transverse colon, and the left-sided colon (the splenic flexure of the colon, the descending colon, the sigmoid colon, and the rectum). Right colonic diverticulosis was defined as a diverticulum, or diverticula, detected on DCBE in the right-sided colon regardless of the involvement of the remaining colon. Left colonic diverticulosis was defined as a diverticulum, or diverticula, detected on DCBE in the left-sided colon regardless of the involvement of the remaining colon. The presence of diverticula in all three colonic segments was defined as pancolonic diverticulosis. Of note, a rectal diverticulum was defined as a diverticulum found below the imaginary line between the sacral promontory and the pubic symphysis on the lateral pelvic view of DCBE. A giant diverticulum was defined as a diverticulum demonstrated on DCBE with a diameter of  $\geq 4$  cm.

## Statistical analysis

All data were prepared and compiled using the Statistical Package for the Social Sciences program version 11.3 for Windows (SPSS Inc, Chicago, IL, United States). The prevalence and distribution of colonic diverticulosis detected at DCBE were analyzed with 95%CI analysis for Windows (Statistics with Confidence, 2nd Edition, BMJ Books, London 2000). The Mann-Whitney *U* test was used to compare the prevalence of diverticulosis between gender, and between symptomatic patients and asymptomatic individuals. Of note, asymptomatic individuals were defined as those without any gastrointestinal tract symptoms. The correlation between age and the presence of colonic diverticular disease was analyzed using a regression analysis. A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

A total of 2877 suitable DCBE examinations were retrospectively reviewed. The mean age of patients was  $59.8 \pm 14.7$  years (range 18-100 years). Of these patients, 1778 (61.8%) were female and 700 (24.3%) were asymptomatic. Colonic diverticulosis was identified on DCBE in 820 patients (28.5%). Right-sided diverticulosis (641 cases; 22.3%) was more frequently found than left-sided diverticulosis (383 cases; 13.3%). Pancolonic diverticulosis and solitary cecal diverticulum were found in 98 cases (3.4%) and 42 cases (1.5%), respectively (Table 1). Rectal diverticulum was seen in 12 cases (0.4%), and it was exclusively associated with the presence of sigmoid diverticulosis. A giant sigmoid diverticulum was demonstrated on DCBE in one case (0.03%). Figure 1 shows the distribution of diverticulosis stratified by colonic segment. Besides colonic diverticula, other major findings included 25 advanced adenomas (0.87%), 76 colorectal cancers (2.64%; 18 in the right-sided colon, 28 in the left-

**Table 1** Percentage and distribution of colonic diverticulosis by location (from total number of 2877 double contrast barium enemas studied)

Location	No. of cases	Percentage of total 820 colonic diverticulosis	Percentage of total 2877 DCBEs studied (95%CI)
Right-sided only <sup>1</sup>	383	46.7	13.3 (12.1-14.6)
Left-sided only	153	18.7	5.3 (4.6-6.2)
Transverse only	10	1.2	0.3 (0.2-0.6)
Extended right-sided (right + transverse)	44	5.4	1.5 (1.1-2.0)
Extended left-sided (left + transverse)	16	2.0	0.6 (0.3-0.9)
Bilateral (right + left)	116	14.1	4.0 (3.4-4.8)
Pancolonic (right + transverse + left)	98	12.0	3.4 (2.8-4.1)
Total	820	100	28.5 (26.9-30.2)
Right colonic diverticulosis	641	78.2	22.3 (20.8-23.8)
Left colonic diverticulosis	383	46.7	13.3 (12.1-14.6)
Transverse colonic diverticulosis	168	20.5	5.8 (5.0-6.8)

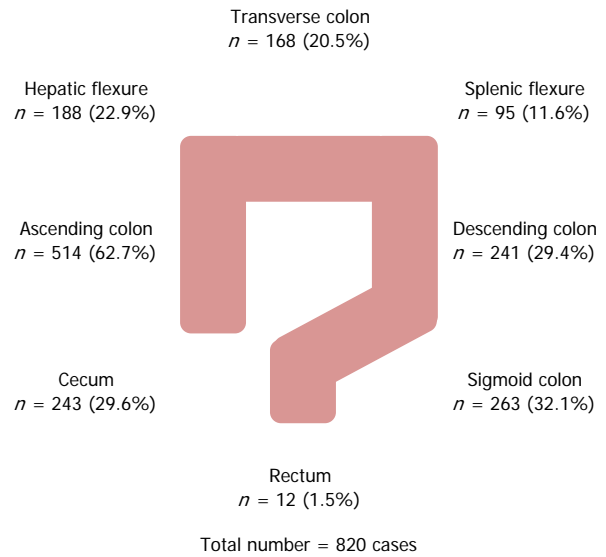
<sup>1</sup>Right-sided only diverticulosis included 42 cases of solitary cecal diverticulum. DCBE: Double contrast barium enema.

sided colon and 30 in the rectum), and 4 ileocecal Crohn's disease (0.14%).

There was no significant difference in the occurrence of colonic diverticulosis between male and female patients (28.3% *vs* 28.6%,  $P = 0.85$ ). However, DCBE examinations performed in patients with some gastrointestinal symptoms revealed the frequent occurrence of colonic diverticulosis compared with those performed in asymptomatic individuals (29.5% *vs* 25.3%;  $P = 0.03$ ). Change in bowel habit was strongly associated with the presence of diverticulosis (RR = 1.39, 95%CI: 1.14-1.70,  $P = 0.005$ ), whereas patients with abdominal pain, constipation and bleeding per rectum had a non-significant increased risk for colonic diverticulosis. The presence of diverticulosis was not significantly correlated with age in symptomatic patients ( $P = 0.62$ ) or asymptomatic persons ( $P = 0.52$ ) (Figure 2).

## DISCUSSION

In this study, colonic diverticulosis was identified in nearly 30% of DCBE examinations performed in Thai adults. Right-sided diverticulosis was found more frequently than left-sided diverticulosis. Our findings of colonic diverticulosis are consistent with other observations; in which right-sided colonic diverticulosis is most commonly involved in Asians, whereas sigmoid diverticulosis predominates in Western populations<sup>[6,7,15]</sup>. Compared with a previous hospital-based study of colonic diverticulosis in Bangkok in the 1980s<sup>[11]</sup>, the present study revealed a markedly higher rate of this condition, but a similar proportion of disease in relatively young individuals. It is difficult to explain why there is a relatively high frequency of colonic diverticulosis in young Thai adults. It is possible that, apart from some differences in dietary intake

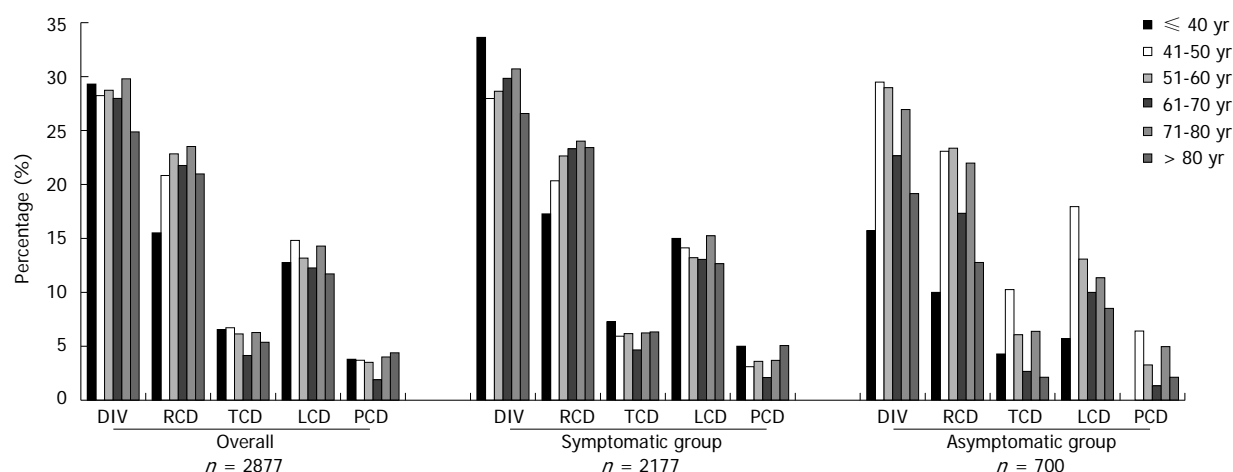


**Figure 1** Distribution of colonic diverticulosis stratified by colonic segment (total number of colonic diverticulosis = 820 cases).

and lifestyle, racial and genetic predisposition could play an important role in the development of colonic diverticulosis<sup>[16]</sup>. Apparently, genetic influences on the development of diverticulosis in Asian populations have a stronger impact than those in Western populations, especially for right-sided colonic diverticulosis<sup>[17]</sup>.

Moreover, we found no significant difference in the rate of colonic diverticulosis detected by DCBE between genders, which is consistent with several recent reviews of the literature<sup>[8,17,18]</sup>. However, there have been a few reports of an increased risk of colonic diverticulosis in males<sup>[19,20]</sup>. In addition, we did not identify a significant correlation between the presence of diverticulosis and age. Notably, the frequency of pancolonic diverticulosis in our study was 3.4%, which was fairly constant among the different age groups. In contrast to these findings, many authors have repeatedly reported that the prevalence of diverticulosis increases with age<sup>[4,21,22]</sup>. An interesting study by Takano *et al*<sup>[13]</sup> also showed that diverticulosis progressed with time from the proximal colon to the distal colon. Although the prevalence and extent of colonic diverticulosis is largely age-dependent, its widespread appearance in the Asian population could be as early as adolescence<sup>[20]</sup> with peak prevalence at the age of 50-60 years<sup>[10]</sup>. This could, in part, explain our findings of a relatively high rate of colonic diverticulosis in fairly young age groups; therefore, we did not identify a significant increment in colonic diverticulosis in advanced age groups.

With regard to cecal diverticulosis which involves multiple lesions, we found 42 cases of solitary cecal diverticulum; accounting for 1.5% of all DCBEs studied. Solitary cecal diverticulum is a fairly rare and asymptomatic lesion unless it becomes hemorrhagic or inflamed (mimicking acute appendicitis). Its incidence in Asian populations seems higher than that in Western popula-



**Figure 2** Pattern and distribution of colonic diverticulosis between asymptomatic individuals and symptomatic individuals stratified by age group. DIV: Diverticulosis; RCD: Right-sided colonic diverticulosis; TCD: Transverse colonic diverticulosis; LCD: Left-sided colonic diverticulosis; PCD: Pancolonic diverticulosis.

tions<sup>[10,23]</sup>. We also identified 12 cases (0.4%) of rectal diverticulum which was exclusively associated with the presence of sigmoid diverticulosis. The true incidence and pathogenesis of rectal diverticulum remain unknown as it is rarely reported<sup>[24]</sup>. As such lesions were present together with sigmoid colon diverticula, rectal and sigmoid diverticulosis may share the same pathogenesis.

More interestingly, we found a single 5-cm diverticulum in the sigmoid colon in a 51-year-old healthy male. The giant diverticulum was first described in 1946, and to date, fewer than 200 cases have been reported in the literature<sup>[25,26]</sup>. It is mainly found in the sigmoid colon, and can be divided into 3 distinct histological types: true diverticulum, false diverticulum, and pseudo-diverticulum (scar tissue without any colonic wall layer)<sup>[27]</sup>. Management of a giant diverticulum depends on the patient's symptoms and underlying disease. Diverticulectomy or segment resection of the affected colon is the favored choice of treatment in symptomatic patients.

Lastly, we demonstrated that the DCBE examinations performed in patients with some gastrointestinal symptoms (*e.g.*, bowel habit change, constipation, abdominal pain and hematochezia) revealed a higher prevalence of colonic diverticulosis than those performed in asymptomatic individuals. It is obvious that many patients with colonic diverticulosis experience chronic gastrointestinal symptoms at some time in their life<sup>[28]</sup>. However, it is difficult to know whether colonic diverticulosis is a cause or a result of such symptoms.

In conclusion, the present study examined the frequency and distribution of colonic diverticulosis from a relatively large number of fluoroscopic DCBEs performed in Thai adults. Colonic diverticulosis was identified in nearly 30% of DCBE examinations. Right-sided diverticulosis was more common than left-sided diverticulosis. There was no association between the presence of diverticulosis and gender or age. Colonic diverticulosis was more commonly reported in patients with some gastrointestinal symptoms, especially those with change in bowel habit.

## COMMENTS

### Background

Colonic diverticulosis is a common gastrointestinal condition. The prevalence and distribution of colonic diverticulosis differ among ethnic groups and lifestyles; left-sided diverticulosis is more common in Western and developed countries, while right-sided diverticulosis is more prevalent in Asian and developing countries. Moreover, the characteristics of colonic diverticulosis have changed over time.

### Research frontiers

Although some data on colonic diverticulosis from Asian countries are available, the information on colonic diverticulosis in Southeast Asia is limited and some are outdated. Double contrast barium enema (DCBE) is a reliable investigation tool for demonstrating the presence and extent of colonic diverticulosis.

### Innovations and breakthroughs

This paper demonstrates that colonic diverticulosis was identified in 28.5% of DCBEs performed in Thai adults. Right-sided diverticulosis was more common than left-sided diverticulosis. Pancolonic diverticulosis was found in 3.4% of patients. There was no association between the presence of diverticulosis and gender or age, however, DCBE examinations performed in patients with some gastrointestinal symptoms revealed the frequent occurrence of colonic diverticulosis compared with those performed in asymptomatic individuals. Change in bowel habit was strongly associated with the presence of diverticulosis.

### Applications

The study results show that the occurrence of colonic diverticulosis in Thailand, a developing country in Asia, is surprisingly prominent and markedly higher than that previously reported from a hospital survey in Bangkok approximately 30 years ago. In addition, the rate of colonic diverticulosis in the present study was equal in the different age groups *i.e.*, its widespread appearance could be seen as early as adolescence. These findings may urge physicians to include or consider colonic diverticular disease as one of the causes of gastrointestinal symptoms in every patient, including young individuals.

### Terminology

Colonic diverticulosis is usually described as the presence of outpouching(s) of the mucosa and submucosa through a weak area of the large intestine. When a diverticulum (or multiple diverticula) becomes symptomatic, infected or bleeding, this gastrointestinal condition may be called "colonic diverticular disease".

### Peer review

This is a good descriptive study in which authors analyze the pattern and distribution of colonic diverticulosis from a third world country, where the frequency of such a condition is expected to be low. In fact, this study showed an unexpectedly high number of colonic diverticulosis in Thai adults. The distribution of colonic diverticulosis is brilliantly shown in great details. The results are also interesting and suggest that colonic diverticulosis can be seen in adolescence as well as its occurrence is not age-dependent. Some findings are different from those shown in Western populations.



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## Intestinal stem cell marker LGR5 expression during gastric carcinogenesis

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cine-rich repeat-containing G protein-coupled receptor 5 (LGR5) in gastric cancer tissues and its significance related to tumor growth and spread.

**METHODS:** Formalin-fixed biopsy specimens of intestinal metaplasia ( $n = 90$ ), dysplasia ( $n = 53$ ), gastric adenocarcinoma ( $n = 180$ ), metastases in lymph nodes and the liver ( $n = 15$ ), and lesion-adjacent normal gastric mucosa (controls;  $n = 145$ ) were obtained for analysis from the Peking University Cancer Hospital's Department of Pathology and Gastrointestinal Surgery tissue archives (January 2003 to December 2011). The biopsied patients' demographic and clinicopathologic data were retrieved from the hospital's medical records database. Each specimen was subjected to histopathological typing to classify the tumor node metastasis (TNM) stage and to immunohistochemistry staining to detect the expression of the cancer stem cell marker LGR5. The intergroup differences in LGR5 expression were assessed by Spearman's rank correlation analysis, and the relationship between LGR5 expression level and the patients' clinicopathological characteristics was evaluated by the  $\chi^2$  test or Fisher's exact test.

**RESULTS:** Significantly more gastric cancer tissues showed LGR5<sup>+</sup> staining than normal control tissues (all  $P < 0.01$ ), with immunoreactivity detected in 72.2% (65/90) and 50.9% (27/53) of intestinal metaplasia and dysplasia specimens, respectively, 52.8% (95/180) of gastric adenocarcinoma specimens, and 73.3% (11/15) of metastasis specimens, but 26.9% (39/145) of lesion-adjacent normal gastric mucosa specimens. Comparison of the intensity of LGR5<sup>+</sup> staining showed an increasing trend that generally followed increasing dedifferentiation and tumor spread (normal tissue < dysplasia, < gastric adenocarcinoma < metastasis; all  $P < 0.001$ ), with the exception of expression level detected in intestinal metaplasia which was higher than that in normal gastric tissues ( $P < 0.001$ ). Moreover, gastric cancer-associated enhanced expression of LGR5 was

### Abstract

**AIM:** To investigate the differential expression of leu-

found to be significantly associated with age, tumor differentiation, Lauren type and TNM stage (I + II vs III + IV) (all  $P < 0.05$ ), but not with sex, tumor site, location, size, histology, lymphovascular invasion, depth of invasion, lymph node metastasis or distant metastasis. Patients with LGR5<sup>+</sup> gastric cancer specimens and without signs of metastasis from the original biopsy experienced more frequent rates of recurrence or metastasis during follow-up than patients with LGR5<sup>-</sup> specimens ( $P < 0.05$ ).

**CONCLUSION:** Enhanced LGR5 is related to progressive dedifferentiation and metastasis of gastric cancer, indicating the potential of this receptor as an early diagnostic and prognostic biomarker.

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**Key words:** Leucine-rich repeat-containing G protein-coupled receptor 5; Cancer stem cell; Gastric cancer; Intestinal metaplasia; Tumorigenesis

**Core tip:** This is the first study to evaluate the expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a putative cancer stem cell marker, in progressive stages of gastric carcinogenesis. The observation of increasing LGR5 expression in human gastric cancer lesions, following the loss of differentiation (from dysplastic to gastric cancer cases) and risk of spread (metastatic cases), suggests that this receptor may represent an important biomarker for early detection of patients at higher risk for gastric tumorigenesis. The observed distinctive expression pattern of LGR5 in intestinal metaplasia suggests that these cells may represent a precancerous condition but not carcinoma precursors.

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## INTRODUCTION

Gastric cancer (GC) is one of the most common cancers worldwide, yet the majority of GC-related deaths occur in less developed countries, including China and other Asian nations<sup>[1,2]</sup>. Studies to elucidate the tumorigenic processes underlying GC development have revealed a multistep sequential process involving normal gastric tissue progression to chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and carcinoma, with or without metastatic potential<sup>[3]</sup>. This model supports the possibility of a stepwise accumulation of genetic alterations affecting expression of key molecules, possibly having direct or

indirect (*i.e.*, signaling pathways) functional effects on cell growth and movement.

The stem cell origin hypothesis of carcinomas has gained much research attention in the recent decade. Cancer stem cells (CSCs), which express a distinctive profile of cell type-specific surface markers<sup>[4]</sup>, have been detected in a broad range of clinical cancer specimens, including hematological malignancies and solid tumors of the breast, lung, ovary, liver, prostate, pancreas, skin, brain and colon<sup>[5-13]</sup>. However, few studies to date have investigated the presence of CSCs in GC lesions, and their role in GC tumorigenesis remains largely unknown.

The leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5, also known as GPR49) has been proposed as a marker of GC-related stem cells. Under normal conditions, LGR5 is expressed primarily on intestinal stem cells, where it functions as a transducer of Wnt signaling<sup>[14,15]</sup>. Murine-based investigations to uncover the role of LGR5 in cancer development and progression have also demonstrated its expression on rare, scattered cells in the eye, brain, stomach, mammary gland and reproductive organs<sup>[16]</sup> and showed that LGR5<sup>+</sup> stem cells were much more effective in producing tumorigenesis than more differentiated (LGR5<sup>-</sup>) cells<sup>[17]</sup>. In humans, LGR5<sup>+</sup> cells have been detected in both the population of crypt stem cells (precursor cells) and gastric mucosal lesions that progressed to cancer<sup>[18]</sup>.

A functional study of LGR5-expressing cells and their age-related distribution using a mouse model revealed that its expression was localized to the base of prospective corpus and pyloric glands in neonatal stomach but predominantly restricted to the base of mature pyloric glands in adult stomach, and demonstrated that a single LGR5<sup>+</sup> cell could efficiently generate long-lived organoids resembling mature pyloric epithelium *in vitro*<sup>[19]</sup>. While the collective findings have increased interest in developing LGR5 as a universal epithelial CSC marker for clinical use<sup>[18]</sup>, the loss of restriction to the stem cell niche is considered an early event in the premalignant transformation of stem cells and suggests that this protein may also be a key contributor to carcinogenesis.

Although many previous studies have investigated the association of perturbed LGR5 expression with tumorigenesis, very few have reported on the differential expression of LGR5 and its role in the multistep sequential process of GC development. Therefore, the present study analyzed LGR5 expression in human clinical specimens of gastric tissues from the non-cancerous condition through gastric adenocarcinoma and in GC-related lymph nodes and liver metastases, and evaluated the relationship between differential LGR5 expression and clinicopathological features. The findings from this study will provide novel insights into the carcinogenic process of GC from the perspective of the stem cell origin hypothesis.

## MATERIALS AND METHODS

### Patients and tissue samples

Formalin-fixed/paraffin-embedded specimens of in-



testinal metaplasia ( $n = 90$ ), dysplasia ( $n = 53$ ), gastric adenocarcinoma ( $n = 180$ ), metastases in lymph nodes and the liver ( $n = 15$ ), and lesion-adjacent normal gastric mucosa (controls;  $n = 145$ ) were obtained for analysis from the Peking University Cancer Hospital's Department of Pathology and Gastrointestinal Surgery tissue archives (January 2003 to December 2011). All specimens had been obtained during endoscopic biopsy or surgical resection. Each specimen was analyzed by routine histopathological analysis and was classified according to the pathological criteria published by the World Health Organization (4<sup>th</sup> edition) and the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer Staging Manual (7<sup>th</sup> edition) and the Japanese Gastric Cancer Association Guidelines (3<sup>rd</sup> edition).

The biopsied patients' demographic and clinicopathologic characteristics (during the clinical management and follow-up periods) were retrieved from the hospital's electronic records database. If a patient had no record of death but lacked follow-up data, the patient's general practitioner was contacted to obtain the information. None of the GC patients had synchronous cancers or previous gastrointestinal diseases, nor had undergone abdominal surgery, chemotherapy or radiotherapy prior to specimen collection.

This study was performed with pre-approval from the Ethics Committee of Peking University Cancer Hospital. Informed consent allowing for investigative use of tissue samples had been provided by each patient.

### Immunohistochemical analysis

Specimen sections (4  $\mu$ m thickness) were mounted on poly-L-lysine coated slides, deparaffinized in xylene, and rehydrated in a descending ethanol-to-water gradient series. Endogenous peroxidase was blocked by exposure to 3% H<sub>2</sub>O<sub>2</sub> for 10 min, followed by antigen retrieval via pressurized heating in EDTA buffer (Zhongshan Biotechnology Inc., Beijing, China) for 5 min. After cooling to room temperature, non-specific sites were blocked by exposure to 10% goat blood serum. LGR5 immunodetection was carried out by incubating with purified rabbit polyclonal antibody (AP2745d; Abgent, San Diego, CA, United States), followed by two-step diaminobenzidine visualization (GK500705; Dako, Glostrup, Denmark).

The immunostained sections were counterstained with hematoxylin for 40 s, rinsed in water, dehydrated in an ascending water-to-ethanol gradient series followed by clearance with xylene, and permanently cover-slipped. Negative controls were created using the same procedure but without addition of primary antibody.

### Evaluation of immunostaining

The processed immunostained sections were examined by light microscopy. Two experienced pathologists (Sun Y and Dong B), working independently and blinded to the corresponding clinical data, evaluated each sample to calculate and score the percentage of LGR5<sup>+</sup> cells [none (negative, -): 0%, 1%-25%: 1, 25%-50%: 2, and > 50%: 3] and to score the intensity of cytoplasmic staining (no

staining: 0, mild: 1, moderate: 2, and strong: 3; with the highest intensity score being assigned when > 10% of cells stained with that intensity). Adding the percentage and intensity scores provided a composite expression score (0-6), which was defined as: weakly positive (+): 1-2, moderately positive (++) : 3-4, and strongly positive (+++) : 5-6. For statistical analysis, a composite score of 0 was classified as negative and 1-6 as positive, with  $\leq 2$  ranked as low expression and  $\geq 3$  ranked as high expression.

### Statistical analysis

All statistical analyses were carried out using the SPSS software statistical package (version 20.0; SPSS Inc., Chicago, IL, United States). The differences in LGR5 expression between the gastric tissue types were analyzed by Spearman's rank correlation analysis. The relationships between LGR5 differential expression and clinicopathological characteristics were evaluated by the  $\chi^2$  test or Fisher's exact test. A two-sided  $P$ -value < 0.05 was considered statistically significant.

## RESULTS

### LGR5 expression and distribution in normal gastric mucosa

Immunostaining of LGR5 showed a predominant localization to the cytoplasm or on the cell membrane in normal gastric mucosa specimens. Morphologically, the LGR5<sup>+</sup> cells were localized to the mucous neck region at the base of the gastric crypts between the foveolae and glands (Figure 1A and B). The positive-staining percentages are presented in Table 1.

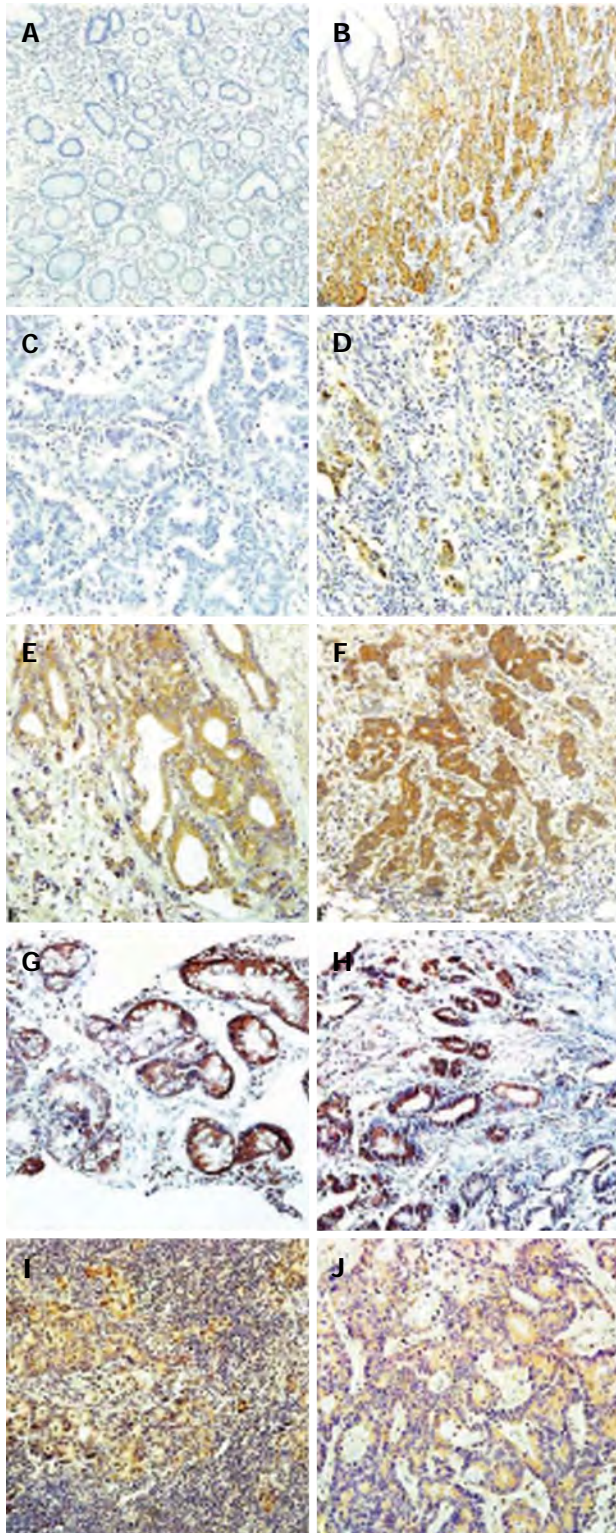
### Differential LGR5 expression in GC-related tissues during tumorigenesis

Immunodetection of LGR5 in GC-related tissues, progressing from non-neoplastic epithelia to gastric cancer and finally metastasis, showed an increasing trend in the number and intensity of LGR5<sup>+</sup> cells (all *vs* normal gastric mucosa specimens and *vs* the different GC-related tissues,  $P < 0.05$ ; Table 1). In addition, the significantly enhanced LGR5 expression in dysplasia specimens ( $P = 0.019$ ) was largely accounted for by the specimens with low grade dysplasia (roughly twice that of the high grade dysplasia specimens). The GC-related enhanced LGR5 expression was also greater in specimens from patients with lower clinical stage (TNM stages I + II > III + IV) and the majority of GC cases showed weak staining (with strong cytoplasmic or membranous immunodetection < moderate staining < weak staining < no staining; Figure 1C-J). Morphologically, the distribution of LGR5<sup>+</sup> cells was uneven and inhomogeneous in the GC-related specimens and occurred in cohesive patches of a variable number of tumor cells.

### Association of immunodetected LGR5 expression with clinicopathological features of GC patients

The patients' demographic and clinicopathologic features





**Figure 1** Immunodetected differential LGR5 expression in gastric tissues, following progression of tumorigenesis, and in distant metastases. Representative samples are shown from leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)<sup>+</sup> (A) and LGR5<sup>+</sup> (B) normal gastric normal tissues, LGR5<sup>+</sup> (C) and LGR5<sup>+</sup> (D-F) gastric cancer (GC) tissues with weak, moderate and strong expression, LGR5<sup>+</sup> gastric intestinal metaplasia and dysplasia tissues (G, H), and LGR5<sup>+</sup> lymph node and liver metastases (I, J). A, C-J: Magnification: × 200; B: Magnification: × 100.

are summarized in Table 2. There were more males than females (130 *vs* 50), but the percentage of LGR5<sup>+</sup> immu-

**Table 1** LGR5 immunostaining in gastric cancer-related gastric tissues and metastases *n* (%)

Pathological type	Total, <i>n</i>	LGR5 expression		<i>P</i> value
		Negative	Positive	
Normal tissue	145	106 (73.1)	39 (26.9)	0.000
Dysplasia grade	53	26 (49.1)	27 (50.9)	0.019
Low	25	8 (32.0)	17 (68.0)	
High	28	18 (64.3)	10 (35.7)	
TNM stage	180	85 (47.2)	95 (52.8)	
I - II	71	27 (38.0)	44 (62.0)	
III-IV	109	58 (53.2)	51 (46.8)	
Metastases	15	4 (26.7)	11 (73.3)	
Lymph node	5	1 (20.0)	4 (80.0)	
Liver	10	3 (30.0)	7 (70.0)	

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; TNM: Tumor, nodes, metastasis.

**Table 2** Association of immunodetected LGR5 expression with clinicopathological features of gastric cancer patients *n* (%)

Clinicopathological feature	LGR5 expression		<i>P</i> value
	Negative	Positive	
Sex			0.072
Male	56 (43.1)	74 (56.9)	
Female	29 (58.0)	21 (42.0)	
Age, yr			0.005
≤ 60	48 (58.5)	34 (41.5)	
> 60	37 (37.8)	61 (62.2)	
Location in stomach			0.657
Upper	15 (40.5)	22 (59.5)	
Mid	17 (47.2)	19 (52.8)	
Lower	45 (49.5)	46 (50.5)	
Lesion size in cm			0.612
≤ 4	33 (42.9)	44 (57.1)	
> 4	32 (47.1)	36 (52.9)	
Differentiation			0.006
Differentiated	31 (36.5)	54 (63.5)	
Undifferentiated	54 (56.8)	41 (43.2)	
Histological type			0.579
Adenocarcinoma	67 (46.2)	78 (53.8)	
Others	18 (51.4)	17 (48.6)	
Lauren type			0.035
Intestinal	48 (41.4)	68 (58.6)	
Diffuse/other	37 (57.8)	28 (42.2)	
Lymphovascular invasion			0.288
No	43 (43.9)	55 (56.1)	
Yes	42 (51.9)	39 (48.1)	
Depth			0.833
T1-T2	19 (48.7)	20 (51.3)	
T3-T4	66 (46.8)	75 (53.2)	
Lymph node metastasis			0.934
No	19 (47.5)	21 (52.5)	
Yes	65 (46.8)	74 (53.2)	
Metastasis			0.160
No	71 (45.2)	86 (54.8)	
Yes	14 (60.9)	9 (39.1)	
TNM			0.046
I - II	27 (38.0)	44 (62.0)	
III-IV	58 (53.2)	51 (46.8)	

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; TNM: Tumor, nodes, metastasis.

nodetection was similar between the two and sex was not found to be significantly correlated with LGR5 expression in the GC-related specimens. The overall patients

**Table 3** LGR5 expression in gastric cancer tissues of various differentiation *n* (%)

Tissue	LGR5 expression		<i>P</i> value
	Negative	Positive	
Intestinal metaplasia	25 (27.8)	65 (72.2)	0.000
Normal tissue	106 (73.1)	39 (26.9)	
Dysplasia with IM			0.004
Yes	3 (18.8)	13 (81.2)	
No	23 (62.2)	14 (37.8)	
Lauren type			0.035
Intestinal	48 (41.4)	68 (58.6)	
Diffuse/other	37 (57.8)	28 (42.2)	
Intestinal type GC			0.019
Metastasis or recurrence	6 (12.5)	21 (31.3)	
No metastasis or recurrence	42 (87.5)	46 (68.7)	

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; GC: Gastric cancer; IM: Intestinal metaplasia.

ranged in age from 22-87 years old (median: 62 years old), and age was found to be significantly associated with LGR5<sup>+</sup> immunodetection in GC-related specimens (*P* = 0.005). In addition, differentiation (*P* = 0.006), Lauren type [*P* = 0.035, with intestinal type having significantly more LGR5<sup>+</sup> cells than the diffuse/other types (58.6% *vs* 42.2%)] and TNM stage (I + II *vs* III + IV, *P* = 0.046) were also correlated significantly with LGR5<sup>+</sup> immunodetection, but tumor site, location, size, histology, lymphovascular invasion and depth of invasion were not.

Analysis of the follow-up data showed that GC patients without metastases at surgery but with LGR5<sup>+</sup> staining specimens experienced a higher rate of recurrence or metastasis than their counterparts with LGR5<sup>-</sup> staining specimens (87.35% *vs* 12.7%, *P* = 0.020). However, the presence of metastases at surgery was not correlated with LGR5<sup>+</sup> immunodetection (both *P* > 0.05; Table 2). The specimens from patients with intestinal type GC also showed a significantly higher rate of LGR5<sup>+</sup> immunodetection than those from patients with diffuse or mixed types GC (*P* = 0.035), and LGR5<sup>+</sup> immunodetection in intestinal type GC was associated with more frequent rates of recurrence or metastasis after surgery (*P* = 0.019; Table 3).

#### Association of LGR5 expression with transformation of intestinal metaplasia tissues

As shown in Table 3, intestinal metaplasia specimens showed a significantly higher rate of LGR5<sup>+</sup> immunodetection than normal gastric tissues (*P* = 0.000). Moreover, dysplasia specimens with intestinal metaplasia had a significantly higher rate of LGR5<sup>+</sup> immunodetection than those without (*P* = 0.004).

## DISCUSSION

Using a standard immunohistochemistry-based method, the differential expression pattern of the putative CSC marker LGR5 in progressively tumorigenic clinical specimens of GC was demonstrated. In particular, an increas-

ing trend was observed in LGR5<sup>+</sup> staining intensity that generally followed increasing dedifferentiation and tumor spread (normal tissue < dysplasia < gastric adenocarcinoma < metastasis).

The adenoma-carcinoma progression sequence is well known in colorectal cancer and esophageal adenocarcinoma, and is becoming more generally accepted as the likely mode of tumorigenesis in the gastrointestinal tract as well<sup>[20-23]</sup>. Recent findings from studies in mammalian (mouse) model systems and with human GC specimens have demonstrated that GC progenitor cells are derived from multipotent stem cells in the highly regenerative and proliferative regions of the stomach, including the isthmus and fundic gland-rich neck<sup>[24,25]</sup>. Indeed, the subpopulation of stem cells with high LGR5 expression were shown to have the capability to reconstitute crypt structures *in vitro*<sup>[26]</sup>, and LGR5 has been detected on progenitor cells in human gastric mucosa crypts<sup>[27,28]</sup>.

As stated in the Introduction, the multitude of signaling factors that comprise this multistep progression model of GC tumorigenesis also represent a plethora of targets for improved detection and treatment methods. The occurrence of gastric epithelial dysplasia is a well-characterized precursor event to GC, and is currently considered the most dependable marker for such cancer risk. A prospective longitudinal study of gastric epithelial dysplasia and development of GC indicated that high grade dysplasia is associated with rapid development of intestinal type GC<sup>[29]</sup>. This finding is in line with the current study's observation of similar LGR5<sup>+</sup> immunodetection rates in dysplasia and gastric carcinoma specimens (with a slightly higher rate in the latter), and higher rates in well to moderately differentiated intestinal type and lower-staged gastric cancers.

The dynamic undulation of immunodetected LGR5 expression observed in the low clinical stage (enhanced in I-II) to the high clinical stage (reduced in III-IV) to metastasis (again enhanced) agrees with a previously reported profile of LGR5 expression in tumorigenesis of endometrial, colorectal and ovarian carcinomas (with the high expression demonstrated during the initial stages, being down-regulated in fully developed tumors)<sup>[30,31]</sup>. Collectively, these findings support the hypothesized clonal selection model of putative stem cells leading to carcinogenesis<sup>[32]</sup>. In particular, the results from the current study suggest that overexpression of LGR5 may be an early event in tumorigenesis and that immunodetection of such protein is achieved with good reproducibility and tracks with differentiation of tumor specimens.

From a mechanistic perspective, the tumorigenic-related expression profile observed in the current study suggests the existence of a potential tumor promoter regulating LGR5. However, it is important to consider the unexpected observation of higher immunodetected LGR5 expression in low grade dysplasia than in high grade dysplasia; similar results were also reported from another study of esophageal dysplasia lesions<sup>[20]</sup>. A possible explanation of this result is the fact that the cur-



rent morphologic criteria for different grade dysplasias include a mix of architectural and cytologic features and do not consider functional characteristics<sup>[33]</sup>. Indeed, low grade dysplasia preserves some of the functions of intestinal metaplasia, which underlies the risk of misdiagnosis for these two conditions<sup>[34]</sup>. Previous studies have addressed this confusing issue, proposing that the increased amounts of high-intensity LGR5<sup>+</sup> cells that are observed in dysplasia may represent a stem cell population that is prone to becoming CSCs<sup>[35,36]</sup>.

Other intriguing findings from the current study are the higher amounts of LGR5<sup>+</sup> cells detected towards the crypt base or in the invasive tumor front during the development and progression of GC (although the change in differential expression did not reach statistical significance) and in metastases (both local and distant). Brablez *et al.*<sup>[37]</sup> hypothesized that tumor progression is mediated by two types of CSCs with distinct functions. The first was proposed as a stationary cancer stem (SCS) cell population, which would be present in the area for cell differentiation but which would not promote metastasis. The second was proposed as a migrating (or mobile) cancer stem (MCS) cell population, which may be derived from the SCS cells and located primarily at the invasive tumor front, and which would drive metastasis. Therefore, the observed shift in distribution of LGR5<sup>+</sup> cells towards the invasive tumor front that accompanied the development and metastasis of GC in the current study may be related to such MCS cells. This notion may also be in line with the current study's observation of GC patients with LGR5<sup>+</sup> intestinal type specimens being at higher risk of recurrence or metastasis after surgery.

Previous studies have demonstrated that Wnt signaling regulates stemness and organ development, as well as the process of epithelial to mesenchymal transition (EMT) that increases the metastatic potential of disseminated cancer cells<sup>[38,39]</sup>. In addition, EMT may also restart the growth and differentiation programs of stem cells at metastatic sites<sup>[37,40]</sup>. Studies of human colorectal cancer have demonstrated that aberrant Wnt signaling not only triggers early steps of intestinal carcinogenesis but also malignant tumor progression towards invasive carcinomas and metastasis<sup>[41-43]</sup>. Therefore, LGR5 (as a Wnt target and a stem cell marker) plays an important role in initiating tumor growth and driving distant metastasis. These functions of LGR5 may also explain the findings in the current study of LGR5<sup>+</sup> GC patients without evidence of metastases during the initial surgical treatment being at a greater risk of recurrence or metastasis.

Interestingly, the LGR5-immunodetected expression had higher intensity in gastric intestinal metaplasia, dysplasia with intestinal metaplasia, and intestinal type GC than in the normal tissues examined in the current study; all of these GC-related lesions have the potential to manifest intestinal type differentiation. Intestinal metaplasia has been shown to originate from stem cells of the isthmus, and the crypts possess multiple stem cells<sup>[44,45]</sup>. Although intestinal metaplasia is regularly detected in the antrum of patients with gastritis and duodenal ulcers

related to *Helicobacter pylori* infection, these patients very rarely develop gastric carcinoma<sup>[46]</sup>. Similarly, Tatematsu *et al.*<sup>[47]</sup> suggested that gastric/intestinal mixed type intestinal metaplasia might be the consequence of abnormal differentiation of stem cells that are capable of producing both gastric and intestinal types of cells.

Only the relatively rare type III intestinal metaplasia has been identified as a risk marker for the development of gastric carcinoma, being classified as "low grade dysplasia"<sup>[34]</sup>. The related findings in our study suggest that intestinal metaplasia may be a precancerous condition, but not a precursor for gastric carcinoma (possibly with the exception of some rare types). Thus, LGR5 may represent a unique and sensitive marker of intestinal stem cells and may be closely related to the intestinal type of GC.

In conclusion, the immunodetectable expression pattern of LGR5, a CSC-related gene, increasing from normal tissues to lesions of dysplasia, gastric carcinoma and finally metastases, suggests potential for this protein to serve as an important biomarker for early detection of patients at higher risk for gastric tumorigenesis. Furthermore, as an intestinal stem cell marker, differential LGR5 expression in conjunction with development of intestinal metaplasia may represent a precancerous condition, but not a carcinoma precursor.

## COMMENTS

### Background

Cancer stem cells (CSCs) may be the source of various carcinomas, including gastric cancer (GC), and are identifiable by clinically detectable profiles of cell type-specific surface markers. The leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a target of Wnt signaling, is primarily expressed on normal intestinal stem cells and has been suggested as a putative CSC marker (and contributor to GC tumorigenesis) according to its differential expression on crypt stem cells (precursor cells) and gastric lesions that progress to cancer. Accumulated evidence has suggested roles for LGR5 in both cancer development and progression. Recent studies have also indicated that LGR5 may be a potential marker of gastrointestinal stem cells in humans and that loss of restriction to the stem cell niche is likely an early event in the premalignant transformation of stem cells.

### Research frontiers

The differential protein expression of LGR5 in normal gastric tissue, intestinal metaplasia and dysplasia specimens, gastric carcinomas, and distant metastases was determined by immunohistochemistry to provide insights into its potential as a clinical marker for early GC detection. Furthermore, the differential LGR5 expression observed in conjunction with development of intestinal metaplasia suggests that this phenomenon represents a precancerous condition, but not a carcinoma precursor.

### Innovations and breakthroughs

An increasing trend in intensity of LGR5 expression was detected in GC-related tissues, following the well-recognized sequential development from normal tissue to dysplasia to gastric carcinoma and finally metastasis, with the exception of the intestinal metaplasia state. The differential expression of LGR5 detected in GC by immunohistochemistry appeared to be significantly associated with age, differentiation, Lauren type, and tumor node metastasis stage. The LGR5<sup>+</sup> cells detected in intestinal metaplasia specimens were more prevalent than those detected in normal gastric tissues, and the data indicated that intestinal metaplasia may manifest from differentiation of a population of abnormal stem cells with high expression of LGR5, but may not represent a carcinoma precursor. Collectively, these data indicate that LGR5 expression may serve as an important biomarker for early detection of patients at higher risk for gastric tumorigenesis, and may be a candidate target for future individualized therapeutic

strategies.

## Applications

The current poor prognosis of GC is largely associated with the low rate of early diagnosis. The findings from this study of human clinical samples of GC lend to a recommendation that LGR5 should be the focus of further studies to develop its potential as a biomarker for early detection of patients at higher risk for GC and as a manipulable intestinal stem cell marker target for improved management of GC cases.

## Terminology

The leucine-rich repeat-containing G protein-coupled receptor 5 is expressed primarily on intestinal stem cells, where it functions as a transducer of Wnt signaling. Cancer stem cells, which express a distinctive profile of cell type-specific surface markers, have been detected in a broad range of clinical cancer specimens and are the basis of the stem cell origin hypothesis of cancer. Gastric cancer development is a multistep sequential process involving normal gastric tissue progression to chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and carcinoma, with or without metastatic potential.

## Peer review

This study determined the GC-related expression profile of the putative CSC marker LGR5, using standard immunohistochemistry to detect expression in human clinical samples of normal gastric tissue, intestinal metaplasia, dysplasia, gastric carcinoma, and distant metastases. The observed increasing trend in differential LGR5 expression following progressive tumorigenesis to metastasis suggests that this protein may serve as an important biomarker for early detection of patients at higher risk for gastric tumorigenesis. The data also implicate a role for LGR5 as an intestinal stem cell marker and suggest that intestinal metaplasia may be a precancerous condition but not a carcinoma precursor. The study is well controlled and provides novel insights into this life-threatening disease.

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## Conservative treatment of early postoperative small bowel obstruction with obliterative peritonitis

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### Abstract

**AIM:** To investigate the effect of somatostatin and dexamethasone on early postoperative small bowel obstruction with obliterative peritonitis (EPSBO-OP).

**METHODS:** This prospective randomized study included 70 patients diagnosed with EPSBO-OP from June 2002 to January 2009. Patients were randomized into two groups: a control group received total parenteral nutrition and nasogastric (NG) tube feeding; and an intervention group received, in addition, somatostatin and dexamethasone treatment. The primary endpoints were time to resolution of bowel obstruction and length of hospital stay, and the secondary endpoints were daily NG output and NG feeding duration, treatment-related complications, postoperative obstruction relapse, and patient satisfaction.

**RESULTS:** Thirty-six patients were allocated to the intervention group and 34 to the control group. No patient needed to undergo surgery. Patients in the intervention group had an earlier resolution of bowel

obstruction ( $22.4 \pm 9.1$  vs  $29.9 \pm 10.1$  d,  $P = 0.002$ ). Lower daily NG output ( $583 \pm 208$  vs  $922 \pm 399$  mL/d,  $P < 0.001$ ), shorter duration of NG tube use ( $16.7 \pm 8.8$  vs  $27.7 \pm 9.9$  d,  $P < 0.001$ ), and shorter length of hospital stay ( $25.8$  vs  $34.9$  d,  $P = 0.001$ ) were observed in the intervention group. The rate of treatment-related complications ( $P = 0.770$ ) and relapse of obstruction ( $P = 0.357$ ) were comparable between the two groups. There were no significant differences in postoperative satisfaction at 1, 2 and 3 years between the two groups.

**CONCLUSION:** Somatostatin and dexamethasone for EPSBO-OP promote resolution of obstruction and shorten hospital stay, and are safe for symptom control without increasing obstruction relapse.

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**Key words:** Dexamethasone; Intestinal obstruction; Parenteral nutrition; Postoperative period; Somatostatin

**Core tip:** This prospective study revealed that somatostatin and dexamethasone, when used in combination, promoted the resolution of small bowel obstruction and shortened length of hospital stay in patients with early postoperative small bowel obstruction due to obliterative peritonitis. Somatostatin and dexamethasone were effective in symptom control in this population.

Gong JF, Zhu WM, Yu WK, Li N, Li JS. Conservative treatment of early postoperative small bowel obstruction with obliterative peritonitis. *World J Gastroenterol* 2013; 19(46): 8722-8730 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8722.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8722>

## INTRODUCTION

Early postoperative small bowel obstruction (EPSBO) with obliterative peritonitis (EPSBO-OP), or “frozen abdomen”, (also known as early postoperative inflammatory small bowel obstruction<sup>[1,2]</sup>), is caused by dense, vascular and inseparable inflammatory adhesions in response to multiple sequential laparotomies, surgery for enterocutaneous fistula (ECF), or extensive adhesiolysis<sup>[3-6]</sup>. Patients with EPSBO-OP may often have a combination of partial mechanical obstruction and diffuse small bowel and colonic ileus. Surgery attempting to lyse the adhesions in these patients is unsuitable due to the high risk of iatrogenic injuries such as ECF or massive small bowel resection<sup>[7]</sup>.

The traditional approach to managing these patients is total parenteral nutrition (TPN) and observation, and most obstructions are relieved spontaneously<sup>[8]</sup>. However, it often takes a long period (*i.e.*, several weeks to months) before the bowel function recovers<sup>[9]</sup>, and it is associated with high costs and high risk of PN-related complications. Patients have to tolerate prolonged nasogastric (NG) suction and fluid loss, which can also create discomfort and complications.

Somatostatin is well known for its antisecretory function in the intestinal epithelium, and clinical studies have suggested that it may be useful for symptomatic relief and treatment of bowel obstruction<sup>[10,11]</sup>. Dexamethasone is a frequently used synthetic corticosteroid that reduces intraperitoneal adhesion and inflammatory edema<sup>[12,13]</sup>, and is effective in promoting the resolution of malignant bowel obstruction or obstruction with encapsulating peritoneal sclerosis<sup>[14,15]</sup>. Based on their mechanisms of action and results of previous studies, we hypothesized that these two drugs, when used in combination, would be beneficial in reducing gastrointestinal secretion and promoting the regression of inflammation and adhesion in patients with EPSBO-OP. However, comparative studies of the effect of somatostatin and dexamethasone in EPSBO-OP are lacking.

In the current study, we prospectively analyzed a consecutive series of patients with EPSBO-OP in our department, a tertiary gastrointestinal referral center in China. The aim of the study was to evaluate the effect of somatostatin and dexamethasone on length of hospital stay and symptom control in patients with EPSBO-OP.

## MATERIALS AND METHODS

### Patients

The diagnostic criteria for EPSBO-OP were: (1) intestinal obstruction that developed 1-4 wk postoperatively after initial recovery of postoperative ileus, as defined previously<sup>[16,17]</sup>; (2) typical operative history with extensive enterolysis or repeated laparotomy over a short period; (3) absence of severe colicky abdominal pain, but with obstipation, abdominal distension, nausea and vomiting; (4) palpation of a subincisional or whole abdominal mass

on physical examination, with only mild or no tenderness on palpation, no peritoneal signs, and low-pitched or no bowel tones; and (5) low or absent air-fluid levels on upright abdominal film, edematous and thickened bowel wall with unclear borders on abdominal computed tomography (CT), and fluid-filled lumen with paucity of gas.

Exclusion criteria included: patients aged < 18 years; patients with terminal disease or presence of metastatic cancer; CT or X-ray film suggesting local adhesions, intussusceptions, volvulus, internal hernia, intra-abdominal abscesses, or hematoma; patients with suspicion of mechanical bowel obstruction, paralytic ileus, or idiopathic pseudo-obstruction; and patients with hypokalemia and retroperitoneal injury that may cause paralytic ileus. On previous laparotomy, all adhesions should have been freed and the possibility of mechanical obstruction, such as anastomotic stenosis or residual malignancy, excluded. All radiographs (X-ray and CT scan) were extrapolated by a specialist in gastrointestinal radiology.

Assignments were based on computer-generated randomizations that were kept in sealed, sequentially numbered envelopes until used. After a diagnosis of EPSBO-OP was made, patients were randomly assigned into one of the two groups: TPN group (T group) or TPN + dexamethasone + somatostatin group (TDS group). The study was approved by the ethics committee of the hospital, and all patients provided written informed consent before enrollment.

### Treatment

Nil by mouth and nasogastric tube were introduced for all patients. For patients in the control group (T group), a central venous catheter was placed on admission. After fluid resuscitation and correction of electrolyte abnormalities, patients were infused with TPN from all-in-one bags. The amount of non-protein calories (NPCs) was 20-25 kcal/kg per day or determined by indirect calorimetry. The NPCs consisted of 60%-70% carbohydrate, with the ratio of NPC: nitrogen = 120-140:1. Parenteral antibiotics were administered when leukocytosis was present. The amount of intravenous fluid was adjusted to maintain optimal hydration and sufficient urine output (> 1 L/d).

The duration of NG tube feeding depended on daily output. If daily NG output was < 200 mL for 2 d, the NG tube was clamped. The NG tube was removed if the patient was able to tolerate for 12 h after clamping. After patients resumed oral intake, gastrointestinal prokinetics (mosapride, 5 mg/8 h, Gasmotin; Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) was given until discharge.

In the intervention group (TDS group), in addition to the treatment protocol in the control group, somatostatin (Stilamin; Merck-Serono S.A., Geneva, Switzerland) was given at 6 mg/d by continuous intravenous infusion. The criteria for stopping NG tube usage were similar to those for the T group, while somatostatin was stopped within 24 h after the patient defecated or passed gas. Dexameth-

asone sodium phosphate (5 mg/mL, Lukang Pharmaceuticals, Shandong, China) was used since the first day of treatment with an intravenous dosage of 5 mg/8 h for seven consecutive days, then 2.5 mg/12 h for 1 d, and stopped. If the patient defecated or passed gas in < 8 d after treatment, dexamethasone was withdrawn within 24 h after resolution of obstruction. During treatment, patients were carefully monitored for abdominal symptoms and systemic complications, such as cholestasis, central catheter infections, and systemic infections. Other complications, such as hypovolemia, electrolyte-fluid imbalance, and hyperglycemia, were corrected during treatment and not documented.

Indications for prompt surgery included suspicion for strangulation (continuous *vs* colicky pain, fever, tachycardia, peritoneal signs, and sustained leukocytosis), or clinical deterioration that implied failure of conservative management for > 3 mo.

The following parameters were recorded in each patient: age and sex; interval between symptom onset and the most recent laparotomy; clinical features including symptoms, presence or absence of fever, white blood cells, nutritional status, and comorbidity; procedures and duration of last operation; and time of previous laparotomy. Complete resolution of obstruction was established when symptoms and signs of obstruction subsided, normal flatus and defecation returned, and there was no relapse of obstructive symptoms after withdrawal of somatostatin. Then, liquid food or enteral nutrition was started. A semiliquid food was usually given 2 d later. Patients were discharged when intravenous fluid was stopped and semiliquid food was tolerated for 3 d.

### Outcome measures

The primary endpoints of the study were time to resolution of obstruction and length of hospital stay, and the secondary endpoints were daily NG output, NG tube placement duration, treatment-related complications, postoperative obstruction relapse, and patient satisfaction.

### Sample size calculation

Sample size calculation was based on our previous data of historical comparison<sup>[18]</sup>, which showed a mean 26.0 d for the intervention group and 30.3 d for the control group, with mean  $\pm$  SD of 9.0 d. Approximately 35 patients in each group were needed to detect a difference in hospital stay with 80% power and a two-sided 5% significance level.

### Long-term follow up

The patients were followed for  $\geq 3$  years after discharge. At each 6-mo visit, patients were given a questionnaire that was completed and returned by mail or they were contacted by telephone with the complete questions answered. Obstruction relapse was defined as abdominal pain with the halt of flatus, and nausea/vomiting, which needed further medical treatment and admission to hos-

pital. At 1, 2 and 3 years, the degree of postoperative satisfaction was evaluated in every patient by using a unified scale (1-4) that indicated very unsatisfied, unsatisfied, satisfied, and very satisfied, respectively. Patient satisfaction was based on the core symptoms of the Gastrointestinal Quality of Life Index such as abdominal pain, feeling of abdominal distension, flatus and stool frequency, anorexia, fatigue, and nausea and vomiting<sup>[19]</sup>. The definition of "very satisfied" was the presence of none of the above-mentioned gastrointestinal symptoms in the past year; "satisfied" was occasional gastrointestinal symptoms; "unsatisfied" was several episodes of abdominal symptoms in the past year, and "very unsatisfied" was frequent abdominal symptoms.

### Statistical analysis

Statistical analysis was performed by per-protocol analysis. Quantitative variables, presented as mean  $\pm$  SD (range), were analyzed by Mann-Whitney *U* test or Student's *t* test if appropriate. Quantitative variables, expressed as a number (percentage), were analyzed by Pearson's  $\chi^2$  test or Fisher's exact test. All analyses were performed with SPSS version 13.0 (SPSS, Chicago, IL, United States). *P* < 0.05 indicated statistical significance.

## RESULTS

### Patient disposition and baseline characteristics

Between June 2002 and January 2009, 82 patients were diagnosed with EPSBO-OP in our department. Six patients were aged < 18 years and two declined to participate in the study, which left 74 patients enrolled in the study. Two patients were eventually confirmed to have mechanical obstruction and two had intra-abdominal abscesses or anastomotic fistulae and withdrew from the study. The dropout patients were eventually proven not to have EPSBO-OP, therefore, we used per-protocol analysis instead of intention-to-treat statistical analysis. Therefore, 70 cases were evaluated (34 in the T group and 36 in the TDS group) (Figure 1).

Patients' demographic data and previous surgeries are listed in Tables 1 and 2. Fifteen patients (10 in the TDS group and 5 in the T group) had a history of malignancy but all underwent radical surgical resection. There were no significant differences between the two groups with respect to laboratory and clinical features at trial entry. The median onset of obstructive symptoms was postoperative day  $9.4 \pm 3.5$  (range: 5-23 d).

Sixty-three patients underwent more than two operations before EPSBO-OP developed. At last surgery, extensive adhesiolysis (including intestinal splinting<sup>[20]</sup>) was performed in 54 (77.1%) of the operations; six (8.6%) received repeated laparotomy within 2 wk, and another six patients had diffuse peritonitis during last laparotomy. Although the mean number of operations ( $2.9 \pm 1.3$  *vs*  $3.0 \pm 1.0$ , *P* = 0.927) and type of operation were similar between the two groups, the operation time was shorter in the TDS group compared with the T group ( $4.1 \pm 1.3$  *vs*



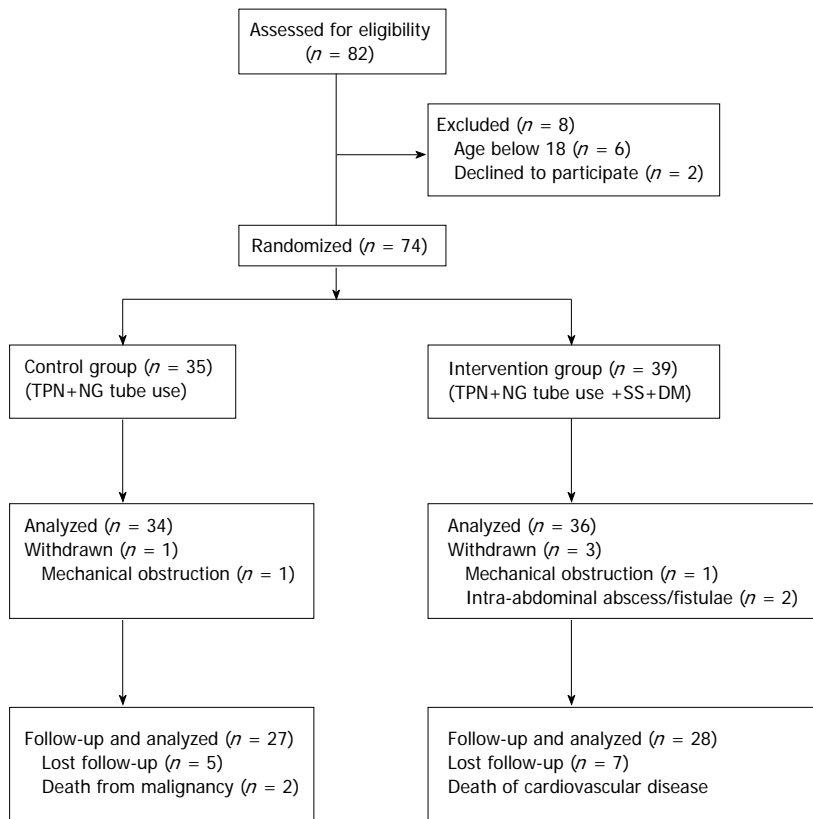


Figure 1 Flow chart of patient inclusion and follow-up. TPN: Total parenteral nutrition; NG: Nasogastric; DM: Dexamethasone; SS: Somatostatin.

Table 1 Demographic data and clinical features *n* (%)

	T group (n = 34)	TDS group (n = 36)	P value
Age (yr)	43.9 ± 10.2 (26-64)	45.4 ± 13.2 (20-78)	0.597
Sex ratio (M/F)	24/10	18/18	0.079
Symptoms onset			
≤ 1 POW	12 (35.3)	13 (36.1)	0.943
1-2 POW	19 (55.9)	21 (58.3)	0.836
2-3 POW	3 (8.8)	1 (2.8)	0.276
3-4 POW	0	1 (2.8)	
Mean POD of symptom onset	9.8 ± 3.4 (5-19)	9.1 ± 3.5 (5-23)	0.412
Symptoms			
Nausea and vomiting	32 (94.1)	33 (91.7)	0.691
Abdominal distension	23 (67.6)	20 (55.6)	0.299
Colic pain	0	0	
Obstipation	34 (100)	36 (100)	
Hyperthermia (> 37.5 °C) <sup>1</sup>	1 (2.9)	1 (2.6)	0.967
Maximum WBC (× 10 <sup>9</sup> /L)	7.0 ± 2.3 (3.7-12.3)	8.0 ± 3.0 (4.8-17.1)	0.148
Neutrophil (%)	71.2 ± 10.4 (48-88)	75.1 ± 8.4 (59-91)	0.084
Nutrition status on admission			
Mean BMI (kg/m <sup>2</sup> )	20.4 ± 2.2 (16.8-26.4)	21.2 ± 2.8 (17.3-28.0)	0.248
Hypoalbuminemia (< 35g/L)	13 (38.2)	8 (22.2)	0.144
Anemia (Hb < 120g/L)	10 (29.4)	4 (11.1)	0.056
Comorbidity	3 (8.8)	4 (11.1)	0.750

<sup>1</sup>One due to pneumonia, and one due to epiglottitis. POW: Postoperative week; POD: Postoperative day; BMI: Body mass index.

4.8 ± 1.1 h, *P* = 0.041). Typical radiographic and intraoperative findings at the last operation are shown in Figures 2 and 3, respectively.

### Efficacy endpoints

Treatment was successful for all patients in both groups.

The mean length of hospital stay was 30.5 ± 10.9 (16-69) d. No patients withdrew because they needed surgery for strangulation or failure of conservative therapy. There were no deaths during treatment. In the TDS group, the mean duration of somatostatin usage was 23.5 ± 9.1 (14-53) d, while dexamethasone was used for 8 d in all

**Table 2 Previous laparotomies *n* (%)**

	T group ( <i>n</i> = 34)	TDS group ( <i>n</i> = 36)	<i>P</i> value
No. of laparotomies			
1	3 (8.8)	4 (11.1)	0.750
2	7 (20.6)	11 (30.6)	0.340
3	15 (44.1)	11 (30.6)	0.241
4	6 (17.6)	4 (11.1)	0.435
≥ 5	3 (8.8)	6 (16.7)	0.327
Mean No. (range)	3.0 ± 1.0 (1-5)	2.9 ± 1.3 (1-6)	0.927
Type of last operation			
Bowel obstruction	12 (35.3)	18 (50.0)	0.214
Enterocutaneous fistula	15 (44.1)	10 (27.8)	0.154
Enterectomy/colectomy	2 (5.9)	1 (2.8)	0.522
Gastroduodenal surgery	0	1 (2.8)	0.328
Hematoma removal	2 (5.9)	1 (2.8)	0.522
Appendectomy <sup>1</sup>	0	2 (5.6)	0.163
Laparotomy after trauma	1 (2.9)	1 (2.8)	0.967
Others	2 (5.9)	2 (5.6)	0.953
Patients with history of malignancy	5 (14.7)	10 (27.8)	0.183
At last operation			
with extensive enterolysis <sup>2</sup>	24 (70.6)	30 (83.4)	0.204
with diffuse peritonitis	4 (11.8)	2 (5.6)	0.354
< 2 wk from previous surgery	3 (8.8)	3 (8.3)	0.971
Last operation time (h)	4.8 ± 1.1 (2.0-6.5)	4.1 ± 1.3 (1.5-7)	0.041

<sup>1</sup>All are perforated appendicitis with diffuse peritonitis; <sup>2</sup>Including with intestinal splinting.

patients.

As shown in Table 3, somatostatin and dexamethasone had a marked effect on the recovery of bowel function, as indicated by earlier passage of stool or gas ( $22.4 \pm 9.1$  vs  $29.9 \pm 10.1$  d,  $P = 0.002$ ). The length of hospital stay in the intervention group was shorter than in the control group ( $25.8 \pm 9.9$  vs  $34.7 \pm 11.2$  d,  $P = 0.001$ ).

The daily NG output and NG duration were evaluated as indicators of symptom control. The daily NG output was  $583 \pm 208$  (150-1050) mL in the TDS group, which was significantly lower ( $P < 0.001$ ) than that in the T group [ $922 \pm 399$  (400-1825) mL]. The need for NG tube use was also significantly shorter in the TDS group ( $16.7 \pm 8.8$  vs  $27.7 \pm 9.9$  d,  $P < 0.001$ ).

### Safety endpoints

Treatment-related complications are shown in Table 4. The rate of overall complications was comparable in the TDS and T group (41.7% vs 38.2%,  $P = 0.770$ ). Cholestasis, as revealed by increased bilirubin, AKP,  $\gamma$ -glutamyltransferase, or biliary sludge on ultrasonography, developed in 13 patients, and percutaneous transhepatic cholecystostomy (PTC) was performed in eight patients presenting with acalculous cholecystitis. The incidence of cholestasis and need for PTC were higher in the TDS group, but not significantly ( $P = 0.419$  and  $0.264$ , respectively). Infectious complications, including catheter-related sepsis, wound infection, and pneumonia, occurred in 15 patients. All blood culture-positive, catheter-related sepsis was cured with antibiotics. Statistical analysis revealed that there was no significant difference in infec-

tious complications between the two groups ( $P = 0.677$ ). Two patients had pneumothorax on catheter insertion.

Treatment with somatostatin and dexamethasone was well tolerated and did not cause any serious or clinical significant adverse reactions except that one patient in the intervention group complained of dry mouth.

### Follow-up outcomes

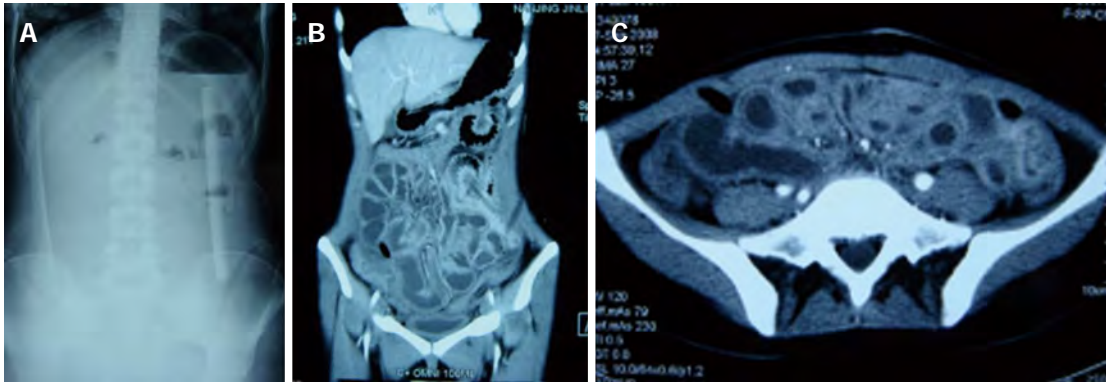
Twelve patients were lost to follow-up (7 in the TDS group and 5 in the T group), and two patients in the T group died of relapse of primary colon cancer and gastric cancer after 12 and 18 mo of follow-up, respectively, while one in the TDS group died of cardiovascular disease (at 30 mo follow-up). Long-term follow-up data indicated that the rates of recurrence of obstruction at 1, 2 and 3 years postoperatively were similar between the two groups (Table 3). In addition, there was no significant difference in postoperative satisfaction at 1, 2 and 3 years between the two groups (Table 3).

## DISCUSSION

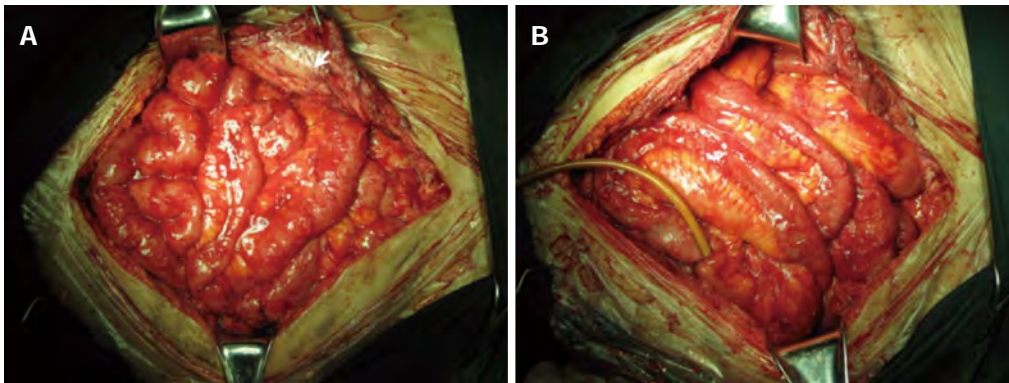
EPSBO-OP is a rare complication after major abdominal procedures, mostly after extensive adhesiolysis. Although conservative therapy was effective in most of our cases, the patients often had to be maintained on long-term NG suction and TPN therapy before recovery of bowel function. Our results suggested that dexamethasone and somatostatin, when added to TPN, decreased the duration of NG suction and daily NG output, and shortened the duration of bowel obstruction as well as the length of hospital stay.

EPSBO-OP was first described by Fazio *et al*<sup>[21]</sup> and Hill *et al*<sup>[22]</sup> in 1983. In contrast to the common causes of EPSBO such as local adhesion, volvulus, or internal hernia, which can be managed surgically after failure of conservative treatment<sup>[23,24]</sup>, OP is caused by formation of dense adhesions and severe peritoneal reaction within the early postoperative period - typically 10 d to 6-8 wk after some major procedures - especially when the bowel has fistulated. The main risk factors include extensive adhesiolysis, multiple sequential laparotomies within a short period (*i.e.*, several days or weeks), peritonitis, and other factors causing extensive intestinal deserosalization<sup>[25,26]</sup>. The acute inflammatory reaction may involve the peritoneal surface and adherence of adjacent loops of bowel, often involving the omentum and mesenteric surfaces. These adhesions are highly vascularized, friable, and immature, thus, surgical separation is impossible. Therefore, recognition of EPSBO-OP is important to avoid serious consequences such as ECF or massive bowel resection because of re-laparotomy attempting to lyse the adhesions<sup>[27]</sup>. The adhesions are extensive, thus, the risk of closed-loop obstruction, volvulus, or strangulation is low, making conservative therapy possible<sup>[4]</sup>.

Resolution of OP after prolonged TPN therapy has been reported previously. Lennard *et al*<sup>[8]</sup> reported two patients with ECF and OP managed with TPN for 8



**Figure 2** Typical radiographic and intra-operative finding in the last operation. A: Typical upright radiograph of early postoperative small bowel obstruction with obliterative peritonitis after extensive adhesiolysis for abdominal cocoon, showing only mild air-fluid levels. No isolated small bowel loops were observed; B, C: Computed tomography scan reveals edematous small bowel filled-up with fluids. The border between the small bowel loops is not clear. No significant discrepancies in small bowel diameter and air-fluid levels were observed.



**Figure 3** Intraoperative photo of a patient who developed early postoperative small bowel obstruction with obliterative peritonitis. The 40-year-old male had a colostomy and patch-repair (arrow) of the abdominal defect after open abdomen due to trauma. He required closure of the stoma 6 months after colostomy. On operation, dense matted adhesions were found, especially beneath the patch, and full enterolysis resulted in extensive intestinal deserosalization (A). An intestinal splinting was performed (B). On postoperative day (POD) 10 the tube was removed, and he had a temporary return of bowel function but early postoperative small bowel obstruction with obliterative peritonitis on POD 15.

and 4 mo, respectively. Selby *et al.*<sup>[9]</sup> reported six patients with EPSBO secondary to dense and vascular benign adhesions that could not be freed by operation. The obstructions all spontaneously resolved within 2-3.5 mo on a TPN program. The authors believe that complete gastrointestinal rest allows adhesions to mature into long avascular collagen fibers in the absence of a persistent inflammatory reaction that accompanies partial or total SBO. In the current study, we also confirmed that with TPN alone, EPSBO-OP could also resolve, but it often takes a long time.

Somatostatin, or its synthetic analog octreotide, inhibits gastrointestinal secretion and release of hormones, and they have been used for treatment of SBO for over a decade, especially for malignant SBO<sup>[28]</sup>. In experimental animal models, somatostatin may be beneficial for the control of intestinal distension, inflammation and necrosis, and bacterial translocation<sup>[29,30]</sup>. Octreotide, the somatostatin analog, may ameliorate intestinal dysmotility and stasis in models of small bowel transplantation<sup>[31]</sup>. Besides its role in symptom control, somatostatin may also promote the resolution of SBO<sup>[32,33]</sup>. Zhang *et al.*<sup>[34]</sup> have

confirmed that octreotide, when combined with water-soluble radiocontrast medium, may accelerate resolution of adhesive SBO by a specific therapeutic effect. This is consistent with the findings of the current study.

Corticosteroids have long been used for their anti-inflammatory effects, which may reduce the edema and fibrin deposition associated with EPSBO-OP, thereby helping to resolve the obstruction<sup>[35]</sup>. In Japan, steroids have been used to reduce the inflammatory state of encapsulating peritoneal sclerosis, in which intraperitoneal inflammation leads to adhesive and inflammatory encapsulation of the intestinal tract, causing bowel obstructive symptoms. In a prospective cohort, 15 of 42 cases (35.7%) of encapsulating peritoneal sclerosis treated with prednisolone alone showed clinical improvement<sup>[14]</sup>. In malignant bowel obstruction, corticosteroids may reduce intestinal inflammatory edema associated with the malignant lesion, thereby aiding resolution of bowel obstruction<sup>[14]</sup>. Extensive dense inflammatory adhesions and intestinal wall edema are characteristics of EPSBO-OP, therefore, we explored the effect of corticosteroids in EPSBO-OP, and our data showed that DM, when combined with so-

**Table 3 Outcomes of patients receiving total parenteral nutrition alone or in combination with somatostatin and dexamethasone**

	TPN ( <i>n</i> = 34)	TDS ( <i>n</i> = 36)	<i>P</i> value
Time to obstruction resolution (d)	29.9 ± 10.1 (17-60)	22.4 ± 9.1 (13-52)	0.002
Length of hospital stay (d)	34.7 ± 11.2 (21-69)	25.8 ± 9.9 (16-57)	0.001
Daily NG output (mL) <sup>1</sup>	922 ± 399 (400-1825)	583 ± 208 (150-1050)	< 0.001
Mean NG duration (d)	27.7 ± 9.9 (7-54)	16.7 ± 8.8 (3-42)	< 0.001
Relapse of obstruction			
1 yr after operation ( <i>n/N</i> ) <sup>2</sup>	3/32	2/34	0.668
2 yr after operation ( <i>n/N</i> ) <sup>3</sup>	6/29	5/31	0.745
3 yr after operation ( <i>n/N</i> ) <sup>4</sup>	8/27	6/28	0.547
Postoperative satisfaction ≥ 3 <sup>5</sup>			
1 yr after operation ( <i>n/N</i> ) <sup>2</sup>	22/32	20/34	0.451
2 yr after operation ( <i>n/N</i> ) <sup>3</sup>	15/29	14/31	0.796
3 yr after operation ( <i>n/N</i> ) <sup>4</sup>	11/27	10/28	0.785

<sup>1</sup>Mean value of the first 2 d after NG tube placement; <sup>2</sup>TPN (*n* = 32, 1 patient was lost to follow-up, and 1 died of malignancy); TDS (*n* = 34, 2 patients were lost to follow-up); <sup>3</sup>TPN (*n* = 29, 2 patients were lost to follow-up, and 1 died of malignancy); TDS (*n* = 31, 3 patients were lost to follow-up); <sup>4</sup>TPN (*n* = 27, 2 patients were lost to follow-up); TDS (*n* = 28, 2 patients were lost to follow-up and 1 died of cardiovascular disease); <sup>5</sup>Patients with satisfaction ≥ 3 were satisfied (3) or very satisfied (4). TDS: TPN + DM + SS; TPN: Total parenteral nutrition; DM: Dexamethasone; SS: Somatostatin; NG: Nasogastric.

**Table 4 Treatment-related complications *n* (%)**

	TPN ( <i>n</i> = 34)	TDS ( <i>n</i> = 36)	<i>P</i> value
Morbidity			
Cholestasis	5 (14.7)	8 (22.2)	0.419
Patients requiring PTC	2 (5.9)	5 (13.9)	0.264
Infectious complications	8 (23.5)	7 (19.4)	0.677
Catheter-related infections	5 (14.7)	4 (11.1)	0.653
Wound infection	2 (5.9)	1 (2.8)	0.522
Pneumonia	1 (2.9) <sup>1</sup>	0	0.300
Pneumothorax	0	2 (5.5)	0.163
Overall complications	13 (38.2)	15 (41.7)	0.770

<sup>1</sup>Patient had tracheostomy. TDS: TPN + DM + SS; TPN: Total parenteral nutrition; DM: Dexamethasone; SS: Somatostatin; PTC: Percutaneous transhepatic cholecystostomy.

matostatin, promoted resolution of the adhesions. Fibrin exudation and intestinal edema were most prominent in the early stage of EPSBO-OP, thus, we recommend the usage of DM immediately after diagnosis.

Cholestasis is a complication of long-term usage of somatostatin<sup>[36]</sup> and TPN. Animal models and human volunteer studies all suggest that the effect of somatostatin is associated with a pronounced decrease in bile flow, bile acid secretion, and increased bile cholesterol saturation<sup>[37-39]</sup>. In the current study, although we observed an increased incidence of cholestasis and need for PTC in patients receiving somatostatin, it did not reach statistical significance. This was possibly due to the small number of cases in our series. However, it could also be that the beneficial effect of dexamethasone on bile excretion partly counteracts the detrimental effect of somatostatin<sup>[40,41]</sup>.

Increased susceptibility to infection and impaired wound healing are the main side effects of systemic corticosteroids. Trésallet *et al*<sup>[42]</sup> have observed that patients on steroids for > 1 mo had a higher incidence of postoperative complications, especially infections after colectomy with rectal anastomosis. We did not observe any difference in the occurrence of infection between the

two groups, which was possibly because we used short-term therapy (7 d). There is currently no direct evidence that dexamethasone promotes relapse of malignancy, therefore, we did not avoid its use in tumor patients.

There were several limitations to our study. First, the diagnosis of OP was made on the basis of clinical presentation, physical examination, and medical history, and was confirmed by plain film radiography and CT, but it could not be definitively proven by laparotomy. Therefore, this may have led to the inclusion of a few cases of obstruction not caused by OP. Second, the mean operation time preceding obstruction was shorter in the TDS group, and this may have influenced the outcome. Third, the study was not blinded and the physicians and patients were aware of which therapy that each patient had received, which would have introduced some bias during evaluation.

In conclusion, our trial indicates that conservative therapy is efficient in EPSBO-OP. Administration of somatostatin and dexamethasone in addition to TPN promotes resolution of obstruction, shortens length of hospital stay, and is efficient for symptom control without increasing complications and obstruction relapse.

## COMMENTS

### Background

Early postoperative small bowel obstruction due to obliterative peritonitis (EPSBO-OP) is a rare complication after abdominal surgery, especially extensive adhesiolysis and enterocutaneous fistula. Traditionally, the only treatment for these patients was total parenteral nutrition, nasogastric tube feeding, and observation. The time to the recovery of bowel function is often long and patients often suffer from low quality of life. Methods to promote resolution and control obstruction-related symptoms are lacking.

### Research frontiers

Somatostatin or its analogs and corticosteroids are effective and safe in patients with inoperative bowel obstruction due to peritoneal carcinomatosis or encapsulating peritoneal sclerosis. Therefore, their clinical role in the management of postoperative OP warrants further investigation.

### Innovations and breakthroughs

Somatostatin and dexamethasone shorten the time to obstruction resolution and length of hospital stay, and decrease nasogastric output and duration of



nasogastric tube usage. They do not increase treatment-related complications and relapse of obstruction.

### Applications

This study revealed that somatostatin and dexamethasone are effective in promoting resolution and controlling symptoms in patients with EPSBO-OP.

### Peer review

This paper provides some useful information on the management of EPSBO-OP.

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## Preoperative biliary drainage in patients with hilar cholangiocarcinoma undergoing major hepatectomy

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### Abstract

**AIM:** To investigate the effect of preoperative biliary drainage (PBD) in jaundiced patients with hilar cholangiocarcinoma (HCCA) undergoing major liver resections.

**METHODS:** An observational study was carried out by reviewing a prospectively maintained database of HCCA patients who underwent major liver resection for curative therapy from January 2002 to December 2012. Patients were divided into two groups based on whether PBD was performed: a drained group and an undrained

group. Patient baseline characteristics, preoperative factors, perioperative and short-term postoperative outcomes were compared between the two groups. Risk factors for postoperative complications were also analyzed by logistic regression test with calculating OR and 95%CI.

**RESULTS:** In total, 78 jaundiced patients with HCCA underwent major liver resection: 32 had PBD prior to operation while 46 did not have PBD. The two groups were comparable with respect to age, sex, body mass index and co-morbidities. Furthermore, there was no significant difference in the total bilirubin (TBIL) levels between the drained group and the undrained group at admission ( $294.2 \pm 135.7$  vs  $254.0 \pm 63.5$ ,  $P = 0.126$ ). PBD significantly improved liver function, reducing not only the bilirubin levels but also other liver enzymes. The preoperative TBIL level was significantly lower in the drained group as compared to the undrained group ( $108.1 \pm 60.6$  vs  $265.7 \pm 69.1$ ,  $P = 0.000$ ). The rate of overall postoperative complications ( $53.1\%$  vs  $58.7\%$ ,  $P = 0.626$ ), reoperation rate ( $6.3\%$  vs  $6.5\%$ ,  $P = 1.000$ ), postoperative hospital stay ( $16.5$  vs  $15.0$ ,  $P = 0.221$ ) and mortality ( $9.4\%$  vs  $4.3\%$ ,  $P = 0.673$ ) were similar between the two groups. In addition, there was no significant difference in infectious complications ( $40.6\%$  vs  $23.9\%$ ,  $P = 0.116$ ) and noninfectious complications ( $31.3\%$  vs  $47.8\%$ ,  $P = 0.143$ ) between the two groups. Univariate and multivariate analyses revealed that preoperative TBIL  $> 170 \mu\text{mol/L}$  (OR = 13.690, 95%CI: 1.275-147.028,  $P = 0.031$ ), Bismuth-Corlette classification (OR = 0.013, 95%CI: 0.001-0.166,  $P = 0.001$ ) and extended liver resection (OR = 14.010, 95%CI: 1.130-173.646,  $P = 0.040$ ) were independent risk factors for postoperative complications.

**CONCLUSION:** Overall postoperative morbidity and mortality rates after major liver resection are not improved by PBD in HCCA patients with jaundice. Preoperative TBIL  $> 170 \mu\text{mol/L}$ , Bismuth-Corlette classification and extended liver resection are independent risk

factors linked to postoperative complications.

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**Key words:** Obstructive jaundice; Hilar cholangiocarcinoma; Preoperative biliary drainage; Major hepatectomy; Surgical outcome

**Core tip:** There is currently no consensus on the use of preoperative biliary drainage (PBD) in jaundiced patients with hilar cholangiocarcinoma undergoing major liver resection. We retrospectively analyzed prospectively maintained database of these patients who underwent PBD or not. The baseline characteristics, perioperative and short-term postoperative outcomes between these two groups were compared and no significant differences were identified. We found that a preoperative total bilirubin level > 170  $\mu\text{mol/L}$ , Bismuth-Corlette classification and extended liver resection are three independent risk factors for postoperative complications. There is a need to undertake well-designed, prospective multicenter studies to inform future practice.

Xiong JJ, Nunes QM, Huang W, Pathak S, Wei AL, Tan CL, Liu XB. Preoperative biliary drainage in patients with hilar cholangiocarcinoma undergoing major hepatectomy. *World J Gastroenterol* 2013; 19(46): 8731-8739 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8731.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8731>

## INTRODUCTION

Hilar cholangiocarcinoma (HCCA), which was first defined by Klatskin<sup>[1]</sup> as an adenocarcinoma of the hepatic duct at its bifurcation within the porta hepatis, is associated with a poor prognosis<sup>[1,2]</sup>. Currently, the only curative treatment is radical surgical resection<sup>[3]</sup>. However, a R0 resection margin is difficult to achieve because the tumor often infiltrates the portal vein, the hepatic artery and liver parenchyma<sup>[4,5]</sup>. In order to obtain negative histological margins and improve survival, many surgeons have adopted a more aggressive surgical approach, namely, extended hepatectomy combined with portal vein or hepatic artery resection and reconstruction, and hepato pancreaticoduodenectomy for the treatment of this malignancy<sup>[6-8]</sup>. However, the majority of patients with HCCA have obstructive jaundice at presentation, which increases the risk of complications, such as sepsis, bleeding and liver failure, especially in patients undergoing major hepatectomy<sup>[9,10]</sup>. Therefore, preoperative biliary drainage (PBD) was introduced with the aim to abrogate these potential complications in patients with jaundice secondary to HCCA, despite that a consensus on an appropriate cut-off level of total bilirubin (TBIL)<sup>[11-14]</sup> and duration of drainage<sup>[8,15,16]</sup> has not been reached yet.

There is still controversy with regard to whether PBD

is essentially needed for jaundiced patients with HCCA undergoing major liver resection. It was shown that PBD reverses cholestasis-associated hepatic and systemic toxicity, and improves liver function, nutritional status and cell-mediated immune function<sup>[17]</sup>. However, concerns were also raised as PBD may associate with an increased incidence of postoperative morbidity and mortality<sup>[18-20]</sup>, although this was not the case for other studies<sup>[21,22]</sup>. Recently, one multicenter European study including patients undergoing major liver resection for HCCA suggested that overall morbidity was not affected by PBD procedure<sup>[14]</sup>. Furthermore, preoperative portal vein embolization (PVE), which is restricted to the treatment of postoperative inadequate residual liver volume and induces hypertrophy of the future remnant liver, has led to a change to PBD strategy<sup>[23]</sup>. PBD followed by PVE prior to major hepatectomy is considered a safe management strategy for HCCA, particularly in patients with remnant liver volume less than 40%<sup>[24-26]</sup>.

The aim of this study was to inform the debate by comparing the perioperative and short-term postoperative outcomes of jaundiced patients with HCCA undergoing curative major liver resection with or without PBD, at a large specialist center in China.

## MATERIALS AND METHODS

### Study population and preoperative management

The prospectively maintained database for a cohort of consecutive HCCA patients treated at the West China Hospital of Sichuan University between January 2002 and December 2012 was retrospectively reviewed. From the database, only patients with HCCA who had jaundice and underwent major hepatectomy for curative resection were included in this study. Jaundice was defined as a serum TBIL level > 85.5  $\mu\text{mol/L}$  (5 mg/dL). HCCA was defined as lesions arising from the common hepatic duct, left, right, or both hepatic duct and intrahepatic bile duct cancer invading the hepatic hilus<sup>[11]</sup>. The tumors were classified according to Bismuth-Corlette classification<sup>[27]</sup>.

In our series, blood sampling for serum biochemistry was completed 2-3 d before drainage or surgery. Color Doppler ultrasound and contrast enhanced computed tomography (CT) were used routinely before surgery. Furthermore, magnetic resonance imaging (MRI) was used in most of patients. If distant metastases were suspected, further investigations with positron emission tomography-CT scan were performed. PVE was carried out at our hospital if the remnant liver volume post surgical resection was expected to be less than 50% of the whole liver volume. PBD was performed if patients fulfilled one of the following criteria: duration of jaundice of more than 4 wk; poor nutritional status (serum albumin < 3 g/dL); signs of cholangitis. PBD procedures in our center were percutaneous transhepatic cholangio-drainage (PTCD), endoscopic biliary stenting (EBS), endoscopic nasobiliary drainage (ENBD) and/or surgical drainage. For patients who had inadequate PBD before admission to our hospital, a further drainage by a percutaneous approach was



adapted. Adequate PBD was evident by a relief of cholangitis, and an improvement in the liver function and/or the nutritional status of the patient.

### Surgical procedures

At our center, curative excision was defined as histologically negative surgical margins with a minimum tumor-free margin of 5 mm at the hepatic stump of the bile duct, the duodenal stump of the bile duct, and the excision surface. It included resection of the gallbladder and extrahepatic bile duct; skeletonization of the vasculature of the hepatoduodenal ligament; and partial hepatectomy, or even removal of the caudate lobe or portal vein or hepatic artery as required. The postoperative biliary drainage was established by a Roux-en-Y hepaticojejunostomy. Major hepatectomy was defined as resection of three or more Couinaud segments. Caudate lobectomy was performed in patients in whom it was considered necessary to achieve complete tumor clearance.

### Postoperative complications

While patients were followed routinely after discharge from hospital, as part of this study, we endeavored to investigate the effect of PBD on in-hospital postoperative outcomes. Hence, postoperative mortality was defined as death prior to hospital discharge. All postoperative complications were defined as events that lengthened hospital stay. Infectious complications were defined according to the study by Hochwald *et al.*<sup>[19]</sup>; these were intraabdominal abscess, wound infection, cholangitis, sepsis and lung infection. Noninfectious complications included liver failure, bile leak, anastomotic leak, abdominal collection, gastrointestinal bleeding, abdominal bleeding, respiratory failure and renal failure. Liver failure was defined as an increased international normalized ratio and concomitant hyperbilirubinemia on or after postoperative day five<sup>[28]</sup>. Bile leak was defined as the drainage of 50 mL or more of bile from the surgical drain or from drainage of an abdominal collection, over a period of three days or more<sup>[29]</sup>. In addition, the complications were graded according to the Clavien-Dindo classification of surgical complications<sup>[30]</sup>.

### Literature search

Existing literature was also reviewed by performing a systematic search in PubMed, Medline and Embase from January 1990 to May 2013. The following search terms were used: “preoperative biliary drainage” or “percutaneous transhepatic biliary drainage” or “endoscopic biliary drainage” or “endoscopic nasobiliary drainage” or “endoscopic biliary stenting” and “hilar cholangiocarcinoma” or “hilar bile duct cancer” or “proximal bile duct cancer” or “Klatskin tumor” or “carcinoma of the hepatic duct confluence” along with their synonyms or abbreviations. The search was restricted to studies conducted on human subjects and in the English language only.

### Statistical analysis

Data are presented as mean  $\pm$  SD or median and inter-

**Table 1 Baseline characteristics *n* (%)**

	Drained ( <i>n</i> = 32)	Undrained ( <i>n</i> = 46)	<i>P</i> value
Age (yr)	59.6 $\pm$ 11.0	58.2 $\pm$ 11.3	0.568
Sex (M/F)	21/11	28/18	0.669
Body mass index (kg/m <sup>2</sup> )	20.3 $\pm$ 1.9	21.0 $\pm$ 2.5	0.190
Concomitant diseases			
Diabetes	2 (6.3)	3 (6.5)	1.000
Hypertension	3 (9.4)	7 (15.2)	0.678
Cardiovascular	2 (6.3)	5 (10.9)	0.765
Previous history of abdominal surgery	9 (28.1)	12 (26.1)	0.842
Serum total bilirubin ( $\mu$ mol/L)			
At admission	294.2 $\pm$ 135.7	254.0 $\pm$ 63.5	0.126
Before surgery	108.1 $\pm$ 60.6	265.7 $\pm$ 69.1	0.000
Time of PBD (d)	15.3 $\pm$ 3.4	-	-
Time between admission and surgery (d)	20.7 $\pm$ 2.1	3.8 $\pm$ 1.6	0.000
Portal vein embolization	5 (15.6)	3 (6.5)	0.355
Bismuth–Corlette classification			
I	1 (3.1)	1 (2.2)	1.000
II	8 (25)	14 (30.4)	0.600
IIIa	6 (18.8)	7 (15.2)	0.680
IIIb	9 (28.1)	15 (32.6)	0.673
IV	8 (25)	9 (19.6)	0.567
Perioperative details			
Hilar bile duct resection	32 (100)	46 (100)	-
Left hepatectomy	17 (53.1)	31 (67.4)	0.203
Extended left hepatectomy	2 (6.3)	1 (2.2)	0.747
Right hepatectomy	8 (25)	10 (21.7)	0.737
Extended right	5 (15.6)	4 (8.7)	0.561
Hepatectomy			
Caudate lobectomy	8 (25)	12 (26.1)	0.914
Pedicule clamping	17 (53.1)	26 (56.5)	0.767
Portal vein resection	6 (18.8)	8 (17.4)	0.878
Hepatic artery resection	2 (6.3)	3 (6.5)	1.000
Number of blood	11 (34.4)	24 (52.2)	0.120
Transfusions			
Intraoperative blood transfusion (mL)	900 (800-900)	800 (600-1100)	0.513

PBD: Preoperative biliary drainage.

quartile range. The  $\chi^2$  test or Fisher's exact test or RxC table analysis was used to compare categorical variables, and the Student's *t* test or Mann-Whitney *U* test was used to compare continuous variables. A statistically significant difference was defined as a *P* value < 0.05. The variables of statistical significance during univariate analysis were included in a follow-up multivariate analysis, by using the logistic regression test. The OR and 95%CI were also calculated for individual factors in the multivariate analysis. All statistical analyses were performed with SPSS software (SPSS version 17.0, Chicago, Illinois).

## RESULTS

### Baseline characteristics

During the study period, 78 patients with jaundice underwent major hepatic resection for HCCA at our hospital. There were 32 patients in the drained (PBD) group and 46 patients in the undrained (no PBD) group. The baseline characteristics of patients are outlined in Table 1. The drained group was comparable with the undrained

**Table 2 Postoperative outcomes of patients undergoing major hepatectomy *n* (%)**

	Drained ( <i>n</i> = 32)	Undrained ( <i>n</i> = 46)	<i>P</i> value
Morbidity	17 (53.1)	27 (58.7)	0.626
Infectious morbidity	13 (40.6)	11 (23.9)	0.116
Intra-abdominal abscess (II-IIIa)	3 (9.4)	2 (4.3)	0.673
Wound infection (I-IIIb)	4 (12.5)	4 (8.7)	0.869
Cholangitis (II)	1 (3.1)	2 (4.3)	1.000
Sepsis (IVa-V)	2 (6.3)	1 (2.2)	0.747
Lung infection (II)	6 (18.8)	5 (10.9)	0.325
Noninfectious morbidity	10 (31.3)	22 (47.8)	0.143
Liver failure (II-V)	3 (9.4)	6 (13)	0.890
Bile leak			
Remnant liver <sup>1</sup> (II-IIIa)	2 (6.3)	4 (8.7)	1.000
Anastomotic leak <sup>2</sup> (II-IIIb)	1 (3.1)	2 (4.3)	1.000
Abdominal collection (I-IIIa)	6 (18.8)	9 (19.6)	0.928
Gastrointestinal bleeding (IIIa-V)	0	2 (4.3)	0.510
Abdominal bleeding (II-IIIb)	1 (3.1)	2 (4.3)	1.000
Respiratory failure (IVa)	0	3 (6.5)	0.265
Renal failure (IVa-V)	3 (9.4)	4 (8.7)	1.000
Mortality (V)	3 (9.4)	2 (4.3)	0.673
Reoperation	2 (6.3)	3 (6.5)	1.000
Postoperative hospital stay (d)	16.5 (13.5-20.5)	15 (12-18)	0.221

Clavien-Dindo grades of surgical complications are within parentheses.

<sup>1</sup>From remnant liver; <sup>2</sup>From hepaticojejunostomy.

group with regards to age, sex, body mass index, comorbidity and previous history of abdominal surgery ( $P > 0.05$  for all). Nine patients in the PBD group had previous abdominal surgery that included 5 cholecystectomies and 4 common bile duct explorations with T-tube drainage. Twelve patients in the undrained group had previous abdominal surgery, which included 6 appendectomies, 4 cholecystectomies and 2 cholecystectomies with common bile duct exploration. Furthermore, there was no significant difference in the TBIL levels at admission between the drained and undrained groups ( $294.2 \pm 135.7$  *vs*  $254.0 \pm 63.5$ ,  $P = 0.126$ ).

### PBD techniques and liver function tests

In the PBD group, 23, 5 and 4 patients underwent PTCD, ENBD, and surgical drainage, respectively. In this study, 4 patients underwent surgical drainage through laparotomy and T-tube placement at the referring hospitals. No patient in this study underwent EBS. Six patients underwent PTCD twice each as a result of previous inadequate drainage. Drainage-related complications occurred in 8 patients (10.3%), with 3 cases of cholangitis and 4 of hemobilia following PTCD, and 1 case of hyperamylasemia following ENBD. All these adverse events were resolved after symptomatic treatment alone before surgery. The mean time between insertion of a biliary drainage catheter preoperatively and surgical resection was  $15.3 \pm 3.4$  (d). PBD significantly improved liver function as evidenced by reduced TBIL ( $294.2 \pm 135.7$  *vs*  $108.1 \pm 60.6$ ,  $P = 0.000$ ), direct bilirubin (DBIL) ( $231.8 \pm 87.0$  *vs*  $85.2 \pm 57.4$ ,  $P = 0.000$ ), aspartate aminotransferase (AST) ( $132.1 \pm 68.6$  *vs*  $86.1 \pm 35.8$ ,  $P = 0.000$ ), alanine aminotransferase (ALT) ( $123.2 \pm 79.1$  *vs*  $97.5 \pm 62.4$ ,  $P = 0.004$ ),

gamma-glutamyl transpeptidase (GGT) ( $531.2 \pm 434.7$  *vs*  $357.6 \pm 268.3$ ,  $P = 0.000$ ) and alkaline phosphatase (ALP) ( $502.1 \pm 356.2$  *vs*  $343.5 \pm 187.6$ ,  $P = 0.001$ ), although albumin (ALB) ( $36.7 \pm 4.8$  *vs*  $34.8 \pm 5.9$ ,  $P = 0.213$ ) levels remained unchanged.

### Perioperative details

All patients in both groups had hilar bile duct resection. There were no significant differences in operation procedure (liver resection) between the two groups. Also, there were no significant differences between the drained and undrained groups in terms of caudate lobectomy, pedicle clamping, portal vein resection, hepatic artery resection, number of patients requiring blood transfusions and intraoperative blood transfusion volume (all  $P > 0.05$ ).

### Postoperative outcomes

Postoperative outcomes are outlined in Table 2. The number of patients with postoperative morbidity in the two groups was comparable (53.1% *vs* 58.7%,  $P = 0.626$ ). No significant difference was found in the number of patients who had either infectious morbidity or non-infectious morbidity. Also, there was no significant difference in the incidence of individual complications. In addition, in a subgroup analysis (data not shown in table), there was a higher morbidity (84.6% *vs* 35.7%,  $P = 0.028$ ) in patients undergoing right-sided hepatectomy without PBD than patients with PBD. However, in the left-sided hepatectomy group, patients had a higher morbidity (78.9% *vs* 40.6%,  $P = 0.018$ ) in the drained group compared to the undrained group. However, there was no difference in the postoperative hospital stay between the two groups (16.5 *vs* 15,  $P = 0.221$ ). Two patients in the drained group and 3 patients in the undrained group underwent reoperation. There was no significant difference in mortality (9.4% *vs* 4.3%,  $P = 0.673$ ) between the two groups. In the drained group, 1 patient died of multiorgan failure (liver failure and renal failure) while another 2 patients died of septic shock. In the undrained group, one patient died from a massive gastrointestinal bleeding while another 1 patient died of multiorgan failure (liver failure and renal failure).

### Logistic regression analyses

Several variables in this study were analyzed for their association with postoperative morbidity (Table 3). Univariate logistic regression showed that PBD was not a risk factor associated with postoperative morbidity. However, preoperative TBIL  $> 170$   $\mu\text{mol/L}$  ( $P = 0.021$ ), preoperative AST  $> 100$  U/L ( $P = 0.036$ ), Bismuth-Corlette classification ( $P = 0.025$ ) and extended liver resection ( $P = 0.018$ ) were risk factors associated with postoperative morbidity on univariate logistic regression analysis. Furthermore, multivariate analysis identified preoperative TBIL  $> 170$   $\mu\text{mol/L}$  (OR = 13.690, 95%CI: 1.275-147.028,  $P = 0.031$ ), Bismuth-Corlette classification (OR = 0.013, 95%CI: 0.001-0.166,  $P = 0.001$ ) and extended liver resection (OR = 14.010, 95%CI: 1.130-173.646,  $P = 0.040$ ) as three independent risk fac-

**Table 3** The risk factors for postoperative complications *n* (%)

Variable	<i>n</i>	Incidence of complications	Univariate <i>P</i> value	Multivariate	
				OR	<i>P</i> value
Age (yr)					
> 60	38	21 (55.3)	0.842		
≤ 60	40	23 (57.5)			
Sex					
Male	49	25 (51)	0.212		
Female	29	19 (65.5)			
PBD					
Yes	32	17 (53.1)	0.626		
No	46	27 (58.7)			
Concomitant diseases					
Yes	20	14 (70)	0.155		
No	58	30 (51.7)			
Previous abdominal surgery					
Yes	21	11 (52.4)	0.663		
No	57	33 (57.9)			
Preoperative TBIL					
> 170 μmol/L	48	32 (66.7)	0.021	13.690 (1.275-147.028)	0.031
≤ 170 μmol/L	30	12 (40)			
Preoperative AST					
> 100 U/L	47	31 (66)	0.036	1.138 (0.157-8.225)	0.898
≤ 100 U/L	31	13 (41.9)			
Preoperative ALT					
> 100 U/L	44	29 (65.9)	0.054	5.664 (0.595-53.905)	0.131
≤ 100 U/L	34	15 (44.1)			
Preoperative ALB					
> 35	41	26 (63.4)	0.189		
≤ 35	37	18 (48.6)			
Bismuth–Corlette stage					
I and II	24	9 (37.5)	0.025	0.013 (0.001-0.166)	0.001
III and IV	54	35 (64.8)			
Extended liver resection					
Yes	12	11 (91.7)	0.018	14.010 (1.130-173.646)	0.04
No	66	33 (50)			
Caudate lobectomy					
Yes	20	12 (60)	0.707		
No	58	32 (55.2)			
Pedicle clamping					
Yes	43	22 (51.2)	0.300		
No	35	22 (62.9)			
Vascular resections					
Yes	19	13 (68.4)	0.225		
No	59	31 (52.5)			
Additional surgery					
Yes	6	3 (50)	0.742		
No	72	41 (56.9)			
Intraoperative blood transfusion					
Yes	35	19 (54.3)	0.644		
No	43	25 (59.5)			

TBIL: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALB: Albumin; PBD: Preoperative biliary drainage.

tors for postoperative complications.

### Results of literature search

Fourteen studies were identified<sup>[5,10,14,18-20,31-37]</sup> using the defined search strategy (Table 4). Seven studies included patients who had curative resections only<sup>[5,14,18,20,31,33,37]</sup>, while the remaining studies included both curative and palliative resection groups.

## DISCUSSION

Currently, the only curative treatment for HCCA is radical

surgical resection<sup>[3]</sup>. Patients with HCCA usually present with concomitant obstructive jaundice, which results in high surgical morbidity and mortality in those undergoing major hepatic resection<sup>[38,39]</sup>. Furthermore, postoperative liver failure is a common cause of in-hospital death after major hepatectomy in patients with obstructive jaundice<sup>[13,40]</sup>. PBD offers the advantage of being able to increase the tolerance of cholestatic liver to ischemia, improve the regeneration capacity of the liver and decrease blood loss, which may contribute to reducing morbidity and mortality. However, there were conflicting conclusions from various studies with regards to the benefits of

**Table 4** Studies including resections for hilar cholangiocarcinoma with and without preoperative biliary drainage *n* (%)

Ref.	Year	Country	Design	Type of PBD	Surgical procedures for included patients	PBD	<i>n</i>	Morbidity	<i>P</i> value	Mortality	<i>P</i> value
Su <i>et al</i> <sup>[10]</sup>	1996	China	Retro	PTCD	CR and PR	Yes	33	17 (51.5)	NS	5 (15.2)	NS
Takada <i>et al</i> <sup>[37]</sup>	1996	Japan	Retro	PTCD	CR	No	16	6 (37.5)	-	0	NS
						Yes	24	NA		3 (12.5)	
Hochwald <i>et al</i> <sup>[19]</sup>	1999	United States	Pro	PTCD	CR and PR	No	12	NA	0.045	6 (50)	NS
						Yes	42	36 (85.7)		2 (4.8)	
Figuera <i>et al</i> <sup>[31]</sup>	2000	Spain	Retro	PTCD	CR	No	29	19 (65.5)	NS	4 (14.3)	NS
						Yes	11	11 (100)		1 (9)	
Parks <i>et al</i> <sup>[36]</sup>	2000	United Kingdom	Retro	PTCD	CR and PR	No	9	6 (66)	NS	2 (22.2)	NS
						Yes	20	11 (55)		1 (5)	
Gerhards <i>et al</i> <sup>[5]</sup>	2000	The Netherlands	Retro	PTCD	CR	No	27	11 (40.7)	NS	1 (3.7)	NS
						Yes	93	59 (63)		16 (17)	
Dinant <i>et al</i> <sup>[33]</sup>	2006	The Netherlands	Retro	PTCD	CR	No	18	13 (72)	NS	3 (17)	NS
						Yes	83	56 (67.5)		14 (16.7)	
Li <i>et al</i> <sup>[32]</sup>	2009	China	Retro	PTCD	CR and PR	No	14	6 (42.9)	NS	2 (14.3)	NS
						Yes	55	20 (36.3)		4 (7.3)	
Ferrero <i>et al</i> <sup>[18]</sup>	2009	Italy	Retro	PTCD	CR	No	56	16 (28.6)	NS	5 (8.9)	NS
						Yes	30	21 (70)		1 (3)	
Ercolani <i>et al</i> <sup>[34]</sup>	2010	Italy	Retro	PTCD	CR and PR	No	30	19 (63)	NS	3 (10)	NS
						Yes	44	25 (56.8)		NA	
El-Hanafy <i>et al</i> <sup>[20]</sup>	2010	Egypt	Retro	PTCD	CR	No	7	2 (28.5)	0.001	NA	NS
						Yes	46	27 (58.6)		5 (10.8)	
Yu <i>et al</i> <sup>[35]</sup>	2012	China	Retro	PTCD with bile re-infusion	CR and PR	No	54	11 (20.3)	0.036	3 (5.5)	NS
						Yes	48	14 (29.2)		1 (2.1)	
Farges <i>et al</i> <sup>[14]</sup>	2013	France and Belgium	Retro	PTCD	CR	No	39	20 (51.3)	NS	2 (5.1)	NS
						Yes	180	123 (68.3)		17 (9.4)	
Present study		China	Retro	PTCD	CR	No	186	128 (68.8)	NS	22 (11.8)	NS
						Yes	32	17 (53.1)		3 (9.4)	
				EBD		No	46	27 (58.7)		2 (4.3)	
				SD							

Retro: Retrospective; Pro: Prospective; PBD: Preoperative biliary drainage; PTCD: Percutaneous transhepatic cholangio-drainage; EBD: Endoscopic biliary drainage; SD: Surgical drainage; CR: Curative resection; PR: Palliative resection; NA: Not available; NS: Not significant.

#### PBD<sup>[14,19,20,31,35,41]</sup>

In our study, the two groups were comparable with respect to demographics, BMI, comorbidities and serum TBIL levels at admission. PBD-associated complications were low, occurring in 8 patients (10.3%), which may be because EBS was not used in our study<sup>[42]</sup>. This also illustrates that the drainage techniques and technology used at our center are feasible. The number of patients undergoing PVE before surgery was comparable between the drained and undrained groups. PBD followed by PVE prior to major hepatectomy is considered a safe management strategy<sup>[24-26]</sup>.

The number of patients with postoperative complications was comparable, with 17 patients (53.1%) in the drained group and 27 patients (58.7%) in the undrained group; there was no significant difference in the number of patients who had either infectious complications or non-infectious complications. We found this result to be consistent with most of the studies that were reviewed in our systematic search. Most recently, a multicenter European study by Farges *et al*<sup>[14]</sup> reported that there was no significant difference in the rate of complications between drained and undrained groups of patients undergoing major liver resection. However, the infectious complications were not compared in this study and the risk factors for the overall complications were not ana-

lyzed. In our study, there was no significant difference in the number of patients with infectious complications between the two groups, which might be because most of them underwent PTCD (71.9%) as compared to endoscopic techniques (ENBD-15.6% and EBS-0%). EBS in particular has been shown to increase the infectious complication rate as compared to other drainage procedures<sup>[18,23,42,43]</sup>. Three studies<sup>[18-20]</sup> from our search reported higher infectious complications in patients who underwent PBD. Four studies<sup>[5,18,19,31]</sup> reported no significant difference in the non-infectious complication rates between the two groups. In our study, patients undergoing right-sided hepatectomy without PBD had a higher morbidity than patients with PBD, whereas contrary results were obtained in the left-sided hepatectomy group. This is consistent with the study carried out by Farges *et al*<sup>[14]</sup>. Furthermore, while some studies have reported a longer stay in the drained group<sup>[20,31]</sup>, other studies have shown no difference between the two groups<sup>[18,19]</sup>. In our study, there was no difference in the postoperative hospital stay, reoperation rate and mortality between the two groups.

While PBD was not a risk factor for postoperative complications, preoperative TBIL > 170  $\mu$ mol/L, a higher Bismuth-Corlette classification and extended liver resection were found to be three independent risk factors for postoperative complications. In our study,



PBD reduced the preoperative serum bilirubin level and other liver function indexes significantly as compared to those on admission. However, this did not translate into a significant reduction in the occurrence of postoperative complications, as compared to the undrained group. Previous studies have shown that preoperative bilirubin levels influence postoperative morbidity and mortality rates<sup>[18,34]</sup>. However, there is no consensus on the serum bilirubin cut-off level before surgery at which PBD should be undertaken. Some studies recommend undertaking PBD at a bilirubin cut-off of 51.3  $\mu\text{mol/L}$  (3 mg/dL) to minimize complications following major surgery<sup>[11,12]</sup>. Other studies recommend a bilirubin cut-off of more than 85.5  $\mu\text{mol/L}$  (5 mg/dL)<sup>[13]</sup>. The serum bilirubin level prior to surgery was  $108.1 \pm 60.6 \mu\text{mol/L}$  in our study. Farges *et al*<sup>[14]</sup> advised that major hepatectomy for jaundiced patients should be delayed until the serum bilirubin level had fallen below 50  $\mu\text{mol/L}$ . Other studies have suggested that PBD should be performed and surgery should be delayed when the preoperative bilirubin level was higher than 171  $\mu\text{mol/L}$  (10 mg/dL)<sup>[10,34]</sup>. Koyama *et al*<sup>[15]</sup> advised that adequate recovery of hepatic function depended not only on the duration of obstructive jaundice prior to decompression, but also on the duration of biliary decompression. Some studies have suggested 3-6 wk of preoperative drainage for obstructive jaundice, with even longer periods proposed with a prolonged biliary obstruction before decompression<sup>[8,15,16]</sup>. In our study, the PBD catheter remained *in situ* for a mean of 15.3 d. In light of the above, it is plausible that postoperative outcomes may have improved further, had we kept the PBD catheter *in situ* longer with a lower preoperative serum bilirubin level. However, we recommend PBD, prior to major hepatectomy, in patients with HCCA with a TBIL above 170  $\mu\text{mol/L}$ .

Gerhards *et al*<sup>[5]</sup> had reported a higher Bismuth-Corlette classification was associated with postoperative morbidity. Also Li *et al*<sup>[32]</sup> reported that while PBD alleviated liver injury caused by hyperbilirubinemia, it did not decrease the postoperative morbidity and mortality and concomitant hepatectomy and Bismuth-Corlette classification were independent risk factors linked to surgical risks. This is explainable as a higher Bismuth-Corlette classification warrants a more extensive surgical resection, which resulted in higher morbidity<sup>[44]</sup>. Indeed, in our study, there were many patients with stage III and IV tumors who underwent extended hepatectomy with caudate lobe resection and vascular resection.

We acknowledge the limitations of our study. First of all, our results derive from a retrospective study and are unavoidably subject to selection bias although a consecutive series was reported. Second, the sample size is relatively small, coming from a single center. Moreover, various factors such as the variable procedures for biliary drainage, treatment of patients at other centers prior to transfer to our center and failure of the initial drainage procedure may have contributed to biases in our study. However, as the baseline characteristics of patients prior to surgery were comparable between the drained and

undrained groups, we hope that the effect of these factors on postoperative outcome was minimized. Currently, there continues to be a lack of consensus and recommendations on the use of PBD prior to major liver resection for HCCA. This has been highlighted by our study and review of literature. While an adequately powered randomized controlled trial at a single center may be currently unrealistic, in view of the rarity of this tumor, a multicenter study would go a long way in informing future practice.

In summary, short-term postoperative outcomes after major liver resection for HCCA are not improved by PBD, which is consistent with most of published evidence. Preoperative TBIL > 170  $\mu\text{mol/L}$  and Bismuth-Corlette classification and extended liver resection might be three independent risk factors for postoperative complications. There is a need to undertake multicenter studies to inform future practice.

## COMMENTS

### Background

Whether preoperative biliary drainage (PBD) should be used in jaundiced patients with hilar cholangiocarcinoma (HCCA) undergoing major liver resection remains unclear.

### Research frontiers

To investigate the role of PBD in patients with HCCA undergoing major liver hepatectomy using prospectively maintained database from a specialty center. A retrospective comparative analysis was performed comparing the perioperative and short-term postoperative outcomes of patients with PBD or not.

### Innovations and breakthroughs

Based on the study, PBD does not improve short-term postoperative outcomes in patients with HCCA undergoing major liver resection. However, Preoperative total bilirubin (TBIL) > 170  $\mu\text{mol/L}$ , Bismuth-Corlette classification and extended liver resection are three independent risk factors for postoperative complications.

### Applications

The advantages of PBD was not found in this study; however, higher preoperative TBIL (> 170  $\mu\text{mol/L}$ ) was indeed a risk factor for postoperative complications. In addition, taking into account the nature of a retrospective study, there is a need to undertake well-designed, prospective multicenter studies to inform future practice.

### Terminology

PBD is an important method for recovery of liver function in patients with obstructive jaundice, which includes the percutaneous transhepatic cholangiodrainage, endoscopic biliary stenting, endoscopic nasobiliary drainage and surgical drainage.

### Peer review

This well-written study investigated the short-time postoperative outcomes and risk factors in jaundiced patients as result of HCCA with PBD or not. It may be of interest for hepatobiliary surgeons worldwide.

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## Vascular resection in pancreatic adenocarcinoma with portal or superior mesenteric vein invasion

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### Abstract

**AIM:** To evaluate long-term survival after the Whipple operation with superior mesenteric vein/portal vein resection (SMV/PVR) in relation to resection length.

**METHODS:** We evaluated 118 patients who underwent the Whipple operation for pancreatic adenocarcinoma at our Department of Hepatobiliary Pancreatic Surgery between 2005 and 2010. Fifty-eight of these patients were diagnosed with microscopic PV/SMV invasion by frozen-section examination and underwent SMV/PVR. In 28 patients, the length of SMV/PVR was  $\leq 3$  cm. In the other 30 patients, the length of SMV/PVR was  $> 3$  cm. Clinical and survival data were analyzed.

**RESULTS:** SMV/PVR was performed successfully in 58 patients. There was a significant difference between the two groups (SMV/PVR  $\leq 3$  cm and SMV/PVR  $> 3$  cm) in terms of the mean survival time (18 mo vs 11 mo) and the overall 1- and 3-year survival rates (67.9% and 14.3% vs 41.3% and 5.7%,  $P < 0.02$ ). However, there was no significant difference in age (64 years vs 58 years,  $P = 0.06$ ), operative time (435 min vs 477 min,  $P = 0.063$ ), blood loss (300 mL vs 383 mL,  $P = 0.071$ ) and transfusion volume (85.7 mL vs 166.7 mL,  $P = 0.084$ ) between the two groups.

**CONCLUSION:** Patients who underwent the Whipple operation with SMV/PVR  $\leq 3$  cm had better long-term survival than those with  $> 3$  cm resection.

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**Key words:** Pancreatic adenocarcinoma; Whipple operation; Vascular resection

**Core tip:** Pancreatic adenocarcinoma can infiltrate the portal vein (PV) or superior mesenteric vein (SMV). In order to achieve negative surgical margins, the Whipple operation combined with SMV/PV resection (SMV/PVR) is usually performed. The long-term survival rate of patients with SMV/PV involvement in relation to the length of resection remains controversial.

Pan G, Xie KL, Wu H. Vascular resection in pancreatic adenocarcinoma with portal or superior mesenteric vein invasion. *World J Gastroenterol* 2013; 19(46): 8740-8744 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8740.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8740>

### INTRODUCTION

Pancreatic adenocarcinoma is a malignant neoplasm that is one of the most common causes of cancer-related death. Unfortunately, there are no symptoms in the early period of the disease, so fewer patients have the chance of achieving negative margin resection. The reason for the lower treatment rate is that many patients have liver metastases, lymph node involvement, invasion of retroperitoneal tissue, and portal vein (PV)/superior mesenteric vein (SMV) invasion when they are diagnosed<sup>[1-4]</sup>. Since the Whipple operation combined with SMV/PV resection (SMV/PVR) and reconstruction for pancreatic adenocarcinoma was first reported in 1951<sup>[5]</sup>, the value



of SMV/PVR has remained controversial<sup>[6,7]</sup>. In the past, tumor invasion of the PV/SMV was considered a contraindication to tumor resection because of the high rate of recurrence and poor prognosis. Recently, some departments have argued that combination of the Whipple operation with SMV/PVR can achieve similar long-term survival to the Whipple operation alone without any increase in morbidity and mortality<sup>[8-10]</sup>.

However, the suitable length of SMV/PVR is under discussion. In this study, we evaluated the outcome in patients who underwent the Whipple operation with SMV/PVR  $\leq 3$  cm compared with  $> 3$  cm. We aimed to clarify long-term survival of patients with SMV/PV invasion in relation to the depth of venous involvement.

## MATERIALS AND METHODS

### Patients and methods

From January 2005 to December 2010, 118 consecutive patients who underwent the Whipple operation for pancreatic adenocarcinoma were analyzed at the Department of Hepatobiliary Pancreatic Surgery, Sichuan University. There were 70 men and 48 women with a median age of 53 years (range, 23-78 years). According to preoperative image evaluation and intraoperative frozen-section examination, 60 patients with pancreatic adenocarcinoma underwent the Whipple operation alone. Twenty-eight patients underwent the Whipple operation combined with SMV/PVR  $\leq 3$  cm. Thirty patients underwent the Whipple operation with SMV/PVR  $> 3$  cm.

### Preoperative evaluation

Preoperative evaluation included a careful physical examination; a series of blood tests such as tumor markers (carcinoembryonic antigen and carbohydrate antigen 19-9), liver function, and thrombin; and chest radiography, abdominal ultrasonography, contrast computed tomography, and electrocardiography. Sometimes magnetic resonance cholangiopancreatography or endoscopic retrograde cholangiopancreatography was selectively performed.

### Indications and operative technique

The exclusion criteria were as follows: (1) extrapancreatic disease such as liver and peritoneal metastases; (2) Whipple operation with SMV/PVR tangential resection; and (3) Whipple operation combined with adjuvant chemotherapy or chemoradiotherapy. We also excluded patients with a previous unsuccessful attempt at pancreatectomy because they could be exposed to different early morbidity or distant prognosis.

The Whipple operation was performed in all of the consecutive patients. Hemigastrectomy was performed, and the bile duct was divided above the cystic duct. An end-to-side pancreaticojejunostomy, end-to-side hepaticojejunostomy, and side-to-side gastrojejunostomy were performed as classic reconstruction after the Whipple operation<sup>[10]</sup>. Vascular consecutiveness was recovered by a direct end-to-end anastomosis. None of the patients in our group used low-molecular-weight heparin after ve-

**Table 1 Comparison of characteristics between the two groups**

Demographics	SMV/PVR $\leq 3$ cm ( <i>n</i> = 28)	SMV/PVR $> 3$ cm ( <i>n</i> = 30)	<i>P</i> value
Sex (M/F)	21/7	20/10	0.082
Age (yr)	64 (range 31-78)	58 (range 38-77)	0.063
Tumor size (cm)	3.1 (range 2-6)	3.7 (range 3-7)	0.051
Tumor stage			0.056
I	5	2	
II	16	6	
III	7	22	
IV	0	0	
Curability			0.067
R0	26	22	
R1+	2	8	
Depth of venous involvement			0.032
Tunica adventitia	4	2	
Tunica media	14	12	
Tunica intima	10	16	
Lymph node invasion	25%	73%	0.043
Operative time (min)	435	477	0.064
Blood loss (mL)	300	383	0.071
Transfusion (mL)	85.7	166.7	0.084

SMV/PVR: Superior mesenteric vein/portal vein resection.

nous reconstruction.

### Statistical analysis

Perioperative data such as pathological data, length of hospital stay, operative blood loss, volume of blood transfusion, morbidity, and mortality were obtained from medical records. The long-term survival outcomes were obtained through postoperative follow-up at outpatient clinics or on the telephone. The outcomes in the two groups were analyzed using the  $\chi^2$  test. All statistical analyses were performed using SPSS version 19.0, when  $P < 0.05$  was considered statistically significant.

## RESULTS

The demographic and operative characteristics of the patients who underwent SMV/PVR  $\leq 3$  cm and  $> 3$  cm are shown in Table 1. The median age of the two groups was 64 years (range, 31-78 years) and 58 years (range, 38-77 years), respectively. The median size of the pancreatic tumors was 3.1 cm (range, 2-6 cm) and 3.7 cm (range, 3-7 cm), respectively. The median length of venous resection was 2.5 cm (range, 1-3 cm) and 3.8 cm (range, 3.5-5 cm), respectively. The mean operation time for patients with SMV/PVR  $\leq 3$  cm and  $> 3$  cm was 435 and 477 min, respectively. The mean blood loss was 300 and 383 mL, respectively. There was no significant difference in operative time, blood loss, transfusion volume, and tumor stage between the two groups. However, there were significant differences in lymph node invasion, depth of venous involvement, and length of SMV/PVR between the two groups. By multivariate analysis, the length of venous resection was the most important prognostic factor.

The postoperative complication and mortality rates

**Table 2** Surgical mortality and morbidity in 58 patients who underwent standard Whipple operation with portal vein resection

Morbidity	SMV/PVR $\leq$ 3 cm ( <i>n</i> = 28)	SMV/PVR > 3 cm ( <i>n</i> = 30)
Hemorrhage	1	2
Hypertension with upper gastrointestinal bleeding	0	1
Pancreatic fistula	2	3
Wound infection	1	1
Reoperation	1	1
Recurrence	21	27
Median hospital stay (d)	18 (range 8-32)	18 (range 11-43)

SMV/PVR: Superior mesenteric vein/portal vein resection.

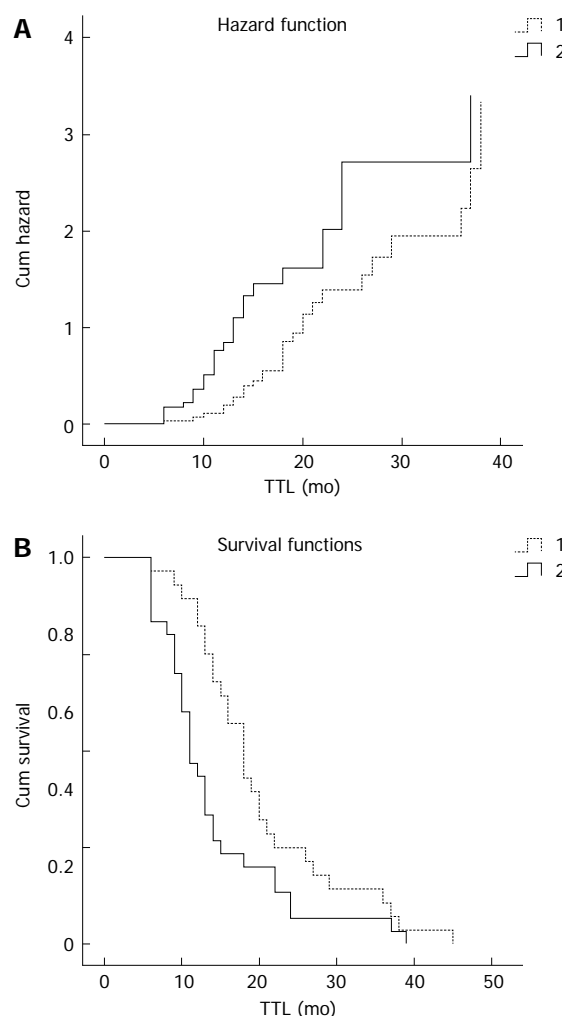
are shown in Table 2. Hemorrhage from the surgical site occurred in three patients after the operation, and one of these died 7 d after surgery. Two patients underwent reoperation. In the patients with SMV/PVR  $\leq$  3 cm, hypertension with upper gastrointestinal bleeding occurred in one patient who underwent spleen vein transection without vascular remodeling.

The overall 1- and 3-year survival rates for patients who underwent the standard Whipple operation combined with SMV/PVR  $\leq$  3 cm (*n* = 28) and SMV/PVR > 3 cm (*n* = 30) were 67.9% and 14.3%, and 41.3% and 5.7%, respectively. The mean survival of patients with SMV/PVR  $\leq$  3 cm and SMV/PVR > 3 cm was 18 and 11 mo, respectively. There was a significant difference in survival between the two groups (Figure 1; *P* = 0.02).

## DISCUSSION

Pancreatic adenocarcinoma is a malignant disease and negative resection margin is still the best treatment option at present. In the past, only 10%-20% of patients with pancreatic adenocarcinoma could undergo surgery because of distant metastases and vascular involvement<sup>[11-13]</sup>. Due to the intimate relationship of the pancreatic head and uncinate, the PV is always infiltrated<sup>[9]</sup>. Surgeons previously considered that pancreatic adenocarcinoma with venous involvement was a contraindication to surgery. They also considered that venous invasion always hindered complete tumor removal. Recent improvements in preoperative imaging and surgical techniques have resulted in the standard Whipple operation with SMV/PVR offering the possibility of achieving negative margin resection in patients with pancreatic adenocarcinoma and SMV or PV involvement, without a relevant increase in morbidity and mortality<sup>[14-16]</sup>. Our study also supports this. However, the suitable length of SMV/PVR is under discussion.

The main conclusion of our retrospective analysis was that patients who underwent the standard Whipple operation with SMV/PVR (Group 1) had similar survival rates and negative resection margins when compared with patients without PV involvement (Group 2). In our study, the median survival time in Group 1 (*n* = 58) and Group 2 (*n* = 60) was 19 and 21 mo, respectively. In addition, the 1- and 3-year survival rates in the two groups were 63.3%



**Figure 1** Survival of patients with portal vein resection. A: Patients with superior mesenteric vein/portal vein resection (SMV/PVR)  $\leq$  3 cm (Dotted line, *n* = 28) had more risk factors compared with patients with > 3 cm resection (solid line, *n* = 30); B: SMV/PVR  $\leq$  3 cm (Dotted line, *n* = 28) was significantly better than > 3 cm resection (solid line, *n* = 30).

and 14.3%, and 69.3% and 18.4%, respectively. There were no significant differences in survival between the two groups (*P* > 0.05). At the same time, the blood loss, volume of transfusion, and surgical mortality and morbidity did not increase obviously in Group 1. However, venous resection combined with reconstruction (Group 1) cost more compared with Group 2, but there was no significant difference in survival between the two groups (*P* > 0.05). Therefore, we considered that patients with SMV/PVR had similar long-term survival to those without SMV/PVR.

Another conclusion is that patients who underwent the Whipple operation with SMV/PVR  $\leq$  3 cm (Group 3) achieved better long-term survival than those with SMV/PVR > 3 cm (Group 4). Median survival time in Group 3 (*n* = 28) and Group 4 (*n* = 30) was 18 and 11 mo, respectively. There was a significant difference in survival between the two groups (*P* < 0.02). Meanwhile, the patients with SMV/PVR  $\leq$  3 cm had more risk factors compared with those with SMV/PVR > 3 cm (*P* < 0.05).

(Figure 1). For example, the ratio of lymph node invasion between the two groups was 25% and 73%, respectively, and this difference was significant ( $P < 0.05$ ). Therefore, we consider that 3 cm is a suitable length of SMV/PVR.

Illuminati *et al.*<sup>[17]</sup> reported that standard Whipple operation combined with PV or SMV resection can be performed when venous involvement does not exceed 2 cm. They have suggested that more complex vein reconstruction could lead to a greater rate of postoperative complications. They have also clarified that 2 cm is the maximal extent that allows one to achieve margin-free resection with simple vascular reconstruction and tension-free anastomosis. However, in our study, we achieved tension-free end-to-end anastomosis by dissociating the root of the SMV when the length of PVR was about 5 cm. Recently, some institutions have suggested that a distance of up to 8 cm can achieve primary anastomosis by this procedure<sup>[6,18]</sup>. Thus, we believe that the main factor influencing the length of venous resection is not the surgical technique itself but the long-term survival. Our study indicated that patients who underwent the Whipple operation with SMV/PVR  $\leq 3$  cm achieved better long-term survival.

Some studies have reported the use of venous interposition graft<sup>[10,19-21]</sup>. They have proposed that SMV/PVR  $> 5$  cm and venous collateral formations should use venous interposition grafting<sup>[14]</sup>. The autogenous venous graft often uses the internal jugular and the greater saphenous veins. The prosthetic venous reconstruction material is usually polytetrafluoroethylene. However, Riediger *et al.*<sup>[6]</sup> considered that the Whipple operation has a risk of abdominal infection, and the use of venous prostheses might increase this complication. In our series, all the patients with PVR had a primary end-to-end reconstruction without any autogenous graft or venous prosthesis.

Shibata *et al.*<sup>[10]</sup> divided SMV/PVR into four types: (1) above and below the level of the splenic vein; (2) above the level of the splenic vein; (3) below the level of the splenic vein; and (4) tangential resection. In our present series, among the 58 patients who underwent the Whipple operation and venous resection, 10 underwent PVR above and below the level of the splenic vein. Among these 10 patients, 4 patients underwent vena lienalis ligatured and transected without splenic vein reconstruction, and six patients had vena lienalis ligation and resection with splenic artery wedge resection in order to reduce the blood flow to the spleen. In the four patients without splenic vein reconstruction, one had local hypertension and upper gastrointestinal bleeding and died in the 14 d after the operation. It has also been reported that division of the splenic vein without splenectomy might lead to portal thrombosis<sup>[22-24]</sup>, but this did not occur in our study. Our experience suggests that splenic vein ligatured and transected with splenic artery reconstruction should be performed when the confluence of spleen vein is involved.

Shibata *et al.*<sup>[10]</sup> have proposed that the degree of venous involvement can be divided into three types: no mural invasion, intramural invasion without tunica intima involvement, and transmural invasion. Several documents

have reported that patients with tunica intima infiltration could not obtain good long-term survival<sup>[10,14]</sup>. In our study, no patient survived beyond 8 mo, when the tunica intima was involved.

Bao *et al.*<sup>[23]</sup> have suggested that mesenteric artery involvement  $> 90^\circ$ , as visualized by computed tomography, implies that we cannot achieve disease-free resection. Today, most surgeons agree that tumor invasion of the mesenteric artery is a contraindication to the Whipple operation<sup>[17,25,26]</sup>. It is considered that the mesenteric artery is often encircled by a neural plexus and lymph nodes. Therefore, artery involvement is always combined with neural plexus and lymph node invasion, and it is difficult to achieve a negative resection margin. It has also been reported that patients with positive lymph nodes have worse overall survival than patients without lymph node invasion, and extensive lymphadenectomy and nerve plexus resection might lead to serious diarrhea and poorer quality of life. Therefore, other treatments such as neoadjuvant and adjuvant chemotherapy could be used in patients with arterial invasion.

In conclusion, we showed that patients with pancreatic adenocarcinoma and venous invasion who underwent the standard Whipple operation with SMV/PVR had similar long-term survival than patients without venous involvement. In addition, patients who underwent the Whipple operation with SMV/PVR  $\leq 3$  cm achieved better long-term survival than those with  $> 3$  cm resection.

## COMMENTS

### Background

Pancreatic adenocarcinoma is a malignant neoplasm. Due to the close relationship between the pancreas and the superior mesenteric vein (SMV) and portal vein (PV), pancreatic cancer can infiltrate the PV/SMV.

### Research frontiers

The Whipple operation and SMV/PV resection (SMV/PVR) has been considered the standard operation for patients with pancreatic adenocarcinoma and PV or SMV involvement. However, the long-term survival rate of patients with PV/SMV involvement in relation to the length of SMV/PVR is under discussion.

### Innovations and breakthroughs

The authors studied 118 patients who underwent the Whipple operation for pancreatic adenocarcinoma between 2005 and 2010. Fifty-eight patients were diagnosed with microscopic SMV/PV invasion by frozen-section examination and underwent SMV/PVR. Twenty-eight of these 58 patients underwent SMV/PVR  $< 3$  cm. Thirty patients underwent SMV/PVR  $> 3$  cm. The authors performed this retrospective study to clarify the long-term survival rate of patients with SMV/PV involvement in relation to the length of SMV/PVR by analyzing the clinical and survival data of those 58 patients.

### Applications

Patients who underwent the Whipple operation with SMV/PVR  $\leq 3$  cm achieved better long-term survival than those with SMV/PVR  $> 3$  cm.

### Peer review

The overall contents are interesting with clinical significance. The data from Whipple procedure only group should be included in tables to compare with SM-PVR groups, in particular for the parameters of time to progress or recurrence and overall survival time. In particular, the comparison of overall survival time between Whipple only and Whipple plus SM-PVR groups.

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## Psychometric hepatic encephalopathy score for diagnosis of minimal hepatic encephalopathy in China

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HE were excluded by the West-Haven criteria and a detailed neurological examination. Based on the nomograms of healthy volunteers, the patients were classified as having MHE when their PHES was less than -4.

**RESULTS:** In total, 146 healthy volunteers completed all the PHES tests. Age and education years were confirmed to be predictors of all five tests. In total, 53 patients with liver cirrhosis completed the PHES. Of the patients with liver cirrhosis, 24 (45.3%), 22(41.5%) and 7(13.2%) had Child-Pugh grades A, B and C, respectively. MHE was diagnosed in 26 patients (49.1%). Compared with compensated cirrhotic patients (Child A), decompensated cirrhotic patients (Child B and C) had a higher proportion of MHE (65.5% vs 29.2%). No differences in age and education years were found between the MHE and non-MHE groups. NCT-A and DST were able to diagnose MHE with a sensitivity of 76.9% and a specificity of 96.3% (AUC = 0.866,  $K = 0.735$ ).

**CONCLUSION:** The proportion of MHE is associated with liver function. NCT-A and DST are simple tools that can be used for the diagnosis of MHE in China.

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**Key words:** Cirrhosis; Minimal hepatic encephalopathy; Neuropsychological tests; Psychometric hepatic encephalopathy score; Number connection test; Digit symbol test

### Abstract

**AIM:** To construct normal values for the tests of the psychometric hepatic encephalopathy score (PHES) and to evaluate its usefulness in the diagnosis of minimal hepatic encephalopathy (MHE) among Chinese individuals with cirrhosis.

**METHODS:** The five tests of PHES, number connection test-A (NCT-A), number connection test-B, serial dotting test, line tracing test and digit symbol test (DST), were administered to all enrolled subjects in a quiet room with sufficient light. Cirrhotic subjects with overt

**Core tip:** The psychometric hepatic encephalopathy score (PHES) has been standardized in several countries, but requires further validation in China. The authors aimed to evaluate the usefulness of PHES for the diagnosis of minimal hepatic encephalopathy (MHE) among Chinese patients with liver cirrhosis. In China, the results of the five neuropsychological tests of PHES were influenced by age and educational status. In total, 49.1% of the patients with cirrhosis were classified as

having MHE, and the proportion of MHE was associated with the severity of liver function. Number connection test-A and digit symbol test are simple and useful tools that can be used for the diagnosis of MHE in China.

Li SW, Wang K, Yu YQ, Wang HB, Li YH, Xu JM. Psychometric hepatic encephalopathy score for diagnosis of minimal hepatic encephalopathy in China. *World J Gastroenterol* 2013; 19(46): 8745-8751 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8745.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8745>

## INTRODUCTION

Minimal hepatic encephalopathy (MHE) is a highly prevalent asymptomatic disturbance in patients with liver cirrhosis. MHE is associated with impaired health-related quality of life and driving capability and can predict the development of overt hepatic encephalopathy (OHE)<sup>[1-4]</sup>. MHE is not detectable by routine physical or neurological examinations, and a specific neuropsychological/neurophysiological test is needed for its diagnosis<sup>[5-7]</sup>. The psychometric hepatic encephalopathy score (PHES) is internationally recommended as the gold standard for the diagnosis of MHE<sup>[8,9]</sup>.

The PHES is composed of five tests, number connection test-A (NCT-A), number connection test-B (NCT-B), serial dotting test (SDT), line tracing test (LTT) and digit symbol test (DST). PHES can be used to assess motor speed, motor accuracy, concentration, attention, visual perception, visual-spatial orientation, visual construction and memory<sup>[10]</sup>, which are related to most of neuropsychological impairments in MHE. The PHES has been standardized in several countries, such as Germany, Italy, Spain, India, Korea and Mexico. However, in China further validation is needed. The aims of this study were to construct and validate a dataset of normal values for the PHES in a healthy Chinese population and to evaluate the usefulness of PHES for the diagnosis of MHE among Chinese patients with liver cirrhosis.

## MATERIALS AND METHODS

### Subjects

**Healthy volunteers:** The healthy volunteers that were recruited for the control group included people who visited the Health Promotion Center at the First Affiliated Hospital of Anhui Medical University in Hefei, China, for routine health examinations, and through word-of-mouth referrals. The following exclusion criteria were applied for the control group: (1) Presence of chronic liver diseases, neurological or psychiatric diseases, or other diseases that can affect cognitive function; (2) A past history of chronic liver disease, neurologic or psychiatric disorders; (3) Consumption of psychotropic drugs; (4) Alcohol consumption > 50 g/d within the past 3 mo;

and (5) Inability to read and write.

**Liver cirrhosis group:** Consecutive inpatients from the Department of Gastroenterology and Hepatology were recruited. Patients with OHE, which was defined according to the West-Haven criteria<sup>[11]</sup>, were excluded. The diagnosis of liver cirrhosis was based on a combination of physical examination, laboratory tests, medical imaging and endoscopic evidence or on liver histology, if available. The following exclusion criteria were applied for the liver cirrhosis group: (1) A history of OHE, upper gastrointestinal hemorrhage or spontaneous bacterial peritonitis during the past 2 wk; (2) Consumption of lactulose, psychoactive drugs or any antibiotics during the past 2 wk; (3) Presence of neurological or psychiatric diseases, such as Alzheimer's disease, Parkinson's disease and nonhepatic metabolic encephalopathy, or a mini-mental status examination (MMSE) score < 25 points; (4) Presence of significant comorbidity, such as heart, respiratory, or renal failure; (5) Presence of hepatocellular carcinoma or other malignancy, previous TIPS or shunt surgery; (6) Alcohol consumption > 50 g/d within the past 3 mo; and (7) Inability to read and write.

All the subjects, both healthy volunteers and patients with liver cirrhosis, were required to have a fair knowledge of numbers and the Chinese alphabet. The research protocol was approved by the ethics committee of the hospital in accordance with the ethical guidelines of the Declaration of Helsinki. Written informed consent to participate was obtained from each subject.

### Neuropsychological tests

All the five tests of PHES were administered to all the enrolled subjects in the same sequence. The tests were conducted on a one-to-one basis in a quiet room with sufficient light. A specially trained medical doctor assisted the enrolled subjects in finishing these tests.

As some of our enrolled subjects were not familiar with the English alphabet, we replaced the alphabet in NCT-B with the Chinese alphabet in the same order<sup>[12]</sup>. The results of the NCT-A, NCT-B, and SDT were measured as seconds, including the time needed to correct any errors, and the result of DST was measured as points. The results of the LTT were measured as both the time needed to complete the test (LTTt, seconds) and as the error score (LTTe),  $LTT = (1 + LTTe/100) \times LTTt$ <sup>[13]</sup>. Accordingly, a higher result of DST equals better performance, and lower results on the other tests equal better performance. Formulas were constructed to predict the expected results of the five neuropsychological tests. These values were then used as references to which the results from the patients with liver cirrhosis were compared.

The result of DST within  $\pm 1$ SD from the mean of the control performance was scored as 0 points. Results between -1 and -2SD, between -2 and -3SD and worse than -3SD were scored as -1, -2 and -3, respectively. A result better than mean + 1SD was scored as +1.

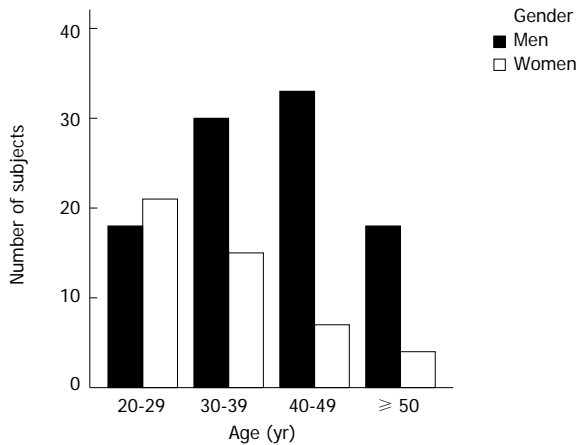


Figure 1 Distribution of volunteers according to age.

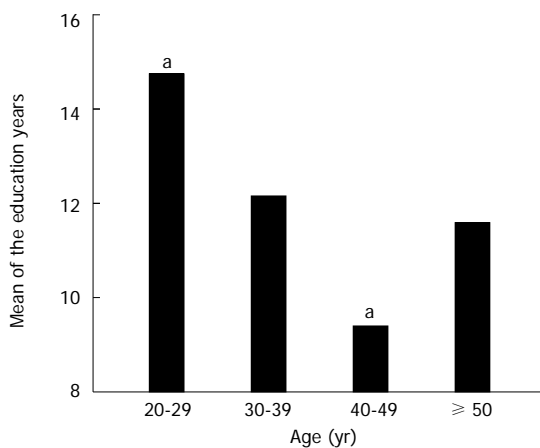


Figure 2 Comparison of education years between healthy volunteers of various age groups ( $^aP < 0.05$ ).

The results (NCT-A, NCT-B, SDT and LTT) within  $\pm 1$  SD from the mean of the control performance were scored as 0 points. Results between  $+1$  and  $+2$ SD, between  $+2$  and  $+3$ SD, and worse than  $+3$ SD were scored as  $-1$ ,  $-2$  and  $-3$  points, respectively. Those better than mean  $-1$ SD were scored as  $+1$  point<sup>[10]</sup>. The final score of PHES was generated from the sum of the scores of five tests, which ranged between  $+5$  and  $-15$ .

### Blood tests and biochemical examinations

On the day of neuropsychological testing, venous blood was taken for routine liver function tests, hematologic parameters and venous ammonia concentration. Venous ammonia was measured within 30 min after blood sampling.

### Statistical analysis

Statistical analyses were performed using the statistical package for the social science (SPSS version 11.0; SPSS, Chicago, IL, United States). Data are expressed as mean  $\pm$  SD or as proportion. ROC analysis was performed with results of NCT-A, NCT-B and DST comparing to PHES. Continuous and categorical variables were

**Table 1** Correlations between psychometric tests and age and education years

	NCT-A	NCT-B	LTT	SDT	DST
Age	0.510	0.478	0.336	0.322	-0.647
Education	-0.409	-0.355	-0.358	-0.374	0.585

The data are presented as Pearson's correlation coefficients;  $P < 0.05$ ; NCT-A: Number connection test-A; NCT-B: Number connection test-B; SDT: Serial dotting test; LTT: Line tracing test; DST: Digit symbol test.

compared using the  $t$  test, the one-way ANOVA test, the Mann-Whitney  $U$ -test and the  $\chi^2$ -test, respectively. Levene's test was used in the evaluation of differences in variance. Non-parametric tests were applied if homogeneity of variance assumptions were not met. Multiple liner regression models were used to predict the value of each test for patients with liver cirrhosis. The difference between the expected and observed results for each test was divided by the corresponding SD of the healthy reference population. Kappa statistics were used to study the agreement between the PHES and NCT-A, NCT-B, DST. A two-sided  $P$  value  $< 0.05$  was considered significant.

## RESULTS

### PHES of healthy volunteers and the relationships between PHES and age and education

Of 154 healthy volunteers who were recruited, 8 were not able to complete NCT-B and as such, only the remaining 146 volunteers were included. The age and education years of the 146 volunteers were  $37.3 \pm 10.5$  (range 20-67) and  $12.0 \pm 4.0$  (range 2-19) years, respectively, and 99 were men (67.8%). The distribution of subjects according to age was as follows: 20-29 years, 39 (26.7%); 30-39 years, 45 (30.8%); 40-49 years, 40 (27.4%); and  $\geq 50$  years, 22 (15.1%) (Figure 1). The education years according to age are presented in Figure 2.

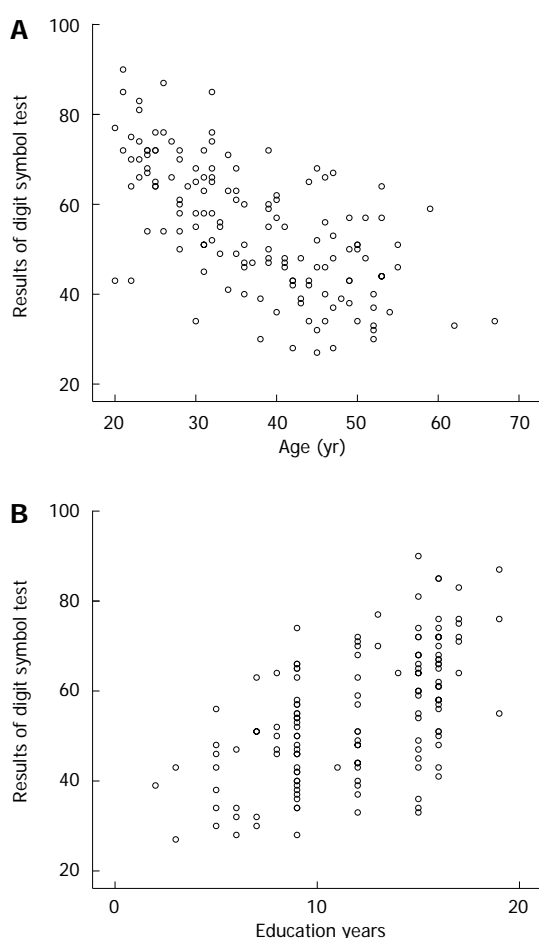
The results of NCT-A, NCT-B, LTT, SDT and DST were  $38.289 \pm 13.694$ ,  $55.846 \pm 17.798$ ,  $33.287 \pm 8.286$ ,  $38.035 \pm 5.774$  and  $55.0 \pm 14.3$ , respectively. The results of the five tests were significantly correlated with age and education, and the Pearson's correlation coefficients are shown in Table 1. In all age categories, the results of all five tests were not significantly correlated with gender ( $P > 0.05$ ). The variables that affected the results of a neuropsychological test were included in the multiple liner regression models, and the final formulas are shown in Table 2. As shown in Table 2, age and education years were predictors of the results of the five neuropsychological tests in healthy volunteers. As shown in Figure 3, younger age and better education were associated with better DST results.

In the healthy volunteer group, the score of PHES was not correlated with education years ( $P = 0.992$ ) or age ( $P = 0.595$ ). Additionally, the PHES did not differ between men and women ( $P = 0.589$ ).

**Table 2** Equations for predicting test results from age and education years

Test	Equation	SD
NCT-A	$27.861 + 0.548 \times \text{age} - 0.821 \times \text{education}$	7.581
NCT-B	$42.816 + 0.672 \times \text{age} - 0.971 \times \text{education}$	9.173
SDT	$38.937 + 0.113 \times \text{age} - 0.423 \times \text{education}$	2.408
LTT	$33.242 + 0.182 \times \text{age} - 0.559 \times \text{education}$	3.455
DST	$63.020 - 0.672 \times \text{age} + 1.421 \times \text{education}$	10.608

NCT-A: Number connection test-A; NCT-B: Number connection test-B; SDT: Serial dotting test; LTT: Line tracing test; DST: Digit symbol test; Age and education are expressed in years.



**Figure 3** Distribution of the results from the digit symbol test in healthy volunteers according to age (A) and education years (B).

### Factors associated with MHE

Of 56 inpatients with liver cirrhosis that were enrolled, 3 were not able to complete NCT-B and thus were not considered further. All tests of the PHES were completed by 53 patients with cirrhosis whose age and education years were  $45.6 \pm 8.2$  years (range 27-62) and  $8.2 \pm 3.6$  years (range 0-15), respectively. The study group comprised 50 (94.3%) men.

The score of PHES in the healthy volunteer group was  $-0.6 \pm 3.7$  (median, 0; range -11 to +5). The score of PHES in the liver cirrhosis group was  $-5.6 \pm 4.9$  (median, -4; range -13 to +4), significantly lower than that in the

**Table 3** Clinical characteristics of patients with liver cirrhosis

	MHE	Non-MHE		P value
Age (yr)	$45.3 \pm 8.0$	$45.9 \pm 8.5$	$t = 0.289$	0.774
Education (yr)	$8.3 \pm 4.4$	$8.4 \pm 2.8$	Mann-Whitney $U = 348.000$	0.956
Ammonia ( $\mu\text{mol/L}$ )	$74.2 \pm 64.2$	$52.9 \pm 24.2$	$t = 1.086$	0.288
Child-Pugh grade			$\chi^2 = 6.943$	0.008
Child A	7	17		
Child B/C	19	10		
Esophageal varices				0.584 <sup>1</sup>
With esophageal varices	15	19		
Without esophageal varices	2	1		
HBV			$\chi^2 = 0.048$	0.827
HBV positive	19	19		
HBV negative	7	8		
Antiviral therapy				$> 0.05^1$
With antiviral therapy	4	4		
Without antiviral therapy	15	15		

<sup>1</sup>By Fisher's exact test; MHE: Minimal hepatic encephalopathy; HBV: Hepatitis B virus.

volunteer group (Mann-Whitney  $U = 1476.00$ ,  $P = 0.000$ ). In the healthy volunteer group, the lower boundary of the 95% range between mean - 2SD and mean + 2SD was -4.0. Using a cutoff for MHE of  $< -4$ , 26 of the 53 patients with liver cirrhosis were diagnosed with MHE (49.1%).

The proportion of patients with MHE increased with the increase in the Child-Pugh grade. Specifically, 7 had Child-Pugh grade A (7/24, 29.2%), 14 had Child-Pugh grade B (14/22, 63.6%) and 5 had Child-Pugh grade C (5/7, 71.4%). Compared with compensated cirrhotic patients (Child A), decompensated cirrhotic patients (Child B and C) had a higher proportion of MHE (19/29 *vs* 7/24;  $\chi^2 = 6.943$ ,  $P = 0.008$ ). No differences in age and education years were found between the MHE and non-MHE groups ( $P > 0.05$ ). Venous ammonia concentration was measured in 26 cirrhotic patients and was found to be similar between the MHE and non-MHE groups ( $t = 1.086$ ,  $P = 0.288$ ). In 37 patients with cirrhosis who underwent endoscopic examination, the prevalence of MHE was not associated with esophageal varices ( $P = 0.584$  by Fisher's exact test). In total, 38 of 53 cirrhotic patients were hepatitis B virus (HBV) positive, while 15 were HBV negative. The prevalence of MHE was similar between the HBV positive and negative groups ( $\chi^2 = 0.048$ ,  $P = 0.827$ ) and the prevalence of MHE was not influenced by antiviral therapy. Table 3 shows the characteristics of patients with or without MHE.

### Comparisons of PHES with NCT-A, NCT-B and DST

International consensus recommends that at least two of the NCT-A, NCT-B, DST and block-design test (BDT) should be used for the diagnosis of MHE<sup>[8]</sup>. Because the BDT is not easy to use, we compared PHES assessment



**Table 4** Comparisons between psychometric hepatic encephalopathy score and number connection test-A, number connection test-B, and digit symbol test

		Sensitivity	Specificity	AUC	K value
Both of the two tests were abnormal	NCT-A + NCT-B	0.538	0.963	0.751	0.505
	NCT-A + DST	0.077	1.000	0.538	0.078
	NCT-B + DST	0.154	1.000	0.577	0.156
All of the three tests were abnormal	NCT-A + NCT-B+DST	0.077	1.000	0.538	0.078
At least one of the two tests was abnormal	NCT-A/NCT-B	0.923	0.741	0.832	0.661
	NCT-A/DST	0.769	0.963	0.866	0.735
	NCT-B/DST	0.769	0.741	0.755	0.510
At least one of the three tests was abnormal	NCT-A/NCT-B/DST	0.923	0.741	0.832	0.661
At least two of the three tests were abnormal	NCT-A/NCT-B/DST	0.615	0.963	0.789	0.582

PHES: Psychometric hepatic encephalopathy score; NCT-A: Number connection test-A; NCT-B: Number connection test-B; DST: Digit symbol test; AUC: Area under the curve.

using NCT-A, NCT-B and DST. Based on the normal range of healthy volunteers, the result of a single test was classified to be abnormal if the score was less than -1 point<sup>[10]</sup>. Using the NCT-A and DST, we were able to diagnose MHE with a sensitivity of 76.9% and a specificity of 96.3% (AUC = 0.866,  $K = 0.735$ ), if at least one of the two tests was abnormal (Table 4).

## DISCUSSION

MHE refers to the cognitive defects in patients with cirrhosis and/or portal-systematic shunting that can be diagnosed after the exclusion of OHE and alternative diagnoses for neuropsychological impairment<sup>[8,11]</sup>. Despite the impact of MHE, most cirrhotic patients are not routinely tested for MHE and remain untreated, because of the lack of standardization of normal values, simple tools and expertise to administer tests<sup>[14]</sup>. Validations of reference norms for neuropsychological tests may increase the likelihood for detection of MHE. The PHES is a neuropsychological test that was specifically designed and recommended for diagnosis of MHE<sup>[8,15]</sup>. The PHES has been validated in Germany, Italy, Spain and other countries. To date, the number of studies focused on the prevalence of MHE in Chinese patients with liver cirrhosis is limited, and the validation of PHES in China is needed. Due to the high prevalence of liver cirrhosis and the impact of MHE, it is important to screen for MHE in China. As such, we sought to construct a normative dataset for PHES in healthy Chinese volunteers and to evaluate the value of PHES in the diagnosis of MHE among Chinese patients with liver cirrhosis. In our study, we found that age and education years were predictors of all five tests included in PHES. However, no differences in age and education years were found between the MHE and non-MHE groups. The proportion of patients with MHE was associated with the severity of liver function.

Age and educational status are widely recognized to be associated with the results of neuropsychological tests and accordingly age- and -education -matched normal values of healthy controls are recommended<sup>[8]</sup>. In the study from Spain, the results of NCT-A and NCT-B were better in males than in females. In our present study,

all five neuropsychological tests of the PHES were influenced by age and education. However, they did not differ between males and females in all age categories. As such, age and education, which affected the results of the neuropsychological tests, were included in the multiple linear regression model and formulas used to establish the expected values. In the healthy volunteer group, the PHES was not affected by age, education and gender. In this study, normative data that were matched for age and education years were used, and no differences were found between patients with and without MHE. Therefore, we conclude that in the Chinese population, age and education influence the neuropsychological tests included in the PHES, but are not associated with the score of PHES and the presence or absence of MHE.

When the cutoff was set at -4, PHES had good sensitivity and specificity for diagnosing MHE<sup>[10]</sup>. This is the same cutoff that was used by the majority of studies focusing on the use of PHES for screening of MHE<sup>[10,12,13,15-21]</sup>. In this study, the lower boundary of the 95% range between mean-2SD and mean+2SD in the volunteer group was -4.0. Accordingly, patients with liver cirrhosis were diagnosed with MHE on the basis of PHES scores lower than -4. MHE was diagnosed in 49.1% of patients with liver cirrhosis. This is similar to a study from India, in which 48% of cirrhotic patients were diagnosed with MHE<sup>[20]</sup>. However, a lower incidence of MHE (25.6%) was reported in a study from Korea<sup>[12]</sup>. One reason might be that the liver function of patients in the studies was different. While 80.6% had Child A in the Korean study, the proportion of Child A in our study and the Indian study were 45.3% and 22.0%, respectively. This higher proportion of Child A may account for the low incidence of MHE diagnosed.

In our study, the proportion of patients with MHE increased with the increase in the Child-Pugh grade as follows: 7 of 24 patients (29.2%) with compensated liver cirrhosis (Child-Pugh grade A) and 19 of 29 patients (65.5%) with decompensated liver cirrhosis (Child-Pugh grades B and C) ( $P = 0.008$ ). This finding is consistent with those of previous studies<sup>[17,22]</sup>. MHE was further confirmed to be affected by liver function. The pathogenesis of HE is multifactorial, and ammonia is considered

an important risk factor<sup>[23]</sup>. However, the relationship between blood ammonia concentration and MHE is still controversial<sup>[12,24-26]</sup>. Ammonia reaches the systemic circulation and accumulates in the central nervous system via esophageal varices<sup>[27]</sup>. In the present study, we found that MHE did not correlate with the presence of esophageal varices and venous ammonia levels. In MHE patients, the blood brain barrier may be breached<sup>[23]</sup>, enabling ammonia to diffuse across the blood-brain barrier into the brain more freely<sup>[28]</sup>. As such, the venous ammonia concentration of patients with MHE may be similar to patients without MHE.

International consensus meetings have recommended the use of the PHES for diagnosing MHE<sup>[8,9]</sup>. The Vienna consensus has also recommended that at least two of four tests (NCT-A, NCT-B, DST and BDT) should be used for the diagnosis of MHE<sup>[8]</sup>. Three of the four tests, NCT-A, NCT-B and DST, have been commonly used for the detection of MHE. The result of a single test was regarded to be abnormal if the result was beyond the 2 SD range of the control norms<sup>[10]</sup>. In some studies, MHE was diagnosed when both of the two tests were abnormal<sup>[1,29,30]</sup>. In others, MHE was diagnosed when at least one of the two tests was abnormal<sup>[24,31,32]</sup>. The present study compared PHES with NCT-A, NCT-B and DST for the diagnosis of MHE. The diagnosis of MHE on the basis of NCT-A and DST showed good agreement with PHES. If at least one of the NCT-A and DST tests was abnormal, MHE could be diagnosed with a sensitivity of 76.9% and a specificity of 96.3% with respect to PHES (AUC = 0.866, K = 0.735). Based on our study, we conclude that NCT-A and DST, which can be completed in minutes, are simple tools for screening MHE among Chinese inpatients with liver cirrhosis.

In summary, the preliminary normal values for all five tests of PHES in Chinese healthy volunteers have been constructed and are influenced by age and educational level. On the basis of a PHES score lower than -4, MHE was detected in 49.1% of the Chinese inpatients with liver cirrhosis. The combination of NCT-A and DST might be a simple and useful tool for the diagnosis of MHE in China.

## COMMENTS

### Background

Minimal hepatic encephalopathy (MHE) is widely prevalent in patients with cirrhosis. MHE is associated with impaired health-related quality of life, driving capability and can predict the development of overt hepatic encephalopathy. MHE is not detectable by routine physical or neurological examinations, and a specific neuropsychological/neurophysiological test is needed.

### Research frontiers

International consensus recommends use of the psychometric hepatic encephalopathy score (PHES) for diagnosing MHE. The PHES has been standardized in Germany, Italy, Spain, India and Korea, but not in China.

### Innovations and breakthroughs

This study constructed normal values for the PHES test in healthy Chinese volunteers and evaluated the usefulness of PHES for the diagnosis of MHE in Chinese patients with liver cirrhosis. In the present study, approximately 49% of patients with liver cirrhosis were classified as MHE. Compared to PHES, NCT-A and DST were able to diagnose MHE with a sensitivity of 76.9% and a specific-

ity of 96.3% (AUC = 0.866, K = 0.735).

### Applications

The results of the five neuropsychological tests of PHES are influenced by age and educational status. Age- and education-corrected nomograms can be used for MHE screening in patients with liver cirrhosis. The proportion of patients with MHE is associated with the severity of liver function. NCT-A and DST are simple and useful tools for the diagnosis of MHE in China.

### Peer review

This is a single-center study from China aiming to validate the use of the PHES for the diagnosis of MHE in cirrhotic patients without overt hepatic encephalopathy. The study, which has created age- and education level-corrected values for the Chinese population, will enable other authors to diagnose MHE in patients with liver cirrhosis and to evaluate interventions.

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## Perirenal space blocking restores gastrointestinal function in patients with severe acute pancreatitis

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### Abstract

**AIM:** To investigate effects of perirenal space blocking (PSB) on gastrointestinal function in patients with severe acute pancreatitis (SAP).

**METHODS:** Forty patients with SAP were randomly allocated to receive PSB or no PSB (NPSB). All the SAP patients received specialized medical therapy (SMT). Patients in the PSB group received PSB + SMT when hospitalized and after diagnosis, whereas patients in the NPSB group only received SMT. A modified gastrointestinal failure (GIF) scoring system was used to assess the gastrointestinal function in SAP patients after admission. Pain severity (visual analog scale, 0 to 100) was monitored every 24 h for 72 h.

**RESULTS:** Modified GIF score decreased in both groups during the 10-d study period. The median score decrease was initially significantly greater in the PSB group than in the NPSB group after PSB was per-

formed. During the 72-h study period, pain intensity decreased in both groups. The median pain decrease was significantly greater in the PSB group than in the NPSB group at single time points. Patients in the PSB group had significantly lower incidences of hospital mortality, multiple organ dysfunction syndrome, systemic inflammatory response syndrome, and pancreatic infection, and stayed in the intensive care unit for a shorter duration. However, no difference in terms of operation incidence was found between the two groups.

**CONCLUSION:** PSB could ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and decrease the mortality of SAP patients.

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**Key words:** Perirenal space blocking; Therapeutics; Severe acute pancreatitis; Gastrointestinal function; Prognosis

**Core tip:** This work aims to investigate the effects of perirenal space blocking (PSB) on the gastrointestinal function and clinical outcome of patients with severe acute pancreatitis (SAP). Our results showed that PSB could commendably improve the gastrointestinal dysfunction or failure during the early stage of severe SAP. Moreover, PSB administration could improve prognosis and significantly decrease the hospital mortality of SAP patients.

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## INTRODUCTION

Severe acute pancreatitis (SAP) has two major clinical stages, early and late. The first (early) stage is characterized by systemic inflammatory response syndrome (SIRS) and lasts for 10 d, whereas the second (late) stage is characterized by infectious complications, which account for most deaths in late-stage SAP patients<sup>[1-3]</sup>. SAP patients present symptoms of flatulence, abdominal distention, nausea, and vomiting related to a disturbance in gastrointestinal motility. Bacterial overgrowth in the ileus plays a major role in the pathogenesis of pancreatic infection<sup>[4-7]</sup>. Therefore, amelioration of intestinal dysmotility and stasis during the early period of SAP is important in reducing the risks associated with serious complications. Recent studies show that early enteral nutrition led to significantly lower incidences of multiple organ dysfunction syndrome (MODS), SIRS and pancreatic infection, and relieved intestinal dysmotility<sup>[8]</sup>. Nevertheless, early enteral nutrition is not usually practiced in SAP patients presenting disturbed gastrointestinal motility<sup>[9]</sup>.

Gastrointestinal tract motor dysfunction in a pathological state is probably associated with muscular and neural dysfunction. For this reason, some researchers considered using epidural anesthesia therapy, which can shorten the duration of the postoperative intestinal paralysis, for patients with early-stage SAP<sup>[10,11]</sup>. Peridural anesthesia is also suggested by researchers but this therapy may not be applicable in all patients, and no rigorous, prospective controlled trials have been able to establish this therapy as a recommended treatment option<sup>[12]</sup>.

The perirenal space is filled with fat. In acute pancreatitis, the perirenal fat and the bridging septa can be involved in the direct spread of inflammation<sup>[13,14]</sup>. This conclusion shows the direct relationship between perirenal space and the peripancreatic area. During SAP, an inflammatory exudate containing pancreatic enzymes leaks out from the pancreas, and its action of dissolving tissue inevitably stimulates the rich splanchnic ganglia and plexuses around the pancreas, which causes adverse reactions and reflection in the visceral nervous system and a series of pathophysiological disorders in the viscera, including gastrointestinal tract motor dysfunction. Considering the physiological and anatomical characteristics of the splanchnic nerves and the pancreas, and the pathological characteristics of SAP, the effect of perirenal space blocking (PSB) of a visceral nerve in the pancreatic region using 1% lidocaine on SAP treatment is studied. A simple, low-cost technique that could lead to short-term hospitalization or clinical treatment will be obtained.

## MATERIALS AND METHODS

### Study design

This is a single-center, prospective, and randomized controlled clinical trial. Patients randomly received either PSB or no PSB (NPSB) upon admission.

**Table 1** The modified gastrointestinal failure score

Item	Points		
	0	1	2
Number of FI symptoms	None	1-2	≥ 3
IAP (mmHg)	12	12-20	> 20 or ACS
Endotoxin concentration (pg/mL)	< 10	10-50	> 50
Computed tomography findings	None	Bowel wall thickening or intestinal extension	Bowel wall thickening and intestinal extension

FI: Food intolerance; IAP: Intra-abdominal pressure; ACS: Abdominal compartment syndrome.

### Patients

All adult SAP patients ( $n = 40$ ) admitted within 3 d after the onset of symptoms to the Department of General Surgery, the First Affiliated Hospital of Henan University of Science and Technology, from January 2012 to March 2013 were included in this study. SAP was defined as the presence of one or more local complications (*e.g.*, pseudocyst, necrosis or abscess) and/or organ failure, and acute physiology and chronic health evaluation APACHE II score > 8 according to the widely used Atlanta criteria formulated in 1992<sup>[15]</sup>. The following criteria were used to exclude patients from the treatments: age (18 years old and below, or older than 75 years), pregnancy, evidence of malignancy, known cardiac morbidity including arrhythmia, severe pre-existing liver or kidney disease, leukopenia, allergic asthma, and known allergies. All the SAP patients received specialized medical therapy (SMT) for SAP<sup>[16]</sup>, such as intensive monitoring, oxygen administration, fluid resuscitation, cessation of oral feeding, exocrine pancreatic suppression, and antibiotic prophylaxis. Patients in the PSB group received PSB + SMT upon hospitalization, whereas patients in the NPSB group only received SMT after a definite diagnosis. This study was conducted in accordance with the declaration of Helsinki, with approval from the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology. Written informed consent was obtained from all participants or their first-degree relatives.

### Evaluation protocol for gastrointestinal function

A modified gastrointestinal failure (GIF) scoring system was used to assess the gastrointestinal function in SAP patients. The system combined food intolerance (FI) symptoms, intra-abdominal pressure (IAP), endotoxin concentration and computed tomography findings into a 3-grade score, which is the modified GIF score (Table 1)<sup>[17]</sup>.

### PSB

One Teflon epidural catheter was placed for intermittent perirenal space blockade under local anesthesia after a definite diagnosis. An epidural transfixion pin was used to puncture the right lumbar region of SAP patients and was positioned into the right capsule of the kidney by the vectoring of B-mode ultrasonic diagnostic equip-

ment. Subsequently, the catheter was placed within the perirenal space through the transfixion pin. The external end of the catheter was fixed to the skin of the lumbar region. The patients were allowed to recover by normal and calm breathing, and lidocaine (100 g/L, 0.08 L/8 h) was intermittently injected into the capsule through the catheter. This regimen was administered for 10 d for the PSB group immediately after the diagnosis, and before randomization.

### Data collection

Upon admission, we recorded the baseline data, including age, gender, etiology, diagnoses, and whether SIRS had been diagnosed. The APACHE II scores<sup>[18]</sup> were recorded daily for 1-3 d. C-reactive protein (CRP) level, and serum endotoxin concentration<sup>[19]</sup> were recorded 1, 3, 7 and 10 d after admission. According to the manufacturer's instructions, we measured serum endotoxin with Gram-negative endotoxin detection reagents (Beijing Gold Mountainriver Technology Development Corporation Limited, China). Contrast-enhancement computed tomography (CECT) was performed 1, 3, 7 and 10 d after admission and the computed tomography severity index<sup>[20]</sup> score was calculated thereafter. We also assessed the image of the gastrointestinal tract. IAP was measured using the bladder technique, according to the method recommended by the World Society of Abdominal Compartment Syndrome in 2006<sup>[21]</sup>. For the duration of hospital stay, we recorded the gastrointestinal functional rehabilitation time (including venting and defecation time), the number of patients that received operation, and the number of patients whose clinical course was complicated by systemic and local complications such as MODS or pancreatic infection. The hospital mortality and length of stay were also recorded. We evaluated the gastrointestinal function of the two groups of SAP patients using the modified GIF score upon admission and for the next 3, 7 and 10 d. Patient abdominal pain was recorded daily using a standard visual analog scale (VAS) ranging from 0 ("no pain") to 100 ("unbearable pain")<sup>[22]</sup>.

### Definitions

The following criteria were used to diagnose pancreatic infection: positive bacterial culture of peripancreatic fluid and repeated increases in body temperature<sup>[3,23]</sup>. IAH was defined by a sustained or repeated pathological elevation in IAP  $\geq 12$  mmHg<sup>[21]</sup>. Abdominal compartment syndrome was defined as a sustained IAP  $> 20$  mmHg associated with new organ dysfunction/failure<sup>[21]</sup>. MODS was defined as the dysfunction of two or more organs. Bowel wall thickening was defined as thickness of 3 mm or greater on CECT, and intestinal extension was defined as a dilatation of more than 2.5 cm on CECT<sup>[24,25]</sup>. Enteral feeding started as early as possible, if the patient had no obvious contraindications such as ileus or intestinal bleeding. FI was diagnosed when enteral feeding was unsuccessful and had to be discontinued because of repeated nausea, vomiting, high gastric residual volume,

**Table 2 Patient characteristics upon admission *n* (%)**

	PSB group ( <i>n</i> = 20)	NPSB group ( <i>n</i> = 20)	<i>P</i> value
Age (yr)	43 (34.5-55)	45 (35-60)	0.589
Sex (male: female)	11:9	12:8	0.749
Etiology			
Biliary origin	10 (50)	11 (55)	0.752
Hyperlipidemia	7 (35)	6 (30)	0.736
Alcohol abuse	2 (10)	1 (5)	0.548
Idiopathic	1 (5)	2 (10)	0.548
BMI	24.6 (23.5-26.8)	25.8 (23.9-28.8)	0.158
APACHEII score	9.5 (8.5-11)	10 (8-11.5)	0.994
CRP (mg/L)	203.5 (188-253)	195 (161-247.5)	0.214
Pain $> 77$ mm (VAS)	13	15	0.654

PSB: Perirenal space blocking; NPSB: No perirenal space blocking; BMI: Body mass index; VAS: Visual analogue scale; CRP: C-reactive protein.

abdominal pain or distension, and diarrhea<sup>[19,26,27]</sup>. We counted the frequency of signs for every patient as the number of symptoms of food intolerance.

### Statistical analysis

All the data are presented as median (interquartile range) if not stated otherwise. Categorical variables are expressed as absolute numbers or in percentages, and were analyzed using the  $\chi^2$  test. Continuous variables were compared by the Mann-Whitney *U* test or Wilcoxon signed-rank test, as appropriate. Statistical package for the social sciences (SPSS, version 17.0, Chicago, IL, United States) software was used for statistical analyses. *P*  $< 0.05$  was considered statistically significant.

## RESULTS

### Baseline data of patients

There were no significant differences between the 2 groups with regard to sex distribution, age, body weight, or cause of pancreatitis. The severity of pain, acute physiology and chronic health evaluation APACHE II, and serum CRP did not significantly differ between the two groups. The demographic data and clinical parameters of the patients upon admission are presented in Table 2.

### Effect on pain

During the 72-h study period, pain intensity decreased in both groups. VAS data were depicted as median values (ranges) for the evaluation of pain intensity at specific time points. The median pain decrease (VAS) was significantly greater in the PSB group (-53) than in the NPSB group (-23) at 24 h; -67 than -46 at 48 h; and -76 than -49 at 72 h. Thus, the magnitude of median pain relief was better in the PSB group compared with the NPSB group (Table 3).

### GIF score

During the 10-d study period, modified GIF score decreased in both groups, from 4.56 to 1.00 in the PSB

**Table 3 Pain intensity between two groups**

	Pain severity: Change from baseline ( $\Delta$ VAS)			
	Baseline (VAS)	At 24 h	At 48 h	At 72 h
PSB group ( <i>n</i> = 20)	78	-53	-67	-76
NPSB group ( <i>n</i> = 20)	77	-23	-46	-49
<i>P</i> value	1.000	0.005	0.018	0.025

PSB: Perirenal space blocking; NPSB: No perirenal space blocking; VAS: Visual analogue scale.

**Table 4 Modified gastrointestinal failure score variables between two groups**

	Before PSB performed	Hospital day			
		1 d	3 d	7 d	10 d
PSB group ( <i>n</i> = 20)	4.56	2.6	2.12	1.43	1.000
NPSB group ( <i>n</i> = 20)	4.34	3.98	3.56	2.58	2.13
<i>P</i> value	1.000	0.042	0.025	0.031	0.012

PSB: Perirenal space blocking; NPSB: No perirenal space blocking.

group and from 4.34 to 2.13 in the NPSB group. The median score decrease was initially significantly greater in the PSB group than in the NPSB group ( $P = 0.042$ ) after hospitalization for 24 h (PSB was performed as soon as PSB group patients were admitted). The variance tendency of the modified GIF score in the two groups is presented in Table 4.

### Comparison of outcome variables between the two groups

As presented in Table 5, patients in the PSB group had significantly lower incidences of hospital mortality, MODS, SIRS, pancreatic infection and shorter intensive care unit stay during hospital stay. However, no difference in terms of operation incidence was found between the two groups.

## DISCUSSION

The celiac plexus is a major interchange for autonomic fibers, receiving many of the thoracic splanchnic nerve fibers as they course toward the abdominal organs. Pain associated with pancreatic morbidity is intense and severe, and for many years, the celiac plexus has been a target for pain block treatments<sup>[28]</sup>. The celiac plexus lies in front of the aorta at the level of the celiac trunk<sup>[29]</sup>. It is composed of a dense network of sympathetic nerve fibers that travel in parallel to the anterior surface of the abdominal aorta and the origin of the celiac artery. The celiac plexus transmits neural signals originating from all abdominal viscera and the majority of pelvic viscera, including the pancreas, liver, gallbladder, stomach, renal pelvis, ureter, and intestine proximal to the transverse colon<sup>[30]</sup>.

Both the pancreas and kidney are retroperitoneal organs and are adjacent to each other. In the retroperitoneal

**Table 5 Clinical outcome variables *n* (%)**

	NPSB group ( <i>n</i> = 20)	PSB group ( <i>n</i> = 20)	<i>P</i> value
Hospital mortality	6 (30)	1 (5)	0.037
ICU stay (d)	12 (8-21)	9 (5-14)	0.033
Pancreatic infection	8 (40)	2 (10)	0.028
MODS	9 (45)	3 (15)	0.038
SIRS	14 (70)	7 (35)	0.027
Surgical operation	4 (20)	2 (10)	0.376

ICU: Intensive care unit; MODS: Multiple organ dysfunction syndrome; SIRS: Systemic inflammatory response syndrome; PSB: Perirenal space blocking; NPSB: No perirenal space blocking.

space, the left and right kidneys and their adipose capsules are next to the pancreas, celiac artery, and superior mesenteric artery root. Thorntons' findings show that the perirenal spaces communicate with each other across the midline, and with the pelvic extraperitoneal spaces. Clinical implications include the potential flow of perinephric collections into the pelvis or across the midline<sup>[31]</sup>. This means that the celiac ganglion and plexus, including the plexus pancreaticus, and the renal and superior mesenteric plexuses, are located in the gallery of bilateral perirenal spaces. During SAP, an inflammatory exudate containing pancreatic enzymes leaks out from the pancreas and its action of dissolving tissue inevitably stimulates the rich splanchnic ganglia and plexuses around the pancreas, which causes adverse reactions and reflection in the visceral nervous system and a series of pathophysiological disorders in the viscera, including gastrointestinal tract motor dysfunction.

Considering the physiological and anatomical characteristics of the splanchnic nerves and the pancreas, and the pathological characteristics of SAP, the effect of PSB of a visceral nerve in the pancreatic region using 1% lidocaine on SAP treatment was studied.

Nutrition support is important in the adjunctive management of SAP patients. Meta-analysis shows that in patients with acute pancreatitis, total parenteral nutrition significantly increases the risk of infective complications, the likelihood of a surgical intervention (to control pancreatic infection) and the length of hospital stay, compared with enteral nutrition<sup>[32]</sup>. Nevertheless, early enteral nutrition is not usually practiced in SAP patients presenting disturbed gastrointestinal motility<sup>[9]</sup>.

This clinical study investigated the effects of PSB on the gastrointestinal function and on the clinical outcome of SAP patients. We found that PSB could commendably ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and significantly decrease the hospital mortality of SAP patients.

Recent studies have shown that early enteral nutrition led to significantly lower incidences of MODS, SIRS and pancreatic infection, and relieved intestinal dysmotility<sup>[8]</sup>. Gastrointestinal tract motor dysfunction in a pathological state is probably associated with muscular and neural dys-



function. For this reason, some researchers considered using epidural anesthesia therapy, which can shorten the duration of the postoperative intestinal paralysis<sup>[11]</sup>, for the patients with early-stage SAP<sup>[10]</sup>. In fact, the beneficial effect of epidural anesthesia has been attributed to blockade of a sympathetic nerve, which contributes to the recovery of gastrointestinal tract motor function<sup>[33]</sup>. Peridural anesthesia has also been suggested previously, but this may not be applicable in all patients and no rigorous, prospective controlled trials have been able to establish this therapy as a recommended treatment option<sup>[12]</sup>. Epidural anesthesia can selectively block sympathetic nerve fibers which supply the pancreas, but in clinical practice, this technique is difficult to implement because of the different effects of anesthesia in individuals and the different classes of nerve fibers. The risks include total spinal anesthesia, blood circulation disorders, respiratory inhibition, deep venous thrombosis, and bedsore. For the patients with SIRS, this method may lead to fatal complications such as intraspinal hematoma, and intraspinal infection. PSB can prevent these problems because of its common use for different treatments, including acute anuria, paralytic ileus, stomach cramps, bronchial asthma, postoperative abdominal distension, and burn shock. In clinical work, the technique of PSB is common, safe, simple, low-cost, exempt from B ultrasound guidance, and easy to implement in all hospitals. Furthermore, the manual operation is easy to replicate. There are several limitations in this study. Due to the small sample size and the single-center design, our results might be insufficient to reach a definite conclusion. Therefore, the accuracy should be tested further using a larger sample size. Moreover, since this study was not based on a pathophysiological model, the precise mechanisms of PSB in SAP patients should be verified by more basic experiments.

In conclusion, PSB could commendably ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and significantly decrease the hospital mortality of SAP patients. However, the precise mechanisms of PSB for SAP are still not clear, and further studies are required to verify our conclusions.

## COMMENTS

### Background

Severe acute pancreatitis (SAP) has two major clinical stages, early and late. The first (early) stage is characterized by systemic inflammatory response syndrome (SIRS) and lasts for 10 d, whereas the second (late) stage is characterized by infectious complications, which account for most deaths in late-stage SAP patients.

### Research frontiers

The paper is for the first time investigated the effects of perirenal space blocking (PSB) on gastrointestinal function in patients with SAP.

### Innovations and breakthroughs

PSB could ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and decrease the mortality of SAP patients.

### Peer review

It is a good study, which showed that PSB was associated with a significant

decrement of pain, hospital mortality, multiple organ dysfunction syndrome, SIRS and pancreatic infection in patients with SAP.

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## Association between *TNF- $\alpha$* and *IL-1 $\beta$* genotypes vs *Helicobacter pylori* infection in Indonesia

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### Abstract

**AIM:** To investigate the correlation between the *Helicobacter pylori* (*H. pylori*) infection and host genetic background of healthy populations in Indonesia.

**METHODS:** In March 2007, epidemiological studies were undertaken on the general population of a city in Indonesia (Mataram, Lombok). The participants included 107 men and 187 women, whose ages ranged from 6 to 74 years old, with an average age of 34.0 ( $\pm$  14.4) ( $\pm$  SD). The *H. pylori* of subject by UBT method determination, and through the polymerase chain reaction with confronting two-pair primers (PCR-CTPP) method parsing the single nucleotide polymorphism of interleukin (IL)-8, IL-4, IL-1 $\beta$ , CD14, tumor necrosis factor (TNF- $\alpha$ ) and tyrosine-protein phosphates non-receptor type 11 (PTPN11) genotypes. The experimental data were analyzed by the statistical software SAS.

**RESULTS:** The *H. pylori* infection rates in the healthy Indonesian population studied were 8.4% for men and 12.8% for women; no obvious differences were noted for *H. pylori* infection rates by sex or age. TC genotypes of IL-4, TC and CC genotypes of TNF- $\alpha$ , and GA genotypes of PTPN11, were higher in frequency. Both CC and TC genotype of TNF- $\alpha$  T-1031C loci featured higher expressions in the healthy Indonesian population Indonesia studied of (OR = 1.99; 95%CI: 0.67-5.89) and (OR = 1.66; 95%CI: 0.73-3.76), respectively. C allele of IL-1 $\beta$  T-31C gene locus was at a higher risk (OR = 1.11; 95%CI: 0.70-1.73) of *H. pylori* infection, but no statistical significance was found in our study.

**CONCLUSION:** We reveal that the association between the TNF- $\alpha$  and IL-1 $\beta$  genotypes may be the susceptibility of *H. pylori* in the studied population.

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**Key words:** *Helicobacter pylori*; Tumor necrosis factor; Interleukin-1 $\beta$ ; Infection; Allele

**Core tip:** We found single nucleotide polymorphism of tumor necrosis factor- $\alpha$  and allele of interleukin-1 $\beta$  having high frequency in the healthy Indonesian population, which may be associated with potential contact with *Helicobacter pylori* (*H. pylori*) infection. Throughout, *H. pylori* studies were conducted in patients, and treatment was based on quadruple antibiotics to eradicate *H. pylori* infection in clinical trials. However, the implications of the individual differences in recurrent infections and drug resistance of *H. pylori* and other issues must be addressed. Therefore, vaccine development for prevention of *H. pylori* will be a topical issue in the coming years.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a microaerophilic (G-) bacteria that colonizes the area of stomach and duodenum, causing chronic inflammation of the gastric mucosa, the development of the stomach ulcers and even gastric cancer<sup>[1]</sup>. *H. pylori* is a class I carcinogen, and has been identified by the WHO as a cancer-causing prokaryotes<sup>[2]</sup>. More than 50% of the world's population infected with *H. pylori*, but 80% of people infected with *H. pylori* show no symptoms<sup>[3]</sup>. The *H. pylori* infection occurs mainly in economically underdeveloped regions, and the *H. pylori* infection rates of China, Japan and Korea were higher than developed countries<sup>[4-6]</sup>, while the infection rates of Thailand and Vietnam were higher than Indonesia in Southeast Asia<sup>[7]</sup>. Regarding the ethnic groups of Singapore, *H. pylori* infection and the incidence of digestive diseases was higher in the Indian and Chinese than the Malay population<sup>[8]</sup>. The above studies have shown that *H. pylori* infection and geographical, ethnic and host genetic background is relational, and that the bacteria play a key role in the development of gastric cancer.

Single nucleotide polymorphism (SNP) is caused by a single nucleotide mutation in the genomic level DNA sequence polymorphisms. It is the most common type of genetic variation in humans. Accounting for more than 90% of all known polymorphisms, SNP is widespread in the human genome and there is a close relationship between the incidences of the disease<sup>[9]</sup>. Interleukin-8 (IL-8) is an important regulatory factor in the development of gastritis for *H. pylori* associated infection<sup>[10]</sup>. The interleukin-4 (IL-4) promotes HLA class II antigen expression in

B-cells<sup>[11]</sup>, and IL-1 $\beta$  protein is an important inflammatory mediator, involved in infected *H. pylori* of the stomach inflammation reaction<sup>[12,13]</sup>. Have a study reported that the cluster of differentiation 14 (CD14) is an important receptor in the submission of *H. pylori* lipopolysaccharide (LPS). The relationship is between CD14 with the weakening of the immune response in the body to LPS of *H. pylori* and to reduce the proinflammatory cytokine secretion levels<sup>[14]</sup>. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is involved in inflammation, immune regulation and tissue repair, and the TNF- $\alpha$  is an important factor in the development of digestive diseases<sup>[15]</sup>. The tyrosine-protein phosphates non-receptor type 11 (PTPN11) gene is located in chromosome 12, and it has been found that the expression product of SHP-2 to participate in the cytotoxin-associated protein A (cagA) deformation caused by gastric epithelial cells eventually causes gastric cancer<sup>[16]</sup>.

The purpose of this study was to investigate the correlation of *H. pylori* infection in a healthy Indonesian population and host genetic background, and to reveal susceptibility genes of *H. pylori*, as well as new strategies for the prevention and treatment of gastric cancer.

## MATERIALS AND METHODS

### Study population

In recent years, we have conducted long-term international cooperation in research, exploring the impact of environmental factors on the risk factors of gastric cancer in Southeast Asia, including the countries of Thailand, Vietnam and Indonesia, as well as Gansu Province in China. In March 2007, epidemiological studies were undertaken on the general population of a city in Indonesia (Mataram, Lombok). The participants included 107 men and 187 women, whose ages ranged from 6 to 74 years old, with an average age of 34.0 ( $\pm$  14.4) ( $\pm$  SD). We detected and analyzed the *H. pylori* of the observation target as well as the genetic background of the host, namely the *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* genotypes. All the subjects' informed consent was approved by the Nagoya City University Graduate School of Medical Ethics Committee.

### Urea breath test

*H. pylori* infection was determined by UBT, UBT-IR300 kits (Otsuka Pharmaceutical Co., Tokyo, Japan) with  $\geq$  2.5‰ considered as positive. All subjects were classified as *H. pylori* -positive (+) or -negative (-) in this study<sup>[7,8]</sup>.

### Genotyping of DQA1 and DQB1

A template of genomic DNA was isolated from 100  $\mu$ l of peripheral blood leukocytes by the Nucleic Acid Purification System (MagExtractor MFX-6000 TOYOBO, Japan). We carried out a single nucleotide polymorphism (SNP) analysis of the *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* genotypes by two pairs of polymerase chain reaction (PCR-CTPP)<sup>[17-21]</sup>.

**Table 1** *Helicobacter pylori* infection by sex and age in Indonesian people *n* (%)

Indonesia	<i>H. pylori</i> (+) <i>n</i> = 33	<i>H. pylori</i> (-) <i>n</i> = 261
Sex		
Male	9 (8.4)	98 (91.6)
Female	24 (12.8)	163 (87.2)
Age, yr		
≤ 30	12 (9.6)	113 (90.4)
31-40	9 (11.5)	69 (88.5)
41-50	7 (14.6)	41 (85.4)
51-60	3 (9.4)	29 (90.6)
≥ 60	2 (18.2)	9 (81.8)
Mean age, yr	36.3 ± 14.6 (SD)	33.7 ± 14.4 (SD)

*H. pylori*: *Helicobacter pylori*.

### Statistical analysis

Differences in distribution by age according to prevalence of *H. pylori* infection were examined by *t*-test, while differences in distribution by sex and genotype were assessed with a Chi-square test. Hardy-Weinberg equilibrium was examined for *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* gene polymorphisms. Multi-comparisons for *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* genotypes were made according to the Bonferroni method. Associations of the *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* genotypes and SNP with *H. pylori* infection were examined by OR and 95%CI using unconditional logistic regression analysis. Statistical significance was determined as *P* < 0.05. All the statistical analyses were performed using the SAS software package (version 9.1).

## RESULTS

The positive *H. pylori* infection rate as a whole was 11.2% in Mataram (Table 1). No obvious differences were noted for *H. pylori* infection rates by sex or age. TC genotypes of *IL-4*, TC and CC genotypes of *TNF- $\alpha$* , and GA genotypes of *PTPN11* were frequent. Individuals carrying TC and CC allele of *TNF- $\alpha$*  was noted to be at higher risk of *H. pylori* infection, compared with those carrying TT allele of *TNF- $\alpha$*  (OR = 1.66, 95%CI: 0.73-3.76) and (OR = 1.99, 95%CI: 0.67-5.85). We also found TT and CT genotypes of *CD14* C-159T (OR = 1.09, 95%CI: 0.37-3.20) and (OR = 1.26, 95%CI: 0.50-3.19), but no statistical significance was found in our study (Table 2). We found C allele had a higher frequency than T allele of *IL-1 $\beta$*  genotype in the studied population (OR = 1.11, 95%CI: 0.70-1.73), but again no statistical significance was found (Table 3).

## DISCUSSION

In 50% of the world's population was infected *H. pylori* infection rates in developing countries were higher than in developed countries, and it has been reported that hosts at an early age have been infected<sup>[22]</sup>. Indonesia, located in Southeast Asia, is a developing country, but

**Table 2** Association between *Helicobacter pylori* infection and interleukin 1 $\beta$ , interleukin 4, interleukin 8, CD14, tumor necrosis factor- $\alpha$ , tyrosine-protein phosphates non-receptor type 11 single nucleotide polymorphism in Indonesian people *n* (%)

Polymorphism	<i>H. pylori</i> (+) <i>n</i> = 33	<i>H. pylori</i> (-) <i>n</i> = 261	OR <sup>1</sup>	95%CI <sup>1</sup>
IL-1 $\beta$ T-31C				
TT	8 (24.2)	59 (22.6)	ref	
CC	8 (24.2)	73 (28.0)	0.82	0.29-2.32
TC	17 (51.5)	129 (49.4)	1.05	0.42-2.59
TC/CC	25 (75.8)	202 (77.4)	0.96	0.41-2.26
IL-4 T-33C				
TT	15 (45.5)	128 (49.0)	ref	
CC	2 (6.1)	19 (7.3)	0.83	0.17-3.99
TC	16 (48.5)	114 (43.7)	1.24	0.58-2.64
CC/TC	18 (54.5)	133 (51.0)	1.18	0.56-2.45
IL-8 T-251A				
TT	14 (42.4)	98 (37.6)	ref	
AA	8 (24.2)	49 (18.8)	1.25	0.48-3.27
TA	11 (33.3)	114 (43.7)	0.74	0.32-1.72
TA/AA	19 (57.6)	163 (62.5)	0.89	0.42-1.88
CD14 C-159T				
CC	7 (21.2)	65 (24.9)	ref	
TT	8 (24.2)	65 (24.9)	1.09	0.37-3.20
CT	18 (54.6)	131 (50.2)	1.26	0.50-3.19
CT/TT	26 (78.8)	196 (75.1)	1.2	0.50-2.92
TNF- $\alpha$ T-1031C				
TT	11 (33.3)	120 (46.0)	ref	
CC	6 (18.2)	36 (13.8)	1.99	0.67-5.89
TC	16 (48.5)	105 (40.2)	1.66	0.73-3.76
CC/TC	22 (66.7)	141 (54.0)	1.74	0.80-3.76
PTPN11 G/A at intron 3				
GG	17 (51.5)	151 (57.9)	ref	
AA	1 (3.0)	17 (6.5)	0.6	0.07-4.86
GA	15 (45.5)	93 (35.6)	1.49	0.70-3.15
GA/AA	16 (48.5)	110 (42.2)	1.37	0.65-2.85

<sup>1</sup>Odds rate with CI adjusted for age and sex by logistic regression model. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; PTPN11: Tyrosine-protein phosphates non-receptor type 11.

we found that the country has an *H. pylori* infection rate which was very low. We investigated associations between SNP of the host *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* gene polymorphisms and *H. pylori* prevalence in an Indonesian population with an *H. pylori* infection rate of 11.2% in people residing in Mataram, Lombok Island. Although SNP of host *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* genotype with *H. pylori* infection were not found to have statistical significance in our study, we saw that an observation target who had the CC and TC genotype of *TNF- $\alpha$*  gene were at a higher risk of contracting *H. pylori* infection. Perhaps, *TNF- $\alpha$*  gene plays a key role in the *H. pylori* infection process.

*H. pylori* is widely present in the environment, and it can be isolated in surface waters<sup>[23]</sup>, *i.e.*, transmitted by the fecal - oral route<sup>[24]</sup>. Studies have shown that through certain digestive diseases and strains of *H. pylori*, Cytotoxin-associated protein A (cagA) is now known as the most important virulence factors of *H. pylori*<sup>[25]</sup>. CagA is an *H. pylori* cag poison island (cag-PAI) flag, and by cag-PAI coded protein is composed of a bacterial type IV secretion system into gastric epithelial cells, which ultimately



**Table 3** Association between *Helicobacter pylori* infection and allele of interleukin 1 $\beta$ , interleukin 4, interleukin 8, CD14, tumor necrosis factor- $\alpha$ , tyrosine-protein phosphates non-receptor type 11 in Indonesian people *n* (%)

Allele	<i>H. pylori</i> (+) <i>n</i> = 66	<i>H. pylori</i> (-) <i>n</i> = 522	OR <sup>1</sup>	95%CI
IL-1 $\beta$ T-31C				
T	33 (50.0)	247 (47.3)	ref	
C	33 (50.0)	275 (52.7)	1.10	0.70-1.73
IL-4 T-33C				
T	46 (69.7)	370 (70.9)	ref	
C	20 (30.3)	152 (29.1)	0.95	0.58-1.56
IL-8 T-251A				
T	39 (59.1)	310 (59.4)	ref	
A	27 (40.9)	212 (40.6)	0.99	0.62-1.57
CD14 C-159T				
C	32 (48.5)	261 (50.0)	ref	
T	34 (51.5)	261 (50.0)	0.95	0.60-1.49
TNF- $\alpha$ T-1031C				
T	38 (57.6)	345 (66.1)	ref	
C	28 (42.4)	177 (33.9)	0.73	0.46-1.15
PTPN11 G/A at intron 3				
G	49 (74.2)	395 (75.7)	ref	
A	17 (25.8)	127 (24.3)	0.94	0.56-1.57

<sup>1</sup>Odds rate with CI adjusted for age and sex by logistic regression model.  
*H. pylori*: *Helicobacter pylori*; IL: Interleukin; PTPN11: Tyrosine-protein phosphates non-receptor type 11.

causes gastric mucosal epithelium, the morphological changes of the cells and the formation of a hummingbird-like structure<sup>[26]</sup>. Host infected cagA-positive *H. pylori* is less likely to cause digestive diseases, but may damage the gastric mucosal barrier and is cagA related. *H. pylori* infection with strains, geographical, ethnic, and environmental and host genetic background was a correlation.

The IL-8 as a neutrophil chemoattractant and activating factor, which relates to *H. pylori* infection, resulting in second messenger of the mucosal inflammatory response in the *H. pylori* pathogen city, plays an important role of intermediary. But what components of *H. pylori* surface play a major role in the induction of IL-8 expression is still one of the main points about *H. pylori* pathogenesis. Of *H. pylori* cytotoxin-associated protein (cagA) and vacuolating cytotoxin (vacA) on gastric epithelial IL-8 secretion, showing expression of cagA and vacA *H. pylori* strains (vacA+, cagA+) direct stimulation of gastric epithelial cell lines IL-8 mRNA expression and protein secretion of IL-8, suggests that expression of the gene product and cagA *H. pylori* strains induced gastric epithelial expression of IL-8 in the main factors<sup>[11]</sup>. In addition to *H. pylori* gastric epithelial cells directly stimulating the production of IL-8, the inflammation locally produced of TNF- $\alpha$ , transcription factor activation of the IL-1, was also an up-regulated expression of IL-8<sup>[11]</sup>. Furthermore *H. pylori*, in addition to the expression of IL-8 induced gastric epithelial cells, also stimulates gastric epithelial cells TNF- $\alpha$ , IL-1 $\beta$  expression<sup>[27,28]</sup>. In *H. pylori* infection, IL-8 chemotaxis of neutrophil infiltration and epithelial damage caused by *H. pylori* vacuoles toxins can promote mucous membrane endocytosis bacterial products and

induction of mucosal phagocytic cells to secrete cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-8; neutrophils are attracted to the infected local, while neutrophils becomes the main source of iL-1, TNF- $\alpha$  and iL-8 induced inflammatory cytokines. Neutrophil elastase also relates to the epithelial cells induced by IL-8 gene expression, suggesting that the neutrophil enzyme release cytokines can induce a continuity of the inflammatory process itself<sup>[29]</sup>, and *H. pylori*-induced IL-8, IL-1 $\beta$  cytokine expression throughout the entire *H. pylori* infection period<sup>[30]</sup>. Studies found that Protein-tyrosine phosphatase, non-receptor-Type11 (PTPN11) encoding Src homology 2 domain-containing pro-Tein tyrosine phosphatase-2 (SHP-2) in CagA-induced gastric epithelial cell deformation, that eventually cause the gastric process, played a very important role, and the genetic background of the PTPN11 shows certain racial difference<sup>[31,32]</sup>. The IL-4 by CD4+ T cell subsets, B cells and mast cells secreted pleiotropic cytokines involved in inflammation, mucosal repair, cell proliferation and apoptosis and other physiological and pathological processes; changes in the expression levels may also affect pathogenesis of *H. pylori* infection, resulting in a host of different clinical results. The *H. pylori* infection caused by non-ulcerative gastritis can lead to local Th0 cells producing and secreting large amounts of cytokines IL-4; however, in patients with peptic ulcers, *H. pylori* infection can be caused by the polarization of Th1 cells<sup>[33]</sup>. Studies suggest that the CD14 gene C/T mutation may lead to the activation of the CD14 promoter enhanced transcription of the CD14 gene, while monocytes' high expression of CD14 and CD14 can regulate the secretion of LPS-induced IL-1 and TNF- $\alpha$ <sup>[15,34]</sup>. TNF-gene coding region mutations may affect TNF- $\alpha$  activity, caused by TNF- $\alpha$  allele or genetic type associated with *H. pylori* associated gastric duodenal disease susceptibility. In an infected *H. pylori* host of Japan, it was found that the genotype of the TNF- $\alpha$ -857 C/C and 1031 C/C group serological detection of *H. pylori* was the lowest positive rate, and in the TNF- $\alpha$ -857 T/T and TNF-B-1031 T/T genotype the serum *H. pylori* positive rate was the highest<sup>[35]</sup>. The C/C and T/C genotypes of TNF- $\alpha$  T-1031C locus were at the highest risk from *H. pylori* infection in our study.

The development of gastric cancer is a complex process, *H. pylori* infection is caused by one of the risk factors of gastric cancer. In addition, there are environmental factors, social factors, host genetic background and lifestyle. Directly use hand grasp to pilaf is very common, and the schistosome liver disease has also been often reported in Indonesia<sup>[36]</sup>; however, *H. pylori* infection and gastric cancer incidence rate were very low. In addition, have also been reports that complications of the esophagus caused by reflux esophagitis after sterilization of cancer have tended to increase<sup>[37]</sup>. And resistant strains of *H. pylori* by sterilization treatment have been reported<sup>[38]</sup>. Therefore, it appears that sterilization treatment is not the best means of prevention of gastric cancer. This study explored *H. pylori* infection with immune response

gene polymorphisms in a healthy Indonesian population. Although there was no statistical significance in SNP of *IL-8*, *IL-4*, *IL-1 $\beta$* , *CD14*, *TNF- $\alpha$*  and *PTPN11* gene polymorphisms, we found SNP of *TNF- $\alpha$* T-1031C locus was the highest risk of *H. pylori* infection. Our study provides the basis for future research data, and a new direction for the prevention of *H. pylori* infection.

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## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) infection in developing countries is high in comparison with developed countries. Indonesia is a developing country located in Southeast Asia, but the prevalence of *H. pylori* in Indonesia is lower than other countries of Southeast Asia.

### Innovations and breakthroughs

Throughout, *H. pylori* studies were conducted in patients, and treatment was based on quadruple antibiotics to eradicate *H. pylori* infection in clinical settings. However, the implications of the individual differences in recurrent infections and drug resistance of *H. pylori* and other issues must be addressed. The authors observed that the object was a healthy crowd, which reveals that in the host genetic background there is a certain association with *H. pylori* infection.

### Applications

This study provided basic vaccine development data for the prevention of *H. pylori*, and for the prevention of gastric cancer through the advancement of new ideas.

### Terminology

*H. pylori* is a Gram-negative, microaerophilic bacterium found in the stomach. It was identified in 1982 by the Australian scientists Barry Marshall and Robin Warren, who found that it was present in patients with chronic gastritis and gastric ulcers, conditions that were not previously believed to have a microbial cause. It is also linked to the development of duodenal ulcers and stomach cancer. However, over 80 percent of individuals infected with the bacterium are asymptomatic, and it has been postulated that it may play an important role in the natural stomach ecology.

### Peer review

The manuscript is interesting, but the absence of statistical significance is an important issue.

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## Silencing Bmi-1 enhances the senescence and decreases the metastasis of human gastric cancer cells

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### Abstract

**AIM:** To evaluate the impact of Bmi-1 on cell senescence and metastasis of human gastric cancer cell line BGC823.

**METHODS:** Two pairs of complementary small hairpin RNA (shRNA) oligonucleotides targeting the Bmi-1 gene were designed, synthesized, annealed and cloned into the pRNAT-U6.2 vector. After DNA sequencing to verify the correct insertion of the shRNA sequences, the recombinant plasmids were transfected into BGC823 cells. The expression of Bmi-1 mRNA and protein was examined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The effects of Bmi-1 knockdown on cell senescence and metastasis were determined by the  $\beta$ -Gal activity assay and Boyden chamber assay, respectively.

**RESULTS:** The double-stranded oligonucleotide fragments of Bmi-1 short interfering RNA (siRNA) cloned

into pRNAT-U6.2 vector conformed to the inserted sequence. RT-PCR and Western blotting indicated that the expression levels of Bmi-1 gene mRNA and protein were markedly decreased in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in transfected BGC823 cells with pRNAT-U6.2-si1104, compared with two control groups (empty vector and blank group). In particular, Bmi-1 protein expression was almost completely abolished in cells transfected with the recombinant vector harboring shRNA targeting the sequence GGAGGAGGTGAATGATAAA (nt1104-1122). Compared with untransfected cells and cells transfected with the empty vector, the mean percentage of senescent cells increased and the number of cells passing through the Matrigel decreased in cells transfected with the recombinant vectors.

**CONCLUSION:** Silencing Bmi-1 by RNA interference can increase the senescent cell rate and effectively reduce the metastasis of gastric cancer cells.

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**Key words:** Bmi-1; Gastric cancer; Senescence; Metastasis

**Core tip:** The overexpression of Bmi-1 contributes to the development of cancers. This study aimed at to evaluate the impact of Bmi-1 on the senescence and metastasis of human gastric cancer. The results demonstrated that inhibition of *Bmi-1* gene expression can enhance the senescence of human gastric cancer cells and inhibit the invasion and metastasis of gastric cancer. This research has provided an indication that Bmi-1 inhibitors might be developed as new agents for gastric cancer.

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senescence and decreases the metastasis of human gastric cancer cells. *World J Gastroenterol* 2013; 19(46): 8764-8769 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8764.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8764>

## INTRODUCTION

Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog), a member of the polycomb group (PcG), functions as a transcriptional repressor and presents with high expression in many tumors, indicating a poor prognosis<sup>[1,2]</sup>. Several lines of evidence suggest that Bmi-1 blocks cell senescence and proliferation<sup>[3,4]</sup>, and the *Bmi-1* gene is also associated with tumor invasion and metastasis<sup>[5]</sup>. Based on a list of genes on a wild-type and Bmi-1-deficient genetic background, Bmi-1 has been identified as a predictor of the response to therapy and survival in multiple types of cancer<sup>[6,7]</sup>. Therefore, this study intended to silence Bmi-1 in BGC823 cells by RNA interference, to observe the role of Bmi-1 in the senescence and metastasis of gastric cancer cells.

## MATERIALS AND METHODS

### Materials

Short interfering RNA (siRNA) vector pRNAT-U6.2 was purchased from GenScript Inc. (Piscataway, NJ, United States), Bmi-1 antibody from Santa Cruz Biotechnology (CA, United States). *Bgl*II, *Hind*III and T4DNA ligase were obtained from Promega. BGC823 human gastric cancer cell lines were received from the Chinese Academy of Science. RPMI 1640 and fetal bovine serum were supplied by Gibco BRL (Grand Island, NY, United States). Liposomes LipofectAmine™2000, G418, Trizol reagent and reverse transcription-polymerase chain reaction (RT-PCR) kit were purchased from Invitrogen (Carlsbad, CA, United States) and senescence  $\beta$ -galactosidase staining kit (Cell Signaling Technology, Beverly, MA, United States).

### Methods

**Selection of siRNA for Bmi-1 target sequence:** The analysis and design of Promega siRNA target sequence scanned human *Bmi-1* gene sequence (NM\_005180) was based on the design principle of siRNA target sequence. The 19bp siRNA target sequences, including 1104nt-1122nt (GGAGGAGGTGAATGATAAA) and 1356nt-1374nt (GAGAGATGGACTGACAAAT), were selected as the target sequence after the BLAST homology analysis. Two oligonucleotide hairpin DNA single strands were synthesized (1104F and 1104R, 1356F and 1356R), adding BamHI and XhoI endonuclease residues at the two ends. Two oligonucleotide hairpin DNA single strands demonstrated the following:

1104F: 5'-GATCCGGAGGAGGTGAATGATAAATCAAGAGATTATCATTCACCTCCTCTTTTTC-3',  
1104R: 5'-TCGAGAAAAAAGGAGGAGGTGAATGATA-

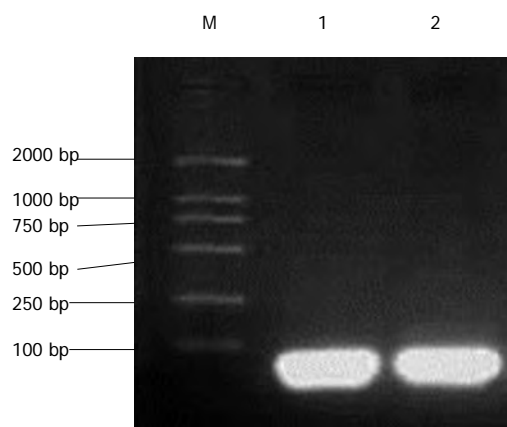
AATCTCTTGAATTTATCATTCACCTCCTCCG-3';  
1356F: 5'-GATCCGAGAGATGGACTGACAAATTTCAAGA  
GAATTGICAGTCCATCTCTCTTTTTC-3',  
1356R: 5'-TCGAGAAAAAAGAGAGATGGACT-  
GACAAATCTCTTGAATTTGTCAGTCCATCTCTCG-3'.

**Reconstruction of siRNA vectors:** The single-stranded DNA oligonucleotide (1104F and 1104R, 1356F and 1356R) was converted into a double-stranded DNA (si1104 and si1356) by conventional annealing, and reconnected overnight at 4 °C, utilizing 2 × reaction reconnected buffer (5  $\mu$ L), linear pRNAT-U6.2 vector (1  $\mu$ L), T4 ligase (1  $\mu$ L) and annealing product (3  $\mu$ L). The two recovered products were incubated at 16 °C for 16 h after addition of Solution I containing DNA ligase, and the resulting ligated products were used to transfect well-prepared competent *E. coli* DH5 $\alpha$ . The whole transfection mix was plated onto a prewarmed LB-ampicillin (AMP) agar plate and then incubated at 37 °C for 12 h. Individual growing colonies were picked out and incubated at 37 °C for 12 h in LB broth containing AMP. Full length plasmid DNA was extracted from positive clones using a plasmid DNA extraction kit and then subject to testing for the presence of Bmi-1 with nuclease digestion using *Bgl* II, *Hind* III and T4DNA ligase.

**Identification of recombinants:** The recombinants were identified by PCR amplification, using primers PRNA-U6.2 FORWARD and PRNA-U6.2 REVERSE. PCR reaction was performed with 3 min of initial denaturation at 94 °C, 35 cycles of 45 s denaturation at 94 °C, 45 s annealing at 55 °C, 45 s extension at 72 °C, and finally 10 min extension at 68 °C. RT-PCR amplification products were electrophoresed and inspected on a 1.1% agarose gel, and recovered and purified by using DNA Gel recovery kit.

**Transfection by liposome-mediated siRNA:** The transfection process was according to the Lipofectamine™ 2000 instructions: a cell suspension containing  $4-8 \times 10^5$  cells was added to 500  $\mu$ L of growth medium with serum but without antibiotics; 0.8-1.2  $\mu$ g DNA was added to 50  $\mu$ L of medium without serum; 2  $\mu$ L of Lipofectamine™ 2000 was added to 50  $\mu$ L OptiMEM® I medium and incubated for 5 min at room temperature; the DNA-Lipofectamine™ 2000 complexes were added and incubated for 4 h at 37 °C in a CO<sub>2</sub> incubator. Finally cells were assayed at 24-48 h post-transfection for the appropriate activity.

**RT-PCR analysis:** RT-PCR was carried out as described previously<sup>[8]</sup>. Cells were harvested and rinsed with phosphate-buffered saline (PBS) at corresponding time points and total RNA in the treated sections was extracted according to the total RNA extracting kit. A solution was added consisting of 10 mmol/L dNTP, 0.5 g/L oligo(dT), 40 U reverse transcriptase (m-mulv), 59 pH 8.3 RT buffer (250 mmol/L Tris-HCl, 250 mmol/L KCl, 20 mmol/L MgCl<sub>2</sub>, 50 mmol/L DTT) and deionized



**Figure 1** Annealing of siRNA hairpin DNA by electrophoresis. M: DNA marker; 1: Hairpin single-stranded DNA products for 1104F and 1104R; 2: Hairpin single-stranded DNA product for 1356F and 1356R.

water. Total sample volume was 20  $\mu$ L. Samples were incubated at 37  $^{\circ}$ C for 1 h and the reaction was stopped by heating at 70  $^{\circ}$ C for 10 min. Reverse transcriptase was used to synthesize the first-strand cDNA from an equal amount of the RNA sample following the manufacturer's instructions. About 35-45 cycles of PCR reaction were used to cover the linear range of the PCR amplification. The Bmi-1 specific primers (forward 5'GGAGACCAGCAAGTATTGTCC 3'; reverse 5'GACCATTCTTCTC-CAGGTAT 3') were used to amplify a 517 bp fragment of the *Bmi-1* coding region.  $\beta$ -actin was used as an internal control to amplify a 268 bp fragment. The band densities were scanned with a densitometer (Bio-Rad, United States). The relative amount of mRNA in each sample was calculated from the densitometry ratio of Bmi-1 OD value/ $\beta$ -actin OD value.

**Western blotting analysis:** Western blotting was conducted according to the manufacturer's instructions. The samples of each supernatant and the final pellets were heat-blocked for 5 min in a loading buffer (125 mmol/L Tris-HCl, 20%glycero1, 10%2-mercaptethanol, 4% SDS, 0.02% bromophenol blue, pH 6.8) and then subjected to electrophoresis on a 10%-20% Tris-glycine sulfate-polyacrylamide gel. The samples were then electronically transferred to a transfer membrane and blocked for 1 h in Tris-HCl buffered saline containing 5% skimmed milk and 0.1% Tween. Primary antibodies were incubated at 4-8  $^{\circ}$ C overnight in a TBS buffer containing 5% bovine albumin. The membrane was rinsed with TBS buffer containing 0.1% Tween 20, incubated with HRP-labeled second antibody for 2 h, and then stained with the detection reagents. Western blot analysis was performed as described previously to assess the protein expression level of Bmi-1 (1:200) and  $\beta$ -actin (1:100). Blots were developed with a SuperSignal ECL Western blotting Dura Substrate kit (Pierce Biotech, Rockford, IL, United States).

**Senescence staining:** Cell senescence  $\beta$ -galactosidase staining was carried out according to the manufacturer's

instructions. Growth medium was removed from the cells and the plate rinsed once with PBS (2 mL for a 35 mm well), followed by addition of 1mL of 1x Fixative Solution to each 35 mm well. Cells were allowed to fix for 10-15 min at room temperature. The plate was rinsed twice with PBS (2 mL for a 35 mm well). After addition of 1 mL of  $\beta$ -galactosidase staining solution to each 35 mm well, the plate was incubated at 37  $^{\circ}$ C overnight in a dry incubator. While the  $\beta$ -galactosidase staining solution was still on the plate, the cells were checked under a microscope ( $\times$  200 total magnification) for the development of blue color. Five visual areas were randomly selected and photographed to record the percentage of the senescent cells.

**Cell migration and invasion assay:** Serum-free 1640 medium containing Matrigel was added to the filter membrane of the upper chamber to prepare a gel at 37  $^{\circ}$ C for 2 h. The 200  $\mu$ L supernatant of serum-free NIH3T3 cells was utilized as chemokines in the lower chamber. After adding 400  $\mu$ L of cells ( $1 \times 10^9$ /L) to the upper chamber, they were cultured at 37  $^{\circ}$ C for 24 h. Five visual areas in the lower chamber were randomly selected and the percentage of senescent cells was recorded with hematoxylin-eosin staining. Each group had five parallel experiments.

### Statistical analysis

Western blotting and RT-PCR results were analyzed with scanning densitometry (Bio-Rad). Quantitative data were documented as the mean  $\pm$  SD. The significance of the differences was analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, United States), with significance at  $P < 0.05$ .

## RESULTS

### Annealing of siRNA hairpin DNA

After annealing of hairpin single-stranded DNA for 1104 and 1356, the electrophoresis showed bright bands below 100 bp, consistent with the design (Figure 1).

### Identification of Bmi-1 siRNA vectors

Two hairpin single-stranded DNA products (si1104 and si1356) were connected with pRNAT-U6.2 plasmid to transfect well-prepared competent *E. coli* DH5 $\alpha$ . More than 10 transfected colonies grew on the Amp + LB culture plate. Ten transfected colonies were randomly selected. The DNA sequence of the inserted fragments was consistent with the designed positive recombinants (pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356) (Figure 2).

### Expression of Bmi-1 mRNA

The expression of Bmi-1 mRNA was inhibited in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in pRNAT-U6.2-si1104 transfected BGC823 cells, while two control groups (empty vector and blank groups) had significantly higher levels of Bmi-1 mRNA ( $P < 0.01$ ) (Figure 3A).

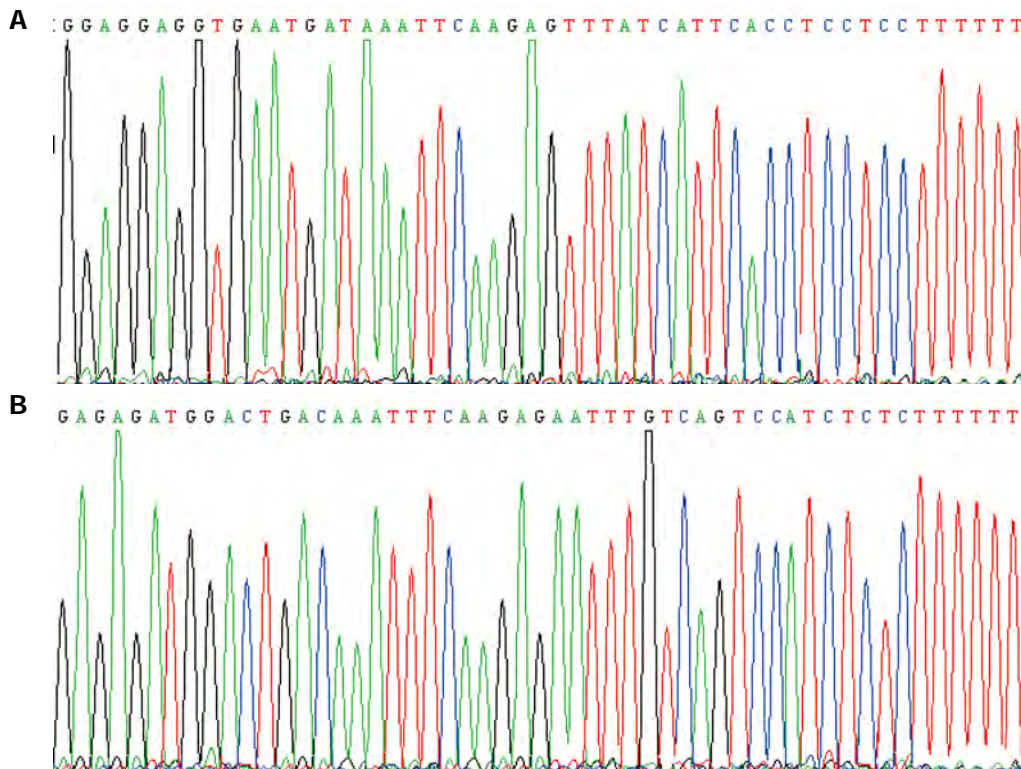


Figure 2 DNA sequence of the inserted fragment by transfected bacteria recombinant plasmid. A: The DNA sequence of the inserted fragment by recombinant plasmid pRNAT-U6.2-si1104. B: The DNA sequence of the inserted fragment by recombinant plasmid pRNAT-U6.2-si1356. The two DNA sequences of the inserted fragment by recombinant plasmids corresponded to the designed sequences.

**Table 1**  $\beta$ -Gal activity assay and Boyden chamber assay to investigate the effects of Bmi-1 on cell senescence and metastasis ( $n = 5$ , mean  $\pm$  SD)

Group	Transfected plasmids	$\beta$ -Gal activity assay	Boyden chamber assay (cell number)
1	pRNAT-U6.2-si1104	28.3% $\pm$ 3.9% <sup>b</sup>	22.4 $\pm$ 4.2 <sup>b</sup>
2	pRNAT-U6.2-si1356	25.9% $\pm$ 4.3%	33.6 $\pm$ 5.5 <sup>b</sup>
3	pRNAT-U6.2	15.6% $\pm$ 2.7%	74.7 $\pm$ 9.3
4	Non-transfected	17.2% $\pm$ 3.1%	68.9 $\pm$ 10.1

Group 1: Transfected BGC823 cells with pRNAT-U6.2-si1104; Group 2: Transfected BGC823 cells with pRNAT-U6.2-si1356; Group 3: Transfected BGC823 cells with pRNAT-U6.2 (empty vector); Group 4: Non-transfected BGC823 cells (blank). <sup>b</sup> $P < 0.01$  vs non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2.

### Expression of Bmi-1 protein

There were high levels of Bmi-1 protein by Western blotting in non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2, compared with transfected BGC823 cells targeting Bmi-1 (pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356), while there was no Bmi-1 expression in the transfected BGC823 cells with pRNAT-U6.2-si1104 targeting Bmi-1 ( $P < 0.01$ ) (Figure 3B).

### Silencing Bmi-1 increased the senescent cell rate and reduced the metastasis of BGC823 cells

The senescent rate of transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356 significantly increased compared with the non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2 ( $P < 0.01$ ). The number of transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356 through

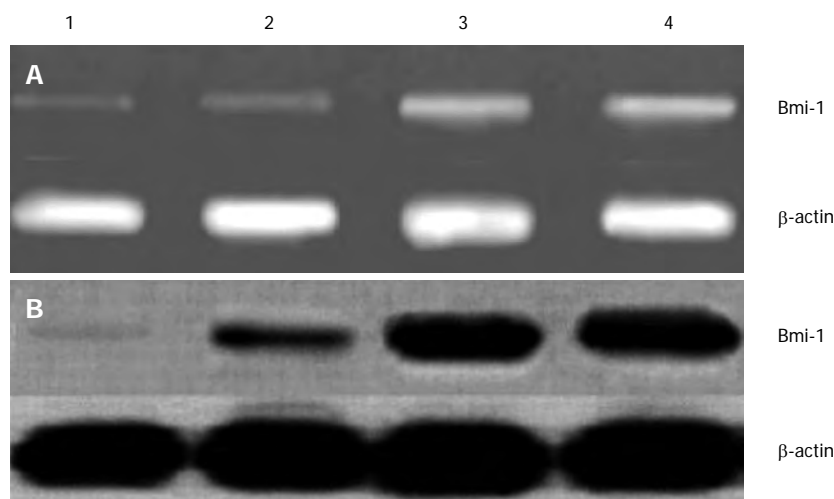
the Matrigel significantly decreased, compared with the non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2 ( $P < 0.01$ ) (Table 1).

## DISCUSSION

This study aimed to investigate the impact of Bmi-1 on the senescence and metastasis of human gastric cancer cells, and our results indicate that inhibition of *Bmi-1* gene expression can enhance the senescence of human gastric cancer cells and limit the invasion and metastasis of human gastric cancer cells.

Gastric cancer, the most common gastrointestinal malignancy, is the fourth most commonly diagnosed malignancy and the second leading cause of cancer-related death in the world<sup>[9]</sup>. Gastric cancer is often either asymptomatic or has nonspecific symptoms in its early stages.





**Figure 3** Levels of Bmi-1 mRNA and protein. A: The Bmi-1 mRNA level decreased in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in pRNAT-U6.2-si1104 transfected BGC823 cells. B: The levels of Bmi-1 protein was higher in non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2, compared with transfected BGC823 cells targeting Bmi-1 (pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356). There was no expression in the transfected BGC823 cells with pRNAT-U6.2-si1104 targeting Bmi-1. 1: Transfected BGC823 cells with pRNAT-U6.2-si1104; 2: Transfected BGC823 cells with pRNAT-U6.2-si1356; 3: Transfected BGC823 cells with pRNAT-U6.2 (empty vector); 4: Non-transfected BGC823 cells (blank).

Once symptoms become apparent, the cancer has often reached an advanced stage and may also have metastasized and spread to other parts of the body. Accordingly, gastric cancer has a relatively poor prognosis since invasion and metastasis are important prognostic factors<sup>[10,11]</sup>. Currently, there is evidence that the incidence of gastric cancer is related to multiple oncogenes, such as *C-myc*, *Ras*, *Hst* and *C-erbB-2*<sup>[12-14]</sup>. The *Bmi-1* gene, a polycomb gene (PcG), has been reported as an oncogene with high expression in cancers, and this may be related to high aggressiveness, such that overexpression of Bmi-1 is associated with poor prognosis<sup>[1,7]</sup>. Compelling research has supported that the expression of Bmi-1 decreases tumor cell senescence and proliferation, and increases tumor invasion and metastasis. The *Bmi-1* gene can be synergistic with *C-myc* to induce cell metastasis and tumor formation<sup>[3,15,16]</sup>. This study demonstrated that the inhibition of *Bmi-1* gene expression can increase the senescence of gastric cancer cells and slow down the invasion and metastasis of gastric cancer cells. It has provided further evidence of a role for Bmi-1 in the pathogenesis of gastric cancer.

The senescence β-galactosidase staining kit is designed to detect β-galactosidase activity at pH 6, a known characteristic of senescent cells not found in presenescent, quiescent or immortal cells<sup>[17,18]</sup>. Boyden chamber assays are used to measure cell invasion and various types of cell migration<sup>[19,20]</sup>. In this study, the incidence of senescent gastric cancer cells was most obvious when Bmi-1 expression was inhibited, according to β-galactosidase activity. Meanwhile, the number of gastric cancer cells through the Matrigel significantly decreased after inhibiting Bmi-1 expression in the Boyden chamber assay, indicating that the inhibition of Bmi-1 expression can limit the invasion and metastasis of gastric cancer cells. These results suggest that inhibition of *Bmi-1* gene expression can enhance cell senescence and reduce the capability for cell invasion and

metastasis.

In conclusion, we documented in the present study that silencing Bmi-1 by RNA interference enhances the senescent cell rate and effectively reduces the metastasis of gastric cancer cells. Many studies have shown that Bmi-1 is essential in multiple pathways in the pathogenesis of gastric cancer. Other reports have suggested that Bmi-1 inhibitors have therapeutic potential for gastric cancer through various mechanisms. The current has provided additional support for the notion that Bmi-1 inhibitors might be developed as new agents for gastric cancer.

## COMMENTS

### Background

Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog) has been reported as an oncogene that plays an important role in several types of cancer. The amplification and overexpression of Bmi-1 contribute to the development of many tumors and cancers, such as skin, prostate, breast, ovarian, and colorectal, as well as hematological malignancies. Whether Bmi-1 influences cell senescence and metastasis of human gastric cancer remains unknown. The aim of this study was to evaluate the impact of Bmi-1 on cell senescence and metastasis of the human gastric cancer cell line BGC823.

### Research frontiers

Bmi-1 is essential in multiple pathways in the pathogenesis of gastric cancer. The role of Bmi-1 on cell senescence and metastasis of human gastric cancer remains unclear.

### Innovations and breakthroughs

The inhibition of *Bmi-1* gene expression can enhance the senescence of gastric cancer cells and limit the invasion and metastasis of gastric cancer cells.

### Applications

Bmi-1 inhibitors have therapeutic potential for gastric cancer through various mechanisms. This research has provided additional support for the notion that Bmi-1 inhibitors might be developed as new agents for gastric cancer.

### Peer review

This study demonstrated that the inhibition of *Bmi-1* gene expression can increase gastric cancer cell senescence and inhibit invasive behavior in a well-accepted Boyden chamber model. The present study focused on the role of Bmi-1 in cell senescence and metastasis. It would help to understand the mechanism



of Bmi contribution to cancer progression. The data presented in this manuscript are quite good and very supportive of the hypothesis tested.

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## Meta-analysis of Barrett's esophagus in China

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### Abstract

**AIM:** To investigate the epidemiology and characteristics of Barrett's esophagus (BE) in China and compare with cases in the west.

**METHODS:** Studies were retrieved from the China National Knowledge Infrastructure and PubMed databases using the terms "Barrett" and "Barrett AND China", respectively, as well as published studies about BE in China from 2000 to 2011. The researchers reviewed the titles and abstracts of all search results to determine whether or not the literature was relevant to the current topic of this research. The references listed in the studies were also searched. Inclusion and exclusion criteria for the literature were appropriately established, and the data reported in the selected studies were analyzed. Finally, a meta-analysis was performed.

**RESULTS:** The current research included 3873 cases

of BE from 69 studies. The endoscopic detection rate of BE in China was 1%. The ratio of male to female cases was 1.781 to 1, and the average age of BE patients was  $49.07 \pm 5.09$  years. Island-type and short-segment BE were the most common endoscopic manifestations, accounting for 4.48% and 80.3%, respectively, of all cases studied. Cardiac-type BE was observed in 40.0% of the cases, representing the most common histological characteristic of the condition. Cancer incidence was 1.418 per 1000 person-years.

**CONCLUSION:** Average age of BE patients in China is lower than in Western countries. Endoscopic detection and cancer incidence were also lower in China.

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**Key words:** Barrett's esophagus; Epidemiology; Cancer incidence; China; Meta-analysis

**Core tip:** Barrett's esophagus (BE) is a precursor of esophageal adenocarcinoma. Western countries have published more research on BE than China has. Thus, epidemiological knowledge of BE in China is inadequate. Diagnosis and treatment of BE in China is based on western criteria, therefore, diagnosis, monitoring, and treatment of BE require more data based on the unique characteristics of patients and clinics in China. The current research analyzed 69 clinical studies to obtain a comprehensive understanding of BE in China. Results provide important guidelines that can help improve the treatment and follow-up of BE patients in China.

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## INTRODUCTION

Barrett's esophagus (BE) is a pathological phenomenon that occurs when the stratified squamous epithelium in the lower esophagus is replaced by a metaplastic simple columnar epithelium. In some cases, BE is accompanied by intestinal metaplasia, which is considered a precursor of esophageal adenocarcinoma<sup>[1]</sup>. Academics in Western countries have conducted more research on the subject than researchers in China. As such, despite the attention BE has drawn in recent years, epidemiological knowledge of BE in China is insufficient. The Digestive Disease Branch of the Chinese Medical Association drafted the Diagnosis and Treatment Consensus<sup>[2]</sup> of BE in 2005 and amended it in 2011, when a consensus amongst clinicians was finally achieved<sup>[1]</sup>. This consensus on BE, however, is based on western standards. Thus, the diagnosis, monitoring, and treatment of BE in China require more data based on Chinese clinics.

Although increasing numbers of researchers in China have focused on BE, the studies published thus far do not feature large samples or prospective designs. A systematic review<sup>[3]</sup> of the clinical characteristics of BE in China was published in 2008. However, in this review, studies that used metaplasia as a necessary standard were not excluded, which contradicts the consensus. In addition, the sample sizes of some included studies are rather small, with reports featuring only one or two cases. The present study aims to obtain a comprehensive understanding of the characteristics of BE in China by conducting a meta-analysis on BE in China and comparing findings with cases in Western countries. Results will help improve the treatment and follow-up of Chinese BE patients.

## MATERIALS AND METHODS

### *Sources of literature and retrieval methods*

Information from the China National Knowledge Infrastructure (CNKI) and PubMed databases were used. Clinical studies on BE published in Chinese between 2000 and 2011 were retrieved from the CNKI database, and those published in English were obtained from the PubMed database using the keywords "Barrett" and "Barrett AND China", respectively. The researchers reviewed the titles and abstracts of all search results to determine whether or not the study was relevant to the current topic. The references listed in the studies obtained were also reviewed to locate additional studies.

### *Inclusion criteria of studies*

The selected studies met the following criteria: (1) all of the cases described were from China; (2) diagnosis of BE conformed to standards set by the Digestive Disease Branch of the Chinese Medical Association in 2011; (3) BE was diagnosed through endoscopy and pathology; and (4) the number of cases included in the sample was > 10.

### *Exclusion criteria of studies*

Studies were excluded if they featured any of the following criteria: (1) only published as an abstract; (2) intestinal metaplasia was used as a necessary diagnostic criteria; (3) the clinical aspects of BE were insufficient; that is, the study lacked at least three of the following aspects: endoscopic detection rate, sex, age, endoscopic manifestations, or histological type; (4) only a short segment of the study focused on BE; or (5) duplicate publication.

### *Data extraction and statistical analysis*

Four researchers independently extracted data from every study, and any ensuing disagreements were resolved through discussion. The following data were extracted: name of the first author; year of publication; region of study; total number of cases; male to female ratio of the patients; average age, endoscopic detection rate; proportion of each endoscopic and histological type; follow-up cases and follow-up duration; and occurrence of esophageal adenocarcinoma during follow-up.

Data were analyzed using SPSS version 17.0. Proportions were evaluated using standard formulas. A mean difference demonstrating a 95% confidence rate was used for continuous data. The total number of person-years during follow-up was calculated by multiplying the number of follow-up cases with the follow-up duration. Cancer incidence was calculated by dividing the number of occurrences of esophageal adenocarcinoma among the follow-up cases by the total number of person-years.

## RESULTS

### *Sources of studies*

A total of 1121 studies were found in the CNKI database, and 108 of these studies met the inclusion criteria. Among these studies, 42 were rejected on the basis of the exclusion criteria; seven<sup>[4-10]</sup> for using intestinal metaplasia as a necessary diagnostic standard; 28<sup>[11-38]</sup> for having insufficient data on the clinical aspects of BE; four<sup>[39-42]</sup> for providing only a small study on BE; and one each for inconsistent data<sup>[43]</sup>, doubts about plagiarism<sup>[44]</sup> and duplicate publication<sup>[45]</sup>. A total of 65 studies were found in the PubMed database; five of which met the inclusion criteria. Among these studies, one<sup>[46]</sup> was excluded because of its duplicate publication in Chinese, and another<sup>[47]</sup> was excluded for its use of intestinal metaplasia as a necessary diagnostic standard. A total of 69<sup>[48-116]</sup> studies were included in the present research. The screening process is summarized using the flow diagram shown in Figure 1.

### *Characteristics of included studies*

The 69 studies included in the present research were conducted in 25 provinces. The total number of samples in these studies was 12404, and the total number of cases was 3873 (Table 1).

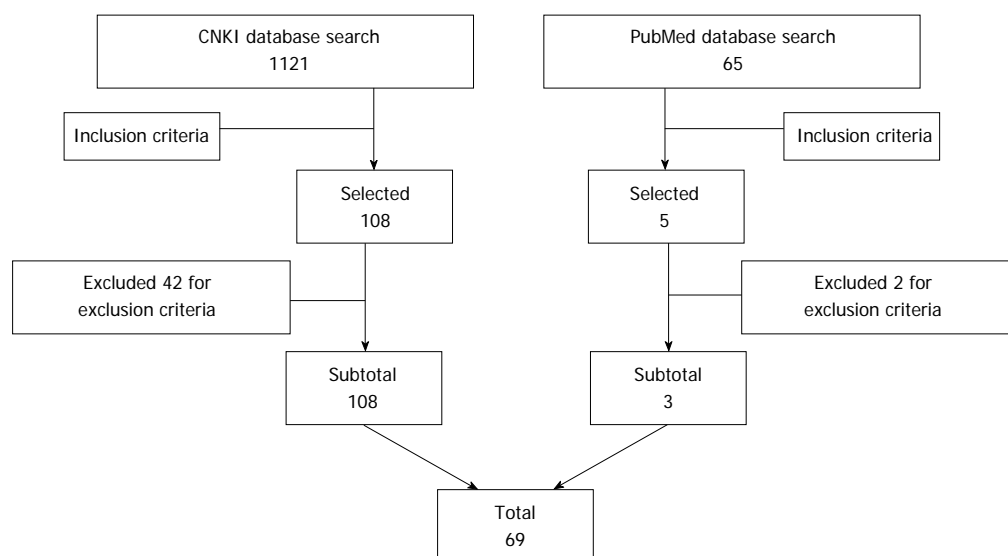


Figure 1 Flow diagram for the literature search. CNKI: China National Knowledge Infrastructure.

### Endoscopic detection rate

A total of 15 studies reported the endoscopic detection rate of BE, which was obtained from all patients who had undergone endoscopy. However, the detection rate varied significantly, with rates ranging from 0.06% to 17.65%. The total endoscopic detection rate was 1.0% (95%CI: 0.1%-1.8%).

### Sex

All studies reported the sex of BE patients (Table 1). One study<sup>[85]</sup> was not included in this analysis because the sum of male and female patients was inconsistent with the reported total number of cases. The remaining 68 studies showed a total of 3829 cases with 2452 male patients, accounting for 64.0% of the sample (95%CI: 61.1%-67.0%), and 1377 female patients, accounting for 36.0% of the sample (95%CI: 33.0%-38.9%). The male to female ratio was 1.781 (95%CI: 1.552-2.009).

### Age

A total of 58 studies reported the age of the BE patients (Table 1), and the average age of the patients was  $49.07 \pm 5.09$  years.

### Endoscopic manifestations

The endoscopic patterns of BE in 49 studies could be categorized into several types based on Herlihy criteria<sup>[117]</sup>: island, tongue, circumferential, and mixed. On the basis of the columnar epithelial length reported in 29 studies, BE was divided into long-segment BE (LSBE) (*i.e.*, columnar epithelial metaplasia cells were involved in the entire circumference of the esophagus and the length of the segment was  $\geq 3$  cm) and short-segment BE (SSBE) (*i.e.*, columnar epithelial metaplasia cells were not involved in the entire circumference of the esophagus or the whole circumference of esophagus was involved but the length of the segment was  $< 3$  cm)<sup>[11]</sup> (Table 2).

### Histological type

The histological type of BE was divided into the gastric-fundic, cardiac, and intestinal metaplasia types<sup>[1]</sup> (Table 3).

### Cancer incidence of BE

Thirty-one studies reported follow-up information. The total number of follow-up cases was 1283, with a follow-up duration ranging from 3 mo to 3 years. The mean follow-up duration was 1.099 years. Three studies<sup>[80,91,95]</sup> focused only on the follow-up of atypical hyperplasia of BE; thus, these studies were not included in this analysis. The total number of person-years during follow-up was 1410. Among 1283 cases, only two developed esophageal adenocarcinoma during the follow-up period; these cases were reported in two studies<sup>[91,103]</sup>. Four cases were also detected with esophageal adenocarcinoma during follow-up, but the number of follow-up cases and follow-up times were not provided. Studies with these cases were excluded from this analysis. The cancer incidence of BE was 1.418 per 1000 person-years.

## DISCUSSION

The present research included 69 studies. The total endoscopic detection rate of BE was 1.0%, consistent with the total BE morbidity rate in Asia (0.9%-1.2%) reported by Hou *et al.*<sup>[118]</sup>. The endoscopic detection rate of BE in the reviewed studies ranged from 0.06% to 17.65%. Tseng *et al.*<sup>[90]</sup> reported that the endoscopic detection rate of BE in Taiwan was 0.06%, which is much lower than the total detection rate observed in China. This variation may be attributed to their inclusion of upper gastrointestinal tract endoscopy in routine health maintenance programs, which can yield more reliable data on the prevalence of BE in local populations. The endoscopic detection rate of BE in most studies is based on patients who have undergone upper gastrointestinal tract endos-



Table 1 Characteristics of the selected studies

Ref.	Year of publication	Region (province)	Cases	Male	Female	Mean age (yr)
Chen <i>et al.</i> <sup>[48]</sup>	2011	Henan	150	60	90	45.42
Chen <i>et al.</i> <sup>[49]</sup>	2011	Hubei	52	31	21	49
Guo <i>et al.</i> <sup>[50]</sup>	2011	Hebei	42	27	15	48
Han <i>et al.</i> <sup>[51]</sup>	2011	Jilin	30	21	9	45
Hao <i>et al.</i> <sup>[52]</sup>	2011	Guangdong	76	58	18	50.6
Lin <i>et al.</i> <sup>[53]</sup>	2011	Zhejiang	41	28	13	58.9
Lv <i>et al.</i> <sup>[54]</sup>	2011	Zhejiang	108	80	28	61
Su <i>et al.</i> <sup>[55]</sup>	2011	Hubei	23	13	10	40.3
Wang <i>et al.</i> <sup>[56]</sup>	2011	Fujian	113	67	46	54.5
Zou <i>et al.</i> <sup>[57]</sup>	2010	Guangxi	23	16	7	50.3
Xia <i>et al.</i> <sup>[58]</sup>	2010	Hubei	56	34	22	45.85
Wu <i>et al.</i> <sup>[59]</sup>	2010	Shanghai	84	50	34	36.3
Liu <i>et al.</i> <sup>[60]</sup>	2010	Chongqing	62	35	27	51
Li <i>et al.</i> <sup>[61]</sup>	2010	Anhui	32	18	14	48.6
Li <i>et al.</i> <sup>[62]</sup>	2010	Guangdong	45	18	27	43
Hao <i>et al.</i> <sup>[63]</sup>	2010	Henan	144	98	46	NA1
Yao <i>et al.</i> <sup>[64]</sup>	2010	Sichuan	21	15	6	40.1
Tang <i>et al.</i> <sup>[65]</sup>	2010	Jiangxi	63	43	20	45
Shi <i>et al.</i> <sup>[66]</sup>	2010	Fujian	57	36	21	53
Jia <i>et al.</i> <sup>[67]</sup>	2010	Shanxi	26	21	5	49
Gao <i>et al.</i> <sup>[68]</sup>	2010	Hubei	32	21	11	NA
Tian <i>et al.</i> <sup>[69]</sup>	2009	Shandong	59	43	16	49.14
Li <i>et al.</i> <sup>[70]</sup>	2009	Guangxi	38	27	11	47.56
Dai <i>et al.</i> <sup>[71]</sup>	2009	Hunan	23	18	5	50.3
Bai <i>et al.</i> <sup>[72]</sup>	2009	Chongqing	67	41	26	50.7
Yang <i>et al.</i> <sup>[73]</sup>	2009	Shaanxi	87	58	29	53.3
Yang <i>et al.</i> <sup>[74]</sup>	2009	Ningxia	51	29	22	49.14
Tan <i>et al.</i> <sup>[75]</sup>	2009	Liaoning	48	31	17	58.4
Qiu <i>et al.</i> <sup>[76]</sup>	2009	Fujian	404	238	166	44.2
Lu <i>et al.</i> <sup>[77]</sup>	2009	Jiangsu	12	9	3	NA
Liu <i>et al.</i> <sup>[78]</sup>	2009	Liaoning	23	18	5	49
Gao <i>et al.</i> <sup>[79]</sup>	2009	Liaoning	42	25	17	NA
Peng <i>et al.</i> <sup>[80]</sup>	2009	Guangdong	27	14	13	48.18
Wu <i>et al.</i> <sup>[81]</sup>	2008	Henan	25	16	9	48.3
Wang <i>et al.</i> <sup>[82]</sup>	2008	Henan	12	10	2	49.5
Wang <i>et al.</i> <sup>[83]</sup>	2008	Ningxia	109	64	45	50.11
Lu <i>et al.</i> <sup>[84]</sup>	2008	Guangxi	32	22	10	52.5
Gao <i>et al.</i> <sup>[85]</sup>	2008	Shandong	44	22	20	50
Zhang <i>et al.</i> <sup>[86]</sup>	2008	Jiangxi	84	51	33	46
Yang <i>et al.</i> <sup>[87]</sup>	2008	Hebei	74	40	34	52.6
Jian <i>et al.</i> <sup>[88]</sup>	2008	Yunnan	68	51	17	52
Ji <i>et al.</i> <sup>[89]</sup>	2008	Jiangsu	51	38	13	52.5
Tseng <i>et al.</i> <sup>[90]</sup>	2008	Taiwan	12	9	3	61.6
Zhang <i>et al.</i> <sup>[91]</sup>	2007	Shandong	30	24	6	52
Meng <i>et al.</i> <sup>[92]</sup>	2007	Liaoning	21	13	8	54.6
Duan <i>et al.</i> <sup>[93]</sup>	2007	Henan	54	38	16	51.6
Zhou <i>et al.</i> <sup>[94]</sup>	2007	Zhejiang	13	7	6	NA
Wang <i>et al.</i> <sup>[95]</sup>	2007	Hubei	88	61	27	47.46
Li <i>et al.</i> <sup>[96]</sup>	2007	Fujian	75	45	30	45.42
Jin <i>et al.</i> <sup>[97]</sup>	2007	Zhejiang	37	22	15	53
Zhou <i>et al.</i> <sup>[98]</sup>	2006	Hubei	128	93	35	NA
Yang <i>et al.</i> <sup>[99]</sup>	2006	Shaanxi	86	58	28	46
Wu <i>et al.</i> <sup>[100]</sup>	2006	Fujian	13	10	3	48
Wang <i>et al.</i> <sup>[101]</sup>	2006	Shaanxi	73	29	44	45.6
Suo <i>et al.</i> <sup>[102]</sup>	2006	Fujian	37	24	13	50
Li <i>et al.</i> <sup>[103]</sup>	2006	Liaoning	54	35	19	49
Li <i>et al.</i> <sup>[104]</sup>	2006	Tianjin	37	25	12	58.3
Dou <i>et al.</i> <sup>[105]</sup>	2006	Guizhou	89	57	32	46.3
Wang <i>et al.</i> <sup>[106]</sup>	2006	Hubei	33	22	11	48
Shu <i>et al.</i> <sup>[107]</sup>	2006	Hubei	13	12	1	NA
Zheng <i>et al.</i> <sup>[108]</sup>	2005	Hubei	45	31	14	NA
Liang <i>et al.</i> <sup>[109]</sup>	2005	Xinjiang	20	14	6	NA
Zhang <i>et al.</i> <sup>[110]</sup>	2004	Shaanxi	69	54	15	56.2
Zhao <i>et al.</i> <sup>[111]</sup>	2003	Shandong	55	38	17	46.8
Dong <i>et al.</i> <sup>[112]</sup>	2003	Zhejiang	32	23	9	48.8
Zhang <i>et al.</i> <sup>[113]</sup>	2001	Anhui	14	11	3	NA
Wang <i>et al.</i> <sup>[114]</sup>	2001	Guangdong	21	16	5	67.3

Zhao <i>et al</i> <sup>[115]</sup>	2000	Shandong	35	26	9	NA
Yang <i>et al</i> <sup>[116]</sup>	2000	Beijing	29	22	7	50

NA: Not applicable (data were either unavailable or not reported).

**Table 2 Endoscopic manifestations of Barrett's esophagus**

Type	Proportion	95%CI
Island	0.448	0.375-0.521
Tongue	0.262	0.204-0.320
Circumferential	0.247	0.190-0.303
Mixed	0.043	-0.006-0.093
SSBE	0.803	0.771-0.835
LSBE	0.197	0.165-0.229

The island type of Barrett's esophagus (BE) accounted for 44.8% of all cases, the tongue type for 26.2%, the circumferential type for 24.7%, and the mixed type for 4.3%. Short-segment BE and long-segment BE accounted for 80.3% and 19.7% of the cases, respectively.

**Table 3 Histological type of Barrett's esophagus**

Type	Proportion	95%CI
Cardiac	0.400	0.310-0.491
Gastric-fundic	0.325	0.227-0.422
Intestinal Metaplasia	0.272	0.226-0.318
Mixed type	0.003	-0.002-0.008

The cardiac type accounted for 40.0% of the cases, the gastric-fundic type for 32.5%, the intestinal metaplasia type for 27.2%, and the mixed type for 0.3%.

copy in a local hospital. These patients mainly suffer from several gastrointestinal symptoms such as regurgitation, heartburn, epigastric discomfort, nausea, vomiting, and eructation. Reports of such symptoms increase the detection rate of BE, so this result cannot represent the prevalence of BE in the general population.

In this research, the total endoscopic detection rate was lower than that reported in a meta-analysis in 2008<sup>[3]</sup> (2.39%), likely because of the increasing number of patients accepting endoscopy in recent years as a means of diagnosing and treating upper gastrointestinal tract diseases. Some patients opt to undergo endoscopic examination when experiencing upper gastrointestinal tract symptoms, while others choose endoscopy for routine health maintenance. Thus, the data do not provide sufficient evidence to conclude that the incidence of BE has declined in China.

The detection rate of BE in Western countries is 3%-8%<sup>[119]</sup>, which is higher than that in China. Variations observed may be due to the following reasons: (1) variations in genetic and environmental factors; (2) western lifestyle and diet-related factors, such as visceral obesity, high-fat diet, and tobacco and alcohol consumption, which are risk factors for BE<sup>[118,120-133]</sup>; and (3) delayed recognition of BE in China, considering that current diagnostic standards are based on western practices and some Chinese doctors experience difficulties when diagnosing patients with BE.

In this research, the number of male BE patients was higher than that of female patients, which is similar to western reports<sup>[133]</sup>. The average age of onset of BE in this study was 49.07 years, whereas the average age in Western countries is 60 years<sup>[134,135]</sup>. Variations observed may be attributed to differences in the Chinese and western lifestyles.

The main types of endoscopic manifestations of BE include the island type and SSBE, which is similar to western reports<sup>[136]</sup>. Experts in the United States believe that LSBE and SSBE represent two different pathological changes and that the former is related to severe gastro-esophageal reflux disease, which is common in older people. Although LSBE tends to increase the risk of cancer, no evidence today correlates the length of BE and cancer risk<sup>[137]</sup>.

The number of cases of cardiac type BE was higher than that of other histological types in this research. Jankowski *et al*<sup>[138]</sup> reported that intestinal metaplasia progresses to cancer, and the Diagnosis and Treatment Consensus of BE of China (2011) regards intestinal metaplasia as a precursor of esophageal adenocarcinoma. Unfortunately, the reviewed studies present limited clinical and pathological data on intestinal metaplasia related to BE, considering Chinese researchers' lack of knowledge on the topic. Some researchers believe that cancer formation is related to atypia of the epithelial instead of columnar epithelial metaplasia<sup>[139]</sup>.

An individual with BE is estimated to be at 25-30 times greater risk of developing esophageal adenocarcinoma<sup>[140-143]</sup> than the general population. Cancer incidence was found to be 1.418 per 1000 person-years in the present study, which is lower than that in England (7.0 per 1000 person-years), United States (6.4 per 1000 person-years), and other European countries (5.6 per 1000 person-years)<sup>[144]</sup>. Thus, the cancer incidence of BE in China is lower than that in Western countries. The spectrum of BE characteristics differs significantly between the two regions.

## COMMENTS

### Background

Barrett's esophagus (BE) is a precursor of esophageal adenocarcinoma. The Diagnosis and Treatment Consensus of BE in China is based on Western criteria. Epidemiological knowledge of BE in China is inadequate.

### Research frontiers

A large number of clinical studies on BE have been conducted in China but they do not feature large sample sizes or prospective designs. A systematic review of the clinical characteristics of BE in China was published in 2008. However, in this review, studies that used metaplasia as a necessary standard were not excluded, which contrasts the consensus.

### Innovations and breakthroughs

The Digestive Disease Branch of the Chinese Medical Association drafted the

Diagnosis and Treatment Consensus of BE in 2005 and amended it in 2011, when a consensus amongst clinicians was achieved. Based on this consensus, the present research analyzed existing clinical studies with the aim of obtaining a comprehensive understanding of the characteristics of BE in China.

### Applications

This research analyzed existing clinical studies with the aim of understanding the characteristics of BE in China. The results obtained will help improve the treatment and follow-up protocols of BE patients in China.

### Terminology

BE is a pathological phenomenon that occurs when the stratified squamous epithelium in the lower esophagus is replaced by a metaplastic simple columnar epithelium. In some cases, BE is accompanied by intestinal metaplasia, which is considered a precursor of esophageal adenocarcinoma.

### Peer review

This research has a high degree of significance. The meta-analysis presented here reviews the characteristics of the BE cases in China, including patient demographics, endoscopic and histological features, and risks for developing adenocarcinoma. The inclusion and exclusion criteria are appropriate, and the information obtained from selected reports is sufficiently analyzed.

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## Smoking, alcohol consumption, and the risk of extrahepatic cholangiocarcinoma: A meta-analysis

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### Abstract

**AIM:** To assess the association between smoking and alcohol consumption and extrahepatic cholangiocarcinoma (ECC) through a meta-analysis of clinical observational studies.

**METHODS:** A literature search was conducted using Embase and MEDLINE databases from inception to 31 May 2013 without language limitations, and by manually searching the references of retrieved articles. Case-control and cohort studies that investigated the association between smoking or alcohol consumption and ECC were included. The quality of these studies was assessed using the Newcastle-Ottawa quality assessment scale. Summary relative risks and corresponding 95%CI were calculated using a random-effects model. Publication bias was assessed by Begg's funnel plot and

Egger's test.

**RESULTS:** A total of 12 eligible articles (11 case-control studies and one cohort study) were included in this meta-analysis. Eleven studies reported the association between smoking and ECC. Pooled analysis indicated that smokers had an increased risk of ECC development as compared with non-smokers (summary RR = 1.23; 95%CI: 1.01-1.50). This correlation was present in population-based studies ( $n = 5$ ; summary RR = 1.47; 95%CI: 1.06-2.05) but not in hospital-based studies ( $n = 6$ ; summary RR = 1.10; 95%CI: 0.88-1.37) and in non-Asian regions ( $n = 7$ ; summary RR = 1.39; 95%CI: 1.03-1.87) but not in Asia ( $n = 4$ ; summary RR = 1.08; 95%CI: 0.85-1.38). Seven studies reported an association between consuming alcohol and ECC. Pooled analysis indicated that alcohol drinkers had a similar risk of ECC development as did individuals who did not drink alcohol (summary RR = 1.09; 95%CI: 0.87-1.37). There was moderate heterogeneity among the studies and no evidence of publication bias.

**CONCLUSION:** Smoking is associated with an increased risk of ECC, but alcohol consumption is not. Further population-based studies, particularly cohort studies, are warranted to enable definitive conclusions.

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**Key words:** Extrahepatic cholangiocarcinoma; Smoking; Alcohol consumption; Meta-analysis; Relative risk

**Core tip:** Little is known about the etiology of extrahepatic cholangiocarcinoma (ECC) because of its rarity and high fatality. Smoking and alcohol consumption are potential risk factors for ECC development. However, reported relations between these two risk factors and ECC are conflicting. Our meta-analysis identified a positive association between smoking and the risk of ECC. The association between alcohol consumption and the



risk of ECC was positive but not significant. Further investigations are required.

Ye XH, Huai JP, Ding J, Chen YP, Sun XC. Smoking, alcohol consumption and the risk of extrahepatic cholangiocarcinoma: A meta-analysis. *World J Gastroenterol* 2013; 19(46): 8780-8788 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i46/8780.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i46.8780>

## INTRODUCTION

An extrahepatic cholangiocarcinoma (ECC) is a malignant tumor that arises from cholangiocytes and involves the biliary tree within the hepatoduodenal ligament<sup>[1]</sup>. It is a relatively rare but often lethal neoplasm that accounts for about 80% of cholangiocarcinomas in the Western world<sup>[2]</sup>. The prognosis of ECC is poor, and the 5-year survival rate for patients with ECC after resection is as low as 20%-40%<sup>[3]</sup>. Hilar cholangiocarcinoma is typically classified as extrahepatic<sup>[4]</sup>. Although the incidence of ECC seems to be constant (annual percent changes = 1%)<sup>[1,5,6]</sup>, it varies across regions, with the highest incidence in Southeast Asia and the lowest in Australia<sup>[7,8]</sup>. Such geographic variation may be associated with different genetic and environmental factors, including dietary patterns and lifestyle effects.

Although little is known about the etiology of ECC, several risk factors have been proposed to be involved in the development of this disease<sup>[9-11]</sup>. Epidemiological studies have found that a history of cholecystectomy, cholecystitis, parasitic infection, or primary sclerosing cholangitis is a risk factor for ECC<sup>[11-13]</sup>. There are other potential factors, such as hepatitis virus infection, obesity, diabetes, and host genetic polymorphisms, but these are less well established<sup>[4]</sup>. The low incidence of ECC precludes carrying out well-designed, single-center, case-control or prospective cohort studies with sufficient size and statistical power to determine the potential risk factors.

Smoking is associated with the risk of nonpulmonary cancer at many sites, including the liver and pancreas<sup>[14,15]</sup>, and consuming alcohol is related to cancer of the upper digestive tract<sup>[16]</sup>. The metabolites of smoking and alcohol have carcinogenic properties<sup>[17]</sup>. However, the reported correlations between these two risk factors and ECC are inconsistent<sup>[10,11,18-27]</sup>. The lack of consistency across studies may be due to the small number of cases, differences in the study populations, differences in methodological designs or exposure definitions, or a shortage of data concerning confounding factors.

To provide a quantitative assessment of the correlations between these two factors and the risk of ECC, we performed a meta-analysis of published studies following the meta-analysis of observational studies in epidemiology guidelines<sup>[28]</sup>.

## MATERIALS AND METHODS

### Data sources and searches

Two investigators (Ye XH and Huai JP) independently performed a computerized search of MEDLINE (from 1 January 1966 to 31 May 2013) and Embase (from 1 January 1974 to 31 May 2013) databases to identify potentially relevant articles. Searches were performed using the following text words and/or Medical Subject Headings: “tobacco”, “smoking”, “alcohol”, “beverages”, “ethanol”, “cholangiocarcinoma”, “extrahepatic”, “bile duct cancer”, and “epidemiologic studies”; the search results were restricted to studies performed after 1990 to avoid any possible inconsistencies in the diagnostic criteria used. The bibliographies of all relevant articles were reviewed manually to identify additional relevant articles. No language restrictions were imposed.

### Study selection

Studies were included if they fulfilled the following criteria: (1) case-control or cohort design and published in manuscript form; (2) smoking or alcohol consumption included as an exposure of interest; (3) ECC included as an outcome of interest; and (4) RR in cohort studies or OR in case-control studies and their 95%CI (or sufficient data to calculate them) reported. If data on the same population were reported in multiple papers, the most informative report was selected. Studies were excluded if the data were not specified for ECC, or if they reported data for another type of cancer. Articles or reports that were not peer reviewed were not included.

### Data extraction

Two investigators (Ye XH and Huai JP) independently extracted the following data from all included studies: first author's last name, publication year, geographic location of the study population, study design, methods used to determine risk factors and ECC, sample size (cases and controls or cohort size), variables adjusted for in the analysis, and RR estimates with corresponding 95%CI. From each study, the risk estimates that indicated the greatest degree of control for potential confounders were extracted, and discrepancies were resolved by consensus.

### Assessment of study quality

The quality of the included studies was assessed using the Newcastle-Ottawa scale<sup>[29]</sup>. The scale consists of nine items that cover three dimensions: (1) patient selection (four items); (2) comparability of the two study arms (two items); and (3) assessment of outcome (three items). A point is awarded for each item that is satisfied by the study. The total score therefore ranges from zero to nine, with higher scores indicating higher quality. Studies that scored seven or more points were considered to be of high quality. The Newcastle-Ottawa scale score was assessed independently by both of the reviewers. Discrepancies in the score were resolved through discussion between the reviewers.

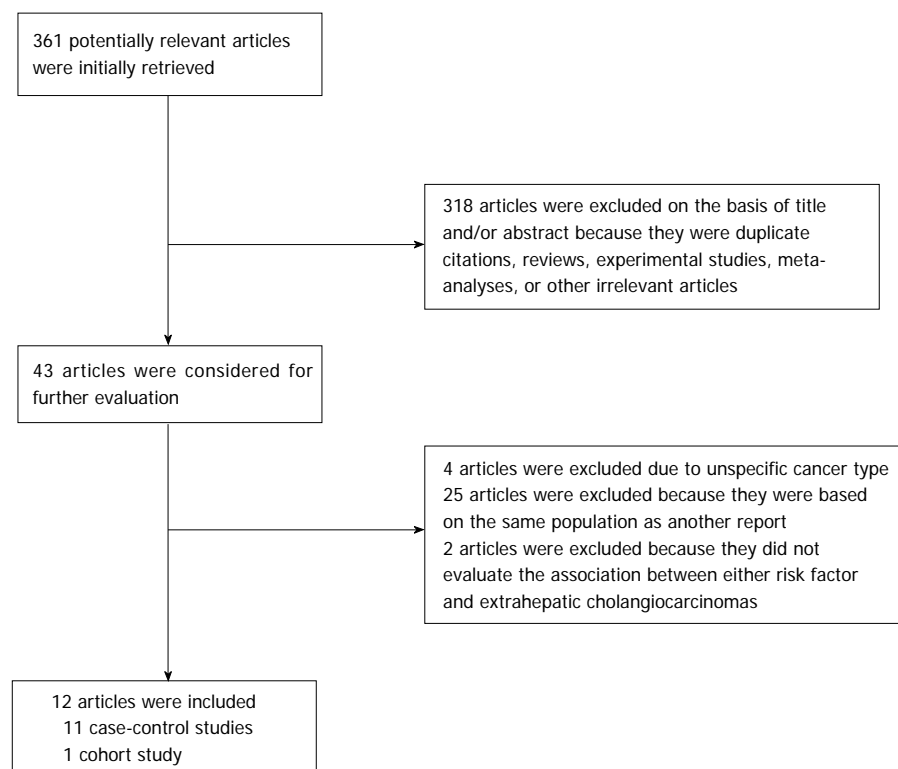


Figure 1 Flow chart of the study selection.

### Statistical analysis

Different measures of RR were included in this meta-analysis: case-control studies (odds ratio) and cohort studies (rate ratio, hazard ratio). In practice, these measures of effect yielded similar estimates of RR because of the low absolute risk of ECC.

Summary RR estimates with their corresponding 95%CI were calculated with a random-effects model using the methods of DerSimonian and Laird, which consider both within- and between-study variations<sup>[30]</sup>. Most of the included studies reported the RR of ECC for smokers *vs* nonsmokers and alcohol drinkers *vs* non-alcohol drinkers. If studies reported separate RRs for males and females or for different levels of alcohol consumption, we calculated the pooled RR and its corresponding 95%CI. We conducted further analyses stratified by study design, geographic region, and adjustment for cholelithiasis.

Heterogeneity was evaluated using the  $Q$ -statistic and quantified by  $I^2$ <sup>[31]</sup>. For the  $Q$  test, a  $P$  value of  $> 0.10$  was considered to indicate no statistically significant heterogeneity.  $I^2$  is the proportion of total variation contributed by between-study variation. In addition, a sensitivity analysis was carried out to estimate the effects of each included study on the overall pooled RR. Publication bias was assessed using Begg's funnel plot and Egger's test<sup>[32,33]</sup>. All statistical analyses were carried out using STATA software (*vs* 12.0; STATA, College Station, Texas, United States). A two-tailed  $P$  value of  $< 0.05$  was considered to be significant.

## RESULTS

### Search results and study characteristics

Twelve articles (11 case-control studies and 1 cohort

study) were included in this meta-analysis (Figure 1). Eleven studies reported the association between smoking and ECC, and seven studies reported the association between alcohol consumption and ECC. Briefly, our initial search identified 361 articles, and 318 were excluded by examining the titles and abstracts. Reasons for the exclusion included duplicate citations, reviews, experimental studies, meta-analyses and other irrelevant articles. Forty-three full-text articles were considered for detailed evaluation. One additional relevant study was identified by manually reviewing the references of all 43 articles. Thirty-one of these 43 articles were subsequently excluded from the meta-analysis: 4 did not specify the cancer type, 25 were duplicate reports based on the same population, and 2 did not evaluate the association between each risk factor and ECC. The remaining 12 studies were published between 1993 and 2013 and included a total of 1834 incident cases (Table 1). The studies were carried out in Asia ( $n = 4$ ), North America ( $n = 6$ ), and Europe ( $n = 2$ ; Table 1). Ten of the 12 studies were of high quality (Newcastle-Ottawa scale score  $\geq 7$ ; Table 2).

Control subjects in the 11 case-control studies were recruited from a population-based<sup>[11,18,19,21,26]</sup> or hospital-based setting<sup>[10,20,23-25,27]</sup> (Table 1). Most studies used a questionnaire or hospital records to evaluate smoking or alcohol consumption status (Table 1). ECC was diagnosed on the basis of histological and imaging methods in 10 studies and according to diagnostic codes in 1 study<sup>[11]</sup>; the method of diagnosis was not reported in 1 study<sup>[18]</sup> (Table 1). Adjustments were made for potential confounders of one or more factors in nine of 12 studies (Table 1).

One study<sup>[18]</sup> reported an increased risk of ECC in

Table 1 Characteristics of the 12 studies that reported an association between smoking or alcohol consumption and the risk of extrahepatic cholangiocarcinoma

Author and year	Country	Design	Source	Number of cases	Number of controls	Risk factor assessment	ECC ascertainment	Smoking RR (95% CI)	Alcohol RR (95% CI)	Adjustments
Ghadirian <i>et al</i> <sup>[18]</sup> 1993	Canada	Case-control	Population	24	239	Questionnaire	NA	2.820 (1.010-7.860)	-	Age, sex, other smoking habits, alcohol consumption, schooling, respondent status
Chow <i>et al</i> <sup>[19]</sup> 1994	United States	Case-control	Population	64	255	Questionnaire	Pathological	1.630 (0.900-2.970) <sup>1</sup>	0.600 (0.290-1.220) <sup>1</sup>	Age, ethnic origin, smoking status (adjusted for alcohol consumption)
Khan <i>et al</i> <sup>[20]</sup> 1999	United States	Case-control	Hospital	31	138	Medical records	Pathological	0.630 (0.210-1.880)	-	Age, female gender, ethnicity, cholelithiasis, socioeconomic status
Zhang <i>et al</i> <sup>[21]</sup> 2004	China	Case-control	Population	99	373	Questionnaire	Cancer registry	1.490 (0.870-2.560) <sup>2</sup>	1.290 (0.780-2.170) <sup>2</sup>	Age, total energy, cholelithiasis, hypertension, history of salty food intake, smoking status (adjusted for alcohol consumption)
Welzel <i>et al</i> <sup>[11]</sup> 2007	United States	Case-control	Population	549	102782	Medical records	Cancer registry	1.700 (1.000-3.000)	-	Age, sex, race, geographic location, state buy-in status
Shaib <i>et al</i> <sup>[10]</sup> 2007	United States	Case-control	Hospital	163	236	Medical records	Pathological + imaging	1.300 (0.800-1.900) <sup>3</sup>	1.290 (0.190-8.910) <sup>1</sup>	Race, age, gender, HBV, HCV markers (adjusted for alcohol consumption)
El-Serag <i>et al</i> <sup>[22]</sup> 2009	United States	Cohort	-	-	-	Registry	Cancer registry	-	1.060 (0.600-1.870)	Age, sex, baseline visit date, type of visit, a preceding visit
Tao <i>et al</i> <sup>[23]</sup> 2010	China	Case-control	Hospital	129	380	Medical records	Pathological + imaging	0.900 (0.500-1.300) <sup>3</sup>	1.200 (0.800-1.900) <sup>3</sup>	-
Cai <i>et al</i> <sup>[24]</sup> 2011 <sup>4</sup>	China	Case-control	Hospital	313	608	Medical records	Pathological	0.900 (0.640-1.248) <sup>3</sup>	-	-
Onal <i>et al</i> <sup>[25]</sup> 2012	Turkey	Case-control	Hospital	89	48	Questionnaire	Pathological + imaging	1.900 (0.900-4.200) <sup>3</sup>	4.010 (0.480-33.62) <sup>3</sup>	-
Brandi <i>et al</i> <sup>[26]</sup> 2013	Italy	Case-control	Population	59	212	Questionnaire	Pathological	0.780 (0.400-1.500)	-	Age, sex, region of residence
Zhou <i>et al</i> <sup>[27]</sup> 2013	China	Case-control	Hospital	239	478	Medical records	Pathological + imaging	1.301 (0.863-1.962)	1.053 (0.670-1.655)	Age, sex, cirrhosis, cholelithiasis, cholecystectomy, DM, family history of other cancer

<sup>1</sup>The summary RRs and 95%CI were derived by pooling the relative risks for each sex; <sup>2</sup>Only males were included in the summary relative risks because of the rarity of smoking in females; <sup>3</sup>Univariate OR was calculated because adjusted ORs were not available; <sup>4</sup>Only hilar cholangiocarcinomas were included; ECC: Extrahepatic cholangiocarcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; DM: Diabetes mellitus; NA: Not available.

smokers as compared with non-smokers, whereas 10 studies reported a similar risk of ECC in smokers and non-smokers (Table 1). All seven studies reported a similar risk of ECC in alcohol drinkers and non-alcohol drinkers (Table 1). One study<sup>[10]</sup> stratified alcohol consumption as moderate/heavy and found an increased risk of ECC in heavy drinkers as compared with non-drinkers (RR = 3.6; 95%CI: 1.5-9.4).

Only one study had a prospective cohort design and evaluated the association between alcohol consumption and the risk of ECC<sup>[22]</sup>. A total of 75 incidence cases of ECC were reported. An increased risk of ECC was observed in alcohol drinkers as compared with non-alcohol drinkers, but this was not significant.

## Smoking and risk of ECC

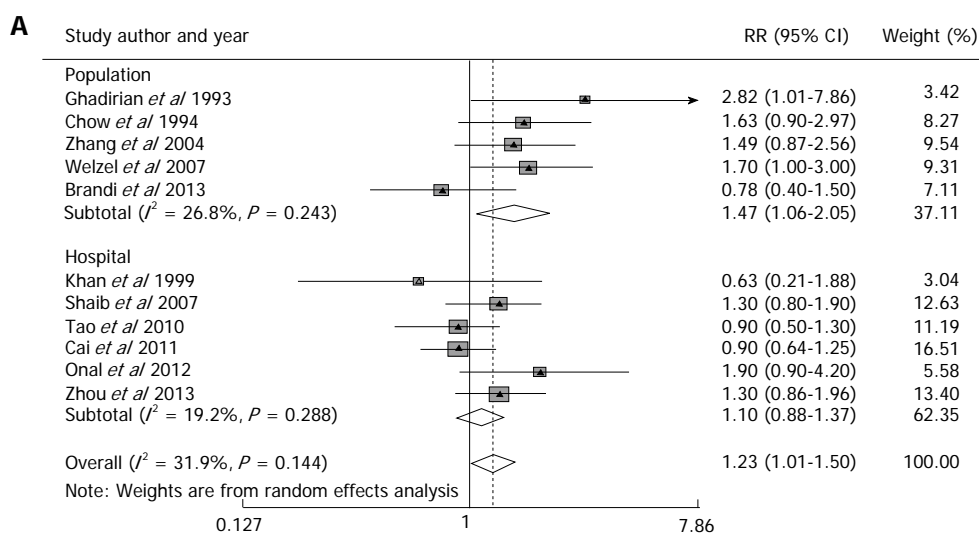
Eleven case-control studies were identified that reported the association between smoking and the risk of ECC<sup>[10,11,18,21,23-27]</sup>. The summary RR for ECC was 1.23 (95%CI: 1.01-1.50) in a random-effects model for smokers vs non-smokers (Figure 2A). There was moderate heterogeneity among studies ( $Q = 14.69$ ,  $P = 0.144$  for heterogeneity,  $I^2 = 31.9\%$ ).

Subgroup meta-analyses were conducted according to geographical region, study design and confounders. The summary RR for ECC was significant for studies conducted outside of Asia<sup>[10,11,18,20,25,26]</sup> ( $n = 7$ ; summary RR = 1.39; 95%CI: 1.03-1.87;  $P = 0.218$  for heterogeneity,  $I^2 = 27.5\%$ ) but not for studies conducted in Asia ( $n = 4$ ; summary RR = 1.08; 95% CI, 0.85-1.38;  $P = 0.278$  for heterogeneity,  $I^2 = 22.1\%$ ; Table 3). The summary RR for ECC was significant for population-based case-control studies<sup>[11,18,19,21,26]</sup> ( $n = 5$ ; summary RR = 1.47; 95%CI: 1.06-2.05;  $P = 0.243$  for heterogeneity,  $I^2 = 26.8\%$ ) but not for hospital-based case-control studies ( $n = 6$ ; summary RR =

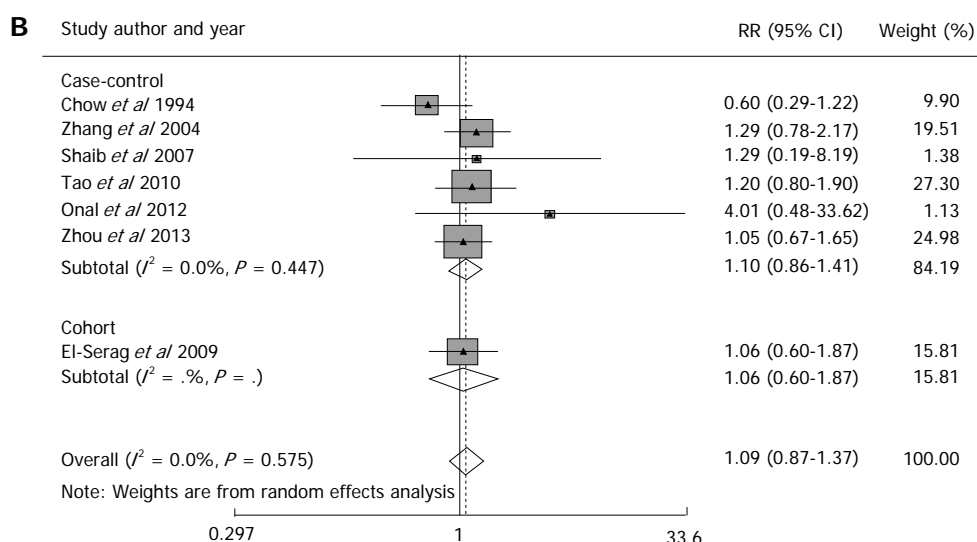
**Table 2** Quality of the studies used in this analysis

Author and year	Quality indicators of Newcastle-Ottawa quality assessment scale									Score
	Selection				Comparability		Exposure/outcome			
	I a	I b	I c	I d	II a	II b	III a	III b	III c	
Ghadirian <i>et al</i> <sup>[18]</sup> 1993	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	6
Chow <i>et al</i> <sup>[19]</sup> 1994	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	7
Khan <i>et al</i> <sup>[20]</sup> 1999	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Zhang <i>et al</i> <sup>[21]</sup> 2004	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	7
Welzel <i>et al</i> <sup>[11]</sup> 2007	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Shaib <i>et al</i> <sup>[10]</sup> 2007	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	7
El-Serag <i>et al</i> <sup>[22]</sup> 2009	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	8
Tao <i>et al</i> <sup>[23]</sup> 2010	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Cai <i>et al</i> <sup>[24]</sup> 2011	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Onal <i>et al</i> <sup>[25]</sup> 2012	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	7
Brandi <i>et al</i> <sup>[26]</sup> 2013	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	6
Zhou <i>et al</i> <sup>[27]</sup> 2013	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	7

For case-control studies; I a: Indicates cases with independent validation; I b: Indicates consecutive or representative cases; I c: Indicates community controls; I d: Indicates controls with no history of ECC; II a: Indicates that study controls were comparable for age and sex; II b: Indicates that study controls were comparable on all additional factor(s) reported; III a: Indicates that the same method of ascertainment was used for cases and controls; III b: Indicates that assessment of exposure was from a secure record; III c: Indicates that the non-response rate was similar in both groups. For cohort studies; I a: indicates that the exposed cohort was representative of the population; I b: Indicates that the non-exposed cohort was drawn from the same population; I c: Indicates that the exposure ascertainment was from secure records or a structured interview; I d: Indicates that ECC was not present at start of study; II a: Indicates that the cohorts were comparable for age and sex; II b: Indicates that the cohorts were comparable on all additional factor(s) reported; III a: Indicates that ECC was assessed from a secure record; III b: Indicates that follow-up was long enough for ECC to occur; III c: Indicates that follow-up was complete. ECC: Extrahepatic cholangiocarcinoma.



**Figure 2** Relative risk of extrahepatic cholangiocarcinoma. A: Smokers as compared with non-smokers in population- and hospital-based case-control studies; B: Alcohol drinkers as compared with non-alcohol drinkers in case-control and cohort studies.





**Table 3** Subgroup analyses of the association between smoking and extrahepatic cholangiocarcinoma and alcohol consumption and extrahepatic cholangiocarcinoma

	No. of studies	RR (95%CI)	Tests for heterogeneity		
			<i>Q</i>	<i>P</i>	<i>I</i> <sup>2</sup>
Smoking					
Geographical region					
Non-Asia	7	1.39 (1.03-1.87)	8.280	0.218	27.5%
Asia	4	1.08 (0.85-1.38)	3.850	0.278	22.1%
Study design					
Population-based	5	1.47 (1.06-2.05)	5.470	0.243	26.8%
Hospital-based	6	1.10 (0.88-1.37)	6.190	0.288	19.2%
Adjustment for cholelithiasis	3	1.28 (0.94-1.76)	1.917	0.383	0.0%
Alcohol drinking					
Geographical region					
Non-Asia	4	0.94 (0.56-1.56)	3.560	0.313	15.7%
Asia	3	1.17 (0.90-1.53)	0.360	0.835	0.0%
Study design					
Population-based	2	0.92 (0.44-1.94)	2.890	0.089	65.4%
Hospital-based	4	1.16 (0.86-1.58)	1.520	0.678	0.0%
Case-control	6	1.10 (0.86-1.41)	4.750	0.447	0.0%
Cohort	1	1.06 (0.60-1.87)	-	-	-

1.10; 95%CI: 0.88-1.37;  $P = 0.288$  for heterogeneity,  $I^2 = 19.2\%$ ; Table 3). The summary RR was not significant for studies that controlled for cholelithiasis<sup>[2,21,27]</sup> (summary RR = 1.28; 95%CI: 0.94-1.76;  $P = 0.383$  for heterogeneity,  $I^2 = 0\%$ ; Table 3).

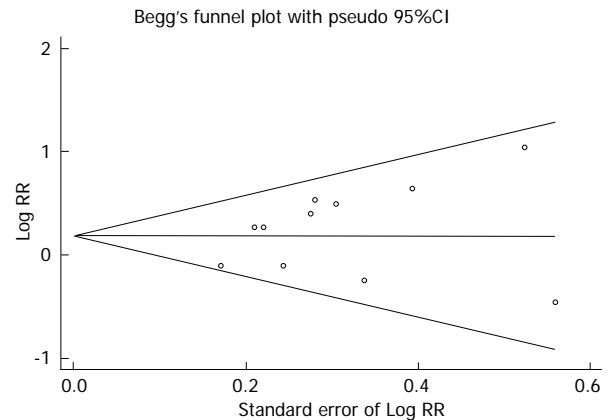
### Alcohol consumption and risk of ECC

Six case-control studies and one prospective cohort study were identified that reported an association between alcohol consumption and the risk of ECC. The summary RR for ECC was 1.09 (95%CI: 0.87-1.37) in a random-effects model for alcoholic drinkers *vs* non-alcoholic drinkers (Figure 2B). There was no heterogeneity among studies ( $Q = 4.76$ ,  $P = 0.575$  for heterogeneity,  $I^2 = 0\%$ ).

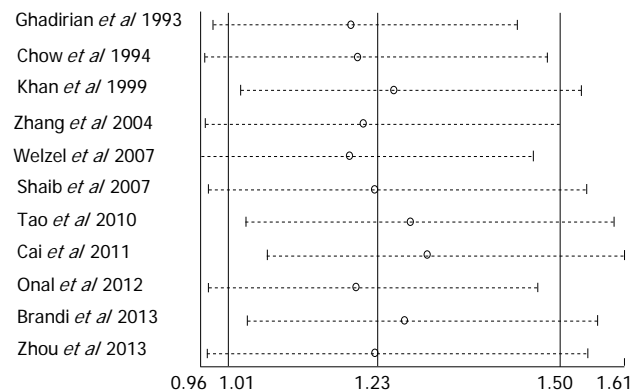
Subgroup meta-analyses were conducted according to geographical region and study design. The summary RR was not significant for studies conducted outside of Asia ( $n = 4$ ; summary RR = 0.94; 95%CI: 0.56-1.56;  $P = 0.313$  for heterogeneity,  $I^2 = 15.7\%$ ) or in Asia ( $n = 3$ ; summary RR = 1.17; 95%CI 0.90-1.53;  $P = 0.835$  for heterogeneity,  $I^2 = 0\%$ ; Table 3). The summary RR was not significant for case-control studies ( $n = 6$ ; summary RR = 1.10; 95%CI: 0.86-1.41;  $P = 0.447$  for heterogeneity,  $I^2 = 0\%$ ) or for the cohort study (RR = 1.06; 95%CI 0.60-1.87; Table 3). The summary RRs for the population-based<sup>[19,21]</sup> ( $n = 2$ ; summary RR = 0.92; 95%CI 0.44-1.94;  $P = 0.089$  for heterogeneity,  $I^2 = 65.4\%$ ) and hospital-based<sup>[10,23,25,27]</sup> ( $n = 4$ ; summary RR = 1.16; 95%CI: 0.86-1.58;  $P = 0.678$  for heterogeneity,  $I^2 = 0\%$ ) case-control studies were not significant (Table 3).

### Publication bias and sensitivity analysis

A funnel plot showed no evidence of publication bias (Figure 3).  $P$  values for Begg's adjusted rank correlation test and Egger's regression asymmetry test were 0.161 and 0.296, respectively, which indicate that publication



**Figure 3** A Begg's funnel plot with pseudo 95% confidence limits showing the symmetrical distribution of included studies. This indicates that there was no publication bias.



**Figure 4** Influence of each individual study on the relative risks of extrahepatic cholangiocarcinoma in smokers as compared with non-smokers. Data show the RR (open circle) and 95%CI (dashed horizontal line) when the study named on the left was omitted. Random-effects estimates (exponential form) were used. RR: Relative risk.

bias probably had little effect on summary estimates.

Sensitivity analysis was performed to assess the influence of individual studies on the overall risk of ECC by excluding each individual study and recalculating the pooled RR. Similar RR and 95%CI were generated with the exclusion of each study, indicating the high degree of stability of the results (Figure 4).

## DISCUSSION

In this meta-analysis, we assessed the association between smoking and the risk of ECC and between alcohol consumption and the risk of ECC. A previous meta-analysis evaluated the association between alcohol consumption and the risk of extrahepatic bile system cancer<sup>[34]</sup>, and a more recent meta-analysis investigated the association between smoking and the risk of gallbladder cancer<sup>[35]</sup>. Although both of these previous studies investigated the risk of extrahepatic bile system cancer, the risk of ECC was not specified. To the best of our knowledge, this is the first study to provide comprehensive evidence of the association between smoking and alcohol consumption and the risk of ECC. In this meta-analysis we found that

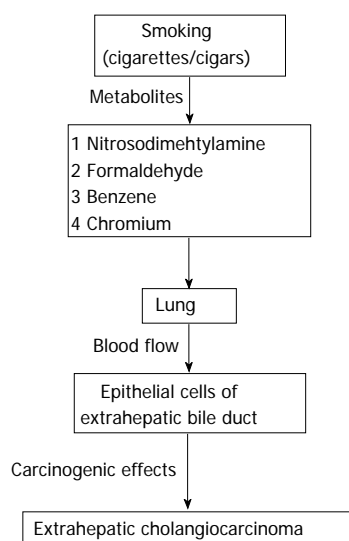


Figure 5 Proposed mechanisms by which smoking may be associated with the formation of extrahepatic cholangiocarcinoma.

smokers had a 23% increased risk of ECC as compared with non-smokers. The association between alcohol consumption and the risk of developing ECC was positive but not significant.

Although the incidence of ECC remained low among smokers and alcohol drinkers, our results carry substantial clinical and public health implications. The incidence of ECC has been on the rise worldwide in recent years, although this type of malignancy is uncommon<sup>[4]</sup>. The number of habitual smokers is rising in spite of current anti-smoking campaigns<sup>[36]</sup>, and a rapid increase in the consumption of alcohol has been documented in many regions<sup>[37]</sup>. It is estimated that there are currently more than 500 million alcohol drinkers in China<sup>[16,36]</sup> and approximately 37% of Chinese adults are heavy drinkers<sup>[37]</sup>.

In the subgroup analysis, we found that smoking was associated with an increased risk of ECC in non-Asian regions but not in Asia. This difference may be associated with ethnicity or with differences in the types of tobacco use between the two areas. For example, cigars contain more nicotine than regular cigarettes<sup>[38]</sup>, and the pH of cigar smoke is higher than that of cigarettes, allowing more complete delivery of nicotine into the bloodstream<sup>[38,39]</sup>. Only 0.3% of the Chinese population use cigars<sup>[40]</sup>, compared with 6.7% of the American population<sup>[40]</sup>. However, most of the studies included in our meta-analysis did not report the type of tobacco use, and thus we could not conduct further analysis. Although the risk of ECC was similar in smokers and non-smokers in Asia, attention should still be paid to the potential association between smoking and ECC in this population. The number of smokers in China increased from 320 to 350 million from 2005 to 2007<sup>[16,36]</sup> and 72% of Chinese citizens aged 15 years or older have been exposed to tobacco<sup>[41,42]</sup>.

The potential mechanism by which smoking and alcohol consumption are associated with ECC remains unknown. Direct carcinogenic properties of smoking might be mediated by various metabolites generated in cigarettes including formaldehyde, benzene, and chromium.

As early as the 1970s, it was suggested that tobacco compounds exert carcinogenic effects on the epithelial cells of the bile ducts as a result of exposure via blood flow<sup>[43]</sup> (Figure 5), and this may underlie the relation between smoking and ECC. For alcohol consumption, it is more likely that there is an indirect and bidirectional effect of carcinogenesis on the development of ECC. Moderate alcohol intake protects against gallstone formation, and gallstones are a risk factor for biliary tract cancer<sup>[17]</sup>. Metabolites of alcohol are produced in the liver and excreted into the bile duct and may interact with cholesterol metabolism. Alcohol also enhances the activation of different precarcinogenic elements<sup>[17]</sup>. Therefore, alcohol may be associated with ECC via co-effects of different mechanisms.

As with all meta-analyses of observational studies, our results have several potential limitations. First, definitions of both smoking and alcohol consumption were not consistent across the included studies. In addition, a dose-response relationship between alcohol consumption and ECC was observed in one study<sup>[10]</sup>, in which the risk of ECC development was higher in heavy drinkers who consumed at least 80 g of ethanol per day. However, we could not further evaluate this dose-response relationship because of a paucity of data. The majority of studies included in this meta-analysis were case-control studies, which are more susceptible to selection and recall bias than are cohort studies. Associations between smoking or alcohol consumption and the risk of ECC in case-control studies may be confounded by changes in lifestyle after the diagnosis of ECC. In addition, 6 of the 11 case-control studies were hospital based, and these cases may not represent the general population of patients with ECC. This may have introduced selection bias into our results. Furthermore, moderate heterogeneity was observed across studies, and this may also bias the results. This heterogeneity results from diversity of the study designs, analysis of populations from different geographic locations, and the selection of participants for the different studies. These biases may distort the true associations, and data provided by this meta-analysis should thus be interpreted with caution.

Confounding effects may also have influenced the results of this meta-analysis. As noted above, moderate alcohol consumption is inversely related to gallstone disease<sup>[17,44]</sup>. When we limited the meta-analysis to studies that were adjusted for cholelithiasis, the association between smoking and ECC was no longer significant, suggesting that a confounding effect may exist. The possibility of residual confounding such as gallstone formation cannot be excluded because of a paucity of data.

Although it is possible that small studies with null results were less likely to be published than large studies with significant results, we found no evidence from funnel plot analysis and formal statistical tests for such bias.

In conclusion, the results from this meta-analysis suggest that smoking, but not alcohol consumption, is associated with a higher risk of ECC. However, the pos-

sibility that the association may be influenced by bias or confounding variables cannot be fully excluded. Further well-designed prospective studies are warranted to clarify the association between smoking and alcohol consumption and the risks of ECC.

## COMMENTS

### Background

Data based on epidemiological studies related to the associations between smoking and alcohol consumption and extrahepatic cholangiocarcinoma (ECC) remain conflicting. The aim of this meta-analysis was to assess the association of each risk factor with ECC.

### Research frontiers

Until now, several studies have assessed the association between smoking and alcohol consumption and the risk of ECC in various regions and ethnicities; however, the results have been mixed and inconsistent. No quantitative summary of the evidence has ever been provided.

### Innovations and breakthroughs

This meta-analysis identified that smoking was associated with an increased risk of ECC, especially in population-based studies and studies conducted in non-Asian regions. A positive but non-significant increased risk of ECC was observed in alcohol drinkers as compared with non-alcohol drinkers.

### Applications

These results suggest that smoking is associated with an increased risk of ECC, especially in population-based studies and in studies conducted in non-Asian regions. Lifestyle changes may contribute to reducing the incidence of ECC.

### Peer review

This was a well-performed meta-analysis of currently available studies on the association between smoking and alcohol consumption and ECC. The authors concluded that smoking rather than alcohol consumption may be associated with increased risk of ECC, with an emphasis on population-based studies and in non-Asian regions. This study was well designed and performed, and the results are well discussed.

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## Acute cholestatic hepatitis caused by amoxicillin/clavulanate

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abdomen, serum tests for infection history, laboratory screening of autoimmune diseases, nuclear magnetic resonance (NMR) of the abdomen with bile duct-NMR and transcutaneous liver biopsy guided by ultrasound. The duration of disease was approximately 4 mo, with complete resolution of symptoms and laboratory changes at the end of that time period. Specific treatment was not instituted, only a combination of anti-emetic (metoclopramide) and cholestyramine for pruritus.

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**Key words:** Hepatology; Hepatitis; Amoxicillin/Clavulanate; Drug reactions; Hyperbilirubinemia

**Core tip:** This report describes a case of acute cholestatic hepatitis caused by the use of amoxicillin/clavulanate. This case presented an unusual evolution, characterized by severe hyperbilirubinemia and cholestatic symptoms without the development of hepatic failure, and with total resolution requiring no specific treatment. There are few case reports in the literature that describe a similar clinical condition due to drug-induced cholestatic hepatitis.

### Abstract

Amoxicillin/clavulanate is a synthetic penicillin that is currently commonly used, especially for the treatment of respiratory and cutaneous infections. In general, it is a well-tolerated oral antibiotic. However, amoxicillin/clavulanate can cause adverse effects, mainly cutaneous, gastrointestinal, hepatic and hematologic, in some cases. Presented here is a case report of a 63-year-old male patient who developed cholestatic hepatitis after recent use of amoxicillin/clavulanate. After 6 wk of prolonged use of the drug, he began to show signs of cholestatic icterus and developed severe hyperbilirubinemia (total bilirubin > 300 mg/L). Diagnostic investigation was conducted by ultrasonography of the upper

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### INTRODUCTION

Amoxicillin/clavulanate is a synthetic penicillin that is currently commonly used, especially for the treatment of respiratory and cutaneous infections. The addition of

clavulanate to amoxicillin provides action against bacteria that produce beta-lactamase, conferring a wide spectrum against gram-positive and -negative bacteria for the drug<sup>[1]</sup>. However, this combination considerably changes the frequency of collateral effects, as described in a study by Francesco Salvo *et al.*<sup>[1]</sup> that examined the frequency of drug reactions in six Italian regions from January 1988 to June 2005. Their study showed that the percentage of gastrointestinal, hepatic and hematological reactions was significantly higher for amoxicillin/clavulanic acid (13%, 4% and 2%, respectively) than for amoxicillin (7%, 1% and 1%, respectively)<sup>[1]</sup>.

With respect to hepatic side effects, cases of drug-induced hepatitis by amoxicillin/clavulanate have been reported since the 1980s, typically with a benign course. Approximately 23% of individuals on amoxicillin/clavulanate experience non-significant increases in hepatic enzymes<sup>[2]</sup>. However, a small number of severe episodes have been described, some of which are characterized by fulminant hepatitis, a disease that leads to death or the need for liver transplant<sup>[3]</sup>.

Presented here is a case report of a 63-year-old male patient who developed cholestatic hepatitis after use of amoxicillin/clavulanate.

## CASE REPORT

The male, a 63-year-old patient was admitted to the Hospital Renascentista on September 1, 2012, with a history of jaundice, choloria, fecal acholia, generalized pruritus, malaise, hyporexia and sporadic nausea without associated vomiting for five days.

The patient had hypertension for 10 years, dyslipidemia for 3 years, a recent diagnosis of altered fasting blood sugar, oligosymptomatic benign prostatic hyperplasia for 3 years and was overweight. He took 50/12.5 mg of atenolol/chlorthalidone once a day. He indicated that it had been approximately 45 d since he had used a topical corticoid for 15 d for acute otitis, and denied using any other medications. He had no history of trauma or surgery, and no epidemiological history of note. He was a non-smoker and drank alcohol on the weekend (three cans of beer on Saturdays and Sundays), but did not drink in the periods preceding the beginning of the symptoms.

Upon physical examination at admission, the patient was jaundiced (2+/4) with small traumatic lesions on the skin, was afebrile, normotensive (AP: 130/80 mmHg) with a heart rate of 80 beats/min, eupneic without changes in pulmonary auscultation and showed normal findings on abdominal examination with no visceromegaly.

The admission tests indicated Hb: 130.6 g/L, leukocytes: 7200 cells/mm<sup>3</sup> (normal differential), platelets: 248000 cells/mm<sup>3</sup>, fasting glycemia: 1100 mg/L, creatinine: 9 mg/L, urea: 290 mg/L, Na: 137 mEq/L, K: 3.8 mEq/L, Mg: 18 mg/L, Ca: 42 mg/L, blood gases: normal, CRP: 66 mg/L, amylase: 40 IU/L, lipase: 351 U/L, AST: 78 IU/L, ALT: 200 IU/L, alkaline phosphatase: 60 IU/L, GGT: 33 IU/L, albumin: 33 g/L, complete coagu-



**Figure 1** Magnetic resonance of abdomen and bile duct-nuclear magnetic resonance. Images of bile duct-nuclear magnetic resonance demonstrating the biliary tree without evidence of obstructive processes.

lation profile: normal, LDH: 2 941 U/L, total bilirubin: 83 mg/L, direct bilirubin: 50.1 mg/L, reticulocytes: 0.9%, haptoglobin: 1440 mg/L (160-2200 mg/L).

Diagnostic investigation began with ultrasonography of the upper abdomen, which demonstrated only cholesterosis of the biliary vesicles. Serological tests for hepatitis A, hepatitis B, hepatitis C, hepatitis E, cytomegalovirus, Epstein-Barr, dengue, leptospirosis and HIV were all negative. Auto-immune analysis demonstrated negative anti-nuclear factor, anti-smooth muscle and anti-mitochondria, and normal serum IgG and serum IgM. Nuclear magnetic resonance (NMR) of the abdomen with bile duct-NMR was subsequently requested, which demonstrated constricted biliary vesicles (Figure 1).

The Council for International Organizations of Medical Sciences (CIOMS) score was +9 points and the Clinical Diagnostic Scale (CDS) score was +11 points.

During hospitalization, the patient developed a progressive worsening of hyperbilirubinemia (Table 1), pruritus, malaise and nausea, where he received symptomatic treatment with cholestyramine 4 g four times per day and metoclopramide when necessary.

Because there was no clinical improvement on day 30, the patient was more extensively questioned. Additionally, the accompanying family members were asked to bring all recent medical documents. A prescription was found for amoxicillin-clavulanate 500 mg 3 times a day for twenty-one days, which had been initially used forty-five days ago, together with a topical corticoid to treat acute otitis. The presence of these drugs led to a suspected diagnosis of drug-induced cholestatic hepatitis, which was confirmed by transcutaneous liver biopsy (Figure 2).

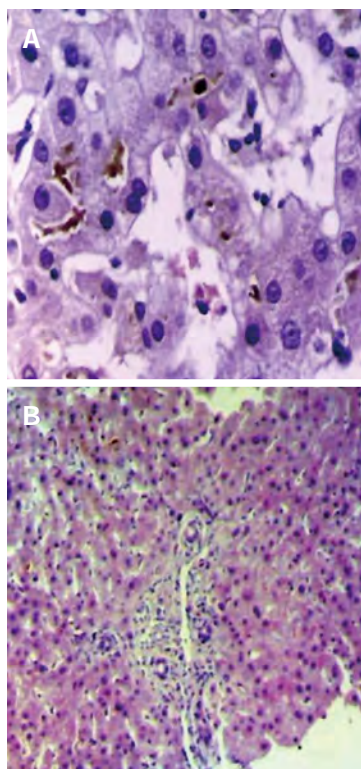
The patient began to show improvement, both clinically and based on laboratory results, after thirty days of hospitalization and was discharged with the use of cholestyramine 4 g four times per day, and for recommendation of outpatient follow-up.

Four months after the onset of symptoms, he became asymptomatic with jaundice and other previous changes resolved, normal routine liver tests, and he began receiving only previous chronic anti-hypertensive therapy.

**Table 1** Results of routine hepatic tests during hospitalization

	9/1	9/3	9/6	9/9	9/10	9/11	9/18	9/22	9/26
AST (IU/L)	78	89	80	75	51	50	63	77	70
ALT (IU/L)	200	245	160	130	69	59	65	89	82
BT/BD (mg/L)	80.3/50.1	100.9/70.0	110.3/70.9	150.6/90.0	180.3/110.0	210.9/140.0	250.9/160.0	280.9/180.0	310.2/200.0
AP (IU/L)	60	80	110	140	193	188	239	300	311
GGT (IU/L)	33	65	90	123	150	134	175	223	226
INR	1.01	1.00	1.12	1.1	1.0	1.2	1.0	1.0	1.0

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BT/BD: Total bilirubin/Direct bilirubin; AP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase.



**Figure 2** Transcutaneous liver biopsy guided by ultrasound. A: Liver biopsy at x 100 magnification - moderate cholestasis is demonstrated associated with discrete parenchymal activity with slight tumefaction of hepatocytes; B: Liver biopsy at x 40 magnification - portal space is shown with discrete increase in periportal lymphocytes, extravasation of lymphocytes towards the interface (spillover), absence of piecemeal necrosis and cholestasis.

## DISCUSSION

Amoxicillin/clavulanate is a widely used antibiotic that is associated with adverse effects, especially of the cutaneous, gastrointestinal, hepatic and hematologic types<sup>[1]</sup>. The incidence of hepatic damage by amoxicillin/clavulanate is greater than that associated with amoxicillin administration alone (1.7 *vs* 0.3 for every 10000 prescriptions)<sup>[1-4]</sup>; predominantly cholestatic lesions, although isolated mixed and hepatocellular lesions also occur<sup>[2,5-7]</sup>. There are also reports in the literature of patterns of granulomatous lesion secondary to the use of the medication in question<sup>[8]</sup>.

Histopathological examination usually reveals centrilobular or panlobular cholestasis and inflammation,

predominantly lymphocytic, portal and periportal, with neutrophils and eosinophils frequently present<sup>[2,3,6]</sup>. Other biopsy findings include degeneration and necrosis of ductal epithelial cells, ductopenia<sup>[4]</sup> and vacuolization and necrosis of hepatocytes<sup>[5,6]</sup>, all in addition to granulomatous inflammatory process<sup>[8]</sup>.

The pathogenic events that cause lesions due to the use amoxicillin/clavulanate require further study<sup>[3]</sup>, but it is believed that idiosyncratic immunoallergic mechanisms are the underlying causes<sup>[3,6,7,9-11]</sup>. The common presence of eosinophils in the inflammatory infiltrate<sup>[3]</sup>, the co-existence of manifestations of hypersensitivity, such as skin rash and hypereosinophilia<sup>[3]</sup>, the documentation of the involvement of specific autoantibodies (anti-mitochondrial type 6, anti-LKM2 and anti-LM antibodies)<sup>[3]</sup> and class II HLA antigens (DRB1\*1501-DRB5\*0101-DQB1\*0602)<sup>[11]</sup> reinforce the hypothesis that immune aggression is involved in the lesions formed due to amoxicillin-clavulanate use.

The risk factors for hepatotoxicity caused by amoxicillin/clavulanate include male sex, associated alcohol consumption, repeated courses of the drug, concomitant consumption of other hepatotoxic substances<sup>[2]</sup> and age over 55 years<sup>[7]</sup>. Treatment duration has been included as a predisposing factor in some reviews<sup>[3]</sup>.

The clinical characteristics are predominantly cholestatic signs and symptoms, which include malaise, hyporexia, nausea, vomiting, jaundice, choloria, fecal acholia, cutaneous pruritus and, less commonly, painful hepatomegaly. Manifestations associated with hypersensitivity can occur, such as skin rash and fever, with an incidence as high as 50%<sup>[12,13]</sup>. The symptoms can begin in any period after the end of treatment, but typically appear between 4 and 10 wk and are self-healing, as they are resolved in 4-16 wk. Reports of chronification, as described by Ryley *et al*<sup>[14]</sup>, are extremely rare.

Severe hyperbilirubinemia, changes in laboratory liver function blood tests and neurological alterations constitute the criteria for a poor prognosis, with the possibility of the development of fulminant hepatitis<sup>[3,6,9]</sup>.

Treatment consists mainly of support and should attend to various aspects of hepatic lesions. It is common for patients to become dehydrated due to decreased fluid intake and vomiting. Therefore, the evaluation of the volemic status is essential and should be corrected rapidly if necessary. Additionally, the cholestatic symp-



toms can become limiting and require prescriptions for symptomatic patients, such as anti-emetics and analgesics, in addition to medications to control pruritus. Generally, cholestyramine, anti-histamines, ursodeoxycholic acid and sertraline are used dependent on the intensity of symptoms and the experience of the service with the use of the drugs.

Due to the likely immunological mechanism of hepatic lesions, including hypersensitivity reactions mediated by eosinophils, some authors advocate the use of a systemic corticoid in the treatment of severe cases and in those with potential severity, such as hyperbilirubinemic individuals<sup>[6]</sup>. However, there is no evidence of reduced morbidity.

This study reported the case of a 63-year-old patient who began to show signs of cholestatic icterus after 6 wk of prolonged use of amoxicillin/clavulanate. The patient developed severe hyperbilirubinemia, but did not meet other criteria of severity. The time of disease was approximately four months, with complete resolution of symptoms and laboratory changes. The patient did not receive any specific treatment, only a combination of anti-emetics (metoclopramide) and cholestyramine for pruritus.

Of the risk factors for hepatotoxicity due to the drugs, advanced age, male sex, alcohol drinking and prolonged antibiotic therapy were all present in this case. The period of the onset of symptoms, the clinical characteristics and the time for complete recovery were in accordance with other reported cases. The degree of hyperbilirubinemia is important in the laboratory profile, with few described cases of total bilirubin reaching values higher than 300 mg/L<sup>[6]</sup>. Although there is a possible relation with severity, our patient did not show any signs of hepatic dysfunction. A complete history was taken, as described, to rule out other causes of hepatotoxicity. Based the Council for International Organizations of Medical Sciences score (CIOMS) (+9 points - very likely association)<sup>[15]</sup>, the Clinical Diagnostic Scale (CDS) score (+11 points - possible association)<sup>[16]</sup> and information from liver biopsy, this case had a high probability of hepatotoxicity due to drug use.

The biopsy findings, besides the cholestasis, were typical of hepatitis due to amoxicillin-clavulanate, periportal lymphocytic inflammation with damage to hepatocytes, alterations found in the pathology<sup>[2-6]</sup>.

A relevant factor in the case, which made the diagnosis difficult, was the denial of the patient to taking the medication. Thus, this report demonstrates the importance of thorough anamnesis and careful verification for antibiotics and/or other drugs use.

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## Pancreatic solid cystic desmoid tumor: Case report and literature review

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tumor; Central pancreatectomy; Pancreaticogastrostomy

**Core tip:** Desmoid tumors (DTs) are rare, representing approximately 0.03% of all tumors and 3% of soft tissues tumors. They are nonmetastatic and locally aggressive, with a high local recurrence rate. The pancreas is an extremely rare location for DTs. Moreover, pancreatic desmoids resembling solid cystic tumors are the rarest form of DTs. We report a 17-year-old patient presenting with a sporadic cystic DT of the pancreas, and subsequent disease management with central pancreatectomy. We report the case for its rarity and emphasize disease management by concerted application of clinical, pathological, radiological and immunohistochemical analyses. Associated English-language literature is also reviewed and summarized.

### Abstract

Desmoid tumors (DTs) are nonmetastatic, locally aggressive neoplasms with a high rate of postoperative recurrence. Pancreatic DTs are especially rare; only a few cases have been reported to date. This paper describes a case of a sporadic cystic DT of the pancreas managed successfully with central pancreatectomy, with no signs of recurrence 40 mo after surgery. According to the literature, this is the first reported case in China of a pancreatic DT presenting as a solid cystic lesion, as well as the first pancreatic DT managed with central pancreatectomy and pancreaticogastrostomy. We report the case for its rarity and emphasize disease management by concerted application of clinical, pathological, radiological and immunohistochemical analyses.

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**Key words:** Pancreatic tumor; Desmoid tumor; Cystic

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### INTRODUCTION

Desmoid tumors (DTs), also known as musculo-aponeurotic fibromatoses, are locally aggressive soft tissue neoplasms histologically characterized by fibroblastic proliferation within a collagen matrix<sup>[1]</sup>. Intra-abdominal DTs are associated with familial adenomatous polyposis (FAP) and Gardner syndrome, and they may also occur sporadically or subsequent to localized trauma (surgical or non-surgical). DTs possess negligible metastatic potential and frequently remain asymptomatic for extended periods of time before a diagnosis can be made on the basis of either vague, chronic symptoms or obvious mechanical complications. Aggressive expansion into adjacent tissues

results in significant morbidity due to nerve and organ damage, and the mortality rate due to DTs approaches 10%. DTs also present a considerable dilemma for clinicians because of their high rates of recurrence after surgery, especially following conservative resection. The pancreas is a rather rare location for DTs, even though sporadic DTs are found within the pancreas more frequently than sporadic intra-abdominal DTs<sup>[2]</sup>. Since 1956 when Wilson reported the first case of a pancreatic DT resembling a pancreatic pseudocyst, only a few similar cases have been reported<sup>[1,3]</sup>.

Here, we report a 17-year-old patient presenting with a sporadic cystic DT of the pancreas, and subsequent disease management with central pancreatectomy. Pertinent English-language literature is also reviewed and summarized.

## CASE REPORT

In June 2009, a 17-year-old boy presented to the gastroenterology department in our hospital with complaints of upper abdominal pain, nausea and vomiting for 5 d. He had not had passage of stools for 2 d. He denied hematemesis, melena, diarrhea, weight loss or fever. Past medical and surgical history was unremarkable and he denied any abdominal trauma. Abdominal auscultation revealed no abnormalities and percussion elicited splashing sounds in the upper abdominal region. A mass of 6 cm × 5 cm was palpated in the left upper quadrant without tenderness or rebound tenderness. Neither abdominal muscle guarding nor enlarged superficial lymph nodes were noted. Admission laboratory data were within normal limits, and serum tumor marker levels [carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9] were not elevated. Abdominal ultrasonography demonstrated a solid cystic mass within the body of the pancreas (Figure 1). Abdominal plain X-rays revealed no apparent signs of bowel obstruction, while gastroscopy demonstrated intragastric fluid retention with luminal compression from a mass within the outer gastric wall (Figure 2). Abdominal computed tomography (CT) with intravenous contrast delineated a cystic mass within the central pancreas, invading the horizontal part of the underlying duodenum (Figure 3). Additionally, endoluminal ultrasonography (EUS) demonstrated a cystic hypoechoic tumor posterior to the stomach of 6.7 cm × 5.5 cm and containing four cystic cavities (Figure 4). Examination of EUS-guided fine needle aspirate only demonstrated inflammatory tissue; however, cystic fluid examination revealed elevated levels of both CEA and CA19-9, at 1378 and 425.4 ng/mL, respectively. In light of the aforementioned pancreatic mass and resultant duodenal obstruction, the patient was transferred to the Department of General Surgery for emergency exploratory laparotomy.

During the surgery, a solid cystic mass of 8.6 cm × 6.0 cm within the pancreatic neck and body invading the adjacent horizontal portion of the duodenum was noted (Figure 5). The mass was adjacent to the posterior

wall of the stomach. No regional lymphadenopathy was noted. The tumor was primarily located within the central pancreas, therefore, central pancreatectomy was deemed in order, accompanied by partial distal stomach and horizontal duodenal *en bloc* resection (Figure 6). Digestive tract reconstruction was performed *via* pancreaticogastrostomy, duodenojejunostomy (side to side) and gastrojejunostomy (Billroth II, Figure 7). The postoperative course was uneventful; total parenteral nutrition was provided for 7 d after surgery and the patient was discharged on postoperative day 12. Follow-up examinations with CT, gastroscopy, gastrointestinal imaging and colonoscopy were performed once every 6 mo. The patient recovered well with no complaints of discomfort, malnutrition, or bowel movement abnormalities and continued his study in school as previously. CT showed no recurrence 20 mo postoperatively (Figure 8).

Pathological examination identified a pancreatic DT with several cystic cavities and adjacent duodenal invasion. Histological sections of the solid tumor showed proliferation of spindle-shaped or stellate cells, growing in fasciculate and storiform patterns within a myxoid intercellular matrix. The cystic lesion was lined predominantly by chronically inflamed fibrous tissue and small areas of benign columnar epithelium. Specimen surfaces from the stomach and duodenum contained a mesenteric plaque composed of well-defined sheets of densely collagenized fibrous tissue, which entrapped fat, blood vessels, nerve fibers, and smooth muscle. In the areas between the solid tumor and pancreatic parenchyma, the mesenteric plaque merged with the desmoid masses, entrapping pancreatic acini and resulting in irregularly dilated ducts. The cystic area was the result of dilatation of entrapped excretory pancreatic ducts (Figure 9). Subsequent immunohistochemical analysis found smooth muscle actin and cytokeratin to be positive, while desmin, CD117 (c-kit), CD34, S-100, calretinin and estrogen receptor (ER) were negative.

## DISCUSSION

DTs are rare and first appeared in the medical literature in the early 19<sup>th</sup> century. The term is derived from the Greek “desmos”, describing a band- or tendon-like attribute. DTs represent approximately 0.03% of all tumors and 3% of soft tissues tumors<sup>[4]</sup>. They are nonmetastatic and locally aggressive, with a high local recurrence rate, and may arise virtually anywhere within the body. The tumors are benign and are characterized by the absence of pleomorphism, atypia, or hyperchromatic nuclei<sup>[5]</sup>. Desmoids are particularly predisposed to muscular fascia, but may occur at any fascia. The most frequent sites for these tumors are the torso and extremities. Recent clinical studies have demonstrated that 37%-50% of DTs arise in the abdominal region<sup>[4,6,7]</sup>.

DTs have recently been classified depending on their point of origin as extra-abdominal, abdominal and intra-abdominal; the latter type further subclassified into mes-

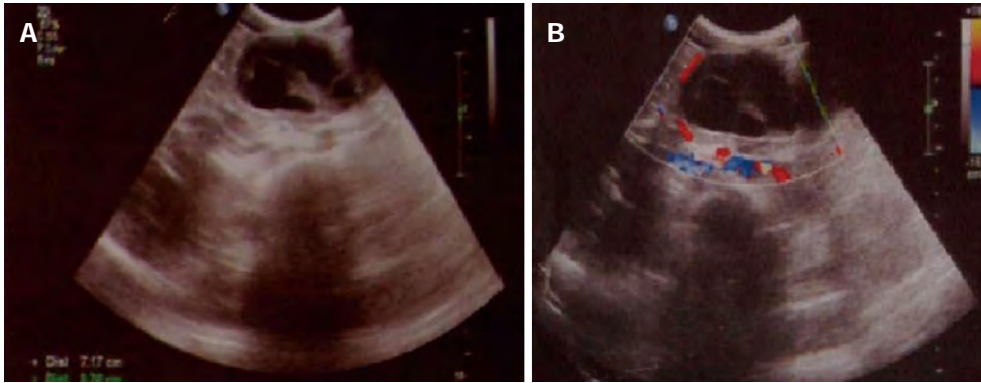


Figure 1 Abdominal ultrasonography demonstrated a solid cystic mass within the body of the pancreas. A: Ultrasonography showed a solid cystic mass within the body of the pancreas; B: Ultrasonography showed that the mass was surrounded by blood vessels.

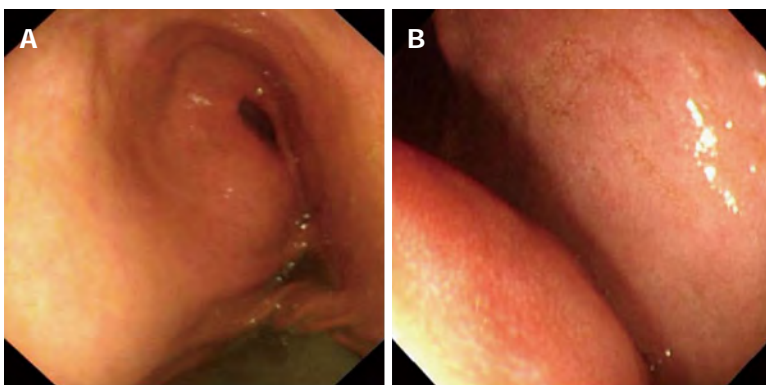


Figure 2 Gastroscopic imaging demonstrating fluid within the stomach and wall compression caused by an external mass. A: Gastroscopy demonstrated intragastric fluid retention; B: Stomach wall compression by an external mass.

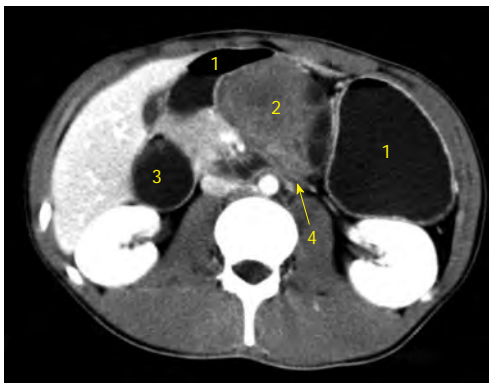


Figure 3 Abdominal computed tomography with contrast (venous phase) demonstrating a solid cystic mass invading the horizontal portion of the duodenum. 1: Stomach; 2: Pancreatic cystic mass; 3: Enlarged duodenum; 4: Horizontal duodenal portion invaded by the tumor.

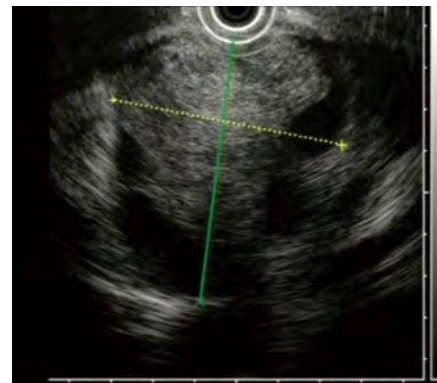


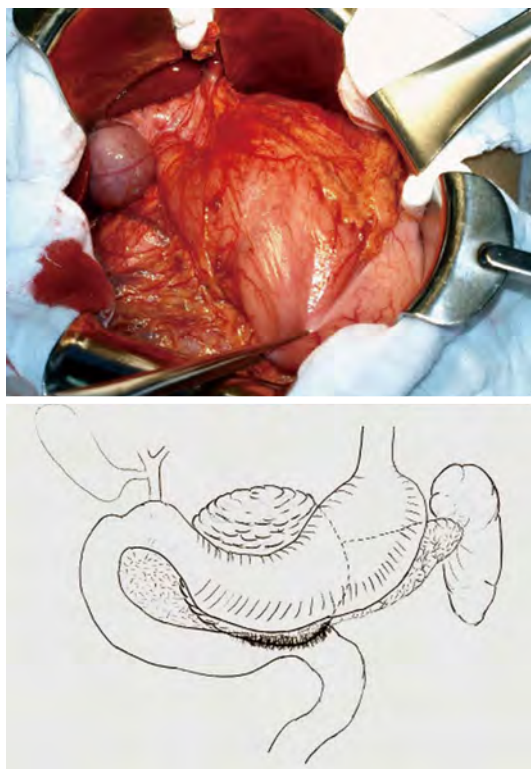
Figure 4 Endoscopic ultrasonography revealing a pancreatic cystic hypoechoic tumor located posterior to the stomach, with four cystic cavities, about 6.7 cm x 5.5 cm in dimension.

enteric and pelvic fibromatoses<sup>[8]</sup>. Associations between abdominal desmoids with FAP and Gardner syndrome have been well documented, suggesting a genetic predisposition to such lesions. Abdominal desmoids occur more frequently in FAP patients, with an incidence of 3.5%-32%, while in the original Gardner syndrome, the incidence was 29%<sup>[4,9]</sup>. In both FAP and familial non-FAP tumors, mutation of the adenomatous polyposis coli

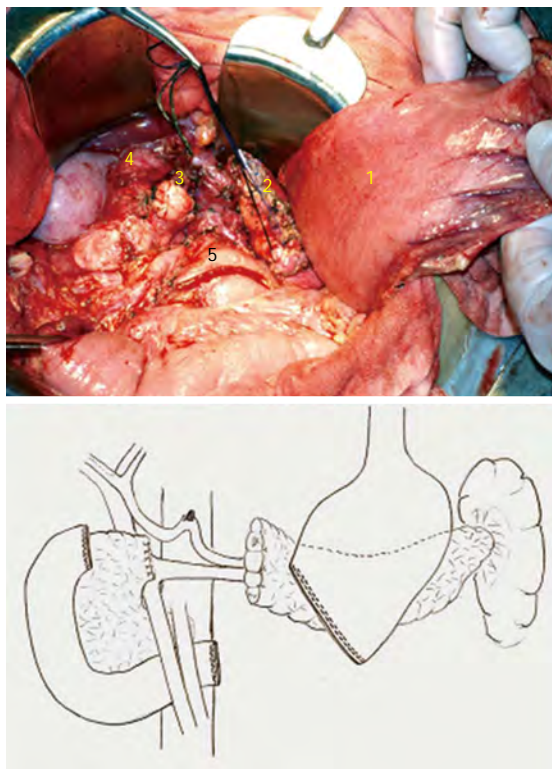
(APC) gene on the long arm of chromosome 5 has been implicated as the primary causative mechanism. As a result of APC mutation,  $\beta$ -catenin degradation markedly decreases, promoting fibroblastic proliferation *via* nuclear signaling pathways<sup>[10]</sup>.

DT etiology has not been well defined. A history of trauma to the site of the tumor, often surgical in nature, may be elicited in approximately 25% of cases<sup>[11,12]</sup>. Anecdotal evidence of tumor regression during menopause<sup>[13]</sup>,

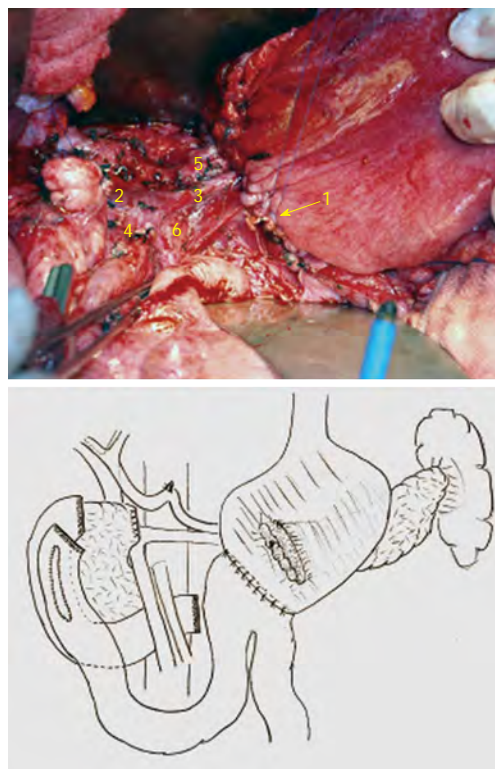




**Figure 5** Exploratory laparotomy revealed a cystic mass of size 8.6 cm × 6.0 cm within the pancreatic neck and body invading the horizontal duodenum.



**Figure 6** The central pancreas (containing the tumor), distal stomach and duodenal portion were all resected. 1: Stomach; 2: Left resected end of pancreas; 3: Right resected end of pancreas; 4: Upper resected end of duodenum; 5: Lower resected end of duodenum.



**Figure 7** Reconstruction was performed via pancreaticogastrostomy, duodenojejunostomy (side to side) and gastrojejunostomy (Billroth II). 1: Pancreaticogastrostomy; 2: Portal vein; 3: Splenic vein; 4: Superior mesenteric vein; 5: Splenic artery; 6: Superior mesenteric artery.

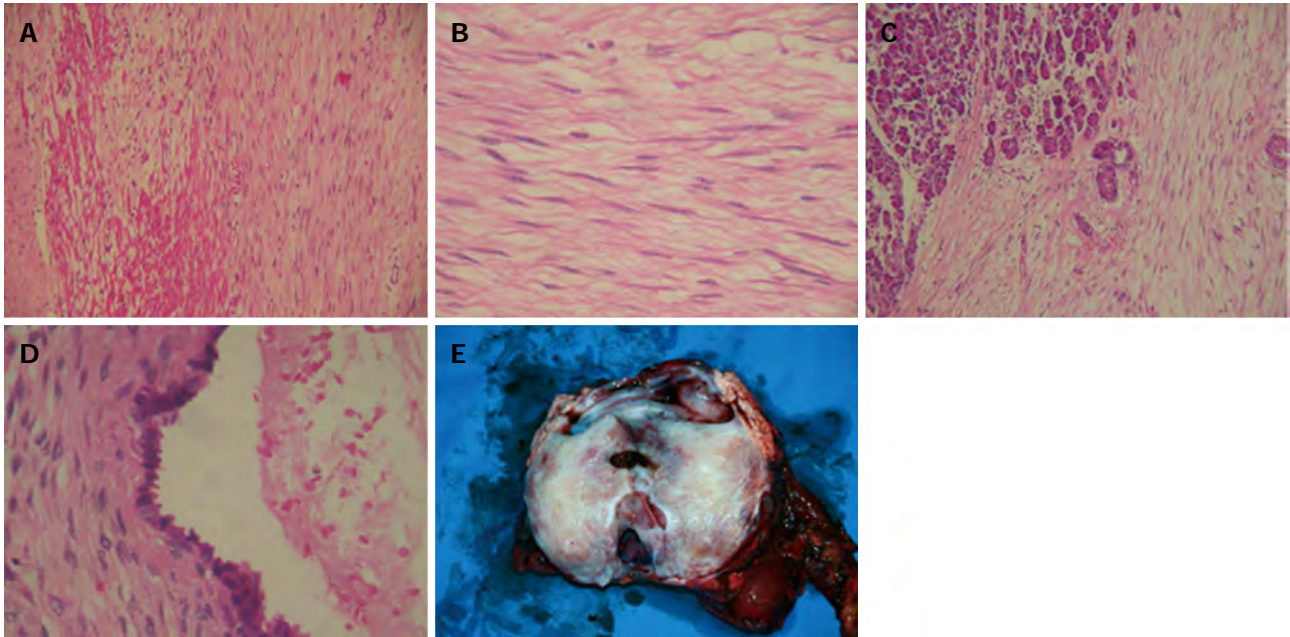


**Figure 8** Contrast computed tomography examination (arterial phase) demonstrated no recurrence 18 mo after operation.

the development of desmoids in patients taking oral contraceptives<sup>[14]</sup>, and reports of tumor regression with tamoxifen treatment<sup>[15]</sup> underline the apparent role of estrogen in the multifactorial pathogenesis of the neoplasm.

The pancreas is an extremely rare location for DT manifestation. Moreover, pancreatic desmoids resembling solid cystic tumors are the rarest form of DTs. Most relevant literature concerns FAP-associated desmoids; only nine cases of pancreatic DTs have been described in the English literature to date<sup>[2,16-22]</sup>. Although intra-abdominal DTs are widely considered to be associated with FAP,





**Figure 9 Pathological examination.** A: Pancreatic desmoid tumor with invasion to the duodenal wall; B: Proliferation of spindle-shaped stellate cells in fasciculate and storiform growth patterns within a myxoid intercellular matrix; C: Pancreatic infiltration by the tumor; D: The cystic area resulted from dilatation of entrapped excretory pancreatic ducts; E: Gross view of resected tumor and pancreas.

such association was noted in only one of the 10 reported cases (including our present case) of pancreatic DTs. Pancreatic DTs occurring after pancreatic surgery or biopsy were noted in three of the 10 reported cases<sup>[18,19]</sup>. Presenting symptoms consisted mainly of epigastric pain (6/10) and weight loss (4/10). The pancreatic DTs were mostly localized to the tail (6/10) and measured 15-85 mm in length.

In the present case, the patient was initially diagnosed with a mucinous cystic or solid pseudopapillary neoplasm. These diagnoses were suggested due to the cystic characteristics of the tumor, and pathological examination revealed the cystic components bearing a resemblance to retention cysts. Only two previously published cases of pancreatic DTs presented as cystic tumors<sup>[21]</sup>. In the first case, the cystic component corresponded to a benign retention cyst. The other case corresponded to an intraductal papillary mucinous neoplasm situated adjacent to a DT, but lacking true intralesional cystic components<sup>[19]</sup>.

Differentiating DTs from other types of soft tissue neoplasms may be difficult based on histological analyses alone. Expression was negative for CD34, CD117, S-100 and desmin, which excluded a gastrointestinal stromal tumor, solitary fibrous tumor, schwannoma, leiomyoma and leiomyosarcoma, respectively<sup>[23,24]</sup>. DTs are theoretically associated with clonal myofibroblastic proliferation and somatic mutation of the Wnt/ $\beta$ -catenin gene, leading to the intranuclear accumulation of  $\beta$ -catenin. Recent efforts in immunostaining of intranuclear  $\beta$ -catenin and the gene responsible for its mutation proved efficient in discriminating DTs from other benign and malignant fibroblastic and myofibroblastic lesions<sup>[25]</sup>.

In the case of intra-abdominal DTs, surgical resection is generally performed in the event of extensive tumor invasion and potentially life-threatening complications. However, resection is usually a difficult procedure because of considerable vascular involvement among the mesenteric and retroperitoneal areas. In this case, the tumor was located in the pancreatic neck and body, invading the horizontal portion of the duodenum and the posterior stomach wall. In order to perform resection with a tumor-free margin, central pancreatectomy with accompanying *en bloc* resection of the distal stomach and horizontal duodenal portion was deemed appropriate (Figure 6). Digestive tract reconstruction mandated three anastomoses for continuity maintenance (Figure 7).

Intra-abdominal DTs possess high rates of local recurrence after surgical resection, particularly in patients with FAP or Gardner syndrome. Intriguingly, frequent recurrences appear to be absent in cases of sporadic pancreatic DTs, according to the limited number of follow-up reports available<sup>[2,16-22]</sup>. A single reported case of recurrence was noted in a patient with FAP by Pho *et al.*<sup>[21]</sup>. The present case remains disease free 40 mo after surgery, consistent with previous reports.

In summary, the pancreas remains a rare location for DTs. Pancreatic DTs presenting as solid cystic tumors are the rarest form of desmoids. There are no notable clinical symptoms, tumor markers or imaging features to aid in diagnosis. Nuclear immunostaining of the  $\beta$ -catenin protein and its corresponding coding gene are efficient in distinguishing DTs from other lesions. Cytotoxic treatments may be utilized for either unresectable neoplasms or those unresponsive to more benign treatment, as radiotherapy may be limited in use due to extensive bowel

geography. Radical surgical resection with tumor-free margins appears to produce an excellent prognosis when applicable.

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## Acute appendicitis: What is the gold standard of treatment?

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### Abstract

McBurney's procedure represented the gold-standard for acute appendicitis until 1981, but nowadays the number of laparoscopic appendectomies has progressively increased since it has been demonstrated to be a safe procedure, with excellent cosmetic results and it also allows a shorter hospitalization, a quicker and less painful postoperative recovery. The aim of this editorial was to perform a review of the literature in order to address controversial issues in the treatment of acute appendicitis.

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**Key words:** Acute appendicitis; Surgery; Laparoscopy

**Core tip:** There are still controversial issues in the treatment of acute appendicitis such as comparison between laparoscopic and open appendectomy and the correct approach in special categories of patients. The aim of this editorial was to perform a review of the literature in order to address controversial issues in the

treatment of acute appendicitis.

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### INTRODUCTION

In 1894, McBurney<sup>[1]</sup> described a new technique for the management of acute appendicitis: this method is still used when an open approach is required.

McBurney's procedure represented the gold-standard for acute appendicitis until 1981, when Semm<sup>[2]</sup> performed the first laparoscopic appendectomy in Germany, a "culture shock" in general surgery since a revolutionary method was discovered by a gynecologist<sup>[3]</sup>. But a real "laparoscopic revolution" took place only in 1985 with the first laparoscopic cholecystectomy performed by Erich Muhe, using Semm's technique and instruments. Laparoscopy was not easily accepted since it was not considered a safe procedure; nowadays laparoscopic surgery is gaining a primary role in many surgical settings.

The number of laparoscopic appendectomies (LA) has progressively increased since it has been demonstrated to be a safe procedure, with excellent cosmetic results; furthermore, LA allows a shorter hospitalization, a quicker and less painful postoperative recovery.

But is laparoscopic surgery the best choice for appendectomy? Which are the correct surgical indications? What are the results from the comparison between LA vs classic open appendectomy (OA)? Are there selected groups of patients in which one of these approaches should be preferred? The aim of this editorial was to perform a review of the literature in order to address these

controversial issues.

## OPEN VS LAPAROSCOPIC APPENDECTOMY

Many comparative studies have already demonstrated the advantages of LA over OA in terms of length of hospital stay, use of postoperative analgesics and earlier return to work<sup>[4]</sup>. The most controversial issues of these studies have been taken into consideration.

### *Surgical-site infection*

Surgical-site infection (SSI) rate was significantly lower in the LA than in the OA group (1.6% *vs* 3.2% respectively) and this gap between the two groups increased in severe forms of appendicitis, such as gangrenous and perforated. Some authors estimated that one wound infection could be prevented for every 23.7 patients treated with LA, instead of OA<sup>[5]</sup>: this can be explained with the use of the extraction bag (endo-bag) in LA, which prevents the direct contact between the infected appendix, the wound edges and the inflamed tissues around the appendix during its removal<sup>[5,6]</sup>.

Other studies found a higher SSI rate in OA, but also a significantly higher intraabdominal abscess (IIA) rate in LA. The difference in the postoperative complications according to the surgical technique were remarkable when inflammation of the appendix was more severe: in fact, when a periappendiceal abscess was present, there were more cases of paralytic ileus (PI) in the LA group and more cases of SSI in the OA group. This result can be due to the leakage of infected substances, the appendiceal stump not being inverted and the resection side being exposed in the intraabdominal cavity during the removal of the appendix in LA<sup>[7]</sup>. Some authors suggest that the use of an Endo-GIA stapler could help minimize these adverse effects<sup>[8]</sup>. Finally, these differences are not statistically significant in case of gangrenous or/and perforated appendicitis<sup>[7]</sup>.

### *Intraabdominal abscess*

In an interesting study that considered 2464 patients, 52 experienced postoperative abscesses. The patients with a diagnosis of complicated appendicitis had a significant correlation with a higher incidence of intraabdominal abscess development (67% in complicated appendicitis *vs* 25% in uncomplicated appendicitis,  $P = 0.01$ ). The majority of abscesses developed in the pelvis (41%), especially in those patients who had complicated rather than uncomplicated appendicitis (63% *vs* 18% respectively,  $P = 0.01$ ). It is interesting to notice how the formation of an IIA in patients with a diagnosis of complicated appendicitis did not differ significantly between those who underwent LA and those who underwent OA (5.9% *vs* 4.1% respectively,  $P = 0.44$ ). Moreover, in patients with complicated appendicitis there was no significant increase in presenting symptoms or in the severity of the case history, quite independently from the surgical approach.

The only remarkable difference was that the patients who underwent OA presented earlier symptoms and received a more timely diagnosis of IIA than the patients who underwent LA (6 d in OA group *vs* 11 d in LA group)<sup>[9]</sup>.

A multivariate analysis has shown that development of abscesses has a higher correlation with the initial diagnosis than with the type of surgical approach. The evaluation of selected patients demonstrated a 30% increase of the risk of IIA for every decade of life. This could be clinically relevant because it suggests the need for careful monitoring of elderly patients who initially presented complicated appendicitis, since they are at higher risk for postoperative IIA<sup>[9]</sup>. Finally an explanation for the formation of IIA could be found in the surgical technique itself: currently, surgeons performing LA tend to apply irrigation more freely; therefore, contaminating the entire peritoneal cavity<sup>[10]</sup>; although irrigation as a cause of IIA is yet controversial.

### *Incisional hernia*

The incidence of incisional hernia is low in both techniques (0.7% in OA group *vs* 1% in LA): the development of post incisional hernias is higher with McBurney's incision, whereas in LA there are incisional hernias only in those patients who undergo conversion<sup>[11]</sup>.

### *Small bowel obstruction*

Finally, as far as long-term complications are concerned, some studies assessed that small bowel obstruction can present many years after surgery, especially for open appendectomy. The prevalence of bowel obstruction after appendectomy increased from 0.63% after 1 year, to 0.97% after 10 years, to 1.30% after 30 years of follow up<sup>[11]</sup>. In a randomized study, a second look laparoscopy was performed on 40 patients who had histological confirmation of acute appendicitis, 3 mo after the first operation: there were adhesions in the 80% of patients that underwent OA, but only in 10% of LA group<sup>[5]</sup>. Therefore, LA seems to be associated with an easier second-look procedure and a minor infertility rate due to less adhesions<sup>[12]</sup>.

Among long-term complications, small bowel obstruction has a very low incidence, between 0.33% and 1.51% in OA. It is known that the risk is higher with negative appendectomy or appendectomy through a midline laparotomic incision. Then, the choice of LA in suspected appendicitis is correct because it avoids unnecessary appendectomy if the appendix is normal and it prevents unnecessary wide incisions<sup>[13]</sup>.

## SUSPECTED APPENDICITIS

The differential diagnosis of most of the surgical abdominal emergencies is based on clinical grounds, laboratory data and diagnostic imaging. The problem, however, is to obtain a correct diagnosis of the exact localization of the lesion to determine surgical indications and to decide the best surgical approach. Laparoscopy is a valuable instru-

ment in the case of suspected appendicitis allowing the surgeon to correctly evaluate the intraperitoneal condition of practically every single patient<sup>[14]</sup>.

At first, considering its exploratory nature and its diagnostic accuracy, besides the advantage of a shorter time of hospitalization and reduction of pain on day 1<sup>[15]</sup>, LA can be considered the first choice in suspected appendicitis, especially in particular categories, such as premenopausal women. In fact, in these patients, in the presence of right lower quadrant pain, differential diagnosis between acute appendicitis, ectopic pregnancy and pelvic inflammatory disease (PID), is necessary. A laparoscopic exploration of the abdominal cavity allows a rapid and safe diagnosis; for the former two affections laparoscopy also represents a therapeutic option, while in the latter one, samples for culture may also be taken, with the advantage of avoiding “negative” appendectomies, with a high diagnostic accuracy (96% in women and 100% in men)<sup>[16]</sup>.

Morino *et al.*<sup>[17]</sup> evaluated, in a prospective, randomized, single-institution trial, the role of early laparoscopy in the management of nonspecific abdominal pain (NSAP) in young women. NSAP was defined as an abdominal pain in right iliac or hypogastric area lasting more than 6 h and less than 8 d, without fever, leukocytosis, or obvious peritoneal signs and uncertain diagnosis after physical examination and baseline investigations including abdominal sonography. Patients were randomly assigned to early (< 12 from admission) laparoscopic group or to clinical observation group. Compared with active clinical observation, early laparoscopy did not show a clear benefit in women with NSAP. A higher number of diagnosis and a shorter hospital stay in the laparoscopic group did not lead to a significant reduction in symptoms recurrences at 1 year.

LA may be performed safely in pregnant patients with appendicitis according to the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) guidelines<sup>[18]</sup>.

## COMPLICATED APPENDICITIS

Excellent results are mentioned in several studies about the use of LA in complicated appendicitis, though a higher incidence of intraabdominal abscesses has been noticed. Some studies have demonstrated that LA is almost totally comparable to OA as far as operating time, hospital stay and postoperative complications are concerned. The rate of postoperative II A was significantly higher in LA when compared with OA (respectively, 14% *vs* 0%), while wound infection and pulmonary complication rate were significantly lower (respectively 2.3% *vs* 8.2% in OA group and 0% *vs* 4.9% in LA group)<sup>[19]</sup>.

The incidence rate of II A increases considerably when a periappendiceal abscess or a postoperative ileus are present. Particularly, the incidence of II A in complicated appendicitis increases remarkably (67% in complicated *vs* 25% in uncomplicated appendicitis): in these patients, there are no significant differences in the postoperative outcome or in the development of the ab-

cess according to the surgical technique; therefore in the presence of an initial diagnosis of complicated appendicitis with a severe clinical background there is a higher probability of developing an abscess regardless of the adopted surgical approach<sup>[9]</sup>.

In another 5-year non randomized study considering 1133 patients of which 244 had a complicated appendicitis (and among them, 175 underwent LA and 69 OA), LA patients had a shorter operative time (55 min *vs* 70 min), reduced length of stay (5 d *vs* 6 d) and a lower incidence of SSI (0.6% *vs* 10%)<sup>[10]</sup>. In the case of complicated appendicitis (gangrenous or perforated), the laparoscopic approach also reduced postoperative pain<sup>[20]</sup>.

## SPECIAL CATEGORIES OF PATIENTS

There are clinical settings in which laparoscopy may be the preferred approach: obese patients, immunocompromised patients and elderly patients.

In obese patients, in fact, laparoscopy is undeniably useful<sup>[21]</sup>, considering at first the difficult exposure of the right lower quadrant during OA, which may require large, morbidity-prone incisions that are at risk of infections and of wound complications<sup>[5,22]</sup>. It is known that BMI is a risk factor for SSI<sup>[23]</sup>. Furthermore, obese patients have a higher risk of incisional hernias: laparoscopic approach reduces the risk of incisional hernia<sup>[24]</sup>.

Immunocompromised patients include heart transplanted patients and those who received immunosuppressive therapy for autoimmune diseases, cancer and AIDS; the risk of infections is higher and the immunity response could be partial and ineffective due to immunodepression. Therefore, these patients may not exhibit the typical signs and symptoms of appendicitis and may only have a barely positive examination<sup>[25]</sup>. In these patients laparoscopic approach represents the best option: compared with OA, LA is characterized by a lower rate of postoperative complications (10.36% in LA group *vs* 22.56% in OA group), a shorter hospitalization (2.9 d *vs* 4.9 d) and a lower mortality (0.16% *vs* 0.61%). These results can be observed in both uncomplicated and complicated appendicitis, with a considerably lower incidence of complications (27.52% in LA group *vs* 57.50% in OA group) and a shorter hospital stay (5.92 d in LA group *vs* 9.67 d in OA group)<sup>[26]</sup>.

Finally, elderly patients might significantly benefit from a laparoscopic approach<sup>[24]</sup>; in these patients it is quite difficult to collect anamnestic data, in addition to a mild abdominal examination and to laboratory and radiological tests which might not be so diriment. Laparoscopy can clarify the diagnosis and also represent a good therapeutic strategy<sup>[27]</sup>.

## INFLAMMED APPENDICEAL STUMP

Stump appendicitis is the acute inflammation of the residual portion of the appendix and is a rare complication of incomplete appendectomy<sup>[28]</sup>.

Due to the relevant recurrence rate, a second appen-



dectomy 3 mo after the outbreak of inflammation, could be necessary. In a histopathological study Gahukamble demonstrated that 13 of the 14 removed appendices had a pervious lumen with a higher risk of recurrent appendicitis. More recently authors focused the problem of a very long stump also on patients undergoing LA; in fact, the presence of an excessively long appendiceal stump could be at risk of recurrence also in these patients. Pain in the lower right abdominal quadrant in a patient that has undergone LA does not rule out a second episode of acute appendicitis<sup>[29]</sup>. The possibility of a recurring appendiceal stump abscess as a complication of LA is high. When performing LA, the appendiceal stump should be as short as possible and its ligation should not determine ischemia of the stump<sup>[30]</sup>.

The tactical modification of appendiceal stump closure, replacing the invaginating suture that nowadays has become the procedure of choice consists in a single endoligature. Alternatively, there are methods which make use of an endostapler, endoligature (endo-loop), metal clips, bipolar endocoagulation and polymeric clips. All the different techniques have advantages and disadvantages depending on the different stages of acute appendicitis; so, the right knowledge about the possible methods and the appropriate choice between them according to every single case allows a safe and efficient management of patients as well as a reduction in hospital costs<sup>[31]</sup>.

Drainage placement, ultrasound and perhaps an exploratory-therapeutical laparoscopy could be very useful in the management of this complication<sup>[30]</sup>. Finally the use of CT imaging allows a precise definition of the surrounding anatomy, in particular of the length of the appendiceal stump<sup>[32]</sup>. Several authors identify the removal of the whole appendiceal stump as the major suggested mean to avoid recurrence of appendicitis<sup>[33]</sup>.

## CONSERVATIVE MANAGEMENT OF ACUTE APPENDICITIS

Acute appendicitis is one of the most frequent conditions seen in a surgical department; urgent appendectomy is considered the treatment of choice because of the low incidence of major complications and the relative rapidity of operation and hospital stay. Nevertheless surgical treatment exposes the patient to risks due to general anaesthesia and other complications such as surgical site infection, adhesions and intestinal obstruction, incisional hernia, infertility in female and pneumonia<sup>[34]</sup>; in this setting, the role of conservative treatment with antibiotics has been investigated in literature.

A recent Cochrane review assessed five low to moderate quality randomized controlled trials<sup>[35]</sup>; with the limit of the analyzed studies, surgical approach remains the gold standard treatment for acute uncomplicated appendicitis. Another large meta-analysis compared the two strategies in the scenario of complicated appendicitis, abscess or phlegmon<sup>[36]</sup>; in this case, radiologic-assisted drainage of appendiceal abscess could be another helpful

conservative strategy. The analysis of seventeen studies revealed that conservative management, with or without interval appendectomy, was associated with less overall complication rates, less reoperations and similar hospital stay compared with urgent appendectomy.

In the absence of high quality studies, laparoscopic or traditional appendectomy is still the treatment of choice for acute appendicitis; some in-progress prospective studies<sup>[34,37]</sup> could be helpful in understanding the role of conservative management.

## NORMAL APPENDIX: LAPAROSCOPIC MANAGEMENT

Negative or white appendectomy refers to the removal of non-inflamed appendix and is performed in about 15%-25% of patients undergoing surgery for suspected acute appendicitis<sup>[38]</sup>. White appendectomy rate is declining over time as cited by large studies, due to the availability of computed tomography and laparoscopy<sup>[39]</sup>; in open surgery, the appendix is generally always removed<sup>[40]</sup>.

Thanks to the widespread use of laparoscopy, laparoscopic management of normal appendix represents a dilemma for the surgeon and no guidelines are available in this field<sup>[41]</sup>. When laparoscopy is performed for suspected appendicitis, exploration is negative in 8%-15% but in up to 27% another condition is diagnosed<sup>[40]</sup>. The risks of leaving in situ an apparent normal appendix are: later appendicitis, misdiagnosed subclinical or "endo"-appendicitis, missed appendiceal malignancy (carcinoid), risk of patient confusion and persisting symptoms<sup>[42]</sup>. At present, the laparoscopic strategy in front of a normal appendix remains controversial.

### Conversions from laparoscopic to laparotomic appendectomy

In case of conversion, it is useful to perform an adequate laparotomic incision and an accurate and complete abdominal toilette. The conversion of perforated appendicitis is often burdened with a higher postoperative morbidity [60% in conversion appendectomy (CA), 22% in LA and 38% in OA]<sup>[8]</sup>.

A recent study in 2011, which included 745 patients that underwent LA or OA, asserts that conversion rate was about 8.6% and mentions that the first cause of conversion was the presence of a severe acute inflammatory process (38.7% of the factors which determine conversion to OA during operation). In this study, 77.42% of the patients that underwent CA had previous abdominal surgery and only 25.81% had a conversion due to adhesions.

Conversion was necessary especially in women over 65 years old (4.30% rather than 4.02% in the rest of patients)<sup>[43]</sup>. It is quite interesting that surgeons who performed at least 50 LA through their study period had a higher CA rate and this could reflect their will to attempt LA in the greatest part of patients, even in not strictly indicated cases. At the same time the number of conver-

sions decreases progressively throughout the career of a surgeon and his equipe<sup>[43]</sup>.

Another study indicates the presence of a generalized purulent peritonitis as the only significant risk factor for conversion. Moreover, although patients with previous abdominal surgery are at higher risk of conversion, this is not significantly correlated with sex and age. Converted patients are at higher risk of relaparotomy and incisional hernia, independently of the duration of the operation<sup>[11]</sup>.

Finally, for patients that underwent LA with complications requiring reintervention following laparoscopy, there is the possibility of a relaparoscopy for a second look: this has the advantage of maintaining the reduced morbidity allowed by the first operation. Relaparoscopy is very useful for abscess drainage, because it provides the accurate identification of the causes, for example in case of appendicular stump insufficiency<sup>[44]</sup>.

## LAPAROSCOPY VS LAPAROTOMY: WHICH FACTORS DETERMINE SURGEON'S DECISION?

It is known that laparoscopic approach is more expensive, as many studies have reported: an American study evaluated hospital cost behaviour in the years 2000-2005, including all patients undergoing both LA and OA. Costs for LA are 22% higher in uncomplicated and 9% higher in complicated appendicitis. They estimate that in 2005 exclusive use of open appendectomy would have saved 93 million dollars: this finding is particularly important because appendectomy is a common routine operation in all hospitals. The authors suggest OA as the gold standard for acute appendicitis, reserving LA only for special categories of patients<sup>[45]</sup>.

Cothren *et al*<sup>[46]</sup> compared the costs for LA and OA, which were significantly higher for LA: the authors noted that the total costs for LA were higher although operative time and stay in hospital were not so different between the two methods. Higher costs for LA might be due to the use of specific disposable surgical material for laparoscopy.

Another important factor for the hospital costs is the severity of illness of the patients at the initial diagnosis<sup>[47]</sup>. Even if more expensive, throughout the years LA has become more common because there are undeniable benefits in hospitalization time and in recovery time: this way, higher costs are balanced out by a more precocious return to work of working patients. Recently, one study found that predicted costs for LA were 1856\$ lower than for OA while the postoperative complication rate did not differ significantly<sup>[47]</sup>.

Another crucial factor which influences the choice between LA and OA is the training and experience of surgical equipe. An interesting study compares the experience in academic-affiliated and community hospitals. The rate of LA and OA in the two kinds of hospitals is quite the same, but in academic-affiliated ones the opera-

tive time is longer both for LA and for OA (47 min *vs* 38 min for LA and 49 min *vs* 44 min for OA): this could be explained considering the intrinsic didactic nature of academic hospitals which inevitably causes a little delay in the operations. Finally in both types of hospitals, hospitalization for LA was shortened by 1 d<sup>[48]</sup>.

A parameter to assess the value of a surgical approach is long-term quality of life. A German study determined how a group of patients - including both LA and OA - perceived their quality of life 7 years after appendectomy, through the administration of a specific questionnaire. The most satisfied patients were those who underwent LA, both for the quick recovery and for the cosmetic result<sup>[49]</sup>. Another work obtained information about overall satisfaction by a telephone interview: the LA group had fewer complications and returned earlier to work (median 13 d for OA *vs* 8 d for LA)<sup>[13]</sup>.

## Laparoscopic appendectomy: Techniques

Recently several methods have been proposed to perform appendectomy in a laparoscopic fashion. In the most popular approach, 3 abdominal wall incisions are performed to insert instruments in the abdominal cavity. According to the patients' demand of scar-free surgery, new minimally invasive methods have been developed.

**Traditional laparoscopic appendectomy [3 port(s) laparoscopic appendectomy]:** In conventional laparoscopic appendectomy, 3 ports are used to place instruments in the abdomen (Figure 1). The laparoscope is inserted in the umbilicus and pneumoperitoneum is induced; the site of the other 2 trocars for operative instruments is variable, according to the surgeon's preference and ability. The most used locations for trocars are: the lower left quadrant and suprapubic or lower left quadrant and lower right quadrant or suprapubic and lower right quadrant or both trocars placed on the "bikini line" (suprapubic)<sup>[50]</sup>. Nevertheless, the trocars are inserted respecting the triangulation rule, with the appendix at the apex of a triangle. The umbilical port is 5-12 mm in diameter while the others are generally 5 mm large<sup>[51]</sup>.

During surgical procedure, many methods are used to amputate and extract the appendix and to perform proper hemostasis; the routinely use of peritoneal irrigation and drainage placement is not recommended<sup>[52]</sup>. The number of trocars can be reduced to 2 using the "puppeteer technique"; in this variant, the appendix is suspended using transabdominal threads<sup>[53]</sup>.

A laparoscopic surgeon must be skilled with the open approach; in fact, open appendectomy represent the first step in the training of an operator who desires to perform laparoscopic appendectomy. But when is the learning curve completed? It is generally accepted that it is completed after 20 operations<sup>[54]</sup>.

To improve the cosmetic result, needlescopic appendectomy has been developed; this term refers to an evolution of conventional laparoscopy. The only difference between the two regards the instruments' diameter,

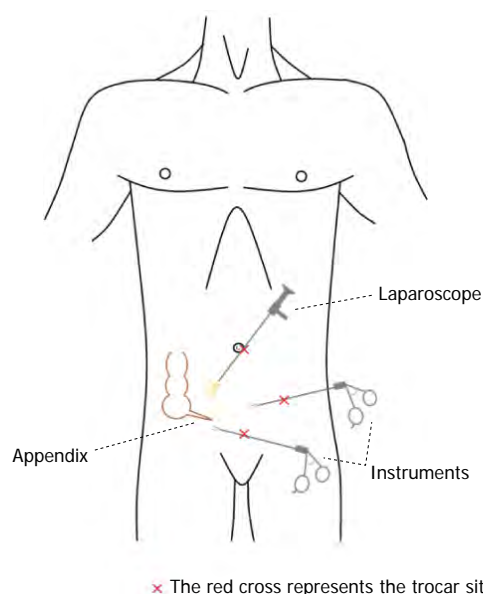


Figure 1 Traditional laparoscopic appendectomy: 3 ports are used to place instruments in the abdomen.

in fact in the needlescopic approach 3-mm or less trocars are used<sup>[55]</sup>. The first needlescopic appendectomy was performed in 1994. The use of smaller trocars potentially reduces postoperative pain and length of hospital stay due to minor abdominal wall incisions<sup>[56]</sup>; patients can quickly return to normal activity. On the other hand, this technique is more challenging for surgeons with a risk of longer duration of surgery and higher conversion rate<sup>[57]</sup>; these disadvantages will probably disappear after an appropriate learning curve and an increase of surgical skill. Needlescopic appendectomy is likely to be more expensive than the traditional approach due to equipment costs<sup>[58]</sup>. This fascinating laparoscopic evolution is not routinely recommended because of the lack of scientific evidence: large randomized controlled trials are necessary. It can, however, represents an option in selected patients, like young women.

**Single-incision laparoscopic surgery:** The continuous evolution of laparoscopic surgery and the ambition of better cosmetic results always tend to less invasive procedures. Single Incision Laparoscopic Surgery (SILS) for acute appendicitis in children began in 1992<sup>[59]</sup>. The development and diffusion of this technique was quite slow due to the lack of adequate instruments; healthcare engineering ideated multilumen ports, special laparoscopes and articulating instruments to facilitate the surgeon's work<sup>[60]</sup>. SILS is now diffused in many surgical specialties and skilled surgeons can perform several operations in this way, *i.e.*, adrenalectomy, Heller myotomy, large bowel surgery, splenectomy, bariatric surgery<sup>[61]</sup>.

In SILS, a multi-luminal and single port device is placed transumbilically: through this device, laparoscope and instruments can reach the abdominal cavity. The proposed advantages of SILS are better cosmetic results, reduced wound infection, postoperative pain, bleeding,

visceral injury and port site hernia due to the presence of a unique abdominal wall incision: for this reason it is known as “scarless” surgery. In a recent randomized controlled trial, SILS was associated with higher post-operative pain and more intravenous analgesics requirement; better wound cosmesis and higher satisfaction scores were also observed<sup>[62]</sup>. On the other hand it also has some technical challenges, like loss of triangulation (the cornerstone of laparoscopy) and instrument crowding (sword fighting)<sup>[63]</sup>. Although it is a technical challenge, in skilled hands, it is considered a safe procedure; patients seem to appreciate when a SILS approach is performed because surgical incisions are hidden in the umbilicus. Recent studies compared SILS and conventional laparoscopic appendectomy: no significant differences in the operative time, length of hospital stay, post operative pain and complication were observed<sup>[64,65]</sup>.

The learning curve of single incision laparoscopic appendectomy is between 5 to 10 cases<sup>[66]</sup>. To reduce the need of special materials and the costs, SILS can be performed using nonarticulating instruments and conventional trocars: early data suggests that it can represent an economic and safe option, even if operative time is longer<sup>[67]</sup>. In this approach, an adequate follow-up to detect the risk of post-incisional hernia is needed because many trocars are inserted in a very small area. There are also original ideas to reduce costs, *i.e.*, the use of a surgical glove like a multi-lumen port where instruments pass via the cutting fingers<sup>[63]</sup>. However, it is very difficult to determine the costs of SILS<sup>[68]</sup>.

Lacking of available evidence, no recommendations can be made on the effectiveness of SILS *vs* conventional multi-incision laparoscopic appendectomy<sup>[69]</sup>.

**Natural orifice transluminal endoscopic surgery:** In 2004 Rao *et al*<sup>[70]</sup> described a new real “scarless” procedure performing a transgastric appendectomy. Natural Orifice Transluminal Endoscopic Surgery (NOTES) represents the forefront of laparoscopic surgery and the next worldwide focus on minimally invasive surgery<sup>[71]</sup>; using a multichannel endoscope, the access to the peritoneal cavity is obtained via natural orifices like vagina, rectum, stomach and bladder. This technique allows to perform many surgical operations without visible scars; avoiding abdominal-wall incisions, postoperative pain is minor and recovery is faster. SILS is considered a bridge between conventional multi-ports laparoscopy and NOTES.

Regarding acute appendicitis, in female patients a transvaginal approach can be used (TVA, TransVaginal Appendectomy); an incision performed in the posterior fornix of vagina permits the access to the peritoneal cavity (Figure 2).

A prospective study comparing TVA to traditional 3-port laparoscopic appendectomy showed significantly less post-operative analgesia demand (Patient Controlled Analgesia morphine utilization) and faster return to normal activity; compared with the conventional laparoscopic approach there were no differences in the length



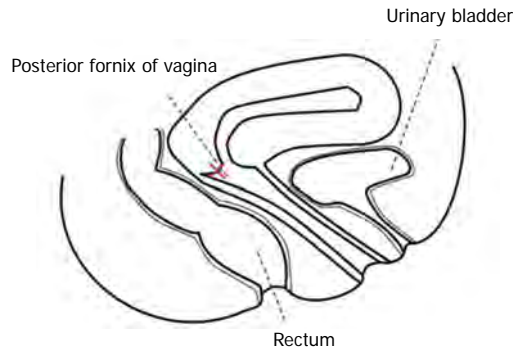


Figure 2 Notes procedure: Transvaginal approach is performed through the posterior fornix of the vagina.

of stay and operative time<sup>[72]</sup>. There were no differences in pre- and post-operatively sexual function; no post-operative dyspareunia was noted and TVA *vs* conventional laparoscopy sexual outcome was comparable. Even though the authors of this prospective study concluded that TVA is a safe and feasible procedure in women with acute non-perforated appendicitis, the authors of this review believe that large randomized controlled trials are necessary before proposing this procedure to a young woman.

## CONCLUSION

Patient selection is important in both LA and OA. LA is the preferred approach in immunocompromised, obese and elderly patients. LA presents longer operative time, but also a shortening of hospital stay, a better and earlier recovery and return to everyday occupations and to work and, last but not least, a better cosmetic result.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Surveillance for early diagnosis of hepatocellular carcinoma: How best to do it?

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cedure. Education of both physicians and patients is of paramount importance in order to improve the surveillance application and its benefits in patients at risk of HCC. The promotion of specific educational programs for practitioners, clinicians and patients is instrumental in order to expand the correct use of surveillance in clinical practice and eventually improve HCC prognosis.

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**Key words:** Hepatocellular carcinoma; Surveillance; Screening; Ultrasonography; Cost-effectiveness

**Core tip:** This article deals with the role of surveillance for early diagnosis of hepatocellular carcinoma in patients at risk. It addresses several topics on this issue, including how to best perform surveillance (tools and interval), its results in terms of cancer stage, patient survival, cost-effectiveness, pitfalls and actual under-(mis-)use.

## Abstract

Surveillance for hepatocellular carcinoma (HCC) is considered a standard of care for patients with chronic liver disease who are at risk of developing this malignancy. Several studies have shown that surveillance can improve the prognosis of patients diagnosed with HCC through an increased likelihood of application of curative or effective treatments. Repetition of liver ultrasonography (US) every 6 mo is the recommended surveillance program to detect early HCCs, and a positive US has to entrain a well-defined recall policy based on contrast-enhanced, dynamic radiological imaging or biopsy for the diagnosis of HCC. Although HCC fulfills the accepted criteria regarding cost-effective cancer screening and surveillance, the implementation of surveillance in clinical practice is defective and this has a negative impact on the cost-effectiveness of the pro-

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## SURVEILLANCE AS A MEANS OF IMPROVING SURVIVAL OF PATIENTS AT RISK OF DEVELOPING HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the leading malignancies worldwide, representing the fifth most common human cancer and the third cause of death from

cancer<sup>[1,2]</sup>. Patients diagnosed with HCC often have a dismal prognosis as it is diagnosed at late stages, when therapeutic approaches are limited, if applicable at all. Conversely, an early diagnosis of HCC allows the application of curative or effective treatments in most cases, improving the survival of these patients<sup>[3]</sup>. Therefore there is a need for early diagnosis of this tumor. Screening and surveillance for HCC applied to patients with chronic liver disease who are at risk of developing this cancer can indeed identify malignancies at early stages and improve patient survival, although there is still debate regarding the optimal screening and surveillance tools and the actual yield of surveillance<sup>[3-5]</sup>. This review addresses the current evidence supporting surveillance programs in patients at risk of developing HCC, the best way to perform surveillance, and the still unsolved nuances of this topic.

## DIFFERENCE BETWEEN SCREENING AND SURVEILLANCE

Screening is the application of a test to detect a disease in a population which has no signs or symptoms of that disease, while surveillance is the periodic repetition of the screening test in the same population. Both screening and surveillance have the aim of detecting a disease before it becomes symptomatic, at an early time point of its natural history and when treatment is more effective, with the ultimate goal of reducing disease-specific mortality. Positive findings of screening or surveillance tests must entrain a pre-defined recall policy aimed at identifying true positive cases with additional diagnostic procedures. Screening and surveillance must fulfill the seven Prorok postulates<sup>[6]</sup> and, as outlined below, this is the case for the surveillance of patients at risk of developing HCC.

### *The disease must be common and with substantial morbidity and mortality*

HCC is a common malignancy worldwide and its incidence is expected to rise in most Western world areas due to the aging of patients with chronic hepatitis C virus (HCV) infection, which is the main etiological factor of this tumor in developed countries<sup>[7,8]</sup>. Moreover, HCC is currently the main cause of death of patients with initially compensated liver cirrhosis<sup>[9,10]</sup>. Noteworthy, the incidence and mortality rates of HCC are very similar all over the world, thus emphasizing the high lethality rate of this tumor in the short term, especially when it is diagnosed at late stages precluding any effective treatment<sup>[2]</sup>, although a favorable mortality trend has recently been observed in Europe<sup>[11-13]</sup>.

### *The target population must be readily identifiable*

More than 90% of HCCs develop in a cirrhotic liver, and the main causes of chronic liver disease in these patients are hepatitis B virus (HBV) or HCV infections, alcohol abuse and non-alcoholic fatty liver disease<sup>[14]</sup>. These diseases can be detected on the basis of patient history and/

or serological tests, thus making the target population for HCC surveillance readily identifiable.

### *Surveillance tests must have low morbidity, high sensitivity and high specificity*

The American and European guidelines for HCC management recommend surveillance to be carried out by ultrasound examination of the liver (US) repeated every 6 mo<sup>[15,16]</sup>. This surveillance schedule has no morbidity and, when US is properly carried out, a fairly high sensitivity and specificity<sup>[17]</sup>. In particular, a recent meta-regression analysis has shown that US can identify subclinical HCCs with a sensitivity of 94%-95%, but this drops to 63% for early HCC, while specificity ordinarily exceeded 90%<sup>[18]</sup>. However, series coming from referral centers reported remarkably higher sensitivity figures (82%), even for early HCC<sup>[19,20]</sup>. Therefore, the availability of sonographers with expertise in this field is a mandatory prerequisite for a useful US-based surveillance for HCC<sup>[21]</sup>. The use of serum alpha-fetoprotein (AFP) as a surveillance test has an acceptable specificity but a poor sensitivity for early HCC since only a small fraction (10%-20%) of early cancers is associated with elevated AFP serum levels<sup>[15,16,19,22-24]</sup>. The combination of US and serum AFP assessment slightly increases (6%) the sensitivity of surveillance but almost doubles the cost for each small HCC detected due to a high number of false positives<sup>[18,25]</sup>.

### *The surveillance test must be acceptable to the target population*

A semiannual repetition of US is a non-invasive, easily-performed, and relatively low-cost surveillance schedule which is not a major obstacle for patient adherence. Rather, physician education and knowledge of the potential benefits of surveillance, and adequate operator training are areas where there is still room for improving the effectiveness of surveillance programs<sup>[26-29]</sup>.

### *There must be standardized recall procedures*

A positive result of the surveillance test must entrain a prompt activation of a pre-defined standardized algorithm (recall procedures) able to provide a definite diagnosis. Recall procedures for suspected lesions identified by US during screening or surveillance have to be consistently defined and involve radiological, contrast-enhanced imaging procedures or pathological evaluation of the lesion(s) relying on precise diagnostic criteria<sup>[15,16]</sup>. The diagnostic yield of these recall procedures has been independently confirmed, and allows an adequate evaluation of tumor extension that, in turn, has a pivotal role in driving the therapeutic strategy<sup>[21,30,31]</sup>. An inappropriate or delayed application of recall procedures is an important cause of surveillance failure<sup>[28]</sup>.

### *There must be an acceptable and effective therapy*

The goal of surveillance for HCC is to identify tiny lesions, amenable to curative treatments with the aim of improving patient survival. Surgical resection, percutaneous



**Table 1** Suggested thresholds of hepatocellular carcinoma incidence for the implementation of surveillance<sup>[15]</sup>

Group of patients	Threshold incidence to implement surveillance (% per year)	Incidence of HCC
Surveillance recommended		
Asian male hepatitis B carriers over age 40	0.2	0.4%-0.6%/yr
Asian female hepatitis B carriers over age 50	0.2	0.3%-0.6%/yr
Hepatitis B carriers with family history of hepatocellular carcinoma	0.2	Incidence higher than without family history
African/North American Blacks with hepatitis B	0.2	Hepatocellular carcinoma occurs at a younger age
Hepatitis B virus carriers, cirrhosis	0.2-1.5	3%-8%/yr
Hepatitis C virus infection, cirrhosis	1.5	3%-5%/yr
Primary Biliary Cirrhosis, stage 4	1.5	3%-5%/yr
Genetic hemochromatosis, cirrhosis	1.5	Unknown, but probably > 1.5%/yr
Alpha 1-antitrypsin deficiency, cirrhosis	1.5	Unknown, but probably > 1.5%/yr
Other cirrhosis	1.5	Unknown
Surveillance benefit uncertain		
Hepatitis B carriers younger than 40 (males) or 50 (females)	0.2	< 0.2%/yr
Hepatitis C virus infection, stage 3 fibrosis	1.5	< 1.5%/yr
Non-cirrhotic non-alcoholic fatty liver disease	1.5	< 1.5%/yr

HCC: Hepatocellular carcinoma.

ous ablation, and liver transplantation (LT) are considered curative options for patients with small HCCs. The results of a randomized study carried out in China in HBV infection active carriers and of several cohort studies carried out in Western and Japanese patients with cirrhosis support the use of surveillance as a way of identifying early tumors amenable to curative treatment, and therefore of improving patient survival<sup>[19,24,32-34]</sup>. Notably, refinements in diagnostic techniques and patient management led to a progressive improvement in the survival of patients diagnosed with HCC during surveillance<sup>[35]</sup>.

### Surveillance should reduce disease-specific mortality

The ideal methodology for confirming that surveillance reduces the disease-specific mortality would be to perform a randomized, controlled trial comparing surveillance *vs* care-on-demand in at-risk patients. Two such studies had been performed in Chinese chronic HBV carriers with contrasting results<sup>[32,36]</sup>. In particular, despite a 40% reduction in the disease-specific mortality, the first trial was affected by a low degree (< 60%) of patient adherence to the semiannual surveillance program and by the LT unavailability, indicating that the reported figure was probably the “minimal” benefit achievable with surveillance in HBV patients<sup>[32]</sup>. The negative study was instead methodologically flawed by the fact that patients diagnosed with early HCC did not receive an effective treatment<sup>[36]</sup>. It is unrealistic to expect results on this topic from new randomized controlled trials, at least in the Western world, due to several reasons: (1) subjects in the control arm would frequently undergo abdominal US due to extra-hepatic or liver disease-related reasons; (2) almost all the patients, if adequately informed on the risk-benefits of surveillance, would refuse to participate in the study<sup>[37,38]</sup>; and (3) this position would likely be shared by most clinicians. Thus, the belief that surveillance for HCC reduces the disease-specific mortality and the pertinent recommendations released by Western and Eastern international

guidelines mainly relies on the available proof-of-concept evidence, showing that US surveillance can detect small, asymptomatic tumors that are amenable to curative treatment while symptomatic HCCs are generally detected at an advanced stage, which greatly limits or even precludes any treatment. Pertinently, Western and Eastern cohort studies comparing the outcome of patients with HCC diagnosed during or outside surveillance programs consistently demonstrate that the assumed surveillance benefit holds true<sup>[4,24,32-34,38,39]</sup>.

### WHO SHOULD BE SURVEILLED?

In the Western world, surveillance is recommended for subjects at high risk of developing HCC such as patients with cirrhosis and certain categories of patients with chronic hepatitis, while Japanese guidelines extend this recommendation to all patients with chronic hepatitis<sup>[15,16,40]</sup>. An essential pre-requisite to perform surveillance is the absence of contraindications to treatment—either curative or palliative—once HCC is diagnosed. Thus, surveillance is useless in patients with Child-Pugh class C cirrhosis not listed for LT<sup>[41]</sup>, as an early detection of HCC does not improve their survival due to the inapplicability of therapeutic options for malignancy other than LT and a strong competitive effect with cancer by liver failure as the death cause<sup>[42]</sup>.

As mentioned before, surveillance should be cost-effective and one crucial determinant of cost-effectiveness (CE) is the disease incidence in the target population (see also the specific chapter below). Therefore, the selection of patients who should enter into surveillance programs for HCC is driven by their oncologic risk, which can be inferred from the incidence of HCC (Table 1). The incidence threshold that should trigger surveillance in patients with cirrhosis is 1.5% per year, while for patients with chronic hepatitis this drops to 0.2% per year<sup>[15]</sup>. It is important to note that these thresholds are not derived

from experimental data but they were proposed considering the results of CE analyses based on the Markov model showing an increase in survival of > 3 mo at a cost of less than 50000 USD per year of life gained<sup>[43,44]</sup>.

### Cirrhosis

According to the above mentioned thresholds, patients with cirrhosis are appropriate candidates for a cost-effective surveillance, as the annual incidence of HCC in cirrhotic patients with HCV or HBV infection is 1.5%-4.5% and 2.2%-4.3%, respectively, and it is approximately 2.6% in both alcoholic and non-alcoholic steatohepatitis cirrhosis<sup>[14,45-47]</sup>. Even cirrhotic patients with genetic hemochromatosis or primary biliary cirrhosis have an HCC risk high enough to implement surveillance, whereas the annual incidence of HCC reported in cirrhotic patients with autoimmune hepatitis is 1.1%, thus questioning the CE of surveillance in this category of patients<sup>[48-50]</sup>.

Patients with HCV-related cirrhosis who cleared the infection with antiviral treatment represent a subset of patients with a decreased, but not abolished, risk of HCC<sup>[51,52]</sup>. Namely, the incidence rate of HCC per 100 person-years in Japanese patients with cirrhosis who achieved a sustained virological response (SVR) to antiviral treatment was 0.5% compared to 5% in patients without SVR and 8% in untreated cirrhotic patients, while a retrospective Italian study showed figures of 0.7% after SVR and 2% in non-responder patients<sup>[51,52]</sup>. It should be pointed out that HCC incidences observed after SVR do not cross the suggested CE threshold for surveillance in cirrhosis. Nevertheless, non-viremic HCV cirrhotic patients represent a peculiar population where mortality due to the complications of cirrhosis or liver failure is negligible and the chance of applying aggressive treatments for HCC is high<sup>[51,52]</sup>. The same applies to HBV patients effectively treated with antiviral nucleos(t)ide drugs in whom the risk of HCC remains as high as 1.3 per 100 person-years despite undetectable viremia<sup>[53,54]</sup>.

To conclude, non-viremic HCV and HBV patients should continue (or start) to undergo surveillance if they were at high risk of developing HCC before starting antiviral treatment.

### Non-cirrhotic chronic liver disease

Among pre-cirrhotic patients, those with chronic HBV infection have the highest risk of developing HCC, especially those with long-standing disease, who more likely acquired the infection perinatally, and those with persistent, high-load viral replication<sup>[55-57]</sup>. These features are frequent in Asian patients, and a study from China showed that patients with chronic hepatitis B without cirrhosis have an annual HCC incidence of 0.8%, thus exceeding the accepted threshold (0.2%) for a cost-effective surveillance<sup>[15,58]</sup>. African active HBV carriers or with a positive family history are also considered good candidates for surveillance, due to high HCC incidence. As the incidence of HCC in HBV-positive Western patients ranges from 0.1% to 0.4% per year<sup>[59,60]</sup>, the latest

European guidelines for HCC management recommend the implementation of surveillance programs in the subgroups of HBV-positive patients with active hepatitis or a family history of HCC<sup>[16]</sup>.

Although Japanese guidelines recommend HCC surveillance in chronic hepatitis C patients whereas American and European guidelines propose this procedure in those with advanced fibrosis, the evidence supporting these suggestions is less robust<sup>[15,16,40]</sup>. Indeed, on the one hand, a study carried out in Japan showed that the annual incidence of HCC in untreated patients with chronic hepatitis C increased with increasing fibrosis stage, being 0.5% in patients without or with mild fibrosis, and 5% in those with severe fibrosis<sup>[52]</sup>. On the other hand, a large, prospective study carried out in the United States to assess the incidence of HCC in patients with bridging fibrosis (Ishak stage 3 and 4) reported an incidence of 0.8% per year<sup>[61,62]</sup>. Importantly, in this cohort the absence of cirrhosis was assessed at enrollment and HCC was diagnosed after a median of 46.5 mo of follow-up, when cirrhosis had developed in 65% of these patients (15/23 patients) and thrombocytopenia was present in all but one patient<sup>[62]</sup>. These findings emphasize the difficulties in identifying a clear hallmark indicating the transition from a low to a high oncologic risk status. In an attempt to overcome this problem, either bed-side clinical scores or transient hepatic elastography have been proposed to stratify patients according to HCC risk<sup>[62-65]</sup>.

Lastly, although HCC may also occur in non-cirrhotic patients with a non-viral chronic liver disease, exhaustive data on its incidence in these categories are not currently available, but it is unlikely for surveillance to be cost-effective in these settings.

## HOW SHOULD SURVEILLANCE BE PERFORMED?

### Imaging tools and expertise

In general, a surveillance test has to have a high sensitivity (to miss very few cancers) and an adequate specificity (to avoid unnecessary confirmatory testing). There is universal agreement that US is the imaging tool to be used for surveillance of HCC. A meta-regression analysis of several cohort studies set the sensitivity of US, as a surveillance test for HCC, at 94% for asymptomatic tumors and 63% for early HCC, with a specificity of > 90%<sup>[18]</sup>. The relatively low sensitivity of US for tiny lesions may be explained by the fact that this technique is highly dependent on both the operator expertise and the quality of US equipment. In fact, the presence of regenerative nodules and fibrous septa conferring a coarse echo-pattern to the cirrhotic liver makes it difficult to identify minute nodules. Therefore, US examination should be performed by skilled operators and with adequate instruments. In this case, the sensitivity for early-stage or small HCC ranges from 82% to 91%, and the mean size of HCCs detected during surveillance is < 2 cm, with only 1.4% of tumors > 3 cm<sup>[19,20,66,67]</sup>.

### Serum AFP

AFP is the serum tumoral marker most widely used in the surveillance for HCC<sup>[22]</sup>. Its levels are influenced by tumor size and aggressiveness, as well as by the etiology and activity of the liver disease<sup>[23,68-74]</sup>. These limitations affect the usefulness of AFP as a surveillance test for HCC, and its principal drawback is a poor sensitivity at cut-off levels ensuring an adequate specificity<sup>[22,75]</sup>. Namely, serum AFP levels are increased in a minority of early HCCs and, when elevated, tend to identify highly malignant cancers with a rapid growth rate<sup>[23,67-69,71-73]</sup>. Furthermore, AFP lacks specificity for HCC since abnormal levels can be caused by hepatitis activity flares in both HBV- and HCV-infected patients<sup>[70,74]</sup>.

The combined use of US and AFP increases the sensitivity for early HCC by 6% compared to US alone, but also enhances the rate of false positive results, with detrimental consequences on direct and indirect costs for each early HCC detected<sup>[76-80]</sup>. In fact, while false positive results occur with US or AFP alone in 2.9% and 5.0% of cases, respectively, the figure rises to 7.5% on combining the two tests, and this drop of specificity translates into a cost of approximately 2000 USD per HCC identified with US alone as compared to 3000 USD with the combination of AFP plus US<sup>[25]</sup>.

Inadequate sensitivity for early lesions and lack of specificity discourage the use of AFP as a screening and surveillance tool for HCC<sup>[23,78]</sup>, so that the use of US alone in this setting has been recommended by Western guidelines for HCC management<sup>[15,16]</sup>. This suggestion, however, is not shared by the recently released Eastern guidelines<sup>[40,81]</sup>, that continue to propose the combined use of US and sero-markers, such as AFP and des-gamma-carboxy prothrombin, aimed at maximizing the sensitivity of surveillance regardless of its negative impact on CE.

### Special subgroups (patients on LT waiting list, patients with coarse liver echo-pattern, obese patients)

Patients on the LT waiting list represent a special subgroup where surveillance for HCC acquires additional clinical significance, as the identification of an HCC in these patients: (1) can hasten the urgency for LT by prioritizing the patient on the list; (2) alternatively, it may represent a reason for waiting list drop-out if the tumor burden exceeds the accepted criteria for LT<sup>[82-84]</sup>; (3) due to these reasons, it also impacts on the probability of the listed non-HCC patients to be transplanted<sup>[82,85]</sup>. Non-HCC patients listed for LT usually have an advanced cirrhosis which associates with a coarse liver echo-pattern, organ shrinkage and ascites, and these features may impair the US ability to detect (small) focal lesions<sup>[86]</sup>. Therefore, although there is no compelling evidence to support this suggestion, an HCC surveillance carried out with multiphasic computed tomography (CT) or magnetic resonance (MR) every 6 mo can be proposed for these patients; considering that the expected surveillance duration seldom exceeds 1 year, the detection of HCC is crucial to define the priority for LT (and hence to fairly allocate a limited therapeutic

resource among oncologic and non-oncologic candidates), and the use of these techniques has been associated with a better CE ratio<sup>[21]</sup>.

Patients with non-alcoholic liver cirrhosis represent a growing population at risk of HCC<sup>[87-89]</sup>, and in most of them the presence of fatty liver and obesity may impair imaging resolution of liver US exploration. Although no formal studies have been carried out to address this issue, in some studies CT was purposely used instead of US for HCC surveillance in a minority of patients (3.3%) due to the presence of suboptimal US resolution because of a coarse liver echo-pattern or extreme obesity<sup>[67]</sup>. Instead, in a study carried out in the United States, the presence of an increased body mass index was not associated with a decreased sensitivity of US for HCC detection, although the robustness of this finding is flawed by the limited statistical power of the study and the overall poor quality of US results<sup>[90]</sup>. Thus, due to the growing prevalence of non-alcoholic liver disease, this is a field where prospective studies comparing US with other surveillance tools are urgently needed<sup>[91]</sup>.

### Optimal interval of surveillance (3 mo vs 6 mo vs 12 mo)

The surveillance interval should be dictated by the expected doubling volume time of the surveyed tumor, and not by the degree of the inherent risk of HCC. Median doubling volume time of untreated HCC is around 170 d, although there is a great inter-individual variability and the growth rate may be not constant over time<sup>[67,92]</sup>. This would indicate that the reference length of the surveillance interval is 6 mo. Increasing the length to 12 mo is indeed associated with a greater likelihood of missing early HCCs, reducing the applicability of effective treatments and thus worsening survival as compared to the semiannual surveillance schedule<sup>[34,93]</sup>. In fact, after correction for the lead-time bias, the survival of Child-Pugh class A or B patients with HCC identified during a semiannual surveillance was significantly improved as compared to patients undergoing 12 mo surveillance<sup>[34]</sup>. Similar findings were obtained in Asian patients, in whom the survival benefit adjusted for lead-time bias was significantly greater when surveillance was carried out with an interval  $\leq 6$  mo as compared to  $> 6$  mo<sup>[93]</sup>. Conversely, a randomized study prevalently including patients with alcoholic cirrhosis demonstrated that shortening the surveillance schedule to 3 mo was detrimental as it did not significantly increase the likelihood to detect small ( $\leq 3$  cm) HCCs (79% *vs* 70%), amenability to curative treatment (62% *vs* 58%) and 5-year survival (85% *vs* 86%), whereas it led to a greater cumulative incidence of detected focal lesions that proved non-malignant during the follow-up, thus leading to an increased cost of recall procedures<sup>[94]</sup>. In this regard, it should be emphasized that the proposal of the Japanese and Asian guidelines to shorten the surveillance interval to 3 mo in patients at very high risk of developing HCC does not rely on experimental results or CE study models<sup>[40,81]</sup>. Thus, on the basis of the currently available evidence, a 6-mo interval should be recommended for HCC



surveillance<sup>[15,16]</sup>.

## DIFFERENCES BETWEEN EFFICACY AND EFFECTIVENESS

Efficacy is a measure of the degree to which one procedure obtains the expected result under standardized conditions, generally chosen to maximize the chance to observe the expected result. Effectiveness, instead, measures the extent of the benefit when the procedure is applied in clinical practice. Effectiveness not only depends upon the efficacy of the procedure but also on “external” non-standardized factors, such as physicians’ (specific knowledge, convincement and recommendation) and patients’ (acceptance and adherence) behavior, health system organization (timeliness of the recall policy, availability and accessibility of appropriate diagnostic tools and treatments, adequate follow-up), as well as economic, cultural and social influences. In the case of surveillance for HCC, it can be optimistically hypothesized that its effectiveness is affected by the following drawbacks: missed/unconvincing doctor recommendation (80%), limitations to surveillance access (90%), patient refusal (90%) or inadequate adherence (90%), untimely recall (by 90%), untimely availability of appropriate diagnostic and therapeutic options (90%) and improper follow-up (90%). Thus, assuming that the mentioned limitations are independent probabilities and the reduction in overall mortality of cirrhotic patients with HCC diagnosed during surveillance is 40% - according to the Italian Liver Cancer data (ITA.LI.CA)<sup>[34]</sup> - it can be calculated ( $0.40 \times 0.80 \times 0.90 \times 0.90 \times 0.90 \times 0.90 \times 0.90$ ) that the actual effectiveness of surveillance in cirrhosis drops to 17%.

Therefore, surveillance for early diagnosis of HCC is a typical example of “clinical nuance”, whose basics tenets are that medical services and providers differ in the clinical benefit provided; hence, the benefit of the service depends on the person using it, as well as where and by whom the service is provided. As previously pointed out by our group, besides limited economical resources, a major flaw of surveillance for HCC is the “behavior hazard” of both clinicians (prescription and organization) and patients (adherence)<sup>[79]</sup>. These shortcomings explain the large gap between efficacy and effectiveness of surveillance of patients at risk of HCC, and indicate the road for reducing this gap and greatly improving the CE of the procedure without the need for diagnostic and therapeutic advancements.

## COST-EFFECTIVENESS OF SURVEILLANCE

The economic aspect of HCC surveillance has also to be considered. Its CE is mainly determined by two features: the gain obtainable with surveillance in terms of quality-adjusted life-expectancy (effectiveness) and its total costs. In turn, these features are determined by two components each. Effectiveness strictly depends on HCC incidence

and the actual possibility to submit patients diagnosed with HCC to potentially curative treatments; total costs result from the sum of the costs of surveillance test(s), tools utilized for tumor diagnosis and staging, and HCC treatment(s).

As mentioned above, from a CE standpoint US surveillance of cirrhotic patients should be started when the annual HCC incidence is expected to be at least 1.5%; however, it cannot be excluded that different surveillance strategies, and different surveillance intervals, can be more cost-effective in different clinical scenarios. For instance, available data suggest that the annual program of US surveillance ( $\pm$  AFP assessment) is cost-effective in patients with a tumor risk up to 3%-3.5% per year, while the semiannual program becomes more cost-effective in patients with a risk above these figures<sup>[44,76,95,96]</sup>. Indeed, the semiannual US strategy has been consistently reported to be the most effective program for an early tumor diagnosis but it inevitably increases direct and indirect costs with respect to programs with longer intervals. Considering this, a reasonable alternative from a CE perspective is the “AFP-triage strategy” that avoids US use in patients with normal AFP values. This strategy has been reported to be more cost-effective than semiannual US but with a lower efficiency in detecting HCC<sup>[97]</sup>.

The second main determinant of surveillance effectiveness is the possibility to timely submit HCC patients to potentially curative treatments. While it is not possible to predict the tumor burden at presentation in the individual patient, it is intuitive that an advanced degree of liver dysfunction strongly limits-or even prevents-the therapeutic approach to the forthcoming HCC. The literature lacks specific analyses comparing the CE of surveillance *vs* no-surveillance in decompensated cirrhotic patients, also because surveillance is not currently recommended in patients with advanced cirrhosis not listed for LT. The only available evidence indicates that semiannual US surveillance can be more cost-effective than annual surveillance only if treatment can ensure a huge survival gain after HCC diagnosis, as in the case of LT<sup>[97]</sup>, indirectly supporting the recommendation to keep, among Child-Pugh class C patients, only candidates for LT under surveillance.

The direct costs of surveillance test(s) are relatively low, as both US examination and AFP dosage are not high-cost procedures. It has been reported that costs for surveillance and tumor diagnosis are around 18000 USD per each potentially curable HCC detected, accounting for only 10%-20% of total costs of cancer management since the main determinant of costs is treatment<sup>[19,98,99]</sup>. Nevertheless, the CE of surveillance programs based on CT, MR or contrast-enhanced US (CEUS) has been tested with Markov model analyses and most of the studies found that their use raised costs, without a parallel significant increase in HCC detection, resulting in a higher incremental cost-effectiveness ratio (ICER) compared to US surveillance<sup>[76,82,100,101]</sup>. Thus, there is not sufficient evidence for adopting CT or MR as surveillance tests,



whereas the use of CEUS, although intriguing, requires further dedicated studies.

Another point that needs to be addressed is the use of AFP as a surveillance test. Sensitivity of AFP is reported to be around 60%, and its specificity is limited by the non-HCC related elevation of the marker due to hepatic necro-inflammation and regeneration occurring in active hepatitis or cirrhosis<sup>[15,16,70,71,74]</sup>. Consequently, the frequent false positive results of a periodic AFP measurement, entraining confirmatory tests, increase the total costs of surveillance based on serum AFP measurement<sup>[96]</sup>.

As mentioned before, the main determinant of surveillance costs derives from the tumor treatment. For example, the inclusion of LT in the treatment algorithm, reimbursement of which can be up to 250000 USD (University of Alabama)<sup>[100]</sup>, results in an up to 10-fold increase of the average cost-effectiveness when compared to scenarios where LT is not an option. Hepatic resection is another high-cost intervention, that can compete with percutaneous ablation in terms of both survival and CE. Available literature suggests a CE advantage for ablation in the case of single tumors  $\leq 2$  cm and 2-3 nodules each  $\leq 3$  cm, while surgery becomes more cost-effective for single tumors  $> 3$  cm<sup>[102]</sup>. Thus, the type of treatment adopted, the proportion of patients undergoing each therapy and, more importantly, costs assumptions are the main sources of uncertainty for simulation models aimed at calculating the CE of surveillance for HCC. Therefore, it is advisable to propose prospective micro-costing analyses to refine this topic. Micro-costing studies collect data and values on the resources utilized for each patient so that, although time- and resource-consuming (expensive record keeping over time and use of database management), they allow a precise definition of costs. Only one prospective micro-costing study has been published, and this was more than 10 years ago<sup>[19]</sup>. Due to the changed scenario of HCC management since then, further similar studies are warranted.

To conclude, semiannual surveillance based on US achieves a higher detection rate of early HCC but at increased costs with respect to the annual program. From a CE perspective, alternative strategies, such as the semiannual AFP + annual US or the annual US ( $\pm$  AFP) schedules, could be proposed and tested in patients with a relatively low HCC incidence, such as young cirrhotic women or patients who have become non-viremic after (HCV- and HBV-infected) or during (HBV-infected) antiviral treatment.

## WHICH IS THE BEST RECALL POLICY?

Recall policy is instrumental to the success of surveillance, since an abnormal surveillance test must promptly entrain a pre-defined strategy aimed at ruling in/out the presence of HCC and staging it. The diagnostic algorithm that composes recall procedures should be carried out within a reasonable time interval to allow timely and adequate treatment. Recall procedures greatly concur in diagnosing HCC at a very early (solitary,  $\leq 2$  cm) or early stage

(meeting the Milan criteria) that, in turn, allows application of curative treatment and eventually improves patient survival. It is recommended that patients are evaluated at a referral center with availability of all diagnostic techniques and therapeutic opportunities.

Importantly, any new lesion identified at screening or during surveillance as well as pre-existing lesions enlarging or changing their echo-pattern should be regarded as malignant unless otherwise demonstrated; however, as most nodules  $< 1$  cm are non-malignant, the institution of recall procedures for these lesions would increase surveillance costs without clinical gain<sup>[94,103]</sup>. Therefore, these lesions should be strictly followed-up with US every 3 mo until an increase in size occurs (allowing a suitable definition of their nature with diagnostic techniques) or for one-two years<sup>[15,16,104]</sup>. This shortening of the interval between US scans ("enhanced" follow-up) is dictated by the knowledge that the volume doubling time of some HCCs may be as short as 30 d, and the main goal of surveillance is to detect HCCs  $\leq 2$  cm<sup>[89]</sup>. It has to be emphasized that the echo-pattern is not predictive of malignancy since, although HCC more often presents as a hypo-echoic lesion, it may be hyper-echoic or have a "target" appearance<sup>[105,106]</sup>.

The recall strategy for lesions  $\geq 1$  cm relies on the use of dynamic, contrast-enhanced, multiphase, imaging techniques with vascular contrast media (CT, MR, CEUS) and overlaps with the diagnostic process. In cirrhotic patients, if the nodule shows the typical vascular pattern *i.e.*, homogeneous contrast enhancement in the arterial phase (wash-in) followed by hypo-enhancement in the portal or venous phase (wash-out) - it can be regarded as HCC with no need for histological confirmation<sup>[20,21,30,31,107,108]</sup>. If the lesion does not display this typical pattern at the first imaging procedure, an alternative imaging technique can be performed, and if an atypical vascular pattern is found again, the lesion should undergo biopsy. It is recommended that histological samples are evaluated by an expert in liver pathology and, in the case of non-diagnostic pathological results, a follow-up with US every 3 mo should be implemented and the recall procedures repeated as soon as a nodule enlargement is observed (Figure 1).

When selecting the most rewarding imaging technique to be firstly performed in a patient with suspected HCC, it should be considered that MR has the highest sensitivity to detect the typical vascular pattern in tiny HCC ( $< 2$  cm) and, using hepatocyte-specific contrast agents, it can provide important additional information in the so called "hepato-biliary phase" (hypo-intensity of the nodule) to suspect malignancy even in the absence of the wash-in phenomenon, a feature quite frequent in tiny lesions<sup>[21,109-112]</sup>. Discovering the malignant nature of nodules  $< 2$  cm is indeed of paramount importance as, above this size, the prevalence of unfavorable prognostic factors, such as microscopic vascular invasion and satellites, greatly increases<sup>[59,112]</sup> (Figure 2).

The inclusion of CEUS among the imaging tech-

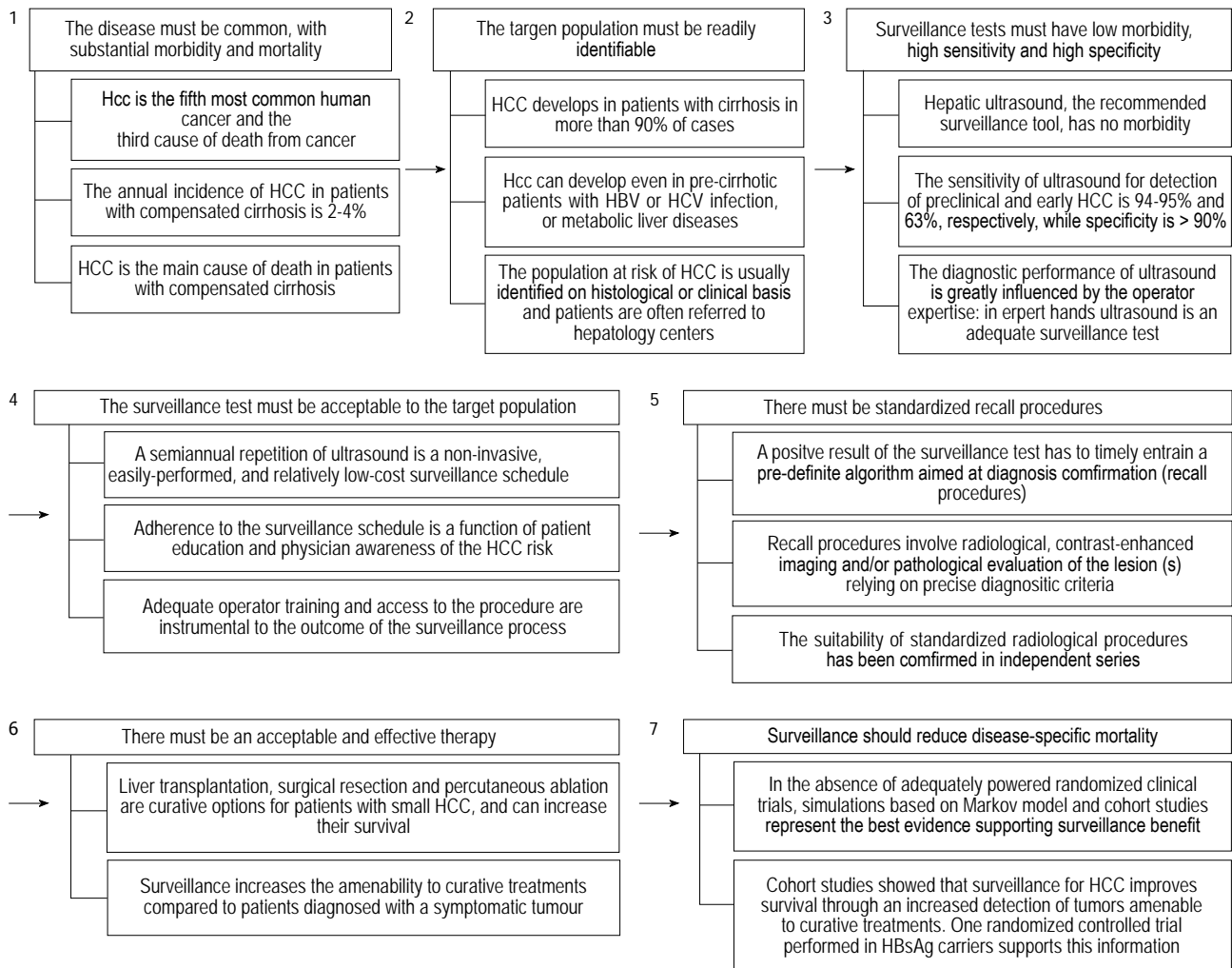


Figure 1 Prorok's postulates: Paradigm of surveillance for early diagnosis of hepatocellular carcinoma.

niques of the recall policy is currently debated, due to the risk of misdiagnosis between HCC and intrahepatic cholangiocarcinoma (ICC)<sup>[15,16,113-115]</sup>. However, the recently released Italian recommendations for HCC management<sup>[104]</sup> have included CEUS in the recall algorithm due to: (1) its positive predictive value for HCC > 95% when a typical vascular pattern is observed; (2) the fairly low incidence of ICC in cirrhosis (1%-3% of newly diagnosed tumors); and (3) the fact that only half of small ICCs display a pattern typical for HCC at CEUS<sup>[115-118]</sup>. From a CE standpoint, however, it should be pointed out that, since a "panoramic" imaging technique is mandatory to correctly stage the tumor, CT or MR should be preferred, using CEUS as a second-line procedure in the case of inconclusive findings at radiological imaging techniques<sup>[105,114]</sup>.

## ACTUAL UPTAKE OF SURVEILLANCE AND LIMITATIONS TO ITS APPLICATION

Despite the available evidence that surveillance increases the survival of patients diagnosed with HCC, expanding the possibility to perform effective therapies, there is still

controversy on its actual usefulness in clinical practice. Some recent studies, coming from the United States, have helped frame the receipt of HCC surveillance in everyday practice in this country and reported the obstacles to its utilization, providing hints on how to improve its uptake and outcome<sup>[116-121]</sup>. Indeed, initial reports showed that no more than 28% of patients diagnosed with HCC underwent at least 1 screening test in the 3 years preceding the diagnosis and, among them, 36% received AFP testing alone as a screening test<sup>[117]</sup>. However, this study did not report a measure of receipt of surveillance in the whole population of patients at risk. A subsequent study, performed on a larger and more representative sample, confirmed a low uptake of surveillance in patients diagnosed with HCC, showing that 17% and 38% of patients received consistent and inconsistent surveillance, respectively, before HCC detection, and demonstrated that being followed up by a gastroenterologist/hepatologist or an academic physician was associated with a higher likelihood of receiving surveillance as compared to patients followed by primary care physicians<sup>[118]</sup>. Thus, being followed by a specialist in liver disease is a key factor for the likelihood of receiving HCC screening and surveillance, a finding indirectly supported by the result of a self-reported use of

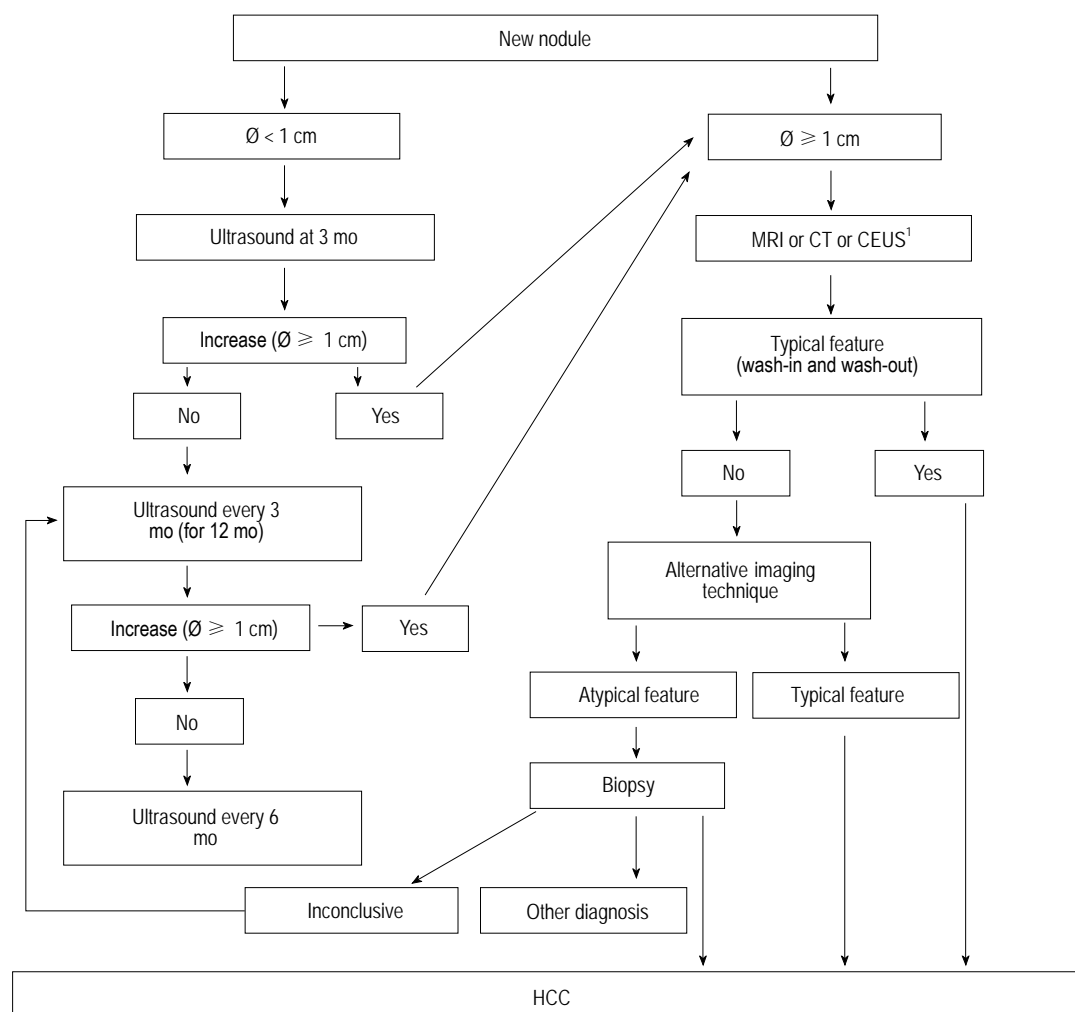


Figure 2 Recall policy and diagnostic algorithm proposed by the Italian Association for the Study of the Liver for cirrhotic patients with a nodule detected during ultrasound surveillance. <sup>1</sup>Note that, since magnetic resonance (MR) or computed tomography (CT) are anyhow needed for staging in the case of hepatocellular carcinoma diagnosis made by contrast-enhanced ultrasonography (CEUS), a pragmatic approach is to perform MR or CT as the first-line imaging technique for diagnosis, and to resort to CEUS when radiological imaging techniques provide inconclusive features (reprinted with permission)<sup>[102]</sup>.

surveillance ranging from 71% to 84% among members of the American Association for the Study of the Liver and the Veteran Health Administration<sup>[121,122]</sup>. Moreover, an adequate surveillance was strongly associated with the local availability of all possible treatments for HCC<sup>[121]</sup>, thus emphasizing the concept that patients at risk should be followed up and managed at referral centers with availability of multi-disciplinary resources to optimize the effectiveness of surveillance. These findings underscore that the patient's probability to be maintained under surveillance is strictly connected with specialist care and the possibility to receive treatment for HCC, and that effectiveness of surveillance is modest in decreasing HCC mortality when surveillance uptake is markedly low<sup>[119,121]</sup>.

Lastly, longitudinal evaluation of the ITA.LI.CA database over 20 years showed an increase in the proportion of patients diagnosed with HCC during surveillance until 2002, followed by stationary figures over the subsequent 6 years, accounting for approximately 53% of these cases, but with a significant continuous shift to preference of the 6-mo interval<sup>[89]</sup>. These data, as well

as those coming from the United States, clearly reveal an insufficient and suboptimal use of surveillance in the real world of health care and should stimulate educational policies aimed at expanding the knowledge and the correct use of this tool for secondary prevention of HCC. Indeed, audits with identification of barriers to the application of surveillance and implementation of measures able to improve physician and patient education, together with system re-design, have led to a great increase in the application of adequate surveillance protocols for an early diagnosis of HCC<sup>[122]</sup>.

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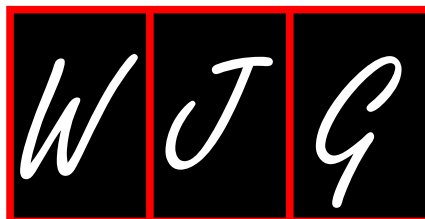
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WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

## Hepatocellular carcinoma in chronic hepatitis B patients under antiviral therapy

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### Abstract

Patients with chronic hepatitis B are at increased risk of hepatocellular carcinoma (HCC), while the inhibition of viral replication can represent a reasonable target for HCC prevention. Interferon- $\alpha$  therapy results in decreased HCC risk, which is more evident in patients with high baseline HCC risk. The majority of chronic hepatitis B patients are treated with a nucleos(t)ide analogue (NA) for several reasons including the non-sustained response after interferon- $\alpha$ . The effect of the first licensed and low genetic barrier NA, lamivudine, on HCC incidence, has been repeatedly evaluated. Lamivudine, compared to no treatment, reduces the HCC incidence, which may increase again in cases with lamivudine resistance. Emerging data with the currently first-line NAs, entecavir and tenofovir, suggest that they also reduce the HCC incidence. The treatment benefit in reduction of the HCC incidence is always greater in patients with high baseline HCC risk, particularly cirrhotics, and without virological remission under entecavir/tenofovir. However, the HCC risk is not eliminated even in the vast majority of patients who remain in virological remission under entecavir/tenofovir. Therefore, patients at increased baseline HCC

risk should continue to undergo HCC surveillance even if they have achieved complete long-term inhibition of viral replication and improvements in liver histology.

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**Key words:** Chronic hepatitis B; Hepatocellular carcinoma; Interferon; Lamivudine; Adefovir; Entecavir; Tenofovir; Virological remission; Cirrhosis

**Core tip:** Antiviral therapy reduces but does not eliminate the risk of hepatocellular carcinoma (HCC) in chronic hepatitis B patients with or without cirrhosis. The reduction of the HCC incidence under a high genetic barrier nucleos(t)ide analogue is higher in the vast majority of patients who will achieve virological remission compared to those who may maintain detectable viral replication. In current clinical practice, however, patients at increased baseline HCC risk should continue to undergo HCC surveillance according to the existing recommendations even if they have achieved complete long-term inhibition of viral replication and improvements in liver histology.

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third most frequent cause of cancer death<sup>[1]</sup>. It represents more than 90% of primary liver cancers and is a major global health problem. In most

cases, HCC develops within an established background of chronic liver disease. Following this, chronic hepatitis B virus (HBV) infection is a significant predisposing factor for the development of HCC and accounts for more than 50% of all cases<sup>[2]</sup>. The relative risk of HCC development is 100-fold higher for patients chronically infected with HBV versus those who are not infected. The risk is even higher for cases with high viral replication and/or HBV related cirrhosis<sup>[3]</sup>.

In patients with cirrhosis, surveillance for HCC increases the possibility of an earlier diagnosis and improved survival<sup>[1]</sup>. However, screening programs are rather unsatisfactory and the prognosis remains poor because therapeutic interventions are rather ineffective in advanced stages<sup>[4]</sup>. Therefore, the development of preventive strategies is mandatory. HCC related to HBV can be prevented by vaccination. Nationwide vaccination of infants in Taiwan reduced the incidence of HCC in children aged 6-9 years from 0.52 per 100,000 for those born between 1974 and 1984 to 0.13 for those born between 1984 and 1986<sup>[5]</sup>. Nevertheless, the incidence of HCC is expected to increase during the next years because approximately 400 million people who are already chronically infected with HBV cannot benefit from immunization<sup>[6]</sup>. In patients with chronic HBV infection and high serum HBV DNA levels, viral replication can be inhibited by antiviral agents that prevent the progression of liver disease and perhaps the development of HCC in the long-term.

The current therapeutic options for patients with chronic hepatitis B include treatment with standard or PEGylated interferon- $\alpha$  (IFN- $\alpha$ ), a drug with antiviral, immunomodulatory and perhaps antitumoral activities, and five oral nucleos(t)ide analogues (NAs) (lamivudine, adefovir, entecavir, telbivudine, tenofovir)<sup>[7]</sup>. In this review, we summarize the data on the impact of antiviral treatment in the prevention of HCC in patients with chronic HBV infection.

## RATIONALE OF ANTIVIRAL TREATMENT FOR HCC PREVENTION

It is believed that persistent viral replication together with the resulting liver injury are key risk factors for HBV-related HCC<sup>[8,9]</sup>. More specifically, a direct linear relationship was reported between viral load and HCC risk<sup>[10]</sup>. Chronic HBV infection promotes viral induced immune response with release of cytokines and genotoxic reactive oxygen species leading to liver cell necrosis as well as to activation of liver fibrosis cascade. The ensuing acceleration of hepatocyte cell cycles and the increased risk of genetic alterations might culminate in malignant transformation of hepatocytes<sup>[11]</sup>.

Moreover, the HBV sequences can integrate into cellular DNA and may modulate the expression of neighboring cellular genes in a cis-acting way<sup>[12]</sup>. The integration of HBV DNA may cause overexpression of those cellular genes which in turn contributes to the develop-

ment of carcinogenesis<sup>[13,14]</sup>. Furthermore, the viral protein HBx may play a crucial role in hepatocarcinogenesis because its trans-activation is involved in the function of a large number of signaling pathways and cellular genes that are involved in oncogenesis, proliferation, inflammation and immune responses<sup>[15]</sup>. Since all of the above mechanisms require the presence and replication of the virus, suppression of viral replication seems to be a reasonable target for the prevention of HCC.

There are additional important viral and host factors that may affect the risk of HCC development. Adequate evidence suggest that HBV genotype C is associated with more active and rapidly progressive liver disease including more frequent HCC development, compared to genotype B<sup>[16]</sup>. HBV genome mutations such as pre-S deletions, enhancer II mutations (T1653) and core promoter mutations (V1753, T1762 and A1764) have also been found to be associated with a higher HCC risk<sup>[17,18]</sup>. Moreover, older age, male gender, alcohol abuse and possibly metabolic syndrome also increase the risk of HCC<sup>[19,20]</sup>.

Lastly, recent data from Eastern Asia showed that high levels of HBV surface antigen (HBsAg) (> 1000 IU/mL) in HBV e antigen (HBeAg) negative patients with low levels of HBV DNA (< 2000 IU/mL) is an independent risk factor for HCC development<sup>[21]</sup>. As HBsAg is mainly produced by the integrated form of HBV DNA, low viremic patients who have high HBsAg level might harbor more hepatocytes with HBV integration thus increasing genomic instability which play an important role in carcinogenesis.

## IFN- $\alpha$ AND HCC

The usefulness of IFN- $\alpha$  in the prevention of HBV-related HCC has been investigated only with traditional IFN- $\alpha$  to date, as PEGylated IFN- $\alpha$  was licensed relatively recently and long-term follow-up studies have not been published yet. The IFN- $\alpha$  data on HCC prevention in patients with chronic hepatitis B have been conflicting so far and thus several meta-analyses have tried to elucidate this issue (Table 1). The first meta-analysis of 7 studies (2 Oriental-5 European) including 1505 patients with cirrhosis suggested a decreased incidence of HCC in IFN- $\alpha$ -treated patients (risk reduction-6.4%,  $P < 0.001$ )<sup>[22]</sup>. However, the pooled estimate in favor of IFN- $\alpha$  was a consequence of the two Oriental trials because the subgroup analysis of the five European studies found no benefit from IFN- $\alpha$  on the prevention of HCC (risk reduction-4.8%, NS). Sung *et al.*<sup>[23]</sup> performed a meta-analysis of 12 randomized, case-control and cohort studies (1292 IFN- $\alpha$  treated and 1450 untreated patients) and showed that the HCC risk was reduced by 34% in IFN- $\alpha$  treated patients (RR = 0.66, 95%CI: 0.48-0.89). Subgroup analysis revealed a significant benefit in patients with early cirrhosis (RR = 0.53, 95%CI: 0.36-0.78) but not in patients without cirrhosis (RR = 0.72, 95%CI: 0.16-3.15). In addition, no difference was found in the HCC incidence in relation to virological response to therapy (RR = 0.76,

**Table 1** Summary of meta-analyses evaluating the effect of antiviral treatment on the incidence of hepatocellular carcinoma in patients with chronic hepatitis B

1 <sup>st</sup> author, year	No. of studies, total (used <sup>1</sup> )	Total No. of patients, treated/untreated	Treatment regimen	HCC cases, total <i>n</i>	HCC incidence	RD or RR	95%CI	<i>P</i> value
Cammà <i>et al</i> <sup>[22]</sup> , 2001	7 (5)	853/652	IFN-α	122	Overall	RD = -6.4	-2.8-10	< 0.001
	(2)				European studies	RD = -4.8	-11.1-1.5	NS
	(2)				Oriental studies	RD = -8.0	-1.4-14.6	< 0.001
Sung <i>et al</i> <sup>[23]</sup> , 2008	12 (6)	1292/1458	IFN-α	190	Overall	RR = 0.66	0.48-0.89	0.006
	(3)				Cirrhotics	RR = 0.53	0.36-0.78	0.001
	(4)				Non-cirrhotics	RR = 0.72	0.16-3.15	NS
	(4)				Virological responders	RR = 0.76	0.08-7.23	NS
	(4)				Non-virological responders	RR = 0.64	0.33-1.26	NS
Yang <i>et al</i> <sup>[24]</sup> , 2009	11	1006/1076	IFN-α	178	Overall	RR = 0.59	0.43-0.81	0.001
Miyake <i>et al</i> <sup>[25]</sup> , 2009	8 (3)	553/750	IFN-α	100	Overall	RD = -5.0	-9.4-0.5	0.028
	(5)				European studies	RD = -0.5	-4.9-4.0	NS
	(5)				Asian studies	RD = -8.5	-13.6-3.6	0.001
	(3)				Incidental rate of HCC ≥ 10%	RD = -9.4	-14.2-4.6	< 0.001
	(4)				Incidental rate of HCC < 10%	RD = -0.2	-4.3-4.7	NS
	(3)				HBeAg positive ≥ 70%	RD = -6.0	-11.8-0.2	0.043
	(3)				HBeAg positive < 70%	RD = -5.4	-15.4-4.6	NS
Sung <i>et al</i> <sup>[23]</sup> , 2008	5 (3)	1267/1022	LAM	152	Overall	RR = 0.22	0.10-0.50	< 0.001
	(2)				Cirrhotics	RR = 0.17	0.04-0.79	0.020
	(3)				Non-cirrhotics	RR = 0.21	0.10-0.47	< 0.001
	(3)				Drug resistance	RR = 0.52	0.28-0.97	0.040
	(3)				Without drug resistance	RR = 0.37	0.17-0.77	0.008
	(3)				HBeAg positive	RR = 0.21	0.10-0.44	< 0.001
	(3)				HBeAg negative	RR = 0.25	0.06-1.06	NS
Papathodoridis <i>et al</i> <sup>[38]</sup> , 2010	21 (3)	3881/534	LAM	202	Treated <i>vs</i> untreated	2.8% (22/779) <i>vs</i> 6.4% (34/534)		0.003
	(3)				Treated in remission <i>vs</i> untreated	2.5% (9/353) <i>vs</i> 6.4% (34/534)		0.015
	(3)				Treated without remission <i>vs</i> untreated	2.8% (12/426) <i>vs</i> 6.4% (34/534)		0.016
	(10)				Treated in remission <i>vs</i> treated without remission	2.3% (23/982) <i>vs</i> 7.5% (64/852)		< 0.001
	(14)				Treated in remission under initial therapy <i>vs</i> treated in remission under rescue therapy	2.3% (23/982) <i>vs</i> 5.9% (19/320)		0.003
Singal <i>et al</i> <sup>[49]</sup> , 2013	49 (6)	10025/3571	LAM or Other NAs <sup>3</sup>	808	LAM <sup>2</sup> <i>vs</i> untreated	RR = 0.48	0.38-0.61	< 0.001
	(49)				No difference between NAs <sup>3</sup> Pooled HCC incidence rate: 1.3 (1.1-1.6) per 100 person-years			

<sup>1</sup>Number of studies included in each analysis; <sup>2</sup>In 6 studies including both LAM treated (*n* = 3306) and untreated patients (*n* = 3571); <sup>3</sup>In the 49 studies, there were 5946 patients treated with LAM, 1929 patients treated with adefovir, 879 patients treated with entecavir, 616 patients treated with telbivudine and 657 patients treated with tenofovir. IFN-α : Interferon-α; LAM: Lamivudine; NS: Non-significant.

95%CI: 0.08-7.23). In a more recent meta-analysis involving 11 studies (1006 IFN-α treated and 1076 controls), IFN-α reduced the risk of HCC in chronic hepatitis B patients by 41% compared to untreated controls<sup>[24]</sup>. Finally, Miyake *et al*<sup>[25]</sup> included 8 studies in a meta-analysis and found a preventive effect of treatment in favor of IFN-α (risk difference, -5.0%, *P* = 0.028) that was more pronounced in Asian patients, in patients with a baseline HCC risk (HCC risk in untreated cohorts) > 10% and in HBeAg positive patients (Table 1).

According to the aforementioned meta-analyses, IFN-α therapy appears to decrease the incidence of HCC, particularly in patients at high baseline risk for

HCC development. It should be noted that the results of the individual studies should be interpreted with caution, as they were usually underpowered to capture relatively infrequent hard end-points such as HCC and they often tended to enroll subjects with less severe disease with low HCC risk. The effectiveness of IFN-α treatment was more evident in HBeAg positive patients suggesting that IFN-α may reduce the HCC risk more easily in patients with high viral replication and perhaps without HBV DNA integration into the host genome by accelerating the HBeAg seroconversion phase. There are no data on the impact of IFN-α-induced HBV DNA elimination in the reduction of HCC risk. In any case,

most of the patients with sustained response to IFN- $\alpha$  still have detectable HBV DNA by sensitive polymerase chain reaction (PCR) assays. However, residual viraemia in the absence of biochemical evidence of necroinflammatory liver activity seems to be of no clinical relevance, as the achievement of sustained biochemical remission in HBeAg negative patients has been associated with a significant decrease of the HCC incidence<sup>[26]</sup>. It should be noted that less than 30%-35% of patients who receive IFN- $\alpha$  achieve sustained responses<sup>[7,27,28]</sup>. Moreover, patients with advanced cirrhosis may experience severe liver decompensation during treatment with IFN- $\alpha$ <sup>[7,28]</sup>. Therefore, patients with contraindications to IFN- $\alpha$  including advanced liver disease as well as cases who do not achieve sustained off-treatment response after a course with IFN- $\alpha$  should receive therapy with a NA<sup>[7,28]</sup>.

## NAS AND HCC

Most patients are currently treated with oral NAs. These agents represent the first-line treatment option for the majority of chronic hepatitis B patients because of the relatively low efficacy and possible contraindications for or poor tolerance of IFN- $\alpha$ . In addition, they are used even in the majority of patients who may start with standard or recently PEGylated IFN- $\alpha$  and fail to achieve a sustained response<sup>[7,29,30]</sup>. Long-term therapy with NAs has improved the overall outcome of chronic hepatitis B and resulted in a substantial reduction in the need for liver transplantation<sup>[31]</sup>. The third generation NAs, entecavir and tenofovir, are currently recommended by the main treatment guidelines as the first-line NAs options<sup>[7,29,30]</sup> due to their high potency and high genetic barrier. Long-term monotherapy with entecavir or tenofovir achieves maintained on-therapy complete viral suppression in the vast majority of patients (> 95%), progressively increasing rates of HBeAg seroconversion in HBeAg positive cases and improvement of liver histology including reversion of histological cirrhosis in most cases<sup>[32-36]</sup>. Nevertheless, the effect of NAs on the prevention of HBV-related HCC is still unclear.

## LOW-MODERATE GENETIC BARRIER NAS

Most of the published data on the effects of NAs on the HCC risk are derived from studies using lamivudine. In the only randomized, controlled clinical trial including 651 chronic hepatitis B patients (58% HBeAg positive) with biopsy-proven cirrhosis or advanced fibrosis, lamivudine was found to significantly reduce the risk of HCC compared to placebo (3.9% *vs* 7.4%,  $P = 0.047$ )<sup>[37]</sup>. When HCC cases diagnosed during the first year of treatment were excluded, the risk reduction was marginally non-significant ( $P = 0.052$ ). It should be noted that the study was terminated early (after a mean duration of 32.4 mo) because of significant beneficial effects in the treatment group (7.8% developed cirrhosis complications *vs* 17.7% in the placebo group,  $P = 0.001$ ). Therefore, it could be

argued that the early termination of the study probably made the effect of HCC prevention less obvious.

Sung *et al.*<sup>[23]</sup> performed a meta-analysis of 5 studies involving 1267 treated patients (mostly with lamivudine) and 1022 controls (Table 1). They showed that the use of NAs reduced the HCC incidence by 78% (2.5% for NAs *vs* 11.7% for controls; RR = 0.22,  $P < 0.001$ ). The HCC risk was found to be significantly reduced in patients with cirrhosis (NAs: 3.9% *vs* untreated controls: 22.4%; RR = 0.17,  $P = 0.02$ ), in patients without cirrhosis (NAs: 1.8% *vs* untreated controls: 8%; RR = 0.21,  $P < 0.001$ ) and even to patients who developed viral resistance (NAs: 3.3% *vs* untreated controls: 6.4%; RR = 0.52,  $P = 0.04$ ). In addition, significantly lower HCC rates reported in treated than untreated HBeAg positive patients (1.7% *vs* 7.9%,  $P < 0.001$ ), while there was only a numerical trend for reduced HCC rates in treated compared to untreated HBeAg negative patients (3% *vs* 10.5%,  $P = 0.06$ ).

Papatheodoridis *et al.*<sup>[38]</sup> performed another systematic review including randomized or observational cohort studies of adult patients with chronic hepatitis B and/or cirrhosis who received treatment with lamivudine and/or perhaps adefovir for a mean/median duration of  $\geq 24$  mo (Table 1). Twenty-one relevant studies (16 with NAs naïve patients-5 with lamivudine resistant patients) were identified including 3881 CHB patients (33% cirrhotics, 49% HBeAg positive). In the analysis of the 3 studies including both treated and untreated patients<sup>[37,39,40]</sup>, HCC was detected significantly more frequently in untreated controls (34/534 or 6.4%) than in all treated patients (22/779 or 2.8%,  $P = 0.003$ ) or in treated patients remaining in virological remission (9/353 or 2.5%,  $P = 0.015$ ) or in treated patients with virological breakthroughs or no response (13/426 or 3%,  $P = 0.016$ ). In the 16 studies including NAs naïve patients, the incidence of HCC was found to be higher in patients with than without cirrhosis (10.8% *vs* 0.5%,  $P < 0.001$ ) and in patients with virological non-response or breakthroughs than in patients remaining in virological remission (7.5% *vs* 2.3%,  $P < 0.001$ ). A higher incidence of HCC was also reported in studies with than those without regular HCC surveillance (6.6% *vs* 2.3%,  $P < 0.001$ ), in studies including patients with a mean/median age  $\geq 50$  than  $< 50$  years (6% *vs* 2.8%,  $P < 0.001$ ) and in studies with predominantly (> 85%) HBeAg negative than predominantly HBeAg positive patients (5.5% *vs* 0.5%,  $P < 0.001$ ).

In the 5 studies including patients with lamivudine resistance<sup>[38]</sup>, HCC developed exclusively in cirrhotics (17.6% *vs* 0%,  $P < 0.001$ ) and more frequently in patients with persistent viremia than in those who achieved virological remission (20.2% *vs* 5.9%  $P < 0.001$ ). However, the induction of virological remission after rescue therapy was not found to be associated with a decreased HCC risk after the exclusion of 13 patients who had already developed HCC at the onset of the adefovir rescue therapy (5.9% *vs* 8.8%,  $P = 0.466$ ). The cumulative HCC rate was significantly higher in patients with lamivudine resistance than in naïve patients regardless of liver disease



severity (7.1% *vs* 3.8%,  $P = 0.001$ ) or among cirrhotics (17.6% *vs* 10.8%,  $P = 0.015$ ).

In a more recent large Greek cohort study published after the latter meta-analysis, 818 HBeAg negative chronic hepatitis B patients with or without cirrhosis starting with lamivudine monotherapy were included<sup>[41]</sup>. During a median follow-up of 4.7 years, the HCC incidence was again higher in older patients and those with cirrhosis at baseline, but virological on-therapy remission was not found to decrease the incidence of HCC in all patients ( $P = 0.322$ ) or in patients with cirrhosis ( $P = 0.327$ ), while there was a trend for lower incidence in non-cirrhotic patients with than without maintained on-therapy remission ( $P = 0.076$ ). In contrast, in another recent Japanese cohort study, maintenance of virological remission under lamivudine was reported to achieve significant reduction in the HCC incidence<sup>[42]</sup>. These seemingly conflicting results may be due to differences in patient characteristics (Caucasian or Asian patients, predominance of HBeAg negative or HBeAg positive patients, older or younger ages) as well as due to differences in the management of lamivudine resistance (prompt or no rescue therapy).

Despite the limitations of most cohort studies including heterogeneous patient populations, variations in treatment regimens and patient monitoring, differences in the definitions of response, wide range in the sensitivity of HBV DNA assays and different durations of follow-up, it is now widely accepted that even the administration of lamivudine, a low genetic barrier NA, significantly reduces the risk of HCC particularly in patients with cirrhosis and in those who achieve maintained virological remission. However, the risk of HCC remains high in patients with cirrhosis even if they achieve virological remission, particularly at older ages<sup>[2,4,38]</sup>. In addition, development of lamivudine resistance appears to be associated with an increased risk of HCC, which may not be reduced by an effective rescue therapy. The latter data in combination with the very high and progressively increasing rates of lamivudine resistance further discourage the use of lamivudine as first-line option for the treatment of chronic hepatitis B<sup>[7,29,30]</sup>.

## HIGH-GENETIC BARRIER NAs

There are only a few recent retrospective or prospective observational cohort studies that provide HCC data for patients treated with the high-genetic barrier NAs. Most of the available studies include patients treated with entecavir and only one patients treated with tenofovir that has been available in chronic hepatitis B for a shorter period.

In a retrospective study from Japan, Hosaka *et al*<sup>[43]</sup> compared the incidence of HCC in entecavir treated patients with a historical cohort of untreated HBV patients. They used a propensity score matching to eliminate the baseline differences resulting in a sample size of 316 patients per cohort (27% cirrhotics). The cumulative HCC incidence at 5 years was significantly lower in the entecavir treated patients than in untreated controls (3.7% *vs*

13.7%,  $P < 0.001$ ). Cox regression analysis showed that entecavir reduced the HCC risk by 63% (HR = 0.37; 95%CI: 0.15-0.91). However, the benefit of entecavir in the reduction of cumulative HCC risk was significant only in cirrhotics (7% *vs* 39%,  $P < 0.001$ ) but not in non-cirrhotics (2.5 *vs* 3.6%,  $P = 0.440$ ).

The favorable effect of treatment with the high-genetic barrier NAs on the risk of HCC was also confirmed in other studies. Wong *et al*<sup>[44]</sup> performed a retrospective-prospective cohort study including 1446 NAs naïve or NAs experienced (28%) patients treated with entecavir and 424 historical untreated controls. Overall, there was no significant difference in the HCC rates between the entecavir treated patients and untreated controls. However, among patients with cirrhosis, entecavir significantly reduced the incidence of HCC compared to untreated cirrhotics (13.8% *vs* 26.4%,  $P = 0.049$ ), while no difference was found in non-cirrhotics (3.3% *vs* 3.0%,  $P =$  non-significant).

In another study, Kim *et al*<sup>[45]</sup> used a prediction model to compare the incidence of HCC in 641 patients treated for 6 years with tenofovir in the tenofovir long-term registration trial with the predicted HCC rate estimated by the REACH-B risk calculator. The authors found that tenofovir reduced the HCC incidence compared to the predicted HCC risk. Specifically, there was a progressive divergence between the predicted and observed number of HCC cases after 3.3 years of follow-up with a standardized incidence ratio of 0.55 (95%CI: 0.32-0.94) at the latest follow-up (median: 5.52 years).

All the data summarized above show that treatment with a high-genetic barriers NA reduces the risk of HCC compared to no treatment with a more profound effect in cirrhotics. The lower benefit on the HCC risk in non-cirrhotic patients seems to be reasonably related to the low baseline HCC risk in this sub-group of patients. Therefore, great numbers of patients and long follow-up periods are required to provide the studies including non-cirrhotic patients with the appropriate power in order to detect a potential benefit on the HCC incidence from these agents.

The effect of entecavir on the risk of HCC has also been compared to the effect of lamivudine in some studies. In the study from Japan by Hosaka *et al*<sup>[43]</sup>, the HCC incidence in the entecavir treated patients was compared to that in a historical cohort of 182 patients treated with lamivudine monotherapy without any rescue therapy in case of resistance. The reduction in the HCC incidence was greater in the entecavir treated than in non-rescued lamivudine treated cirrhotic patients (7% *vs* 22%,  $P = 0.043$ ) but such an effect was not seen in non-cirrhotics (2.5% *vs* 4.9%,  $P > 0.05$ ). On the contrary, an advantage of entecavir over lamivudine in the reduction of HCC risk was not confirmed in other studies. In a prospective study from Japan as well, Kobashi *et al*<sup>[46]</sup> assessed the incidence of HCC in 129 naïve patients (22% cirrhotics) treated with entecavir and 127 patients (27% cirrhotics) treated with lamivudine. After a mean follow-up of 4.25

years, HCC developed in 35 patients (11 on entecavir and 24 on lamivudine) with the 5-year cumulative HCC incidence being similar (12.4%) in the two groups ( $P = 0.680$ ). Lamivudine resistance was developed in 60 (47%) of the 127 lamivudine treated patients and was associated with a significantly increased risk of HCC compared to patients without lamivudine resistance ( $P = 0.035$ ). In a large nationwide prospective cohort study from Greece, Papatheodoridis *et al.*<sup>[47]</sup> estimated the incidence of HCC in 321 HBeAg negative chronic hepatitis B patients (25% cirrhotics) treated with entecavir (86% naïve, 14% experienced) and compared it with the HCC incidence in a historical cohort of 818 patients treated with lamivudine and perhaps adefovir upon lamivudine resistance (26% cirrhotics). After a mean follow-up of 30 mo, 1.2% (4/321) of entecavir treated patients developed HCC with a trend for lower 5-year cumulative HCC incidence in the entecavir compared to the lamivudine group (4.8% *vs* 5.6%,  $P = 0.096$ ). In the multivariate analysis, however, the HCC risk was independently associated with older age, male gender and cirrhosis but not with type of initial therapy. Finally, in a relatively small study from Turkey, Köklü *et al.*<sup>[48]</sup> retrospectively analyzed the data from 227 patients (86% naïve, 14% experienced) with HBV cirrhosis (46% decompensated) who were treated with tenofovir ( $n = 72$ , 36% decompensated), entecavir ( $n = 77$ , 47% decompensated) or lamivudine ( $n = 74$ , 54% decompensated). The incidence of HCC was not statistically different between patients treated with newer antivirals (entecavir/tenofovir: 4% after 2 years of follow-up) and those treated with lamivudine (9% after 3 years of follow-up).

Given that the newer high-genetic barrier NAs achieve more potent and durable suppression of HBV replication and that lamivudine resistance has been associated with an increased risk of HCC, one would expect an advantage over lamivudine in the prevention of HCC development. However, the data from the currently available studies are limited and the findings appear to be inconsistent. Only one study reported a significant benefit in the reduction of the HCC incidence from entecavir over lamivudine without any rescue therapy upon resistance<sup>[43]</sup>. In contrast, three other studies and a recent meta-analysis reported no difference in the HCC rates between entecavir and lamivudine treated patients (Table 1)<sup>[46-49]</sup>. All these findings should be seen with caution, as they come from studies with low statistical power or different strategies for the management of lamivudine resistance (no rescue therapy, perhaps delayed rescue therapy, prompt onset of rescue therapy) that may be critical for the HCC risk. Moreover, these comparisons have limited practical value, as a high-genetic barrier NA should be used in any chronic HBV patient anyway because of their high potency and negligible risk of long-term resistance<sup>[7,28]</sup>.

Other studies usually including NAs naïve and NAs experienced patients assessed the impact of entecavir on HCC development according to the induction of virological remission. Yang *et al.*<sup>[50]</sup> investigated the risk of

HCC in 487 chronic hepatitis B patients (34% NAs experienced, 40% cirrhotics) treated with entecavir for  $\geq 12$  mo. HCC developed in 36 patients (7.4%). The risk of HCC was lower in patients with than without virological remission in both cirrhotics (HR = 0.21, 95%CI: 0.07-0.60) and non-cirrhotics (HR = 0.08, 95%CI: 0.01-0.50). In a multicenter European cohort (VIRGIL) study<sup>[51]</sup> including 372 entecavir-treated patients (26% cirrhotics, 63% NAs experienced), virological remission reduced the probability of a clinical event (HCC, hepatic decompensation or death) by 71% (HR = 0.29, 95%CI: 0.08-1.00,  $P = 0.05$ ). The benefit of virological remission was significant only in patients with cirrhosis (HR = 0.22, 95%CI: 0.05-0.99,  $P = 0.04$ ). Lastly, Kim *et al.*<sup>[52]</sup> assessed the risk for development of HCC in 324 entecavir treated patients with HBV cirrhosis (32% decompensated). The 5-year cumulative incidence of HCC was 28.5% and patients with virological remission had significantly lower probability for development of HCC (RR = 0.056,  $P < 0.001$ ).

There is a considerable amount of evidence that suppression of viral replication improves the outcome of chronic hepatitis B patients<sup>[7,29,30]</sup>. Since the risk of HCC is related to the viral load, reduction of viral load with therapy should presumably reduce the incidence of HCC<sup>[10]</sup>. This hypothesis is further supported by the results of the above single-arm studies in which long-term virological remission under entecavir was associated with a significant decrease in the incidence of HCC<sup>[50-52]</sup>. Again, the benefit on the reduction of the HCC incidence was more obvious in patients with cirrhosis who are at a high HCC risk if they remain untreated.

## CONCLUSION

It is currently clear that antiviral therapy reduces but does not eliminate the risk of HCC in chronic hepatitis B patients with or without cirrhosis. Based on the standard IFN- $\alpha$  data, the currently used PEGylated IFN- $\alpha$  is also expected to reduce the incidence of HCC. Patients without a sustained off-treatment response after (PEGylated) IFN- $\alpha$  therapy should be treated with a NA, which represents the treatment option for the majority of chronic hepatitis B patients for several reasons<sup>[7,28]</sup>. Many data have shown that even treatment with lamivudine reduces the incidence of HCC, which may increase again in cases with untreated lamivudine resistance. Emerging data with the currently first-line NAs, entecavir and tenofovir, suggest that the risk of HCC is also reduced under long-term therapies with these agents. The treatment benefit in the reduction of the HCC incidence is always greater in patients with high baseline HCC risk, particularly those with cirrhosis. In addition, the reduction of the HCC incidence under a high genetic barrier NA is higher in the vast majority of patients who will achieve virological remission compared to those who may maintain detectable viral replication. Whether therapy with a high-genetic barrier NA offers an additional benefit on the reduction

of the HCC incidence compared to other NAs with low-moderate genetic barriers remains unclear, but it has no particular clinical interest, as monotherapy with entecavir and tenofovir represent the first-line NA choice for chronic hepatitis B patients anyway due to superiority of these agents in potency and resistance profile<sup>[7,28,32-36]</sup>.

Since the risk of HCC is not eliminated even in patients who remain in virological remission under a high-genetic barrier NA, it has been suggested that HBV DNA might have already been integrated into the host genome before the onset of treatment resulting in genomic alterations and/or chromosomal instability<sup>[53]</sup>. Thus, the oncogenic process may have started before therapy and the liver may contain clones of cells carrying genetic abnormalities that predispose to cancer<sup>[54]</sup>. Given that the duration of most studies with the high-genetic barrier NAs does not exceed 4–6 years, it remains to be seen whether the HCC incidence will remain stable over time after 5–6 years of NA therapy. In current clinical practice, however, patients at increased baseline HCC risk should continue to undergo HCC surveillance according to the existing recommendations even if they have achieved complete long-term inhibition of viral replication and improvements in liver histology.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Hierarchical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation of hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) represents a global health problem. Infections with hepatitis B or C virus, non-alcoholic steatohepatitis disease, alcohol abuse, or dietary exposure to aflatoxin are the major risk factors to the development of this tumor. Regardless of the carcinogenic insult, HCC usually develops in a context of cirrhosis due to chronic inflammation and advanced fibrosis. Galectins are a family of evolutionarily-conserved proteins defined by at least one carbohydrate recognition domain with affinity for  $\beta$ -galactosides and conserved sequence motifs. Here, we summarize the

current literature implicating galectins in the pathogenesis of HCC. Expression of "proto-type" galectin-1, "chimeric-type" galectin-3 and "tandem repeat-type" galectin-4 is up-regulated in HCC cells compared to their normal counterparts. On the other hand, the "tandem-repeat-type" lectins galectin-8 and galectin-9 are down-regulated in tumor hepatocytes. The abnormal expression of these galectins correlates with tumor growth, HCC cell migration and invasion, tumor aggressiveness, metastasis, postoperative recurrence and poor prognosis. Moreover, these galectins have important roles in other pathological conditions of the liver, where chronic inflammation and/or fibrosis take place. Galectin-based therapies have been proposed to attenuate liver pathologies. Further functional studies are required to delineate the precise molecular mechanisms through which galectins contribute to HCC.

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**Key words:** Galectins; Hepatocellular carcinoma; Inflammation-associated liver injury; Hepatitis B or C virus infection-associated hepatocellular carcinoma; Fibrosis-related liver pathologies

**Core tip:** Galectins, a family of glycan-binding proteins, are involved in the pathogenesis of hepatocellular carcinoma (HCC). Up-regulation of galectin-1, galectin-3 and galectin-4 is observed in HCC cells, whereas galectin-8 and galectin-9 appear to be down-regulated in tumor hepatocytes. This altered expression correlates with tumor growth, HCC cell migration and invasion, tumor aggressiveness, metastasis, postoperative recurrence and poor prognosis. These galectins are also implicated in inflammation- and fibrosis-related liver pathologies.

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archical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation of hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(47): 8831-8849 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/8831.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.8831>

## INTRODUCTION

Hepatocellular carcinoma (HCC) represents a global health problem. It is the fifth most common solid tumor and the third cause of cancer-related mortality per year<sup>[1]</sup>. HCC is most prevalent in Eastern Asia and sub-Saharan Africa; whereas the incidence in Europe and North America is considerably lower<sup>[2-4]</sup>. The etiology of HCC includes major risk factors such as infection with Hepatitis B or C virus (HBV, HCV), alcohol abuse or dietary exposure to aflatoxin<sup>[5-7]</sup>. Regardless of the carcinogenic insult, HCC usually develops in patients with cirrhosis due to chronic inflammation and advanced fibrosis<sup>[8]</sup>. Non-alcoholic steatohepatitis (NASH), a metabolic disorder resulting from insulin resistance syndrome that underlies fibrosis and cirrhosis, is emerging as another important risk factor for HCC<sup>[9,10]</sup>.

During the past decade the management of HCC has significantly improved<sup>[11]</sup>. New advances in the field have led to a better knowledge and an earlier detection of this disease. Additionally, current therapies such as, resection, transplantation, ablation and chemoembolization, have provided benefit to patients diagnosed at early HCC stages improving and extending their survival<sup>[12-14]</sup>. However, most patients are diagnosed at advanced stages and therefore, they are not amenable to surgical treatment. Even after resection or transplantation, the prognosis remains unsatisfactory due to recurrence, metastasis and the development of new primary tumors<sup>[15-17]</sup>.

Recent progress toward a better understanding of the molecular biology of HCC has allowed the development of molecular targeted therapies and has shed light on new systemic therapies for HCC. Several intracellular signaling pathways involved in abnormal proliferation, survival, differentiation, invasion and metastasis have been found to be dysregulated in HCC. Clinical trials are currently testing the potential use of inhibitors of the Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK), phosphatase and tensin homolog deleted on chromosome 10/phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin, transforming growth factor  $\beta$  (TGF- $\beta$ ), Wnt/ $\beta$ -catenin and epidermal growth factor receptor (EGFR) pathways, among others<sup>[18-20]</sup>. Sorafenib, a receptor tyrosine kinase inhibitor targeting vascular endothelial growth factor, platelet-derived growth factor and Raf signaling pathways prolongs survival in patients with advanced unresectable HCC<sup>[21,22]</sup>. Simultaneously, new immunotherapy strategies are being developed for the treatment of HCC, which could be administered in combination with conventional therapies in order to obtain a more favorable clinical

outcome<sup>[23]</sup>. Undoubtedly, the approval of oral administration of sorafenib highlights the importance of elucidating the molecular mechanisms underlying HCC progression for the development of novel therapies.

Recently, there has been increasing evidence highlighting the involvement of galectins, a family of glycan-binding proteins, in the pathogenesis of HCC. In this review, we present emerging data showing that expression of some members of this family is altered in HCC cell lines and tissues compared to normal liver. These observations led to the proposition that galectins are potential prognostic biomarkers and therapeutic targets in HCC. We will discuss the possible roles of these proteins in HCC tumor transformation, progression, aggressiveness and metastasis. Moreover, we will highlight the involvement of galectins in other pathological settings of the liver, where chronic inflammation and/or fibrosis take place.

## GALECTINS

Galectins are a family of evolutionary conserved glycan-binding proteins or lectins that recognize multiple N-acetylglucosamine (Gal $\beta$ 1,4GlcNAc) units on cell surface glycoconjugates. These animal proteins are defined by at least one carbohydrate recognition domain (CRD) with affinity for  $\beta$ -galactosides and conserved sequence motifs<sup>[24]</sup>. To date, fifteen galectins have been described in mammals and according to their structural characteristics they are classified into three groups: “proto-type” galectins (galectin-1, galectin-2, galectin-5, galectin-7, galectin-10, galectin-11, galectin-13, galectin-14 and galectin-15) contain one CRD and can dimerize; “tandem repeat-type” galectins (galectin-4, galectin-6, galectin-8, galectin-9 and galectin-12) contain two distinct CRD in tandem, connected by a linker peptide; and “chimera-type” galectin-3 which consists of unusual proline- and glycine-rich short stretches fused onto the CRD<sup>[25,26]</sup>.

Some galectins (*e.g.*, galectin-1, galectin-3 and galectin-9) are widely expressed among different tissues including, immune cells, endothelial and epithelial cells, and sensory neurons (reviewed by<sup>[27-29]</sup>); whereas other family members have a more restricted tissue localization and compartmentalization (*e.g.*, galectin-7 is preferentially found in the skin, galectin-12 is abundantly expressed in adipose tissue, galectin-5 is restricted to rat reticulocytes, and galectin-10 is strongly represented in human but not mouse eosinophils)<sup>[27]</sup>.

These lectins do not possess a signal peptide for export through the classical secretory pathway (Golgi-endoplasmic reticulum); however they are secreted to the extracellular milieu *via* a non-conventional poorly understood secretory pathway<sup>[30-32]</sup>. For instance, non-classical secretion of galectin-1 has been observed in skeletal muscle during *in vivo* development and in cultured myoblasts during differentiation<sup>[33]</sup>. Besides, secretion of galectin-3 from macrophages, renal and polarized intestinal epithelial cells has been detected<sup>[34,35]</sup>. There is also

evidence for secretion of galectin-9 in activated Jurkat T cells<sup>[36]</sup> and CD4 T cells expressing galectin-9 on the cell surface upon T cell receptor stimulation<sup>[37]</sup>.

Through its binding to *N*-acetylglucosamine sequences, galectins form multivalent complexes with cell surface glycoconjugates and thus, transmit signals inside the cell<sup>[38-40]</sup>. Remarkably, it has also been demonstrated that galectin-1 can be internalized by Jurkat T cells in a carbohydrate-dependent mechanism, following dual pathways involving clathrin-coated vesicles and raft-dependent endocytosis<sup>[41]</sup>. Within the intracellular milieu, galectins bind to their ligands preferentially through protein-protein interactions, and regulate intracellular processes, including mRNA splicing, cell cycle progression, apoptosis, and cell proliferation<sup>[42]</sup>.

Galectins have emerged as pivotal regulators of cellular physiology. Over the past decade, multiple biological functions have been reported for this protein family including roles in cell adhesion, migration, cytokine synthesis, and survival<sup>[43,44]</sup>. In fact, different members of the family have shown critical roles as mediators of acute and chronic inflammation<sup>[45,46]</sup>. Galectins are often aberrantly expressed in many different tumor types including astrocytoma, melanoma and prostate, thyroid, colon, head and neck, bladder, kidney, stomach, lung, bladder, uterine, breast and ovary carcinomas<sup>[27,47,48]</sup>. Moreover, mounting evidence indicates that these proteins play fundamental roles in cancer biology including tumor transformation, tumor growth, angiogenesis, migration, metastasis and tumor-immune escape<sup>[49-52]</sup>. Given these pleiotropic activities in the tumor microenvironment, galectins are being increasingly recognized as molecular targets for innovative cancer therapy<sup>[26,52-56]</sup>.

In this review, we summarize the current data implicating galectins in HCC. Particularly, we focus our discussion on selected members of the family, including galectin-1, galectin-3, galectin-4, galectin-8 and galectin-9, which roles in HCC biology have been demonstrated.

## GALECTIN-1

The first protein discovered within the galectin family was galectin-1. This galectin possesses one CRD and can form homodimers *via* non-covalent binding, which confers the ability to cross-link specific glycoconjugates<sup>[26,28]</sup>. Galectin-1 displays features of typical cytoplasmic proteins; it has been described in nucleus and cytoplasm and can translocate to the intracellular face of cellular membranes. Although galectin-1 lacks a recognizable secretion signal sequence, it is secreted through a non-conventional secretory pathway<sup>[31,32]</sup>, thus being detected on the extracellular side of cellular membranes as well as in the extracellular matrices (ECM) of various normal and neoplastic tissues<sup>[57]</sup>.

While the role of galectin-1 within the intracellular milieu is often independent of its lectin activity, its extracellular functions are mostly dependent on the binding to *N*-acetylglucosamine units on cell surface glycoconjugates<sup>[28]</sup>. Intracellularly, galectin-1 is engaged in funda-

mental processes such as pre-mRNA splicing; and also it interacts with oncogenic H-RAS and contributes to its membrane anchorage, evidencing a key role for this galectin in driving tumor transformation (reviewed by<sup>[49,58]</sup>). In the extracellular space, galectin-1 binds to glycoconjugates on the cell surface, including different members of the integrin family and glycoproteins of the ECM such as laminin and fibronectin<sup>[59,60]</sup>. It is likely that the local abundance of galectin-1 in the tumor microenvironment may play a critical role during attachment or detachment of cancer cells throughout cancer progression<sup>[43]</sup>. Furthermore, galectin-1 promotes cell migration, a function that correlates with the ability of this protein to influence tumor progression, invasion and angiogenesis. However, the biological roles of galectin-1 appear to be tissue-specific as it also decreases cell migration of most immune cells providing a rational basis for its anti-inflammatory properties<sup>[43,45,55,61]</sup>.

Expression of galectin-1 has been well documented in many different tumor types including astrocytoma, melanoma and prostate, thyroid, colon, bladder and ovary carcinomas<sup>[57,62]</sup>. Moreover, preferential accumulation of galectin-1 in the peritumoral stroma has been described for thyroid, head and neck, colon, ovary and prostate carcinoma<sup>[57]</sup>. Functions of galectin-1 during tumor progression have been largely documented in the literature. High levels of galectin-1 correlate with aggressiveness of tumors<sup>[63-67]</sup>, and the acquisition of a metastatic phenotype<sup>[68-71]</sup>. This lectin plays a fundamental role in tumor angiogenesis by modulating endothelial cell biology<sup>[72,73]</sup> and its expression is induced by hypoxia<sup>[74,75]</sup>. Importantly, galectin-1 has been proposed to be a major immunosuppressive factor which contributes to tumor immunoevasive programs<sup>[76,77]</sup>. In fact, galectin-1 expression by tumor cells or by their surrounding stroma can regulate the function, fate and viability of infiltrating tumor-specific T cells<sup>[78]</sup>.

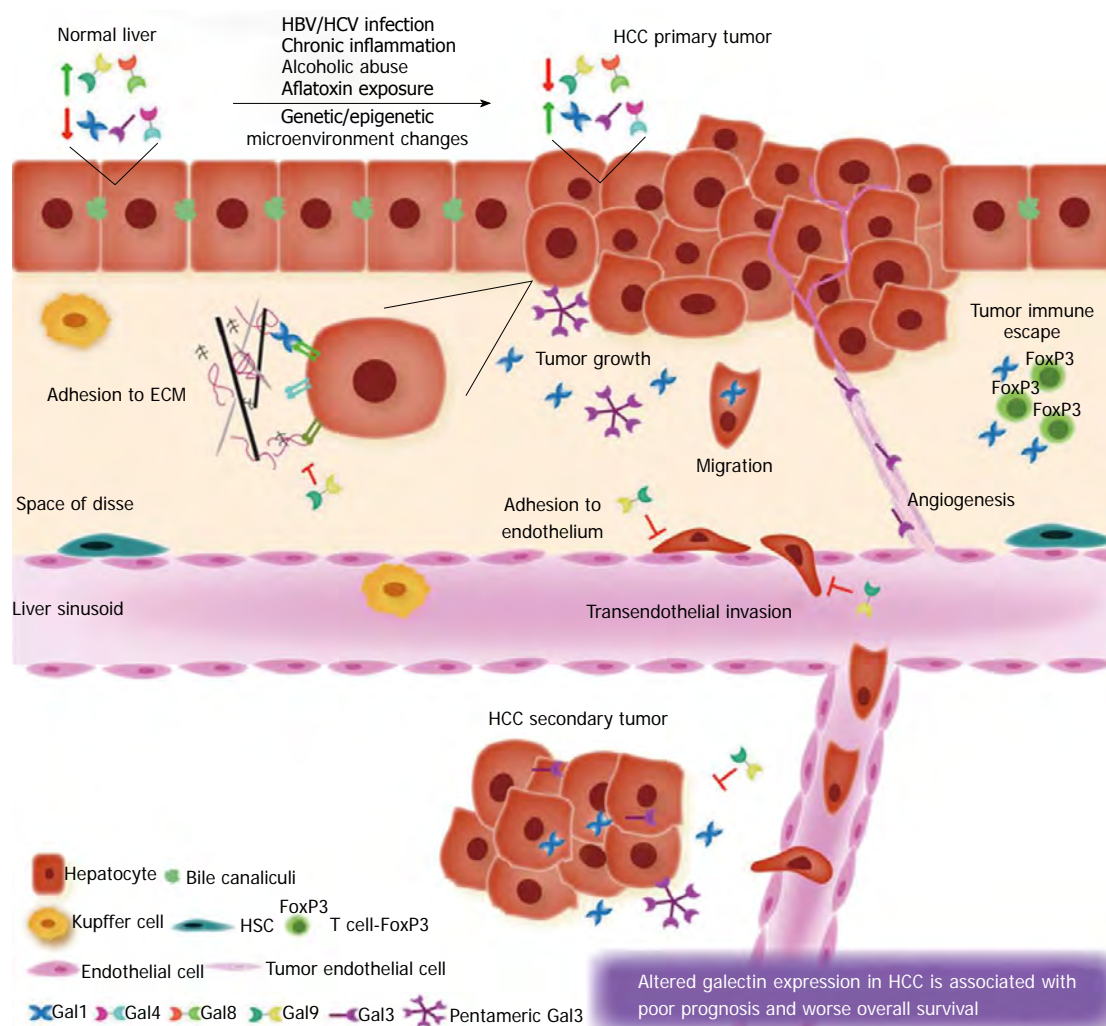
## Galectin-1 in HCC and in inflammation-associated liver injury

Galectin-1 gene (*LGALS1*) regulation was extensively studied using the well characterized system hepatoma x fibroblast hybrids. Activation of gene expression was achieved by treatment of galectin-1-non-expressing cells with the DNA demethylating agent azacytidine. The methylation status of the galectin-1 gene promoter was identified as a central mechanism that controls gene expression in normal tissues and also in transformed cells and tumors<sup>[27]</sup>.

While in normal liver galectin-1 is expressed at low constitutive levels, in HCC its expression is dramatically up-regulated<sup>[79-82]</sup>. Gene expression profiling of normal and HCC human tissues using cDNA microarrays allowed the identification of *LGALS1* as one of the hallmark genes that are over-expressed in HCC, a phenomenon which was further confirmed by RT-PCR<sup>[79]</sup>.

Kondoh *et al*<sup>[80]</sup> elucidated the molecular mechanism governing *LGALS1* gene expression in liver malignancy. This group investigated the methylation states of the





**Figure 1** Galectins in hepatocellular carcinoma. In normal liver, galectin (Gal)-8 and galectin-9 are expressed in hepatocytes whereas galectin-1, galectin-3 and galectin-4 are not detectable. This expression pattern is altered in hepatocellular carcinoma (HCC) as galectin-1, galectin-3 and galectin-4 are up-regulated, whereas galectin-8 and galectin-9 are down-regulated in transformed hepatocytes. This aberrant expression favors tumor growth and hepatocyte adhesion to extracellular matrix (ECM), migration, adhesion to the endothelium, transendothelial invasion and metastasis. Galectin-3, normally absent in sinusoid endothelial cells, is up-regulated in tumor capillary endothelial cells, probably promoting angiogenesis. Increased expression of galectin-1 and lack of galectin-9 expression also contribute to tumor-immune escape. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

galectin-1 gene promoter in human HCC and adjacent non-tumor liver tissue, and in different HCC cell lines. Analysis of the methylation profile revealed that certain CpG dinucleotides surrounding the transcription start site of *LGALS1* promoter were frequently methylated in non-tumor liver, whereas these sequences were hypomethylated in HCC tissues. Interestingly, using a mobility shift assay with nuclear extracts from three HCC cell lines (HLF, HuH7, and HepG2) as well as human embryonic primary liver (PL) cells, the authors showed specific interaction of a methylation-sensitive factor to the upstream and downstream regulatory elements which appear to be essential for the activation of the *LGALS1* gene in HCC cells<sup>[80]</sup>. Northern blot analysis demonstrated that galectin-1 mRNA was up-regulated in primary HCC in comparison to adjacent non-tumor liver tissues and human normal liver tissues. In fact, galectin-1 mRNA level was higher in the HuH-7 and HLF HCC cell lines as compared to HepG2 and PL cells<sup>[80]</sup>.

Although over-expression of galectin-1 was observed in HCC *in vivo* as well as *in vitro*, the precise function of this endogenous lectin in liver pathophysiology remained uncertain for many years. However, emerging findings shed light to the role of the leading role of galectin-1 in HCC development and progression. Spano *et al*<sup>[81]</sup> reported that galectin-1 expression was significantly increased in HCC samples from patients with metastatic disease compared to those harboring a non-metastatic primary tumor. However, no significant associations were found with other parameters, although a trend toward an association between increased galectin-1 expression in HCC and vascular invasion was observed. Moreover, galectin-1 expression profile was also examined in human HuH-7 and JHH-6 HCC cells and human normal liver, cirrhotic tissue and HCC specimens using tissue microarrays. In all cases, increased expression of the *LGALS1* gene was confirmed in HCC. Furthermore, immunohistochemical analysis revealed a preferential accumulation of galectin-1

**Table 1** Involvement of galectins in the pathogenesis of hepatocellular carcinoma

Galectin member	Expression	Function and/or effect	Model	Ref.
Galectin-1	Up-regulated (mRNA and protein) in HCC, secreted by tumor hepatocytes and accumulated in stroma surrounding HCC	Correlates with tumor aggressiveness, metastases and enhanced risk of post-operative recurrence	Human HCC tissues	[79-82]
		Favors HCC cell adhesion to ECM, cell migration and invasion	Human HCC cell lines	[81,85]
		Increases tumor growth and metastasis in draining-tumor lymph nodes	Nude mice injected with galectin-1 over-expressing HepG2 cells	[85]
		Possible role in the suppression of antitumor immune responses	Human HCC tissues	[82]
Galectin-3	Up-regulated (mRNA and protein) in HCC. Transactivation of murine <i>LGALS3</i> promoter can occur by HBV-X protein. High nuclear expression	Correlates with histological differentiation and vascular invasion	Human HCC tissues	[79,124,125]
		Probably promotes angiogenesis	Tumor-associated endothelial cells isolated from rats	[126]
Galectin-4	Up-regulated in HCC-associated capillary endothelial cells		Human HCC tissues and cell lines	[128]
Galectin-8	Higher expression in HCC than normal tissues		Human HCC tissues and cell lines	[154]
Galectin-9	Diminished expression in hepatoblastoma and hepatocarcinoma		Human HCC tissues	[159]
Galectin-9	Downregulated in HCC	Galectin-9 suppression promotes cell proliferation and adhesion to ECM, tumor cell-endothelial cell adhesion and trans-endothelial invasion of HepG2 cells.	Human cell lines	[181]
		Downregulation of galectin-9 represents a risk factor for patient survival, correlates with tumor histopathological grade, vascular invasion and metastasis	Human HCC tissues	[181]

HCC: Hepatocellular carcinoma; ECM: Extracellular matrix; HBV: Hepatitis B virus.

in the delicate stroma tissue surrounding tumor hepatocytes of HCC tumors. The authors hypothesized that neoplastic hepatocytes secrete galectin-1 which is then accumulated in the stroma surrounding HCC (Figure 1 and Table 1).

The correlation between increased expression of galectin-1 in HCC and the presence of metastasis was validated by *in vitro* functional studies. Expression of *LGALS1* gene and secretion of galectin-1 protein were substantially up-regulated in JHH-6 (undifferentiated cells) and HuH-7 (differentiated cells). Notably, galectin-1 over-expression increased the migratory and invasive capacities of HuH-7 cells, and both processes were mediated by the stimulation of the Sky receptor tyrosine kinase (RTK) phosphorylation. Thus, similar to breast cancer<sup>[68]</sup>, neuroblastoma<sup>[83]</sup>, oral squamous cell carcinoma and lung adenocarcinoma<sup>[84]</sup>, galectin-1 expression correlates with HCC tumor aggressiveness (Figure 1 and Table 1).

Under this scenario, we have focused our attention on the role of galectin-1 and its contribution to HCC development. In this regard, we examined the involvement of this galectin in HepG2 HCC cell adhesion and tumor growth<sup>[85]</sup>. We found that galectin-1 acts as a glycan-dependent matricellular modulator of HepG2 cell adhesion. We observed that galectin-1 favored cell adhesion to laminin, a polylactosamine-enriched glycoprotein and a major component of the ECM and basement membranes. Moreover, we demonstrated that the pro-adhesive effects of galectin-1 are specifically mediated by

$\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_v$ , and  $\beta_1$  integrins and involve PI3K and/or ERK1/2 signaling pathways. Besides, galectin-1 over-expressing HepG2 cells showed an increased secretion of this lectin to the extracellular compartment and remarkably, we also found that exogenously added recombinant galectin-1 was internalized by HepG2 cells<sup>[85]</sup>. Hence, in accordance with Spano *et al.*<sup>[81]</sup>, galectin-1 secreted from HCC cells might exert its biological functions either by engaging cell surface receptors and transmitting signals inside the cell or through receptor-mediated internalization and endocytosis. However, because intracellular functions have also been described for this protein<sup>[42]</sup> a cell surface-independent mechanism responsible for galectin-1 functions cannot be excluded. We also found that galectin-1 up-regulation in the tumor microenvironment favored HCC growth *in vivo* and promoted a considerable increase in tumor metastasis. This effect was evident in draining-tumor lymph nodes of mice injected with galectin-1 over-expressing HepG2 cells<sup>[85]</sup>. Collectively, these results suggested the involvement of galectin-1 in neoplastic and inflammatory processes of the liver (Figure 1 and Table 1).

Compelling evidence indicates that high expression of galectin-1 predicts poor patient outcome in a variety of tumors. However, the prognostic value of this endogenous lectin in HCC patients remained elusive for many years. Recently, Wu *et al.*<sup>[82]</sup> reported that elevated galectin-1 expression in HCC is significantly associated with tumor aggressiveness (vascular invasion, incomplete encapsulation, poor differentiation, and large tumor size) and enhanced

Table 2 Galectins in inflammation-associated liver injury

Galectin member	Experimental model	Role	Effects	Ref.
Galectin-1	Hepatitis induced by injection of Con A	Protective	Prevents both liver injury and T-helper cell liver infiltration, induces apoptosis of Con A-activated T cells, suppresses plasma levels of TNF and IFN- $\gamma$	[89]
	Inflammation-induced chronic cholestatic hepatitis at an early age, and HCC at later age (Mdr2-KO mice)	Protective	Galectin-1 is up-regulated in Mdr2-KO/B6 strain at early age	[91]
	Galectin-1-KO mice in the context of Con A-induced autoimmune hepatitis	Protective	Con A up-regulates galectin-1 in galectin-1-KO/B6 and Mdr2-KO/FVB strains. Endogenous galectin-1 selectively protects liver in the B6, but not in the FVB genetic background. It probably determines strain-specific differences in the course of chronic hepatitis and HCC development in the Mdr2-KO model	[91]
Galectin-3	NASH model Galectin-3-KO mice	Protective	Develops NAFLD/NASH spontaneously with aging	[140,141]
	CDA A diet-induced	Protective	Galectin-3 deficiency causes more severe hepatic injury and alterations in the expression of genes associated with carcinogenesis and lipid metabolism	[142]
	NAFLD/NASH in galectin-3-KO mice			
	Atherogenic diet-induced NASH in galectin-3-KO mice	Promotes disease severity	Attenuates NASH: inhibits HSC-driven fibrosis, reduces inflammatory-cell infiltration and hepatocyte apoptosis, acts as a major scavenger receptor involved in ALE/AGE uptake by the liver	[143]
	Human liver tissues	Protective	Negative expression of galectin-3 in normal hepatocytes, strong staining for galectin-3 in hepatocytes from patients with steatosis hepatitis, hepatitis, cholestasis and cirrhosis	[145]
Galectin-9	Acute liver failure induced by APAP-hepatotoxicity in galectin-3-KO mice	Perpetuates liver injury	In wild type mice, galectin-3 is up-regulated in liver infiltrating macrophages. In galectin-3 deficient mice the pro-inflammatory M1-type macrophages subpopulation, the classical macrophage activation markers iNOS, TNF and IL-12 and pro-inflammatory chemokines are reduced	[147,148]
	Hepatitis induced by injection of Con A in galectin-3-KO mice	Pro-inflammatory	Galectin-3 deficiency reduces the number of T lymphocytes, B lymphocytes, dendritic cells, NK and NKT cells and enhances apoptosis of mononuclear cells	[149]
	Con A-induced liver injury in wild type mice pretreated with a selective inhibitor of galectin-3 (TD139)	Pro-inflammatory	TD139 attenuates liver injury, reduces the number of CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells, favors the influx of IL-10-producing CD4 <sup>+</sup> T cells in the liver, decreases serum levels of IFN- $\gamma$ , IL-17 and IL-4	[149]
	Blockade of the TIM-3/galectin-9 pathway using an anti-TIM-3 or anti-galectin-9 mAb in a context of liver IRI	Protective	Blockade of the TIM-3/galectin-9 pathway increases hepatocellular damage, local neutrophil infiltration, T cell and macrophage accumulation and liver cell apoptosis. Increases IFN- $\gamma$ production by Con A-stimulated spleen T cells and augmented TNF and IL-6 production by Con A-stimulated macrophages/T cells	[190]
	Single injection of galectin-9 in the murine model of liver injury induced by Con A	Protective	Eliminates activated CD4 <sup>+</sup> effector T cells, prevents the synthesis and/or release of proinflammatory cytokine	[191]
	Mouse model of diet-induced NAFLD treated with galectin-9	Limits the inflammatory response	Induces apoptosis of NKT cells, also interacts with TIM-3-expressing Kupffer cells to induce secretion of IL-15, thus promoting NKT cell proliferation	[195]

ALE/AGE: Advanced lipoxidation and glycation end products; APAP: Acetaminophen; CDA A: Choline-deficient L-amino-acid; Con A: Concanavalin A; HSC: Hepatic stellate cells; IFN- $\gamma$ : Interferon  $\gamma$ ; IL: Interleukin; iNOS: Inducible isoform nitric oxide synthase; IRI: Ischemia and reperfusion injury; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; NK: Natural killer; NKT: NK T cells; TIM-3: T-cell immunoglobulin mucin domain 3; TNF: Tumor necrosis factor; HCC: Hepatocellular carcinoma; KO: Knockout.

risk of post-operative recurrence. Additionally, galectin-1 expression in HCC was also associated with early tumor recurrence ( $\leq 24$  mo) and dissemination of primary tumor cells. Furthermore, a positive correlation was observed between galectin-1 expression and tumor-infiltrating FoxP3<sup>+</sup> regulatory T cells (Tregs) in HCC samples from a large, random HCC cohort. In line with this evidence, it has been demonstrated that galectin-1 is a key regulator of murine CD4<sup>+</sup>CD25<sup>+</sup> regulatory Tregs<sup>[86]</sup> which play an essential role in suppression of anticancer immunity<sup>[87]</sup>. Taken this information into account it is possible to speculate that interaction between galectin-1 and Treg cells might play a

role in the suppression of antitumor immune responses against HCC (Figure 1 and Table 1).

The immunomodulatory activities of galectin-1 in the liver were also investigated in a model of hepatitis induced by injection of concanavalin A (Con A) into mice, which leads to a dose-dependent injury in the liver<sup>[88]</sup>. T-cell activation is a crucial event in this model as shown by resistance to this inflammatory disease of mice lacking T and B lymphocytes. Furthermore, pretreatment with anti-interferon  $\gamma$  (IFN- $\gamma$ ) or anti-tumor necrosis factor (TNF) monoclonal antibodies conferred protection against Con A-induced liver injury, indicating that Th1-

**Table 3** Galectins in fibrosis-related liver pathologies

Galectin Member	Expression	Function and/or effect	Model	Ref.
Galectin-1	Over-expressed in activated HSCs	Induces proliferation of HSCs <i>via</i> ERK 1/2 through CRD domain	HSCs activated <i>in vitro</i> (cultured on plastic for several days) and <i>in vivo</i> (isolated from rats treated with CCl <sub>4</sub> or with bile duct ligation)	[93,94]
	Positive in ICC cells, intracellular expression and secretion	Correlates with histologic dedifferentiation, vascular invasion, and lymph node metastasis	ICC tissue samples, CCKS1 cholangiocarcinoma cell line	[96]
Galectin-3	Over-expressed in activated HSCs	Induces proliferation <i>via</i> ERK 1/2 involving PKA and PKC pathways Dependent on CRD domain	HSCs activated <i>in vitro</i> (cultured on plastic for several days) and <i>in vivo</i> (isolated from rats treated with bile duct ligation)	[94]
		Intracellular Gal3 is required for activation of HSCs <i>via</i> TGF- $\beta$	HSCs activated <i>in vivo</i> (isolated from rats treated with CCl <sub>4</sub> )	[132]
		Extracellular Gal3 required for activation of HSCs. Integrin and CRD dependent effect	HSCs activated <i>in vivo</i> (isolated from rats with bile duct ligation)	[134]
		NF $\kappa$ B induces expression and secretion of Gal3 in activated HSCs		
	Up-regulated in injured/cirrhotic hepatocytes	Poor liver function	Human fibrotic liver samples and extracts from rats treated with CCl <sub>4</sub>	[124,133,136]
		Related to the preneoplastic and early neoplastic stages of ICC	ICC tissue samples	[96,137]
	Positive in ICC cells	Intracellular expression is associated with anti-apoptotic activity and resistance to chemotherapeutic agents	ICC cell lines	[138]

HSC: Hepatic stellate cells; CRD: Carbohydrate recognition domain; ERK: Extracellular signal-regulated kinase; ICC: Intrahepatic cholangiocarcinoma; PKA: Protein kinase A; PKC: Protein kinase C.

dependent cytokines are involved in this inflammatory disease. Interestingly, it has been demonstrated that galectin-1 exerts a protective role on Con A-induced autoimmune hepatitis in mice (Table 2)<sup>[89]</sup>.

Recently, the protective role of galectin-1 in the liver inflammatory response was investigated using the Mdr2-knockout (Mdr2-KO) mice as a model of inflammation-induced chronic cholestatic hepatitis at an early age, and HCC at a later age, which together mimic the evolution of human disease<sup>[90]</sup>. Potikha *et al.*<sup>[91]</sup> demonstrated that HCC development was retarded in Mdr2-KO/B6 strain compared to Mdr2-KO/FVB mice. Interestingly, up-regulation of galectin-1 transcript in the liver of Mdr2-KO/B6 mice was observed<sup>[91]</sup>. To highlight the relevance of the endogenous protein galectin-1-KO/B6 mice were used in the context of Con A-induced autoimmune hepatitis. The results demonstrated that endogenous galectin-1 selectively protects against Con A-induced liver injury in B6 mice (Table 2)<sup>[91]</sup>.

Collectively, these data indicated that galectin-1 has an important role in HCC tumor growth, aggressiveness and metastasis (Figure 1 and Table 1). Moreover, they suggest that galectin-1 may act as a protective anti-inflammatory agent at early stages of the chronic liver pathology during inflammation-induced hepatocarcinogenesis, but as a pro-tumorigenic agent at late stages of the disease.

### Galectin-1 in fibrosis-related liver pathologies

Hepatic fibrosis is the physiological result of the wound-healing response of the liver to repeated injury. This

process is associated with an inflammatory response and a limited deposition of ECM. If the hepatic injury persists (*e.g.*, chronic viral hepatitis), and eventually the liver regeneration fails, hepatocytes are substituted with abundant ECM, including fibrillar collagen<sup>[92]</sup>. Kristense *et al.*<sup>[93]</sup> conducted a proteome analysis on cellular and secreted proteins of normal (quiescent) and activated rat hepatic stellate cells (HSCs), the main ECM-producing liver cells. These researchers found that galectin-1 was up-regulated in both *in vivo* and *in vitro* activated HSCs, and in fibrotic liver tissues<sup>[93]</sup>. When the biological role of galectin-1 was investigated in HSCs, it was found that this lectin stimulated the proliferation rate and migratory activity of cultured HSCs through carbohydrate-dependent mechanisms (Table 3)<sup>[94]</sup>. These data clearly indicated that galectin-1 has an important role in the development of liver fibrosis.

By immunohistochemistry, galectin-1 expression was also assessed in the intrahepatic biliary tree. The intrahepatic biliary epithelial cells or cholangiocytes are involved in modifying the bile of canalicular origin. Cholangiocarcinoma occurs frequently associated with inflammation and fibrosis of bile ducts, and is caused by multiple factors including autoimmune, bacterial, congenital, drug, or viral agents<sup>[95]</sup>. In normal livers, Shimonishi *et al.*<sup>[96]</sup> observed that intrahepatic bile ducts and hepatocytes did not express galectin-1. Remarkably, 73 % of the intrahepatic cholangiocarcinoma (ICC) samples analyzed were positive for galectin-1<sup>[96]</sup>. Expression of this lectin significantly correlated with histologic dedifferentiation of ICC,



vascular invasion, and lymph node metastasis of ICC<sup>[96]</sup>. These results suggest that galectin-1 over-expression in ICC cells is associated with neoplastic progression and tumor cell proliferation (Table 3).

These results highlight an important role of galectin-1 in chronically injured liver and its involvement in inflammation and fibrosis of bile ducts, thus providing the basis for the development of effective therapies based on the modulation of galectin-1-glycan interactions.

## GALECTIN-3

Galectin-3 is the unique “chimera-type” galectin containing three structurally distinct domains, an atypical N-terminal domain that includes a serine phosphorylation site, important for the regulation of intracellular signaling, a collagen-like sequence sensitive to proteolysis by MMP-2 and MMP-9 matrix metalloproteinases and a C-terminus containing one carbohydrate-recognition domain (CRD) containing an Asp-Trp-Gly-Arg motif. This sequence motif is also present in members of the B-cell lymphoma 2 (Bcl-2) family of apoptosis regulators, and is responsible for the antiapoptotic activity of galectin-3<sup>[97]</sup>. In solution, galectin-3 largely occurs as a monomer<sup>[98]</sup>. Although in the absence of its binding partners it can form homodimers by self-association through its CRDs<sup>[99]</sup>, in the presence of carbohydrate ligands, galectin-3 can polymerize up to pentamers through its N-terminal domain<sup>[99,100]</sup>.

Galectin-3 is mainly localized at the cytoplasmic compartment, but it is also present within the nucleus, in the cell surface or in the extracellular space<sup>[29,101]</sup>. Translocation of this lectin from the cytoplasm to the nucleus is mediated by its N-terminal domain<sup>[102]</sup>, whilst translocation from nucleus to the cytoplasm involves a nuclear export sequence located within its CRD<sup>[103]</sup> and occurs through nucleoporin NP98<sup>[104]</sup>. Notably, the N-terminal domain is also required for the secretion of the lectin to the extracellular milieu<sup>[105]</sup>.

Galectin-3 has multiple and complex functions. In the cytoplasm, galectin-3 can bind to Bcl-2 and inhibit cellular apoptosis<sup>[97]</sup>. Also, it can interact with the activated K-Ras (K-Ras-GTP)<sup>[106,107]</sup> and affect Ras-mediated Akt signaling<sup>[108,109]</sup>. On the other hand, nuclear galectin-3 acts as a pre-mRNA splicing factor and is involved in spliceosome assembly<sup>[110]</sup> by forming protein complexes with Gemin4<sup>[111]</sup>. In the nucleus, Galectin-3 can also regulate gene transcription by enhancing transcription factor association with Spi1 and CRE elements in gene promoter sequences<sup>[29]</sup>. In addition,  $\beta$ -catenin, a molecule involved in Wnt signaling pathway, was also identified as a novel binding partner of galectin-3 in the nucleus<sup>[112]</sup>.

On the other hand, extracellular galectin-3 mediates cell adhesion and activation and also acts as a chemoattractant for certain cell types<sup>[29]</sup>. It often forms multimers and thus, it cross-links cell surface ligands forming lattice-like structures which trigger cell signaling<sup>[29]</sup>. Galectin-3 has been shown to bind glycosylated components of the extracellular matrix, and cell-surface adhesion molecules like integrins<sup>[43]</sup>. Pro-apoptotic activity of extracellular ga-

lectin-3 was observed in several cell types, such as human T leukemia cell lines, human peripheral blood mononuclear cells, and activated mouse T cells<sup>[113]</sup>.

Galectin-3 is widely expressed in human tissues, including immune cells, epithelial cells and sensory neurons (reviewed by<sup>[29]</sup>). This lectin regulates immune cell activities and contributes to immunosuppression as it induces monocyte and T-cell apoptosis, suppresses IL-15 production and inhibits B-cell differentiation<sup>[114,115]</sup>. In general, galectin-3 is a powerful pro-inflammatory signal as demonstrated by both *in vitro* and *in vivo* assays<sup>[29,116]</sup>. Extracellular galectin-3 has been demonstrated to activate and modulate the viability of immune and inflammatory cells, although the effects of Galectin-3 in T-cell survival are dependent on whether the protein is produced endogenously (anti-apoptotic) or is secreted to the extracellular medium (pro-apoptotic)<sup>[114,116]</sup>.

Expression of galectin-3 and its intracellular distribution are frequently altered in cancer and pre-cancerous conditions<sup>[26]</sup>, and it is evident that this lectin plays multiple roles in cancer pathogenesis, proliferation and spreading of metastasis<sup>[29,62,117]</sup>. Pre-clinical and clinical data indicate that expression of galectin-3 is associated with the carcinogenesis and malignant potential in melanoma, head and neck, thyroid, gastric, colon, uterine, and renal cancers<sup>[118]</sup>. In fact, galectin-3 contributes to tumorigenesis and tumor progression through several different mechanisms, including promotion of oncogenesis, angiogenesis, adhesion, invasion and metastasis<sup>[101,115]</sup>.

The mechanisms of regulation of galectin-3 expression are still poorly understood. The promoter region of the human galectin-3 gene (*LGALS3*) contains several regulatory elements for activation by the SP1, AP-1, CREB, and NF- $\kappa$ B transcription factors<sup>[119]</sup>. In this regard, c-Jun, CREB, and NF- $\kappa$ B have been implicated in activation of the *LGALS3* gene<sup>[29,49]</sup>. Galectin-3 expression is also regulated by methylation of CpG islands in the promoter region. It has been demonstrated that demethylation of *LGALS3* promoter induces expression of galectin-3 in thyroid carcinoma<sup>[120,121]</sup>. Recently, Margadant *et al.*<sup>[122]</sup> demonstrated that, in cells from epithelial origin, integrin  $\beta_1$  specifically triggers transcriptional activation of galectin-3 through a mechanism that involves demethylation of the *LGALS3* promoter. Further, it has been shown that the cell-surface glycoprotein MUC1 controls galectin-3 expression in an epigenetic manner in cancer cells, through a miRNA-dependent mechanism<sup>[123]</sup>.

## Galectin-3 in HCC

Hsu and colleagues demonstrated using immunohistochemistry and immunoblot analysis, that normal hepatocytes do not express galectin-3; however this galectin is prominently up-regulated in HCC tissues and in HCC cell lines<sup>[124]</sup>. Increased expression of galectin-3 in HCC was independent of whether the patients were previously exposed to hepatitis B virus (HBV). However, galectin-3 expression in HCC was positively influenced by HBV infection through a mechanism that included transactivation of the murine *LGALS3* gene promoter<sup>[124]</sup>.

Accordingly, using cDNA microarray and gene expression profiling, Chung *et al.*<sup>[79]</sup> reported the up-regulation of Galectin-3 in HCC human tissues with respect to their normal counterparts. Moreover, by analyzing gene expression patterns, Luo *et al.*<sup>[125]</sup> also reported the over-expression of galectin-3 gene in HCC tissues respect to normal liver and adjacent non-tumoral tissues.

Interestingly, expression of galectin-3 correlated with histological differentiation and vascular invasion in HCC patients<sup>[126]</sup>. In particular, higher expression rate of nuclear galectin-3 denoted worse prognosis in this pathology and serum galectin-3 levels were found to be increased in HCC patients compared to those suffering chronic liver disease<sup>[126]</sup>. These results highlighted a central role for galectin-3 in HCC development and progression (Figure 1 and Table 1).

HCC is a hypervascular tumor in which angiogenesis plays a critical role. Tumor-associated capillary endothelial cells (TECs) in HCC are known to originate from liver sinusoid endothelial cells (SECs), which then undergo a capillarization process to become morphologically and functionally different TECs<sup>[127]</sup>. Using two-dimensional gel electrophoresis coupled to mass spectrometry, Jia *et al.*<sup>[128]</sup> observed that galectin-3 is up-regulated in TECs, respect to SECs. This result validated by immunoblot and immunohistochemistry, demonstrated that galectin-3 is generally absent in liver SECs, but is significantly up-regulated in HCC TECs (Table 1)<sup>[128]</sup>. Further investigation is required to reveal whether galectin-3 produced in HCC TECs could influence HCC angiogenesis.

EGFR family is an important mediator of cancer cell transformation, proliferation, maintenance, and survival<sup>[129]</sup>. Paradoxically, high concentrations of epidermal growth factor (EGF) initiates different signaling cascades and mainly induces apoptosis of tumor cells expressing high levels of EGF receptor<sup>[130]</sup>. Recently, the role of galectin-3 in EGF-induced apoptosis on HepG2 cells was investigated<sup>[131]</sup>. Indeed, high concentrations of EGF inhibited proliferation and induced apoptosis of these cells, concomitantly with a reduced expression of galectin-3 at both mRNA and protein levels<sup>[131]</sup>. Also, high levels of EGF down-regulated the expression of cytoplasmic galectin-3. Remarkably, the reduced expression of galectin-3 in EGF-treated cells was associated with reduced phosphorylation of Akt and ERK. Moreover, over-expression of galectin-3 in HepG2 cells blocked EGF-induced growth inhibition and apoptosis<sup>[131]</sup>. Thus, cellular proliferation and/or apoptosis induced by EGF signaling pathway in HCC cells might rely on the expression levels of galectin-3.

Collectively, these results demonstrate that galectin-3 over-expression correlates with HCC progression (Figure 1 and Table 1) and suggest that this lectin could serve as a novel biomarker and therapeutic target in HCC.

### Galectin-3 in fibrosis-related liver pathologies

Expression of galectin-3 is increased in liver fibrosis regardless of the initiating agent or disease process<sup>[94,132]</sup>.

*In vitro* experiments and different experimental models of liver injury and fibrosis demonstrated that galectin-3 stimulated the proliferation rate of cultured activated HSCs and is also involved in myofibroblast activation, identifying galectin-3 as a potential therapeutic target in the treatment of liver fibrosis (Table 3)<sup>[94,132-134]</sup>.

Liver fibrosis leads to progressive liver insufficiency, portal hypertension and ultimately to cirrhosis and/or HCC<sup>[135]</sup>. In patients with liver cirrhosis galectin-3 is not extracted by the liver<sup>[136]</sup>, and also, its expression is induced in hepatocytes of cirrhotic liver<sup>[124,136]</sup>. Furthermore, galectin-3 was negatively associated with liver function in patients with alcoholic liver cirrhosis, an effect which might be partly explained by the impaired hepatic removal and/or by higher hepatic synthesis of galectin-3 (Table 3)<sup>[136]</sup>.

As mentioned before, cholangiocarcinoma frequently occurs in a context of inflammation and fibrosis of bile ducts. Shimonishi *et al.*<sup>[96]</sup> examined galectin-3 expression pattern in intrahepatic cholangiocarcinoma (ICC), and found that 93% of the ICC samples analyzed were positive for this lectin. The expression was more intense in well-differentiated ICC, and was significantly decreased in dedifferentiated areas or poorly differentiated ICCs, indicating that galectin-3 expression is rather related to the preneoplastic and early neoplastic stages of ICC, and tends to disappear at later stages of ICC (Table 3)<sup>[96,137]</sup>. Also, it has been demonstrated that galectin-3 played a role in apoptosis and response to chemotherapy in cholangiocarcinoma cell lines (Table 3)<sup>[138]</sup>. These results highlight the possibility of targeting galectin-3 as an alternative therapeutic approach in cholangiocarcinoma.

### Galectin-3 in inflammation-associated liver injury

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as a condition in which excess fat accumulates in hepatocytes. NASH, a severe form of NAFLD in which inflammation and fibrosis of the liver take place, may eventually progress to end-stage liver disease and ultimately, to HCC<sup>[139]</sup>. Controversial results have been published on the effect of galectin-3 deficiency in models of hepatic steatosis/inflammation, with studies indicating either protection or increased disease severity in galectin-3 knock-out (KO) mice (Table 2)<sup>[140-143]</sup>. On one hand, it has been demonstrated that in choline-deficient L-amino-acid (CDAA) diet-induced NAFLD/NASH hepatic injury was more severe in galectin-3 KO mice, as compared to wild type mice<sup>[142]</sup>.

On the other hand, Iacobini *et al.*<sup>[143]</sup> reported a complete prevention or marked attenuation of NASH induced by an atherogenic diet in galectin-3 KO mice. In these animals, the earlier steps of NASH, *e.g.*, steatosis, hepatocyte injury, and inflammation, were dramatically influenced<sup>[143]</sup>. Further research is needed to elucidate the protective or promoting roles of galectin-3 in liver steatosis and inflammation.

Excess fatty acid oxidation and generation of reactive carbonyls with formation of advanced lipoxidation and

glycation end products (ALEs and AGEs, respectively) are involved in NASH. Several AGE-binding proteins have been identified including galectin-3, which has been widely recognized as an AGE receptor (AGE-R3)<sup>[144]</sup>. Butscheid *et al.*<sup>[145]</sup> explored the expression of galectin-3 and RAGE, a member of the immunoglobulin superfamily which also serves as a receptor for AGEs, in specific cell types and histological structures of human liver biopsy specimens from patients with varying degrees of hepatic impairment (steatosis hepatitis, hepatitis, cholestasis and cirrhosis). They observed that when liver function is impaired and AGE levels rise, overexpression of galectin-3 appears to contribute to tissue protection (Table 2)<sup>[145]</sup>.

Acetaminophen (APAP)-induced hepatotoxicity is a major cause of acute liver failure<sup>[146]</sup>. Evidence suggests that activated macrophages contribute to the pathogenic response to APAP and, two major phenotypically distinct subpopulations have been identified: classically activated (M1-type) macrophages which show pro-inflammatory function and alternatively activated (M2) macrophages which often display anti-inflammatory wound repair activities<sup>[147]</sup>. It appears that the outcome of tissue injury depends on which macrophage subpopulation predominates. In wild type mice, galectin-3 is markedly up-regulated in macrophages infiltrating the liver 48-72 h after APAP administration<sup>[147]</sup>. Interestingly, loss of galectin-3 resulted in reduced hepatotoxicity and decreased expression of proinflammatory mediators<sup>[148]</sup>. Taken together, the data suggest that galectin-3 plays a key role in promoting late pro-inflammatory responses, classical macrophage activation and perpetuating injury in the liver following APAP intoxication (Table 2).

Supporting these findings, Volarevic *et al.*<sup>[149]</sup> showed that galectin-3 deficiency leads to a marked attenuation of Con A-induced hepatitis. This effect was associated with a decreased number of effector cells in the liver. Moreover, pretreatment of wild type mice with a selective inhibitor of galectin-3 (TD139) attenuated Con A-induced liver injury and reduced the number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Table 2)<sup>[149]</sup>. Hence, galectin-3 plays an important pro-inflammatory role in Con-A-induced hepatitis and may function as a potential target for therapeutic intervention in acute liver diseases.

## GALECTIN-4

Galectin-4 is a “tandem-repeat” galectin, which possesses two CRDs and is primarily expressed in epithelial cells along the gastrointestinal tract<sup>[150]</sup>. Recently, this lectin has been reported as a major component of lipid rafts in brush border membranes of small intestinal epithelial cells<sup>[151]</sup>. In a human colon adenocarcinoma cell line, galectin-4 has been proposed to play an important role in the apical delivery of proteins<sup>[152]</sup>.

Galectin-4 expression is altered in human malignancies<sup>[46,62,150]</sup>. Although controversial data has been published, it is apparent that galectin-4 is significantly down-regulated in colon adenocarcinoma. In fact, it has

been recently demonstrated that galectin-4 functions as a tumor suppressor in this type of malignancy<sup>[153]</sup>. In contrast, galectin-4 expression is higher in HCC<sup>[154]</sup> and gastric cancer cell<sup>[155]</sup>, as compared to their corresponding normal tissues, suggesting a context-dependent role of galectin-4 in tumor development and progression. Kondoh *et al.*<sup>[154]</sup> identified several cDNAs that were differentially expressed in surgically resected human HCC as compared to non-tumor liver and normal liver tissues<sup>[154]</sup>. Non-tumor liver tissues were obtained from patients that suffered cirrhosis associated with HCV infection and, from patients suffering liver cirrhosis but in the absence of HCV or HBV infection. Normal liver tissues that were used as controls were obtained from patients who died of pancreatic carcinoma and subarachnoid bleeding. Interestingly, one of the genes differentially expressed was the galectin-4 gene (*LGALS4*). Northern blot analysis revealed that galectin-4 mRNA was more abundant in HCCs than in adjacent non-tumor liver tissues or normal liver tissues from non-HCC patients<sup>[154]</sup>. When HCC cell lines were analyzed (HuH-7 and HepG2 cells), the levels of galectin-4 mRNA were undetectable or low in rapidly growing cells. However, the levels of this lectin increased considerably in HuH-7 cells growing at a higher cell density, although the expression of galectin-4 did not increase in HepG2 cells. Furthermore, the expression of galectin-4 mRNA was also induced in HuH-7 cells cultured with low concentration serum (0.1%)<sup>[154]</sup>. Thus, although the precise roles of galectin-4 in HCC remained to be elucidated, these results show a possible association between galectin-4 expression and liver malignancy. Functional studies will provide insight to further understand the role of galectin-4 in HCC biology.

## GALECTIN-8

Galectin-8 is another member of the “tandem-repeat”-type family of galectins, which possesses two CRDs and thus, behaves as a bivalent molecule. The galectin-8 gene (*LGALS8*) encodes numerous mRNAs (most likely seven) generated through alternative splicing, mostly in intron VIII<sup>[156]</sup>. Because the N-terminal domain of galectin-8 intrinsically dimerizes<sup>[157]</sup>, cleavage of the linker region between galectin-8N and galectin-8C may allow the possibility to dissect potential signaling pathways initiated by each separate domain<sup>[46]</sup>.

This lectin has been initially cloned from a rat liver cDNA library<sup>[158]</sup>. Using Northern analysis it was established that galectin-8 mRNA is highly expressed in lungs and, to a lesser extent in the liver, kidneys, spleen, hind-limb and cardiac muscles in the rat<sup>[158]</sup>. The role of galectin-8 has been mostly investigated in relation to tumor malignancy<sup>[62,156]</sup> in a variety of different tumors from different origin<sup>[62,159]</sup>. Immunohistochemical studies revealed that galectin-8 expression is increased in cancerous versus normal tissues in the lung, bladder, kidney, prostate and stomach. However, in the liver and also in large intestine, pancreas, larynx and skin, immunohistochemical analysis revealed decreased expression of this lectin in cancerous



versus normal tissues, suggesting tissue-specific regulation of galectin-8 expression in cancer<sup>[159]</sup>. In normal and cirrhotic livers, the staining intensities of galectin-8-positive cells appeared to be moderate to strong. On the contrary, in hepatoblastomas and hepatocarcinomas the staining intensity of positive cells was weak to moderate. Collectively, these experiments revealed tissue-specific regulation of galectin-8 expression upon malignant transformation of various tissue types of epithelial origin. Further investigation is necessary to further delineate the functional roles of galectin-8 in liver carcinogenesis and to determine if galectin-8 downregulation is associated with poor prognosis of HCC.

## GALECTIN-9

Galectin-9 is a “tandem-repeat” galectin originally isolated from mouse embryonic kidney cells<sup>[160]</sup>. Galectin-9 consists of two different CRDs joined by a flexible peptide linker, with 39% amino acid sequence homology. The C-terminal CRD and the N-terminal CRD share high affinity for both branched N-glycans and repeated oligo-lactosamines. Further, the N-CRD exhibits striking affinity for the Forssman pentasaccharide and polymerized *N*-acetylglucosamine<sup>[161,162]</sup>. Alternative splicing leads to the formation of three splice variants that vary only in the length of the peptide linker. The 35.9 kDa medium-sized isoform (galectin-9M) corresponds to authentic galectin-9 whereas the long and small-sized isoforms (galectin-9L and S) have a 32-amino acid insertion and a 12-amino acid deletion, respectively in the linker peptide<sup>[36]</sup>. The length of this region influences the rotational flexibility of the two CRDs in the space, impacting on galectin-9 valency<sup>[163]</sup>.

Human galectin-9 was first identified as a tumor antigen in Hodgkin's lymphoma, a condition characterized by abundant blood and tissue eosinophilia<sup>[164]</sup> and it is widely distributed within the immune system. This galectin is known to play a variety of cellular roles, including modulation of cell differentiation, adhesion, aggregation, and cell death<sup>[165]</sup>. Through modulation of cell signaling, this lectin can regulate multiple physiological and pathological processes such as immunity, inflammation, and cancer.

Galectin-9 has been identified as a ligand for the T-cell immunoglobulin mucin domain 3 (TIM-3), a membrane glycoprotein expressed on the surface of Th1, Th17 and cytotoxic T cells, as well as in natural killer (NK) cells, monocytes, dendritic cells, macrophages and mast cells (reviewed by<sup>[166]</sup>). The galectin-9/TIM-3 pathway plays a dual role in immunity. On one hand, it favors a pro-inflammatory response, induces maturation of monocyte-derived dendritic cells, and through this process, enhances Th1-type immune responses<sup>[167]</sup>. On the other hand, galectin-9 contributes to apoptosis of thymocytes and peripheral T cells, implicating a dual role of the Galectin-9/TIM-3 axis in both T-cell maturation and negative regulation of T-cell-mediated immune reactions<sup>[168,169]</sup>.

Blocking or activation of the Galectin-9/TIM-3 signaling pathway has been found to affect the evolution of many diseases, including autoimmune diseases, allergic disorders, graft rejection and anti-viral immunity (reviewed by<sup>[170]</sup>). Due to its potent roles in T cell suppression, galectin-9 has been considered as a therapeutic candidate for autoimmune and inflammatory diseases<sup>[167,171]</sup>.

Although most studies indicate that TIM-3 is involved in galectin-9 mediated signaling in T cells, multiple mechanisms and alternative receptors have been also proposed for this lectin<sup>[163,172,173]</sup>. More recently, a publication by Leitner *et al*<sup>[174]</sup> suggested that TIM-3 does not act as a receptor for galectin-9. These controversial results emphasize the involvement of distinct glycosylated receptors in galectin-9 effects.

In spite of considerable evidence indicating the role of galectin-9 in tumor biology and inflammation, the mechanisms governing expression of this protein are poorly understood. So far, IFN- $\gamma$  has been shown to induce galectin-9 expression in fibroblasts<sup>[175]</sup>, endothelial cells<sup>[176]</sup> and on Kupffer cell<sup>[177]</sup>. Additional modulators of galectin-9 include interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-5 (IL-5) in astrocytes<sup>[178]</sup> and eosinophils<sup>[179]</sup> respectively. Interestingly, decreased galectin-9 expression typically correlates with tumor progression and metastasis formation in various types of cancer<sup>[166]</sup>.

## Galectin-9 in HCC and in HCV/HBV infection-associated HCC

Galectin-9 has been identified as a possible prognostic marker in breast cancer, melanoma, and oral squamous cell carcinoma<sup>[180]</sup>. Most recently, Zhang *et al*<sup>[181]</sup> examined the relationship between galectin-9 expression and HCC, using an *in vitro* approach and immunohistochemistry on HCC tissues. The authors found that silencing galectin-9 expression in HepG2 HCC cells through siRNA-mediated strategies resulted in a weakened cell aggregation and increased proliferation and adhesion to ECM<sup>[181]</sup>. Also, galectin-9 suppression increased tumor cell-endothelial cell adhesion and trans-endothelial invasion of HepG2 cells. Additionally, downregulation of galectin-9 in human HCC tissue specimens represented a significant risk factor for patient survival and significantly correlated with the histopathologic grade of the tumor, lymph node metastasis, vascular invasion and intrahepatic metastasis<sup>[181]</sup>. These results emphasized an anti-metastatic role for galectin-9 in HCC (Figure 1 and Table 1).

T-cell responses are regulated by multiple mechanisms to maintain homeostasis and to prevent exuberant tissue inflammation and autoimmune disease. Whilst these regulatory mechanisms are critical to terminate excessive inflammatory responses, they can excessively constrain antiviral immunity in settings of persistent viral infection<sup>[182]</sup>. Galectin-9 is present at significantly higher levels in sera from patients infected with HCV or HBV compared to normal healthy controls<sup>[177,182,183]</sup>. Galectin-9 is expressed mainly in Kupffer cells<sup>[177,182]</sup>, but is also present in inflammatory leucocytes and hepatocytes<sup>[183]</sup>. Re-



cently, it has been reported that progression to persistent infection of HCV was accompanied by increased plasma levels of galectin-9<sup>[184]</sup>.

In patients chronically infected with HCV or HBV, multiple regulatory mechanisms act in concert to induce failure of the immune response and facilitate viral persistence. Interestingly, it has been demonstrated that galectin-9 plays a key role in limiting T-cell responses in the liver and facilitating the establishment of viral persistence. Galectin-9 induces the secretion of pro-inflammatory cytokines from monocytes and macrophages<sup>[177]</sup> that can further amplify immunopathology associated with HCV/ HBV infection. As a counter-effect, galectin-9 induces TIM-3-mediated apoptosis of effector T cells<sup>[177,182]</sup> and favors the expansion of Tregs<sup>[177,184,185]</sup> thereby attenuating adaptive immune responses.

Li *et al.*<sup>[186]</sup> studied the relevance of galectin-9 in patients with HBV-associated HCC. By flow cytometry analysis, the authors found that tumor cells and T cells expressed low amounts of galectin-9 while dendritic cells expressed moderate levels of this protein and Kupffer cells showed the highest expression in HBV-associated HCC tissues in comparison to non-tumor adjacent tissues<sup>[186]</sup>. The authors also observed that in HBV-positive patients the percentage of galectin-9<sup>+</sup> Kupffer cells was higher in tumor tissues than in normal adjacent tissues. However, in HBV-negative patients the expression of galectin-9 in Kupffer cells was negligible in both HCC and adjacent tissues. Interestingly, IFN- $\gamma$  derived from tumor-infiltrating T cells contributed to the increased galectin-9 expression in the HCC microenvironment<sup>[186]</sup>. In addition, high numbers of TIM-3<sup>+</sup> T cells were detected in HBV-associated HCC, which expressed senescence markers and exhibited decreased proliferative ability and impaired effector function when compared with TIM-3<sup>-</sup> T cells. Therefore, the TIM-3/galectin-9 signaling axis mediates T-cell dysfunction and predicts poor prognosis in patients with HBV-associated HCC<sup>[186]</sup>.

Although these data indicates a major role for galectin-9 in regulating liver immune responses, the observation that this galectin predominantly dampens immune function seems hard to reconcile with the poor outcome in patients with low galectin-9 expression. Possibly, galectin-9 expression is lost during the course of tumorigenesis, enabling tumor cells to metastasize more easily while alternatives modes of escape are being developed<sup>[180]</sup> (e.g., the up-regulation of galectin-1) (Figure 1 and Table 1). A better understanding of the mechanisms underlying galectin-9 functions is required to elucidate its possible role as a promising target in HCC.

### Galectin-9 in inflammation-related liver pathologies

The ischemia and reperfusion injury (IRI), an inflammatory event controlled by an exogenous antigen-independent insult that stimulates innate immunity, remains a critical problem in clinical organ transplantation. Liver IRI occurs frequently after major hepatic resection or liver transplantation. It has been demonstrated that

CD4<sup>+</sup> T cells are the key mediators of IRI-triggered liver inflammation<sup>[187]</sup>. Kupffer cells release pro-inflammatory mediators such as TNF and IL-6<sup>[188]</sup>, and CD4<sup>+</sup> T cells amplify Kupffer cell activity<sup>[189]</sup>. In this context, blockade of the TIM-3/galectin-9 pathway exacerbated local inflammation and liver damage (Table 2)<sup>[190]</sup>. These results suggest the importance of TIM-3/galectin-9 signaling in the maintenance of liver homeostasis and controlling dysregulated liver immune response, for example during IRI.

Similar results were observed in the murine model of liver injury, Con A-induced hepatitis, where T cell activation plays a crucial role. Blockade of TIM-3 using an anti-TIM-3 Ab resulted in more severe liver damage. On the contrary, biochemical and histopathological data indicated that a single injection of galectin-9 was sufficient to protect mice against Con A-induced hepatitis (Table 2)<sup>[191]</sup>.

Another progressive inflammatory liver disorder is autoimmune hepatitis (AIH), where a defective control of CD4<sup>+</sup> T cells takes place. Liberal *et al.*<sup>[192]</sup> showed that patients with AIH had reduced levels of TIM-3 and galectin-9 on effector CD4<sup>+</sup> T cells and Treg cells, respectively, as compared to healthy individuals<sup>[192]</sup>. Reduced signaling of the TIM-3/galectin-9 axis contributed to impaired control during AIH by rendering effector cells less prone to Treg cell control and Tregs less capable of suppressing effector responses.

A distinct subset of cells, referred as NKT cells has been characterized by the expression of a semi-invariant T cell receptor (TCR) and surface antigens typical of natural killer (NK) cells. These cells exhibit features of both cell types and act as a bridging system between innate and adaptive immunity<sup>[193]</sup>. NKT cells are particularly enriched within the liver and regulate immune responses through rapid secretion of large amounts of both Th1 and Th2 cytokines following stimulation<sup>[194]</sup>. The TIM-3/galectin-9 signaling pathway also plays a critical role in the homeostasis of hepatic NKT cells. It has been demonstrated that galectin-9 limits the inflammatory response in a mouse model of diet-induced nonalcoholic fatty liver disease (NAFLD) (Table 2)<sup>[195]</sup>.

In summary, these observations validated the relevance of the TIM-3/galectin-9 signaling axis in maintaining a balanced local immune microenvironment in the liver. Dysregulation of this axis can lead to a chronic inflammatory liver disorder which can eventually develop into an HCC.

## CONCLUSION

Because of their roles in tumor progression, galectins have evolved as promising targets for cancer therapy. A variety of studies revealed the involvement of this evolutionarily conserved protein family in murine and human cancers<sup>[26,52-56]</sup>. Modified citrus pectin, peptides, anti-galectin neutralizing antibodies and chemical inhibitors that antagonize galectins CRDs have been demonstrated the ability to reduce tumor volume, metastasis, angiogenesis,

potentiate immune responses and increase host survival in various tumor-type models<sup>[73,75,196-198]</sup>.

Current literature shows that the “proto-type” galectin-1, the “chimera” galectin-3 and “tandem-repeat” galectin-4 are increased in HCC cells compared to their normal counterparts. On the other hand, expression of “tandem-repeat” galectin-8 and galectin-9 is decreased in tumor hepatocytes. The aberrant expression (up- or down-regulation) of these galectins correlates with tumor growth, HCC adhesion, migration and invasion, tumor aggressiveness, metastasis, postoperative recurrence and poor prognosis (Figure 1 and Table 1). It is noteworthy that galectins also play key roles in other liver pathologies associated with chronic inflammation and fibrosis (Tables 2 and 3). Although research in this field is just beginning, the role for these galectins in HCC biology is substantiated by a wide range of accumulating evidence from animal models and human samples. Further functional studies are crucial to delineate the precise mechanisms by which galectins promote liver carcinogenesis, HCC progression, aggressiveness, inflammation and metastasis. Hopefully, in a near future, galectin-based therapies can be developed for the treatment of HCC, liver-associated fibrosis and liver chronic inflammatory disorders.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Cellular reprogramming and hepatocellular carcinoma development

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## Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers, and is also the leading cause of death worldwide. Studies have shown that cellular reprogramming contributes to chemotherapy and/or radiotherapy resistance and the recurrence of cancers. In this article, we summarize and discuss the latest findings in the area of cellular reprogramming in HCC. The aberrant expression of transcription factors OCT4, KLF4, SOX2, c-MYC, NANOG, and LIN28 have been also observed,

and the expression of these transcription factors is associated with unfavorable clinical outcomes in HCC. Studies indicate that cellular reprogramming may play a critical role in the occurrence and recurrence of HCC. Recent reports have shown that DNA methylation, miRNAs, tumor microenvironment, and signaling pathways can induce the expression of stemness transcription factors, which leads to cellular reprogramming in HCC. Furthermore, studies indicate that therapies based on cellular reprogramming could revolutionize HCC treatment. Finally, a novel therapeutic concept is discussed: reprogramming control therapy. A potential reprogramming control therapy method could be developed based on the reprogramming demonstrated in HCC studies and applied at two opposing levels: differentiation and reprogramming. Our increasing understanding and control of cellular programming should facilitate the exploitation of this novel therapeutic concept and its application in clinical HCC treatment, which may represent a promising strategy in the future that is not restricted to liver cancer.

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**Key words:** Reprogramming; Hepatocellular carcinomas; Cancer stem cells; Transcription factor; Therapeutics

**Core tip:** Cellular reprogramming contributes to chemoresistance and radioresistance and cancer recurrence in hepatocellular carcinoma (HCC). Recent findings on cellular reprogramming in HCC are summarized and discussed, including stemness transcription factors, DNA methylation, miRNAs, tumor microenvironments, and signaling pathways. The novel therapeutic concept of reprogramming control therapy is also described, which may be a promising strategy for HCC therapy in the future.

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## INTRODUCTION

Liver cancer is one of the most common tumors worldwide. An estimated 749000 new liver cancer cases and 695000 cancer deaths occurred worldwide in 2008<sup>[1]</sup>. Half of these cases and deaths were estimated to have occurred in sub-Saharan Africa and Southeast Asia. Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, which accounts for 70%-85% of the total liver cancer burden worldwide<sup>[2]</sup>.

Reports have shown that tumor recurrence<sup>[3]</sup> and patient survival<sup>[4,5]</sup> are correlated with HCC differentiation. Based on the Edmondson-Steiner's classification, HCC can be graded from I to IV: well-differentiated (grade I), moderately differentiated (grade II), poorly differentiated (grade III), and undifferentiated (grade IV) HCC<sup>[6]</sup>. The prognosis of poorly differentiated carcinoma is worse than that of well-differentiated carcinoma<sup>[4]</sup>, and the five-year survival of patients with poorly differentiated HCC is significantly worse than that of patients with moderately or well-differentiated HCC<sup>[7]</sup>. Ample evidence demonstrates that the poor prognosis and low five-year survival with poorly differentiated carcinoma are correlated with the expression of specific genes<sup>[4,8,9]</sup> and signal pathway activation<sup>[10,11]</sup>, which can increase the resistance to chemotherapeutic drugs and the frequency of HCC recurrence.

Evidence shows that aggressive poorly differentiated human cancers express high levels of embryonic stem cell-like genes, suggesting that reprogramming to a more dedifferentiated state occurs during tumor progression<sup>[12]</sup>. Moreover, if different reprogramming factors are activated, cancer cells can form well-differentiated and poorly differentiated sarcomas<sup>[13]</sup>. Poorly differentiated cancers have a higher content of prospectively isolated cancer stem cells than well-differentiated cancers<sup>[14]</sup>. These data support the view that cancer is a reprogramming-like disease and that cancer stem cells (CSC) may arise through a reprogramming-like mechanism before initiating tumor formation and progression in HCC. Therefore, understanding the role of cellular reprogramming may facilitate the development of new therapeutic strategies for HCC.

## CELLULAR REPROGRAMMING AND CANCER STEM CELLS

### Cancer stem cells

Classical tumor formation theory, *i.e.*, clonal evolution theory, suggests that each cell in a tumor is biological homogeneous<sup>[15]</sup>, whereas the alternative theory considers that the cells within a tumor are not identical, which is also known as tumor heterogeneity<sup>[16]</sup>. In the alternative

**Table 1 Expression of transcription factors in various cancer types**

Type of cancer	Transcription factors
Breast cancer	NANOG <sup>[22]</sup> , SOX2 <sup>[23]</sup> , OCT4 <sup>[24]</sup> and KLF4 <sup>[22]</sup>
Colorectal cancer	NANOG <sup>[25]</sup> , SOX2 <sup>[26]</sup> and OCT4 <sup>[26]</sup>
Gastric cancer	NANOG <sup>[27]</sup> , SOX2 <sup>[27]</sup> and OCT3/4 <sup>[27]</sup>
Hepatic cancer	NANOG <sup>[28]</sup> , SOX2 <sup>[29]</sup> , OCT4 <sup>[29]</sup> and KLF4 <sup>[30]</sup>
Lung cancer	NANOG <sup>[31]</sup> , SOX2 <sup>[32]</sup> and OCT4 <sup>[33]</sup>
Esophageal cancer	NANOG <sup>[34]</sup> , SOX2 <sup>[35]</sup> , OCT3/4 <sup>[35]</sup> and LIN28 <sup>[36]</sup>
Ovarian cancer	OCT4 <sup>[37]</sup> and LIN 28 <sup>[38]</sup>

theory, all of cell types can arise from a signal cell, known as a CSC, which has the potential for self-renewal and differentiation<sup>[17]</sup>. Ample evidence supports a major role for the CSC model in tumor heterogeneity. Lapidot *et al*<sup>[18]</sup> first demonstrated a critical role for CSC in human acute myeloid leukemia, where leukemic stem cells (LSC) initiated human acute myeloid leukemia after transplantation into SCID mice. The existence of LSC prompted further research into other types of cancer. CSC have recently been identified in several solid tumors, including breast, brain, colorectal, pancreas, liver, melanoma, and prostate cancers<sup>[19]</sup>. CSC possess the properties of normal stem cells, *i.e.*, self-renewal and differentiation. Self-renewal enables CSC to produce another CSC with essentially the same developmental and replication potential, which can increase the capacity for self-protection against drugs, toxins, and radiation. Differentiation involves the production of different types of cancer cells that trigger tumor initiation, maintain tumor growth, and finally form a bulk tumor.

### Cancer development

Studies have shown that reprogramming factors have specific expression signatures in human tumors (Table 1) and that the expression levels of these factors are correlated with the differentiation grades of tumor. Ben-Porath *et al*<sup>[12]</sup> found that poorly differentiated tumors preferentially overexpressed embryonic stem cell (ESC) genes. Moreover, the activation targets of reprogramming factors, such as NANOG, OCT4, SOX2 and *c-MYC*, are more frequently overexpressed in poorly differentiated tumors than well-differentiated tumors<sup>[12]</sup>. Chiou *et al*<sup>[20]</sup> reported that the expression levels of NANOG, OCT4 and CD133 were correlated with a poor survival prognosis in patients with oral squamous cell carcinoma. Reprogramming factors also play essential roles in maintaining the properties of CSC in tumors. Silencing the expression of Oct-4 in CD133<sup>+</sup> lung cancer can significantly inhibit the capacity for self-renewal, enhance CD133<sup>+</sup> cell differentiation into CD133<sup>-</sup> cells, and reverse the effects of chemotherapy or radiotherapy<sup>[21]</sup>. These data suggest that reprogramming factors play critical roles in the origin and development of CSC.

### Origin of CSC

Studies have shown that the occurrence of CSC is related

to cellular reprogramming, but the origin of CSC remains a conundrum. However, important new evidence has demonstrated that there are two possible routes for CSC emergence.

First, CSC may arise from normal stem cells (SC) that lose the ability to regulate proliferation. Kim *et al*<sup>[39]</sup> showed that SC are more readily reprogrammed into induced pluripotent stem cells (iPS) compared with somatic cells. *OCT4* and either *KLF4* or *c-MYC* are sufficient to generate iPS from neural SC<sup>[39]</sup>, which suggests that SC can be reprogrammed, and the process may be much easier than reprogramming mature cells. Riggi *et al*<sup>[40]</sup> successfully reprogrammed mesenchymal SC (MSC) into Ewing sarcoma cancer SC by inducing the expression of the ESC genes *OCT4*, *SOX2* and *NANOG* using the *EW5-FLI1* fusion gene. Chiba *et al*<sup>[41]</sup> reported that normal SC can be transformed into CSC after overexpressing the *BMI-1* gene, which had the potential for tumor formation.

The alternative theory hypothesizes that CSC may be reprogrammed from somatic cells, which acquire the capacities for self-renewal and tumor initiation after genetic lesions. After forcing the expression of exogenous OSKM (*OCT4*, *SOX2*, *KLF4*, *MYC*) in the human somatic fibroblast line TIG1, Nagata *et al*<sup>[42]</sup> isolated induced cancer SC (iCSC) from cell populations with the capacity for self-renewal. The lack of a functional RB1 can also trigger reprogramming, which generates cells with the properties of CSC from mouse fibroblasts<sup>[43]</sup>. Therefore, studies suggest that CSC can be reprogrammed from somatic cells. Moreover, the dedifferentiation of tumor cells may also lead to stemness property of cells. Recent studies suggest that tumor cells could also be a source of CSC. The expression of the reprogramming factors, *OCT4* and *NANOG*, was detected in poorly differentiated lung adenocarcinoma, whereas ectopic expression of *OCT4* and *NANOG* increased the proportion of the CD133-expressing subpopulation, sphere formation, and enhanced drug resistance in lung adenocarcinoma<sup>[44]</sup>. Similar results were also observed in melanoma and colon cancer<sup>[45,46]</sup>. For example, exogenous expression of the *OCT4* gene or the transmembrane delivery of *OCT4* protein promoted the dedifferentiation of melanoma cells into CSC-like cells by the induced expression of endogenous *OCT4*, *NANOG* and *KLF4*<sup>[45]</sup>. Su *et al*<sup>[46]</sup> showed that HT29/CD44<sup>+</sup> cells can be reprogrammed into CSC with significantly increased expression levels of *c-MYC*, *STAT3*, *SOX2* and *OCT4* by the CD44-SRC-integrin axis.

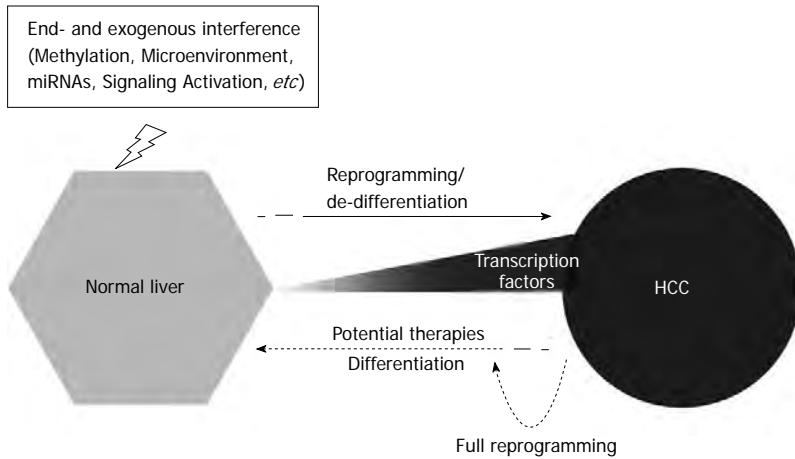
## CELLULAR REPROGRAMMING OF HCC

### Related factors

**Transcription factors:** Recently, it was demonstrated that forced expression of combinations of four transcription factors, *i.e.*, *OCT4*, *KLF4*, *SOX2*, and *c-MYC* or *OCT4*, *SOX2*, *NANOG* and *LIN28*, can reprogram somatic cells into iPS that closely resemble ESC<sup>[47-50]</sup>. In-

creasing evidence has demonstrated that aberrant expression of reprogramming factors may confer primitive and aggressive traits, which are associated with unfavorable clinical outcomes in HCC. *OCT4*, *NANOG* and *SOX2* have been detected in HCC cell lines and in tumor specimens from patients with HCC, and *Oct4* could play a significant role in activating the Wnt/ $\beta$ -catenin and transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathways<sup>[51]</sup>. Huang *et al*<sup>[29]</sup> demonstrated that *SOX2*- and *OCT4A*-positive expression were significantly associated with an aggressive phenotype in HCC. *SOX2* or *OCT4A* are independent prognostic factors for HCC, but the coexpression of *SOX2/OCT4A* has the poorest prognosis in HCC<sup>[29]</sup>. Increased expression of *Nanog* is also correlated with a poorer clinical outcome in HCC, whereas the overexpression of *NANOG* in *NANOG*<sup>+</sup> cells increases the capacity for self-renewal by the insulin-like growth factor receptor (IGF1R) signaling pathway in HCC<sup>[28]</sup>. Of interest, expression of the pluripotent transcription factor *KLF4* is decreased or lost in primary HCC<sup>[30]</sup>. The loss of *KLF4* expression is also significantly associated with poor survival in HCC<sup>[30]</sup>. Evidence suggests that *KLF4* is a putative tumor suppressor gene. The enforced restoration of *KLF4* expression markedly inhibits cell migration, invasion, and growth *in vitro*, and significantly attenuates tumor growth and metastasis in HCC animal models<sup>[30,52]</sup>. Reprogramming factors are expressed preferentially in hepatocellular carcinoma SC (HCSC). Expression levels of *CD44*, *OCT4* and *BMI1* were specifically upregulated in CD45<sup>+</sup>CD90<sup>+</sup> cells isolated from the tumor tissues and blood samples of patients with HCC compared with those in CD45<sup>+</sup>CD90<sup>+</sup> cells isolated from normal livers<sup>[53]</sup>. Ma *et al*<sup>[54]</sup> found that CD133<sup>+</sup> HCC cells expressed consistently higher mRNA levels of  $\beta$ -catenin, *OCT-3/4*, *BMI*, *SMO*, and *NOTCH-1* than CD133<sup>-</sup> HCC cells.

**DNA methylation:** Epigenetic studies have demonstrated that specific DNA methylation patterns, including global hypomethylation and promoter hypermethylation, may be early events in HCC<sup>[55]</sup>. A genome-wide DNA methylation microarray analysis showed that side population (SP) cells had a different DNA methylation status compared with non-SP cells in HCC<sup>[56]</sup>. Recent discoveries have shown that DNA methylation is an essential epigenetic mechanism during iPS reprogramming<sup>[57]</sup>. Demethylating agents and demethylase proteins may activate pluripotent gene promoters, thereby facilitating cellular reprogramming and ultimately enhancing the efficiency of iPS generation. Wang *et al*<sup>[58]</sup> found that chemoresistant cells exhibited increased expression levels of *OCT4* in HCC, whereas the expression of *OCT4* was regulated by DNA methylation. More recent reports have shown that the expression of *OCT4* is associated with the protein level of lipid storage droplet (LSD) in pluripotent cancer cells and human testicular seminoma tissues<sup>[59]</sup>. CD133 expression is also regulated by DNA methylation in HCC<sup>[60]</sup>. The elevated expression of CD133 is associated with the demethylation of *Line-1* in HCC<sup>[60]</sup>. More-



**Figure 1** The process of cellular reprogramming and potential therapies in hepatocellular carcinoma. The endogenous and exogenous interferences such as DNA methylation, microenvironment factors, microRNAs (miRNAs) and signaling activation (see text for details) could induce the reprogramming of hepatic cells and stem/progenitor cells, result in tumor initiation, an excess of self-renewal and chemo/radio-resistance, and form HCC. Conversely, the differentiation induction including demethylation, miRNAs, RNAi and signaling inhibition, will be the potential therapies for HCC. Additionally full reprogramming induction might offer us a novel way to treat HCC. The reprogramming approach would help to induce the partially reprogrammed cells to transform in full reprogrammed cells, like induced pluripotent stem cells, which can be induced to various types of differential somatic cells. HCC: Hepatocellular carcinoma.

over, TGF- $\beta$ -1 can inhibit the expression of DNA methyltransferases (DNMT)1 and DNMT3 $\beta$ , thereby leading to significant demethylation in the CD133 promoter-1 in CD133<sup>+</sup> Huh7 cells<sup>[61]</sup>. Studies of MSC have shown that methylation of the tumor suppressor genes, *HIC1* and *RASSF1A*, is sufficient to successfully reprogram the MSC into cancer stem/initiating cells<sup>[62]</sup>. These studies suggest that the demethylation of reprogramming factors and/or methylation of tumor suppressor genes contribute to reprogramming in HCC and to the origination of HCSC.

**MicroRNAs:** MicroRNAs (miRNAs) are well-characterized regulators of development and differentiation<sup>[63]</sup>. Studies have demonstrated that specific miRNAs have high expression levels in ESC and that they play a critical role in the control of pluripotency-related genes<sup>[64,65]</sup>. The clusters of miRNA-302s/367s<sup>[66]</sup> or miRNA-302s/369s/200c<sup>[67]</sup> can directly reprogram mouse and human somatic cells to pluripotency and increase the expression levels of OCT4 and SOX2. Studies have shown that miRNA-302 is a direct target of OCT4 and SOX2 in human ESC<sup>[68]</sup>, whereas miRNA-302 and OCT4/SOX2 may work as a positive feedback system in cellular reprogramming. Moreover, the reprogramming miRNA-302 is highly expressed in a rare subpopulation of glioma cell lines. miR-302 expression causes tumorsphere formation and significant upregulation of pluripotent genes<sup>[69]</sup>. Results indicate that miRNAs participate in the neoplastic transformation of HCSC in HCC. In total, 68 miRNAs have been found to be overexpressed, whereas 10 miRNAs were underexpressed in a SP of HCC cells compared with fetal liver cells<sup>[70]</sup>. miRNA can also regulate the expression of cancer SC markers in HCC. OCT4 was regulated by miRNA-145 in T3A-A3, which are CSC-like cells<sup>[71]</sup>, whereas miRNA-148 attenuated the expression of CD90 and CD44 in HCC<sup>[72]</sup>. miRNA-181 family members were highly expressed in (epithelial cell adhesion molecule<sup>+</sup> (EpCAM<sup>+</sup>AFP<sup>+</sup>) HCC cells, and the inhibition of miRNA-181 led to a reduction in the quantity of EpCAM<sup>+</sup> HCC cells and their tumor-initiating ability<sup>[73]</sup>. These reports suggest that miRNAs are potential factors in the reprogramming of HCC (Figure 1).

**Microenvironment:** Microenvironment plays a role in HCC, although its role during cellular reprogramming remains unclear. Hypoxia is a well-known characteristic of the tumor microenvironment, including HCC. In the emerging field of induced pluripotency, Yoshida *et al.*<sup>[74]</sup> have shown that hypoxia can significantly improve the generation of iPS colonies following reprogramming. Seven hypoxia-related prognostic genes, *i.e.*, *CCNG2*, *EGLN3*, *ERO1L*, *WDR45L*, *FGF21*, *MAT1A* and *RCL1*, which were dysregulated in HCC, were associated with chronic hypoxia, and were correlated with a poor prognosis in HCC<sup>[75]</sup>. *CCNG2*<sup>[76]</sup> and *EGLN3*<sup>[77]</sup> were upregulated in CSC, whereas *MAT1A* deficiency increases the expression of CD133<sup>+</sup> HCSC<sup>[78]</sup>. Mathieu *et al.*<sup>[79]</sup> showed that hypoxia by hypoxia-inducible factor (HIF) could induce a hESC-like transcriptional program, including induction of the reprogramming factors, *OCT4*, *NANOG*, *SOX2*, *KLF4*, *cMYC* and miRNA-302, in 11 cancer cell types, including HCC. Haraguchi *et al.*<sup>[80]</sup> reported that CD13 is a marker for semiquiescent CSC in human liver cancer cell lines, where the expression of CD13 is accompanied by the expression of carbonic anhydrase 9 (CA9), a hypoxia marker in HCC.

The tumor environment is always characterized by inflammation. Interleukin (IL)-6, an inflammatory cytokine, led to HCC from an IL-6-driven transformed SC with inactivated TGF- $\beta$  signaling<sup>[81]</sup>. Moreover, a subset of highly chemoresistant and invasive HSC were screened that had aberrant expression levels of cytokine IL-6 and TWIST. The secretion of IL-6 and TWIST can significantly increase the expression levels of let-7 and miR-181, which contribute to chemoresistance and cell invasion in HCC<sup>[82]</sup>.

Both of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are the major etiological agents of chronic liver disease and HCC. *In vitro* and *in vivo* studies have shown that OCT4, NANOG, KLF-4,  $\beta$ -catenin and (EpCAM) are activated by HBx, and the upregulated expression of multiple stem genes demonstrates that HBx contributes to hepatocarcinogenesis, at least partly, by promoting changes in gene expression, which are characteristics of CSC<sup>[83]</sup>. Moreover, HCV can also induce the cancer stem cell-like signatures in cell culture and mouse



model.

### Signaling pathways

Reprogramming is likely to induce drastic molecular changes that involve the upregulation of pluripotent genes and the repression of differentiation genes. Thus, signaling pathways have profound effects on the reprogramming of somatic cells into iPS<sup>[84]</sup>. A class comparison analysis showed that 793 genes were differentially expressed in hepatic stem cell-like HCC (HpSC-HCC) and mature hepatocyte-like HCC (MH-HCC)<sup>[85]</sup>. A pathway analysis indicated that differentially expressed genes were significantly associated with SC signaling pathways, including Wnt/ $\beta$ -catenin, TGF- $\beta$  and ERK/MAPK signaling<sup>[85]</sup>. These results suggest that signaling pathways have significant effects on cell reprogramming in HCC.

**Wnt/ $\beta$ -catenin:** It is well-known that Wnt/ $\beta$ -catenin signaling can control ESC self-renewal and the maintenance of stemness<sup>[86]</sup>, and it also regulates the expression of ESC genes<sup>[87]</sup>. Furthermore, it may contribute to the reprogramming of somatic cells in pluripotent cells<sup>[88]</sup>. Yamashita *et al.*<sup>[89]</sup> identified a novel prognostic HCC subtype based on EpCAM expression, which resembled hepatic progenitor cells with activated stem cell markers and Wnt/ $\beta$ -catenin signaling. The expression of EpCAM was associated with the activation of Wnt/ $\beta$ -catenin signaling<sup>[89]</sup>. Similar results were reported by Yang *et al.*<sup>[90]</sup> who found that OV6<sup>+</sup> cancer cells could endogenously activate Wnt/ $\beta$ -catenin signaling in HCC. Expression of OV6 increases after the activation of Wnt/ $\beta$ -catenin signaling, whereas inhibition of Wnt/ $\beta$ -catenin signaling leads to a decrease in the proportion of OV6<sup>+</sup> cells in HCC<sup>[90]</sup>. Moreover, the activation of Wnt/ $\beta$ -catenin signaling could be inhibited by silencing the expression of *OCT4*, with a reduction in *WNT-10b* and  $\beta$ -catenin and an increase in TCF3<sup>[51]</sup>. These results indicate that Wnt/ $\beta$ -catenin signaling may be an essential part of cellular reprogramming and the maintenance of stem-like characteristics in HCC.

**TGF- $\beta$ :** TGF- $\beta$  signaling pathway has been reported in many cellular processes in adult organisms and the developing embryo, including cell growth, differentiation, apoptosis, and homeostasis. Ichida *et al.*<sup>[91]</sup> demonstrated that TGF- $\beta$  signaling is involved with cellular reprogramming. The inhibition of TGF- $\beta$  signaling can promote the completion of reprogramming by the induction of Nanog<sup>[91]</sup>. Recent studies have shown that the TGF- $\beta$  signaling pathway can regulate cellular reprogramming in HCC. HCSC exhibit the unexpected loss of Transforming growth factor beta receptor II, which could lead to inactivation of the TGF- $\beta$  signaling pathway<sup>[81]</sup>. Toll-like receptor 4/NANOG-dependent tumor-initiating stem-like cells (TICs) were also detected with an inactivated TGF- $\beta$  signaling pathway. Restoration of the TGF- $\beta$  signaling pathway can inhibit the expression of pluripotent genes, including *NANOG*, *CD133*, *OCT4* and *SOX2*, as

well as tumorigenesis and abrogate the chemoresistance of TICs<sup>[92]</sup>.

### Mitogen-activated protein kinase/ERK kinase:

The mitogen-activated protein kinase/ERK kinase (MAPK/ERK) signaling pathway has been detected in mouse ESC<sup>[93]</sup>. During reprogramming, the inhibition of MAPK/ERK could promote the transformation of pre-IPS into ground state pluripotent SC, which are cells associated with inhibition of the glycogen synthase kinase-3 (GSK3) signaling pathway<sup>[94]</sup>. It has been reported that CD133<sup>+</sup> HCC exhibit a substantial increase in MAPK/ERK pathway activation<sup>[95,96]</sup> and that activation of the MAPK/ERK pathway can enhance proliferation, tumor angiogenesis, and initiate tumors in CD133<sup>+</sup> HCC. Moreover, MAPK inhibition using the MAPK kinase 1 (MEK1) inhibitor PD98059 leads to a significant increase in TGF- $\beta$ -induced apoptosis in CD133<sup>+</sup> HCC<sup>[97]</sup>.

In addition to these signaling pathways, the BMI-1 and Insulin-like growth factor-1 signal pathways also play key roles during cellular reprogramming in HCC. BMI-1 expression was highly correlated with the CSC phenotype in CD133<sup>+</sup> HCC cells, and a modification in BMI-1 expression resulted in a similar change in the maintenance of a CD133 subpopulation in HCC<sup>[98]</sup>. Insulin-like growth factor (IGF2) and IGF1R can be upregulated in NANOG<sup>+</sup> CSC, and a specific inhibitor of IGF1R signaling may significantly inhibit self-renewal and NANOG expression in HCSC, thereby indicating that IGF1R signaling participates in NANOG-mediated cellular reprogramming in HCC<sup>[28]</sup>.

## POTENTIAL THERAPIES BASED ON CELLULAR REPROGRAMMING

The detection and treatment of HCC have greatly improved with the advances in medicine; however, HCC remains largely incurable due to tumor recurrence. Conventional anticancer approaches, surgical resection, chemotherapy, and radiotherapy are primarily directed at bulk tumor populations. However, these strategies are frequently ineffective because of resistance to drugs and/or radiation<sup>[99]</sup>. Increasing evidence indicates that cellular reprogramming is involved with self-renewal, drug and/or radiation resistance, and tumorigenicity in HCC, and the concept of using precancerous cells and their progeny, CSC, in cancer therapy could provide unique insights into early cancer diagnosis, treatment, and preventive therapy<sup>[100]</sup>. Cellular reprogramming could also be a potentially useful therapeutic target in HCC.

### Inhibition of reprogramming

**Methylation:** Given the essential role of DNA methylation during cellular reprogramming in HCC, DNA methylation may be a therapeutic target in HCC. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that catalyzes the addition of methyl groups to H3K27, and the blocking of H3K27 methylation leads to a sig-

nificant reduction in TF-induced reprogramming<sup>[101]</sup>. 3-deazaneplanocin A, an S-adenosylhomocysteine hydrolyase inhibitor, is an efficient inhibitor of the function of EZH2, which reduces the levels of H3K27 me3 in HCC cells, thereby reducing the number of EpCAM<sup>+</sup> cells and the self-renewal capacity of these cells<sup>[102]</sup>. Lysine-specific histone demethylase 1 (LSD1) is a histone demethylase, and specific small bioactive inhibitors of LSD1 can enhance H3K4 methylation, derepress epigenetically suppressed genes, and inhibit the proliferation of pluripotent cancer cells, including teratocarcinoma, embryonic carcinoma, seminoma, and ESC<sup>[59]</sup>. All these studies suggest that methylation of histone 3 may be a potential target in HCC therapy.

**miRNA:** It is known that miRNAs are involved with the reprogramming of HCC and that they directly regulate the expression of reprogramming factors; however, miRNA can also act as a barrier during reprogramming. Evidence suggests that miRNA-34 is a reprogramming suppression miRNA, which can repress the expression of pluripotent genes, including *NANOG*, *SOX2* and *MYCN*<sup>[103]</sup>. The expression of pluripotent genes in HCC can also be downregulated by miRNAs. miRNA-145 can directly target OCT4 to arrest the cell cycle and inhibit the tumor growth of T3A-A3<sup>[71]</sup>. Moreover, miRNAs can regulate self-renewal, differentiation, and chemoresistance in HCSC. The inhibition of let-7 increases the chemosensitivity to sorafenib and doxorubicin by directly targeting SOCS-1 and Caspase-3, whereas silencing of miR-181 expression leads to a reduction in the motility and invasion by directly targeting RASSF1A, TIMP3, and nemo-like kinase in CD133<sup>+</sup> HCC<sup>[82]</sup>. Zhang *et al.*<sup>[104]</sup> demonstrated that overexpression of miR-150 downregulates c-Myb protein levels and leads to a significant reduction in CD133<sup>+</sup> cells, which is accompanied with significant inhibition of cell growth and tumorsphere formation. Ma *et al.*<sup>[105]</sup> reported that antagonizing miR-130b reduces the resistance to chemotherapeutic agents, leads in the loss of *in vivo* tumorigenicity, and inhibits self-renewal in CD133<sup>+</sup> TICs through TP53INP1 silencing.

### Silencing of transcription factors

Using chemotherapeutic drugs to select chemoresistant cancer cells in HCC, Wang *et al.*<sup>[58]</sup> showed that chemoresistant cells exhibit CSC features with dramatically increased Oct4 levels and a highly activated OCT4-TCL1-AKT-ABCG2 pathway. OCT4 knockdown and/or AKT pathway inhibition can reduce the resistance to chemotherapy both *in vitro* and *in vivo*<sup>[58]</sup>. Oikawa *et al.*<sup>[106]</sup> focused on Sal-like protein 4 (*SALL4*) and found that elevated expression of *SALL4* in tumors is associated with poor survival in HCC. The silencing of *SALL4* expression significantly inhibits *in vitro* and *in vivo* tumor growth with increased differentiation<sup>[106]</sup>. Yamashita *et al.*<sup>[85]</sup> suggested that RNAi-mediated knockdown of EpCAM can reduce self-renewal, tumorigenicity, migration, and drug resistance in HCC cells. Haraguchi *et al.*<sup>[80]</sup> demonstrated that CD13 could ROS-induced DNA damage after genotoxic

chemotherapy or radiation stress and protect cells from apoptosis. The combination of a CD13 inhibitor and the genotoxic chemotherapeutic agent fluorouracil (5-FU) drastically reduces the tumor volume in mouse xenograft models<sup>[80]</sup>.

### Regulating signaling pathways

Reports have shown that the abnormal activation and/or inhibition of signaling pathways in CSC, as well as the regulation of signal pathways, may be effective approaches to HCC therapy. Yamashita *et al.*<sup>[89]</sup> found that TCF/ $\beta$ -catenin binding inhibitors were much more sensitive to EpCAM<sup>+</sup> HCC than EpCAM<sup>-</sup> HCC, and they significantly inhibited the growth of EpCAM<sup>+</sup> HCC. CD133<sup>+</sup> HCC cells that survived chemotherapy had increased preferential expression levels of proteins involved with the AKT/PKB and BCL-2 pathways. AKT/PKB pathway-related cell survival proteins significantly reduce after treatment with an AKT1 inhibitor. Coincubation of an AKT1 inhibitor with DOX or 5-FU almost completely inhibits the preferential survival effect induced by CD133<sup>+</sup> cells in HCC<sup>[107]</sup>. HCSC also exhibit an inactivated TGF- $\beta$  signaling pathway<sup>[81]</sup>. A CD133<sup>+</sup> population demonstrated significant resistance to TGF- $\beta$  induced apoptosis compared with CD133<sup>-</sup> cells in HCC, whereas the MEK1 inhibitor PD98059 leads to a significant increase in TGF- $\beta$ -induced apoptosis in CD133<sup>+</sup> cells<sup>[97]</sup>.

### Differentiation induction

Given that the formation of tumors involves various cancer cells that differentiate from CSC, it is expected that CSC will become less malignant if forced to differentiate into mature cells. Tang *et al.*<sup>[81]</sup> demonstrated that IL-6 can drive the differentiation of HCC from hepatic stem/progenitor cells with inactivated TGF- $\beta$  signaling. Chow *et al.*<sup>[108]</sup> found that MYC-driven tumors contains a subset of cells (SP cells), which are characterized by Hoechst 33342 efflux. SP tumor cells exhibit markers of hepatic stem cells and chemoresistance, whereas chemoresistance is lost when SP tumor cells differentiate into non-SP tumor cells<sup>[108]</sup>. This suggests that the differentiation of hepatic CSC may be a possible therapeutic approach. Recently, Yamashita *et al.*<sup>[109]</sup> identified an oncostatin M (OSM) receptor in EpCAM<sup>+</sup> HCSC. OSM treatment induced hepatocytic differentiation in EpCAM<sup>+</sup> HCSC with a reduction of SC-related gene expression and an increase in albumin expression. Furthermore, a combined treatment with OSM and 5-FU eliminated HCSC and non-CSC subpopulations in an efficient manner<sup>[109]</sup>. A recent study showed that bone morphogenetic protein 4, a critical molecule in hepatogenesis and hepatic stem cell differentiation, can also promote differentiation and inhibit self-renewal in CD133<sup>+</sup> HCSC with a high exogenous dose<sup>[110]</sup>.

### Full reprogramming induction

iPS can be generated from normal tissues by the expression of defined transcription factors, as well as from malignant cells<sup>[111]</sup>. After transformation with four ectopic

reprogramming factors, *i.e.*, OCT4, KLF4, SOX2 and c-MYC, the chronic myeloid leukemia (CML) cell line KBM7 could be reprogrammed into iPS<sup>[112]</sup>. Moreover, Kumano *et al.*<sup>[113]</sup> induced iPS in samples isolated from patients with CML sensitive to imatinib. This report was the first example of the reprogramming of human primary cancer cells into iPS. In principle, CSC can also be reprogrammed into iPS using four or less reprogramming factors. Kim *et al.*<sup>[39]</sup> showed that iPS could be reprogrammed from adult neural SC using only two reprogramming factors. This indicates that the number of reprogramming factors could be reduced using somatic cells that express appropriate levels of complementary factors endogenously. Studies have shown that HCSC exhibit the endogenous expression of *SOX2*, *C-MYC*, *NANOG* and *OCT4*, and that these endogenous reprogramming factors could facilitate the reprogramming of CSC into iPS, which may reduce the recurrence of HCC.

## PERSPECTIVE

In this study, we reviewed the expression of transcription factors detected in HCC and summarized the complex mechanisms that contribute to cellular reprogramming in HCC, which then lead to the acquisition and maintenance of self-renewal and stemness features by a population of cancer cells, thereby resulting in the generation of HCSC. There are numerous potential applications of cellular reprogramming in regenerative medicine and cancer therapy. However, we showed that the knowledge obtained through studies of the molecular and cellular mechanisms that underlie reprogramming in HCC will also have deep implications for our understanding and the treatment of HCC, as well as other types of cancer. Furthermore, we also should refine the theory for application since the non-stem cell mediated, mature hepatocyte-derived HCC emerged in mice<sup>[114-116]</sup>.

Recognizing the role of cellular reprogramming in HCC suggests a novel therapeutic concept: reprogramming control therapy. Based on reprogramming in HCC studies, a possible reprogramming control therapy could be developed that targets two opposing: differentiation (or dereprogramming) and reprogramming (or dedifferentiation). The differentiation approach would focus on the differentiation of reprogrammed cells in HCC. Reprogrammed cells exhibit stem cell-like characteristics, including the expression of stemness genes and the activation of specific signaling pathways. Modifications of gene expression and/or signaling pathways could induce the reprogrammed cells to differentiate into mature somatic cells with impaired self-renewal and reversed chemoresistance and/or radioresistance. The reprogramming approach would help to induce the partially reprogrammed cells in HCC to transform in full reprogrammed cells, such as iPS, which can be redifferentiated into various types of mature cells. *In vitro* experiments and mice model studies have shown that these theoretical therapeutic approaches may have applications in future

HCC therapy. Increased knowledge and control of cellular programming could lead to the development of this novel therapeutic concept and its application in clinical HCC therapy, which may be a promising strategy in the future.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Anti-viral therapy to reduce recurrence and improve survival in hepatitis B virus-related hepatocellular carcinoma

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improved the survival rate of patients with hepatocellular carcinoma (HCC). However, hepatitis B virus (HBV)-related HCC has a much higher recurrence rate. In this article, we describe strategies for reducing recurrent HCC using anti-viral therapy for HBV infection.

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## Abstract

Hepatocellular carcinoma (HCC) is the most common malignancy and the third leading cause of cancer death worldwide. Chronic infection with hepatitis B virus (HBV) and hepatitis C virus accounts for approximately 75%-80% of HCC cases worldwide. In particular, chronic HBV infection is a predominant risk factor for HCC in Asia and Africa. Hepatic resection and radiofrequency ablation are increasingly used for the curative treatment of HCC, and good local control can be achieved. However, the high rate of recurrence is a major obstacle to improving prognosis. A high viral load of HBV DNA is the most important correctable risk factor for recurrence. Furthermore, interferon and/or nucleotide analogues may decrease HBV DNA. Therefore, these drugs may decrease recurrence. In this article, treatment strategies for HBV-related HCC are described in order to reduce recurrence and improve survival.

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**Key words:** Hepatocellular carcinoma; Hepatitis B virus; Recurrence; Nucleotide analogues; Interferon

**Core tip:** Recent advances in treatment modalities have

## INTRODUCTION

Hepatic resection or liver transplantation provide a complete curative treatment for hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. In addition, regional ablation therapy including radiofrequency ablation (RFA) is now increasingly used for the curative treatment of HCC, and good local control can be achieved<sup>[3-5]</sup>. However, these techniques are unsatisfactory due to a high post-treatment recurrence rate<sup>[6]</sup>. It was reported that up to 70% of patients relapse within 5 years after curative treatment<sup>[7]</sup>.

This high rate of recurrence is a major obstacle to improving prognosis. Therefore, antiviral and anti-inflammatory therapies both before and after curative treatment may be crucial in preventing HCC recurrence and improving survival. Current approved medications for chronic hepatitis B treatment are interferon- $\alpha$  (IFN $\alpha$ ) and nucleotide analogues (NAs), including lamivudine (LVD), entecavir (ETV), tenofovir disoproxil fumarate, adefovir-dipivoxil (ADV), and telbivudine<sup>[8]</sup>. However, despite curative treatment of HCC, the 5-year recurrence rate remains high, at 70%-80%<sup>[9]</sup>. The mechanisms of HCC recurrence differ greatly from those of other carcinomas in terms of the high rate of intrahepatic metastases and multicentric carcinogenesis against a background



of viral liver disease. Whether antiviral therapy after treatment of HCC can prevent recurrences is thus an important issue. Interferon (IFN) therapy in hepatitis C virus (HCV)-related HCC has been reported to reduce recurrence rates and contribute to survival, and its significance in preventing secondary carcinogenesis<sup>[10-14]</sup> including improvement of hepatic functional reserve<sup>[15]</sup> has been established.

The treatment of hepatitis B virus (HBV)-related HCC has centered on nucleic acid analogues to reduce viral load and inactivate hepatitis, however, treatment with IFN, similar to that in type C hepatitis, has recently attracted attention. Nucleic acid analogues and IFN may act together, but therapeutic strategies for preventing secondary carcinogenesis after treatment of HBV-related HCC remain unclear. This paper reviews the clinical evidence regarding treatment from the perspective of preventing secondary carcinogenesis, including reducing recurrence rates and improving prognosis after curative treatment of HBV-related HCC.

## ROLE AND MECHANISM OF HBV DNA LOAD IN RECURRENT HCC

The mechanism of hepatocarcinogenesis by HBV includes direct malignant transformation and other indirect effects. With regard to direct malignant transformation, HBV gene integration into the host hepatocyte genome causes changes in host gene expression and properties, facilitating hepatocarcinogenesis<sup>[16,17]</sup>.

Hence, as an indirect effect, persistent infection by HBV leads to hepatocyte destruction and regeneration, increasing genetic instability<sup>[18]</sup>. Epidemiological studies have examined differences in carcinogenesis due to HBV DNA load<sup>[19]</sup>, however, the mechanisms by which HBV DNA load causes differences in malignant transformation remain unclear.

HBV DNA load has been shown to play a role in carcinogenesis in patients with type B chronic liver disease, and more recently, HBV DNA load has also been reported to be involved in recurrence after curative treatment of HCC.

In a retrospective study of 72 patients with hepatic resection for HBV-related HCC, Hung *et al*<sup>[20]</sup> reported that patients with a high serum HBV DNA load at the time of tumor resection showed a significantly higher recurrence rate, compared to patients with a low viral load. Multivariate analysis showed that a high HBV DNA load, alpha-fetoprotein level, tumor size, and age were factors contributing to recurrence. Xia *et al*<sup>[21]</sup> reported that high serum hyaluronic acid and HBV viral load are the main prognostic factors of local recurrence after complete radiofrequency ablation of hepatitis B-related small HCC.

Because HBV DNA load changes with the administration of antiviral drugs, patients with a high viral load at the time of HCC treatment who receive antiviral drugs subsequently show differences in HBV DNA load com-

pared to those who do not receive such treatment. Kim *et al*<sup>[22]</sup> analyzed the patients excluded from antiviral drug therapy. After the patients treated with antiviral drugs were excluded, recurrence-free survival rates in a total of 157 patients with HBV-related HCC who underwent hepatic resection were compared between 89 patients with a persistently low HBV DNA load and 68 patients with a persistently high viral load. Recurrence-free survival rates were better in the persistently low HBV DNA load group compared to the high level group.

## MECHANISM OF ANTIVIRAL DRUGS IN PREVENTING RECURRENT HCC

### NAs preparations

The direct antitumor activity of nucleotide analogues has not been reported. Lamivudine has no inhibitory effects on integrated HBV DNA, thus there is no suppressive effect on de novo carcinogenesis due to HBV gene integration into the host genome<sup>[23,24]</sup>.

Considering that HBV DNA load is related to HCC recurrence, the prevention of recurrence by antiviral drugs, rather than direct antitumor effects, is due to a reduction in HBV DNA load which improves hepatocyte destruction and regeneration and reduces genetic instability, thus decreasing HCC recurrence rates. Hosaka *et al*<sup>[25]</sup> reported that HBV core-related antigen levels were independent risk factors for HCC recurrence. In addition, Chuma *et al*<sup>[26]</sup> reported that recurrence was significantly lower in patients who received lamivudine before the development of HCC.

In a retrospective study by Kubo *et al*<sup>[27]</sup> of 24 patients with HBV-related HCC who underwent liver resection, a difference in recurrence-free survival rates was seen between 14 patients who received lamivudine and 10 patients who did not. Multivariate analysis also showed that lack of antiviral therapy and multiple tumors were factors related to recurrence-free survival rates.

In another retrospective study of 49 patients who underwent curative treatment for HBV-related HCC (liver resection, 31 patients; RFA, 18 patients), Kuzuya *et al*<sup>[28]</sup> examined cumulative recurrence rates of HCC in 16 patients who received lamivudine and 33 patients who did not. There was no significant difference between the two groups. Although there was no significant difference in HCC recurrence rates, hepatic functional reserve was improved and survival was better in the lamivudine group. In the lamivudine group, hepatic functional reserve was significantly better at the time of HCC recurrence, a higher percentage of patients were able to undergo curative treatment, and prognosis tended to be better (Table 1).

Other studies<sup>[29,30]</sup> have reported significantly larger remnant liver volume and better prognosis after liver resection in lamivudine-treated groups, and that lamivudine improves liver function and reduces deaths due to liver failure. Lamivudine after treatment of HCC may not prevent cancer recurrence, but may contribute to an improved prognosis by maintaining hepatic functional

**Table 1 Studies in which Nucleoside analogues were administered after treatment for hepatitis B virus-related hepatocellular carcinoma**

Authors	Treated vs Untreated	Treatment	Observation time	HCC Tx	Recurrence	Survival
Kubo <i>et al</i> <sup>[27]</sup>	14 vs 10	LVD	1117 d (median)	Ope	NA	Tumor-free survival (P = 0.0086)
Kuzuya <i>et al</i> <sup>[28]</sup>	16 vs 33	LVD	38.0 mo vs 32.6 mo (median)	Ope/RFA	NS (P = 0.622)	NS (P = 0.623)
Li <i>et al</i> <sup>[29]</sup>	43 vs 36	LVD with/without ADV	12 mo	Ope	NS (P = 0.077)	Overall survival (P = 0.0094)
Piao <i>et al</i> <sup>[30]</sup>	30 vs 40	LVD	24 mo	Ope/RFA	NS	NS (P = 0.12)
Wu <i>et al</i> <sup>[31]</sup>	518 vs 4051	LVD/ETV/Telbivudine	2.64 yr	Ope	P < 0.001	P < 0.001

HCC: Hepatocellular carcinoma; LVD: Lamivudine; ETV: Entecavir; ADV: Adefovir-dipivoxil; NS: Not significant; NA: Not analyzed; Tx: Treatment.

**Table 2 Studies on the effects of interferon on hepatitis B virus-related hepatocellular carcinoma after treatment**

Authors	Treated vs Untreated	Treatment	Observation time	HCC Tx	Recurrence	Survival
Someya <i>et al</i> <sup>[38]</sup>	11 vs 69	IFN $\alpha$	16 yr	Ope/RFA	P = 0.013 (High AST group)	NA
Lai <i>et al</i> <sup>[39]</sup>	35 vs 36	IFN $\alpha$	30 mo	Inoperable	P = 0.001 (Tumor regression)	P = 0.047
Lo <i>et al</i> <sup>[40]</sup>	40 vs 40	IFN $\alpha$	60 mo	Ope (Stage III / IVA)	P = 0.031	NS (P = 0.311)
Sun <i>et al</i> <sup>[41]</sup>	118 vs 118	IFN $\alpha$	36.5 mo (median)	Ope	P = 0.048	P = 0.0003
Chen <i>et al</i> <sup>[42]</sup>	106 vs 109	IFN $\alpha$	63.8 mo (median)	Ope	NS (P = 0.766)	NS (P = 0.826)

HCC: Hepatocellular carcinoma; IFN: Interferon; NS: Not significant; NA: Not analyzed; Tx: Treatment.

reserve<sup>[28]</sup>. Wu *et al*<sup>[31]</sup> recently reported that NAs were important in preventing recurrences after liver resection (Table 1).

At present, opinion is divided regarding whether administration of NAs after HCC treatment prevents HCC recurrence<sup>[32]</sup>. However, NAs may improve prognosis by improving hepatic functional reserve. NAs treatment was able to improve survival post-HCC treatment compared with no NAs therapy<sup>[33]</sup>. Recently, ETV therapy was found to be more effective with a rapid reduction in viral load compared with LVD. ETV is safe and well-tolerated during long-term treatment<sup>[34]</sup>. Furthermore, ETV has a higher genetic barrier to resistance<sup>[35]</sup>. ETV treatment might have potent protective effects against recurrence of HCC.

## EFFECTS OF IFN IN PREVENTING RECURRENCE AFTER CURATIVE TREATMENT FOR HCC

Basic research has shown that IFN has antiviral effects, antitumor effects against HCC<sup>[36,37]</sup>, and inhibits the proliferation of cancer cells. In a retrospective study by Someya *et al*<sup>[38]</sup> evaluating IFN therapy in patients after curative treatment for HCC who also had HBV-related cirrhosis, uni- and multivariate analysis showed that IFN prevented recurrences, especially in the group with high aspartate transaminase. In addition, in a randomized controlled trial (RCT) of high-dose IFN in patients with HCC who could not undergo surgery, the IFN-treated group showed a significantly higher rate of  $\geq 50\%$  tumor size reduction compared to the control group<sup>[39]</sup>

(Table 2).

Furthermore, RCTs have been conducted to investigate the effects of IFN in preventing recurrences in patients after treatment for HCC. Lo *et al*<sup>[40]</sup> conducted a RCT in 40 patients with HBV-related HCC after curative hepatic resection. They compared a group treated with IFN- $\alpha$ 2b 10 MU/m<sup>2</sup>, three times weekly, for 12 wk and a non-treated control group. The 1- and 5-year survival rates in the IFN group were 97% and 79%, respectively, compared to 85% and 61% in the control group (P = 0.137). Multivariate analysis showed that IFN therapy may lower the risk of death. In a subgroup analysis, the 5-year survival rate in stage I / II patients did not differ between the IFN and control groups, but with IFN therapy in stage III/IVA patients, early recurrence of HCC was prevented, and the 5-year survival rate improved from 24% to 68% (P = 0.038). Sun *et al*<sup>[41]</sup> also compared an IFN group and control group after HCC surgery in a randomized study. IFN therapy was reported to be useful, with significant increases both in median overall survival and median disease-free survival times. However, the results of a recent phase III randomized study of IFN- $\alpha$ 2b after curative resection for HBV- and HCV-related HCC conducted in Taiwan showed no prevention of HBV or HCV recurrence<sup>[42]</sup> (Table 2).

Therefore, the effects of IFN therapy after curative treatment of HCC remain unclear. Pegylated (PEG)-IFN has superseded conventional IFN due to a higher response rate and once weekly administration instead of daily or three times a week. Recently, it was reported that high levels of hepatitis B surface antigen (HBsAg) increase HCC development among hepatitis B envelope an-

tigen (HBeAg)-negative patients with a low viral load<sup>[43]</sup>. A recent study clearly showed that the rates of HBsAg clearance after PEG-IFN treatment are substantial and durable in HBeAg-negative patients. Rates of HBsAg clearance were shown to increase further during long-term follow-up, with 12% of patients achieving HBsAg clearance at 5 years post-treatment<sup>[44]</sup>. Better results are anticipated in the future using PEG-IFN.

## CONCLUSION

Patients with high HBV DNA levels at HCC onset show significantly higher HCC recurrence rates compared to patients with low HBV DNA levels. In patients with high HBV DNA levels, the administration of antiviral drugs relatively early during treatment is recommended to prevent HCC recurrences. However, to more accurately evaluate the effects of antiviral therapy in preventing HCC recurrence, large-scale studies in more patients should be conducted.

Persistent viral suppression by antiviral therapy can inhibit carcinogenesis. Treatment with PEG-IFN results in a higher virological therapeutic response compared with conventional IFN. In addition, ETV, which has become a drug of first choice instead of LVD, has a very low resistance mutation rate, thus long-term viral suppression is possible. The long-term therapeutic effects of PEG-IFN and ETV are currently uncertain, but equal or better efficacy than conventional IFN or lamivudine for the prevention of carcinogenesis is expected. Future research should be aimed at clarifying the effects of antiviral therapy in HBV-related HCC.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Risk prediction of hepatitis B virus-related hepatocellular carcinoma in the era of antiviral therapy

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## Abstract

Hepatocellular carcinoma (HCC) is a grave primary liver cancer that has a limited therapeutic option because it is generally diagnosed later in an advanced stage due to its aggressive biologic behavior. The early detection of HCC has a great impact on the treatment efficacy and survival of patients at high risk for cancer. Potential host, environmental, and virus-related risk factors have been introduced. Hepatitis B virus (HBV) is a major cause of end-stage liver diseases such as liver cirrhosis or HCC in endemic areas, and its serologic or virologic status is considered an important risk factor. HCC risk prediction derived from the identification of major risk factors is necessary for providing adequate screening/surveillance strategies to high-risk individuals. Several risk prediction models for HBV-related HCC have been presented recently with simple, efficient, and readily available to use parameters applicable to average- or unknown-risk populations as well as high-risk individuals. Predictive scoring systems of risk estimation to assess HCC development can provide the way to an evidence-based clinical approach for cost- and effort-

effective outcomes, capable of inducing a personalized surveillance program according to risk stratification. In this review, the concepts and perspectives of the risk prediction of HCC are discussed through the analysis of several risk prediction models of HBV-related HCC.

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**Key words:** Hepatocellular carcinoma; Hepatitis B virus; Chronic hepatitis B; Risk prediction; Risk factors

**Core tip:** This review shows the concepts and perspectives of the risk prediction of hepatitis B virus-related hepatocellular carcinoma. Accurate risk scoring systems to predict hepatocellular carcinoma (HCC) development, derived from independent risk factors integrated in aspects of host, environment, and virus, are necessary for performing the strategic processes such as screening/surveillance, diagnosis, and treatment in high-risk individuals of HCC. Globally standardized consensus for HCC risk prediction models should be established on the basis of simplicity, assessability, and reproducibility of the model characteristics available in real clinical setting.

Song IH, Kim SM, Choo YK. Risk prediction of hepatitis B virus-related hepatocellular carcinoma in the era of antiviral therapy. *World J Gastroenterol* 2013; 19(47): 8867-8872. Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/8867.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.8867>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most aggressive malignant neoplasms and a leading cause of cancer-related morbidity and mortality<sup>[1]</sup>. During the last

few decades, the incidence rate of HCC has increased in most developed countries, but its mortality rate has decreased<sup>[2]</sup>. Tumor diagnosis at an advanced stage and accompanying chronic liver diseases, including liver cirrhosis, are major limitations of curative management in many cases. The accurate selection of high-risk groups and adequate screening/surveillance programs for HCC detection at an early stage may provide clinical strategies capable of overcoming “tumor diagnosis at an advanced stage”<sup>[3]</sup>. The early detection of HCC in populations and individuals at high risk is critical in providing curative treatments and in consequently acquiring a survival benefit, which has been validated through a randomized controlled trial of screening for HCC<sup>[4]</sup>.

The hepatitis B virus (HBV) genome consists of partially double-stranded DNA of approximately 3200 base pairs with four overlapping open reading frames encoding the envelope (S), core (C), polymerase (P), and X proteins (Figure 1).

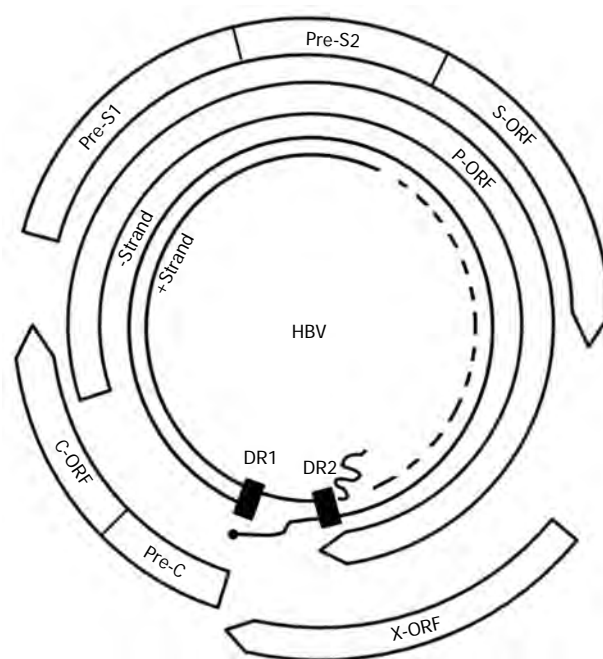
Chronic HBV infection is usually characterized by the presence of hepatitis B surface antigen (HBsAg) in the serum for at least 6 mo after exposure to the virus. Patients with chronic HBV infection have a more than 100-fold increased risk of HCC occurrence compared with uninfected individuals<sup>[5]</sup>. Therefore, HBV-infected patients have been considered a high-risk group of HCC and regarded as candidates for a precise application of screening/surveillance strategies scheduled by using risk weight-based stratification. In addition to the possession of HBsAg itself, the following HBV-associated biomarkers affecting liver disease progression to cirrhosis and HCC have been suggested: serum titer of HBsAg, hepatitis B e antigen (HBeAg), serum level of HBV DNA, HBV genotype, and HBV mutations<sup>[6]</sup>. In recent years, the evolution of antiviral therapeutics for chronic HBV infection is a result of the clinical efforts to reduce the development of HCC.

In this article, we discuss the host, environmental, and virus-related risk factors associated with the development of HBV-related HCC and present the risk prediction systems for the development of HCC based on stratification of scoring estimation derived from independent risk factors.

## RISK FACTORS

### Host factors

The following potential host factors for HCC occurrence in HBV-infected individuals have been suggested based on demographic, clinical, and epidemiologic investigations: male gender, increasing age, genetic susceptibility and family history of HCC, obesity, diabetes, coexistent alcohol consumption or smoking, high serum alanine aminotransferase (ALT) activity, low serum albumin, low platelet counts, high serum alpha-fetoprotein level, and accompanying liver cirrhosis<sup>[6]</sup>. In relation to these risk factors, several study groups have strongly recommended HCC surveillance strategies in men > 40 years old and



**Figure 1** Representative scheme of the hepatitis B virus genome. Hepatitis B virus (HBV) genome consists of partially double-stranded DNA with four overlapping open reading frames. ORF: Open reading frame; DR: Direct repeat.

women > 50 years old with chronic hepatitis B who have a family history of HCC and accompanying cirrhosis, considering different ethnicities<sup>[3,7-10]</sup>. Liver cirrhosis, irrespective of etiology, is the most important and independent risk factor for the development of HCC, accounting for 73% to 85% of patients with HCC in HBV-endemic areas<sup>[2]</sup>. Among the biochemical risk factors, ALT is the most readily available in clinical fields, and its serum level above the upper limit of normal (ULN) is considered an independent risk factor for HCC even in average-risk populations without HBsAg, to say nothing of high-risk subjects seropositive for HBsAg. Subjects in the upper range ( $0.5-1 \times \text{ULN}$  or approximately 25-40 IU/L) of the normal limit of serum ALT levels were reported to be at an increased risk of HCC compared with subjects in the lower range ( $< 0.5 \times \text{ULN}$  or  $< 25 \text{ IU/L}$ )<sup>[11,12]</sup>. The risk factors mentioned previously are simple to measure, easy to administer, and convenient to apply clinical parameters useful for constructing risk prediction models of HCC.

### Environmental factors

Environmental risk factors are difficult to define clearly in clinical settings. Ethnic susceptibility, alcohol consumption, cigarette smoking, co-infection with other viruses, and chemical carcinogens including aflatoxin were representative of environmental factors. Ethnicity is considered a relative risk factor for HCC. Africans, African Americans, and Asians are included in populations for HCC surveillance in HBV-endemic regions<sup>[3,7-9]</sup>. In these regions, virus infection mainly occurs through perinatal transmission vertically or horizontally<sup>[13]</sup>, resulting in sus-

ceptibility to disease chronicity and relative intractability to antiviral therapy because of long-standing periods of infection. Simultaneous co-infections by other viruses such as hepatitis C virus, hepatitis D virus, and human immunodeficiency virus may be additional risk factors, but they have not been established definitely. Aflatoxin is well known to be a carcinogen capable of developing HCC. Aflatoxin B1 is a representative genotoxic hepatocarcinogen that induces the transversion of guanine (G) to thymine (T) in codon 249 of exon 7 of the *p53* tumor suppressor gene in human hepatocytes (the so-called stop-codon mutation), resulting in the substitution of arginine (A) to serine (S). Mutations of *ras* oncogenes are also found in aflatoxin B1-induced HCC, but are less frequent than the *p53* mutation<sup>[14]</sup>. These environmental factors are not usually involved in constructing risk prediction models of HCC because of the lack of quantitative assessment as independent risk factors.

### Virologic factors

Virologic risk factors have been considerably investigated in cases with hepatitis virus-associated HCC. The clinical implications of the serum HBV DNA level for liver disease progression are recognized in HBV-infected patients. A stepwise increase of the serum HBV DNA level is associated with a corresponding linear increase in the cumulative incidence of HCC as well as the progression of HBV-related liver disease to liver cirrhosis or hepatic decompensation regardless of the serum ALT activity, HBeAg status, and presence of cirrhosis<sup>[15,16]</sup>. Therefore, the serum HBV DNA level is a major independent virologic risk factor. Furthermore, inactive carriers with chronic HBV infection, who are seronegative for HBeAg have serum levels of HBV DNA less than 4 log copies/mL and serum ALT activity within the normal limit and do not have chronic hepatitis, cirrhosis, or HCC either histologically or clinically, are at risk for HCC and liver-related death compared with individuals not infected with HBV<sup>[17]</sup>. Hepatitis B viral load has been reported to be a risk factor for post-treatment recurrence of HCC<sup>[18]</sup>. In these backgrounds, antiviral therapy with nucleoside/nucleotide analogs in patients with HBV-associated liver disease is a main pivot to control HCC development and recurrence. In fact, lamivudine therapy has reduced the incidence of HCC in patients with compensated cirrhosis when viral suppression was sustained<sup>[19]</sup>. Recently, the quantitative assessment of serum HBsAg has been suggested as a new tool for determining HCC development and for predicting the response to antiviral therapy<sup>[20,22]</sup>. However, circulating HBsAg in blood, a component of the HBV envelope proteins, is originated from non-infectious viral particles as well as intact Dane particles with viral infectivity; the clinical impact of the serum HBsAg level on HCC development and the antiviral response in patients with chronic HBV infection should be ascertained prospectively. On the other hand, the serum level of HBsAg, like the HBV DNA level, may fluctuate in the natural course of chronic HBV infection<sup>[23,24]</sup>, and

changing patterns of the serum HBsAg level through long-term regular monitoring might determine whether the changes could affect the disease progression in HBV-infected patients. Besides these virologic factors, HBeAg/anti-HBe status, HBV genotype, basal core promoter mutations/precure mutations or mutations relevant to deletions within pre-S region, and co-infection with other viruses can be considered risk factors for HCC<sup>[25-28]</sup>. Among these virologic risk factors, the serum HBV DNA level and HBeAg status are the most valuable and available parameters capable of constructing risk prediction models of HCC. However, the serum HBsAg level, HBV genotype, and HBV mutations are not readily available in clinical settings. The measurement of these factors tends to be required for specific situations such as academic approaches to antiviral therapy, epidemiologic investigations, or scientific interest. Therefore, the application of virologic factors for building risk prediction models of HCC should be granted as evidence-based as a matter of prudence even if most virologic factors provide important information for HCC risk stratification.

## RISK PREDICTION SYSTEMS

Risk prediction systems capable of estimating the strength of HCC development are clinically important for identifying patients at high risk who should participate in a scheduled surveillance program. To construct readily available prediction systems of HCC, the risk factors for HCC mentioned previously should be independently established through statistical verification. Statistical techniques adopted in the process should be reliable and reasonable to identify an objective recognition. Next, selected risk factors should be integrated and organized under the consideration of demographic and epidemiologic differences of developing HCC, inducing a systematic stratification of scoring estimation derived from independent risk factors for HCC. Finally, constructed risk prediction systems should be validated internally or externally, which makes individualized surveillance strategies possible. Cancer risk weighed-oriented scoring estimation could provide the advantage in aspects of cost- or effort-effectiveness through a tailored approach to cancer surveillance.

Several predictive scoring systems for the development of HBV-related HCC have been introduced recently (Table 1). Yuen *et al.*<sup>[11]</sup> for the first time deduced and validated the risk score (*i.e.*, the GAG-HCC score) with sensitivity > 84% and specificity > 76% to predict the 5- and 10-year risks for the development of HCC based on age, gender, HBV DNA levels, core promoter mutations, and cirrhosis; they concluded that the risk score could be used to identify high-risk patients with chronic hepatitis B (CHB) for screening and treating HCC. They emphasized the significance of this study with a valuable approach excluding the patients who received any type of established management for CHB capable of affecting the occurrence of HCC. Wong *et al.*<sup>[29]</sup> included two prospective



**Table 1 Risk prediction models of hepatocellular carcinoma in patients with chronic hepatitis B virus infection**

Ref.	No. of participants/ validation	Parameters of HCC prediction	Risk weights for parameters	Range of weights	Year of risk prediction
Lee <i>et al</i> <sup>[32]</sup> , 2013	2227/1113	Age Sex ALT Family Hx of HCC HBeAg/HBV DNA/ HBsAg/Genotype	[0-6] [0-2] [0-2] [0-2] [0-7]	0-19	5, 10 and 15
Wen <i>et al</i> <sup>[12]</sup> , 2012	<sup>1</sup> 298051	Age Sex Smoking Alcohol Physical activity DM ALT AST HBV AFP	M1, 2, 3, 4: [0-6] M1, 2: [0-2], M3, 4: [0-1] M1, 3, 4: [0-1] M1, 3, 4: [0-1] M1, 3, 4: [-1-0] M1: [0-2], M3, 4: [0-1] M2: [0-2], M3, 4: [0-1] M2: [0-13], M3: [0-12], M4: [0-7] M4: [0-4] M4: [0-8]	M1: -1-12 M2: 0-23 M3: -1-23 M4: -1-30	5 and 10
Yang <i>et al</i> <sup>[30]</sup> , 2011	3584/1505	Age Sex ALT HBeAg HBV DNA	[0-6] [0-2] [0-2] [0-2] [0-5]	0-17	3, 5 and 10
Yang <i>et al</i> <sup>[31]</sup> , 2010	2435/1218	Age Sex Alcohol ALT Family Hx of HCC HBeAg HBeAg/HBV DNA HBeAg/HBV DNA/ Genotype	M1, 2, 3: [0-6] M1, 2, 3: [0-2] M1: [0-1], M2, 3: [0-2] M1: [0-3], M2: [0-2], M3: [0-1] M1, 2, 3: [0-2] M1: [0-3] M2: [0-6] M3: [0-7]	M1: 0-17 M2: 0-20 M3: 0-20	5 and 10
Wong <i>et al</i> <sup>[29]</sup> , 2010	1005/424	Age Alb Bil HBV DNA Cirrhosis	[0-3] [0-20] [0-1.5] [0-4] [0-15]	0-43.5	5 and 10
Yuen <i>et al</i> <sup>[11]</sup> , 2009	<sup>2</sup> 820	Age Sex HBV DNA Core promoter mutations Cirrhosis	With core promoter mutations Age (in years) + 16 sex (male = 1; female = 0) + 3 HBV DNA levels (Log copies/mL) + 19 core promoter mutations (mutant = 1; wild-type = 0) + 30 cirrhosis (presence = 1; absence = 0) Without core promoter mutations Age (in years) + 14 sex (male = 1; female = 0) + 3 HBV DNA levels (Log copies/mL) + 33 cirrhosis (presence = 1; absence = 0)	-	5 and 10

<sup>1</sup>The number of participants in a subcohort without hepatitis C virus test results was 298051, with being randomly and equally split into a training set and a validation set. <sup>2</sup>The risk score was assessed by the leave-one-out cross-validation. HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; DM: Diabetes mellitus; AFP: Alfa fetoprotein; Bil: Bilirubin; Alb: Albumin; M: Model.

cohorts in their study: a training cohort (1005 patients) and a validation cohort (424 patients). A predictive scoring system ranging from 0 to 43.5 was constructed by using five independent risk factors: age, albumin, bilirubin, HBV DNA, and cirrhosis. They concluded that the classification of HCC risk to low-, medium-, and high-risk groups based on this scoring system was accurate in predicting HCC development. They insisted that the score was derived from clinical parameters routinely measurable in large prospective cohorts for a long-term period, and the validation was established with high accuracy in another sizable cohort. Yang *et al*<sup>[30]</sup> enrolled 3584 pa-

tients from the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) study cohort as a development cohort of risk estimation. Male gender, older age, elevated serum ALT activity, HBeAg positive, and higher serum HBV DNA titer were identified as independent risk factors for HCC; a 17-point risk score from these risk factors was developed. They interpreted that this simple-to-use risk score based on noninvasive clinical variables could accurately predict the risk of HCC in patients with chronic hepatitis B. They also depicted easy-to-use nomograms to accurately predict the risk of HCC in CHB patients,

facilitating risk communication between clinicians and patients<sup>[31]</sup>. This Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B study suggested that clinicians could make evidence-based decisions about clinical management. Wen *et al*<sup>[12]</sup> developed five simple risk prediction models with one-by-one escalating manner based on clinically available data from a prospective cohort of 428 584 general subjects. Age, sex, health history, HBV and hepatitis C virus (HCV) status, and serum ALT, aspartate aminotransferase, and alpha-fetoprotein levels were determined to be statistically significant independent predictors of HCC risk. They concluded that prediction models using transaminase data were best able to predict HCC risk even among subjects with unknown or HBV- or HCV-negative infection status. The significance of this study is that setting up a simple, easy-to-administer risk prediction model applicable even in low-risk, average-risk, or unknown-risk subjects as well as a high-risk population was attained. Lee *et al*<sup>[32]</sup> most recently developed risk prediction models of HCC by integrating host and HBV profiles after identifying independent risk factors such as older age, male, HBeAg, HBV genotype C, and increasing levels of ALT, HBV DNA, and HBsAg associated with an increased risk of HCC. They concluded that the categorization into low, medium, and high HCC risk could enable physicians to estimate the 5-, 10-, and 15-year risk of HCC with excellent accuracy and discriminatory ability. Two points are noteworthy in this study. One was the introduction of the quantitative serum HBsAg level in the analytic process to derive HCC risk models. The serum HBsAg level was determined to be an independent risk factor of HCC development as well as a response assessment to antiviral therapy. The other was the construction of a prediction model of cirrhosis risk, which was not developed before in patients with chronic HBV infection. Because cirrhosis is a precancerous lesion, the development of a cirrhosis risk prediction model has a valuable impact on the selection of candidates for a scheduled surveillance program according to the risk stratification of HCC.

In summary, accurate prediction models of HCC development constructed from readily available clinical and laboratory variables are necessary for performing strategic processes such as screening/surveillance, diagnosis, and treatment in high-risk patients of HCC. A globally standardized consensus for cancer risk prediction models should be established based on simplicity, assessability, and reproducibility of the model characteristics available in real clinical settings. Hereafter, generalized authorization of risk prediction models needs to be confirmed by using internal and external validation with a prospective manner in different populations of regions with epidemiologic versatility of HCC.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Target genes discovery through copy number alteration analysis in human hepatocellular carcinoma

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oxidative stress play critical roles in HCC tumorigenesis. Nevertheless, because there are few druggable genes used in HCC therapy, the identification of new therapeutic targets through integrated genomic approaches remains an important task. Because a large amount of HCC genomic data genotyped by high density single nucleotide polymorphism arrays is deposited in the public domain, copy number alteration (CNA) analyses of these arrays is a cost-effective way to reveal target genes through profiling of recurrent and overlapping amplicons, homozygous deletions and potentially unbalanced chromosomal translocations accumulated during HCC progression. Moreover, integration of CNAs with other high-throughput genomic data, such as aberrantly coding transcriptomes and non-coding gene expression in human HCC tissues and rodent HCC models, provides lines of evidence that can be used to facilitate the identification of novel HCC target genes with the potential of improving the survival of HCC patients.

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**Key words:** Copy number alteration; High-density single nucleotide polymorphism arrays; Driver genes; Hepatocellular carcinoma

## Abstract

High-throughput short-read sequencing of exomes and whole cancer genomes in multiple human hepatocellular carcinoma (HCC) cohorts confirmed previously identified frequently mutated somatic genes, such as *TP53*, *CTNNB1* and *AXIN1*, and identified several novel genes with moderate mutation frequencies, including *ARID1A*, *ARID2*, *MLL*, *MLL2*, *MLL3*, *MLL4*, *IRF2*, *ATM*, *CDKN2A*, *FGF19*, *PIK3CA*, *RPS6KA3*, *JAK1*, *KEAP1*, *NFE2L2*, *C16orf62*, *LEPR*, *RAC2*, and *IL6ST*. Functional classification of these mutated genes suggested that alterations in pathways participating in chromatin remodeling, Wnt/ $\beta$ -catenin signaling, JAK/STAT signaling, and

**Core tip:** In addition to detecting somatic mutations in cancer genomes with high-throughput short-read sequencing technologies, analysis of copy number alteration in hepatocellular carcinoma (HCC) cancer genomes genotyped by high density single nucleotide polymorphism arrays is a cost-effective approach to reveal genome-wide somatic alterations accumulated during tumorigenesis. Integration with other genomic data from HCC tissues derived from high-throughput short-read sequencing, proteomics, epigenomics and transcriptomics could provide lines of evidence to identify common and novel HCC genes for potential clinical applications.



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## INTRODUCTION

Human hepatocellular carcinoma (HCC) is the fifth leading cause of cancer mortality, causing an estimated half a million deaths annually<sup>[1,2]</sup>. Risk factors for developing HCC include hepatitis infection, obesity, alcoholism and consumption of aflatoxin-contaminated food. Due to the rising incidence of hepatitis C infection, HCC is one of the fastest-growing cancers in the United States and Western countries, and the incidence is expected to continue to increase<sup>[3]</sup>. Surgical resection is the most successful treatment for early stage HCC. However, fewer than 30% of HCC patients are qualified for curative resection owing to liver dysfunction and cirrhosis. Moreover, frequent tumor recurrence is observed even after curative resection.

Recent successes in cancer targeted therapy arising from the identification of somatic alterations and their specific inhibitors are associated with reduced side effects and prolonged patient survival. Many of these FDA-approved inhibitors are small molecules or monoclonal antibodies against cancer-specific tyrosine kinase mutations, including Imatinib mesylate (Gleevec) for fusion oncogene Bcr/Abl-positive chronic myelogenous leukemia<sup>[4]</sup>, Gefitinib (Iressa) or Erlotinib (Tarceva) for epidermal growth factor receptor mutated non-small cell lung cancer<sup>[5]</sup> and Trastuzumab (Herceptin) for HER2/neu receptor amplified and overexpressed breast cancer patients<sup>[6]</sup>. Although no specific drug target has been identified for HCC, FDA approved the multi-kinase inhibitor sorafenib for treatment of advanced HCC, due to a favorable overall patient survival<sup>[7]</sup>. However, HCC patients receiving sorafenib showed marginal benefits, with a prolonged survival of 3-4 mo on average<sup>[8,9]</sup>. With limited improvement of HCC patient survival, identification of recurrent and altered somatic genes through integrated genomic approaches is vital to better understand HCC molecular tumorigenesis, to develop early diagnostic markers and methods, and to find additional druggable targets for the improvement of HCC management.

## MUTATED HCC GENES WITHIN RECURRENT ALTERED CHROMOSOME LOCI

In HCC, many tumor suppressor genes and oncogenes were identified based on recurrent genetic lesions, including loss of *TP53* (17p13)<sup>[10]</sup>, *RB* and *BRCA2* (13q)<sup>[11]</sup>, and amplification of *c-myc* (8q24)<sup>[12]</sup> and *ERBB2*

(17q12-q21)<sup>[13]</sup>. Epigenetic mechanisms also contribute to HCC progression, such as CpG island hyper-methylation of *p16* (*INK4a*) and *COX2*<sup>[14-16]</sup>, as well as altered expression of microRNAs<sup>[17,18]</sup>. Conventional point mutation is another common mechanism to alter cancer gene functions. In HCC, frequent point mutations of *TP53* and *β-catenin* are involved in key pathways of hepatocarcinogenesis<sup>[19,20]</sup>. Other studies have reported mutations in *M6P/IGF2R*<sup>[21]</sup>, *BRCA2*<sup>[22]</sup>, *Smad2/4*<sup>[23]</sup>, *HCCS1*<sup>[24]</sup>, *PTEN*<sup>[25]</sup> and *Axin1*<sup>[26]</sup>.

Recently developed high-throughput short-read sequencing technologies were used to identify somatic mutations in HCC cancer genomes at genome-wide scales. These studies confirmed that *TP53* and *CTNNB1* (encoding for *β-catenin*) are the most frequent recurrent mutations in human HCC. In addition, moderate mutation frequencies were identified in multiple HCC cohorts for several novel genes, including epigenetic and chromatin remodeling genes (*ARID1A*, *ARID2*, *MLL* and *MLL3*) and members of a number of oncogenic pathways (*RPS6KA3*, *JAK1* and *KEAP1*)<sup>[27-32]</sup>. These results suggested that aberrant pathways involved in cell cycle regulation, oxidative stress, chromatin remodeling and oncogenic signaling, such as Wnt/*β-catenin*, JAK/STAT and Akt/mTOR, play critical roles in the process of HCC tumorigenesis. Nevertheless, HCC remains a highly lethal cancer due to the lack of biomarkers for early diagnosis, molecular classification and efficient therapeutic interventions. Efforts to develop specific inhibitors for these aberrant pathways and reveal better therapeutic targets in HCC are urgently needed.

## HIGH DENSITY SINGLE NUCLEOTIDE POLYMORPHISM ARRAYS FOR ANALYSIS OF RECURRENT COPY NUMBER ALTERATIONS

Copy number alterations (CNAs), distinguished from germ line transmitted copy number variations, account for some of the genetic diversity of populations, in addition to the accumulated genomic DNA changes during tumor progression. CNAs are important subclasses of somatic mutations, with aberrant chromosomal regions of amplification or deletion commonly associated with overexpressed oncogenes or loss of tumor suppressor genes, respectively<sup>[33]</sup>. With the comprehensive annotation of human genome in the last decade, the mutated cancer genes could be aberrant protein-coding and non-coding genes such as small microRNAs or long non-coding RNAs within the CNA regions<sup>[34]</sup>.

Copy number alterations in cancer cells can be detected by conventional karyotyping and chromosomal in situ hybridization technologies. To profile CNAs in cancer genomes compared to the genomes of adjacent normal cells, comparative genome hybridization (CGH) technology was used to identify copy number changes in karyotypes from breast cancer cell lines and primary blad-

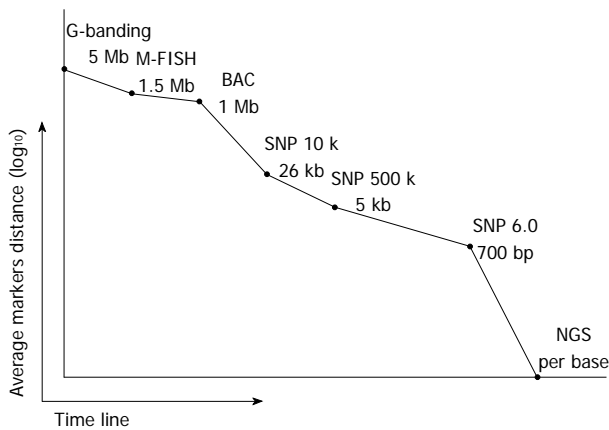


Figure 1 Timeline and average marker distance of technologies for the detection of copy number alterations.

der tumors<sup>[35]</sup>. With the availability of genomic resources (*e.g.*, BAC clones) and array technologies (*e.g.*, high density oligonucleotide probes), array-based CGH (aCGH) technologies not only examine genome-wide CNAs in high resolution but also allow researchers to pinpoint and profile the non-random CNAs for identification of novel aberrant cancer genes (Figure 1)<sup>[36-38]</sup>. The recently developed high-throughput short-read sequencing technology might become an alternative and effective approach to simultaneously detect CNAs and other classes of somatic mutations at the single nucleotide level<sup>[39,40]</sup>. Nevertheless, the availability of thousands of cancer genomes genotyped by high-density SNP arrays from various tumor samples and cancer cell lines at both NCBI GEO (Gene Expression Omnibus) and EBI ArrayExpress databases is a critical resource for *in silico* analysis of CNAs<sup>[41,42]</sup>. Moreover, integrated genomic analysis with both high-throughput short-read sequencing technology and high-density SNP genotyping arrays to comprehensively profile and validate recurrent CNAs of cancer genomes are promising approaches for the identification of novel cancer genes<sup>[40]</sup>.

## DIFFERENT TYPES OF CANCER MUTATIONS EMBRACED IN CNAS LOCI

To identify novel diagnostic and therapeutic target genes, CNA analysis of cancer genomes genotyped using commercial high-density SNP arrays from your own experiments or downloaded from public domains is a powerful and cost-effective approach. First, to discover putative tumor suppressor genes, we overlapped homozygous deleted regions from multiple samples to narrow down the common deleted regions by using high-density SNP genotyping arrays. As shown in Figure 2, the homozygous deleted region at chromosome 13q12.11 in SK-hep1 cells could be refined from 1.88 to 1.46 Mb to facilitate the identification of candidate tumor suppressor genes<sup>[38,43]</sup>. Second, for the identification of candidate oncogenes in HCC, the most common approach is to integrate data

Table 1 Copy-number altered regions in genomes of hepatocellular carcinoma cell lines

Cytoband	Start (Mb)	End (Mb)	Known cancer genes	Novel candidates
Amplicons				
1q21.2-22	150.07	151.89	<i>SHC1, CKS1B, ADAM15</i>	<i>CREB3L4, RAB1, mir190b, S100A14, LMCD1</i>
3p26.1-25.3	6.90	9.43		
3q26.2-26.31	170.07	170.24		
	170.28	170.99	<i>EV11, MDS1, TERC</i>	
	171.21	173.50		<i>FNDC3B</i>
5p15.33-12	0.40	45.14	<i>TRIO, AMACR, DAB2</i>	<i>LPCAT1, SEMA5A, CDH12</i>
7p22.2-14.3	4.15	32.10	<i>RAC1, ETV1, CHN2</i>	
7p12.1-11.2	52.79	55.17	<i>EGFR</i>	
	56.00	56.53		
8p11.21	40.44	40.62		
8q24.21	129.21	129.29		
11q13.2-13.3	65.85	66.44	<i>RIN1, BRMS1</i>	<i>SLC29A2</i>
	67.58	67.71		
	67.91	69.35	<i>LRP5, CCND1, ORAOV1</i>	<i>FGF4, FGF3</i>
12p12.1	24.36	25.54	<i>BCAS1, K-ras</i>	
20q13.31	53.94	53.96		
Homozygous deletions				
2q22.1	141.72	141.80	<i>LRP1B</i>	
7q21.11	77.96	78.04		<i>MAGI2</i>
9p23	9.42	9.46		<i>PTPRD</i>
	11.90	12.00		
9p21.3	21.85	21.90	<i>MTAP, CDKN2A</i>	
	24.27	24.84		
13q12.11	18.98	20.44	<i>TPTE2, Tg737</i>	

from genomic experiments in order to reveal genes residing in overlapping amplicons with up-regulated gene expression. For instance, *FNDC3B*, *SLC29A2*, *Ago2*, *IER3* and many others were identified as putative oncogenes due to their genomic DNA amplification and mRNA overexpression in HCC tissues<sup>[38,44-47]</sup>. When ectopically expressed, these putative oncogenes in HCC cells commonly show malignant phenotypes using various functional assays and facilitated tumor progression *in vitro* and *in vivo*.

Third, CNA analysis allows the identification of HCC genes with attributes of genomic DNA amplification, mRNA overexpression and recurrent point mutations, such as the putative metastatic HCC oncogene with LMCD1 mutations at E135K (in 3/48 cases) and K237R (in PLC/PRF/5 cells)<sup>[44]</sup>. When these mutations were expressed in HCC cells, HCC cell migration capability was enhanced in association with cortical actin accumulation and lamellipodial extension. Moreover, the overexpression of the LMCD1 E135K mutation in HCC cells significantly promoted systemic lung metastasis in a murine tail vein injection model. Table 1 summarizes some novel HCC genes in association with overlapping amplicons and homozygous deletions in HCC cell lines. Finally, CNA analysis detects differences in copy number (*i.e.*, dosages), such as amplifications and deletions. Therefore,

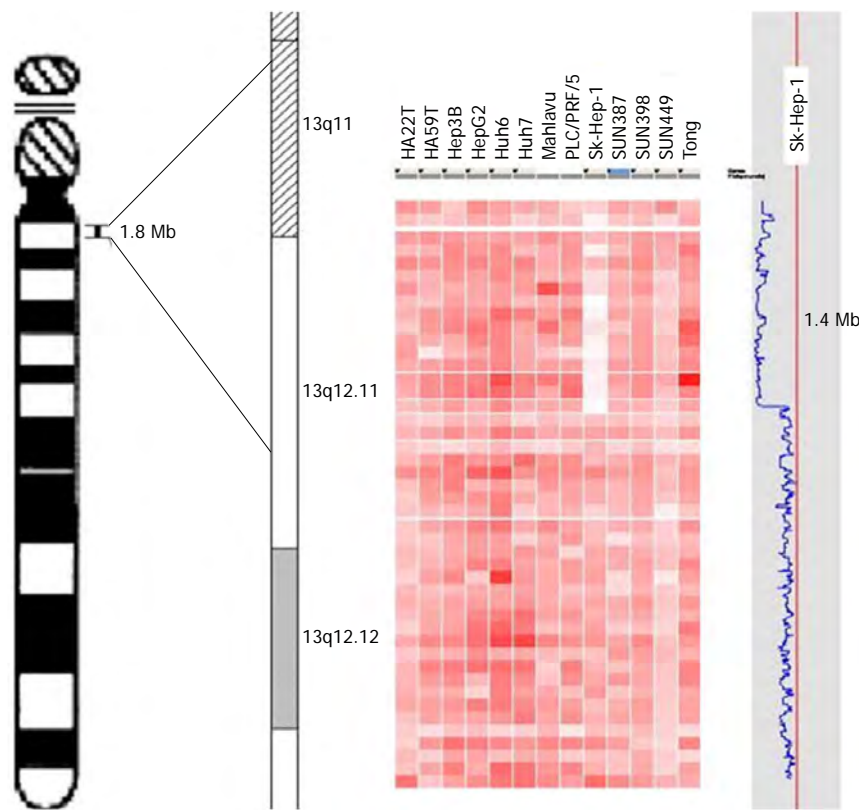


Figure 2 Refinement of homozygous deletion by copy number alteration analysis at chromosome 13q12.11 in hepatocellular carcinoma cells.

Table 2 List of some integrated cancer genomic databases			
Database	Project	Website	Ref.
cBioPortal for cancer genome	Project provides visualization, analysis and download of large-scale cancer genomic data sets	<a href="http://www.cbioportal.org/public-portal/">http://www.cbioportal.org/public-portal/</a>	Cerami <i>et al</i> <sup>[57]</sup>
COSMIC	Catalogue of somatic mutations in cancer	<a href="http://cancer.sanger.ac.uk/cancer-genome/projects/cosmic/">http://cancer.sanger.ac.uk/cancer-genome/projects/cosmic/</a>	Forbes <i>et al</i> <sup>[58]</sup>
ICGC	International Cancer Genome Consortium provides tools for visualizing, querying and downloading the data.	<a href="http://dcc.icgc.org/">http://dcc.icgc.org/</a>	Joly <i>et al</i> <sup>[59]</sup>
TCGA data portal	A platform for researchers to search, download, and analyze data sets generated by TCGA	<a href="https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp">https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp</a>	
Tumorscape	High-resolution copy number data collected from multiple cancer types	<a href="http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf">http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf</a>	
UCSC cancer genome browser	A set of web-based tools to display, investigate and analyze cancer genomic data and associated clinical information	<a href="https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/">https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/</a>	Goldman <i>et al</i> <sup>[60]</sup>

COSMIC: Catalogue of somatic mutations in cancer.

it will not reveal balanced translocation but will detect sudden dosage changes for unbalanced translocation. Using CNA analysis and high-density SNP arrays, *PAX5* fusion genes were identified with a variety of partner genes, including *ETV6*, *FOXP1*, *AUTS2*, and *C20orf112*, in pediatric acute lymphoblastic leukemia (ALL)<sup>[48]</sup>.

### INTEGRATED HCC CANCER GENOMIC DATABASES WITH CNAS

Integrated data derived from multiple genomic approaches could potentially avoid pitfalls of data inconsistency usual with the single genomic approach and provide lines

of evidence to validate target genes embraced in the aberrant genomic loci from the level of DNA and RNA to protein. For these advantages, several user-friendly HCC databases were constructed, including OncoDB.HCC, HCCnet, dbHCCvar, CellMinerHCC, HCC-M, and EHCO<sup>[49-54]</sup>. However, only OncoDB.HCC integrated genomic alteration data to prioritize HCC cancer genes for further expression and functional validations in HCC cell lines and tissues. Nevertheless, recent international efforts at applying high-throughput short-read sequencing technologies and CNA analysis of cancer genomes in multiple cancer types, including HCC, comprehensively cataloged different types of somatic mutations and revealed genetic heterogeneity even from the same subtype

of cancer. Table 2 lists common open-access integrated cancer genome databases for downloading and visualizing cancer genomic data<sup>[55,56]</sup>.

## CONCLUSION

As discussed in this review article, an integrated genomic approach is an effective and essential method of identifying novel HCC genes. With the availability of a tremendous amount of high-throughput short-read sequencing data and SNP array data from cancer genomes deposited in the public domain, integrated genomic approaches, including CNA analysis, are the most cost-effective approach for revealing HCC driver genes for improving HCC therapy.

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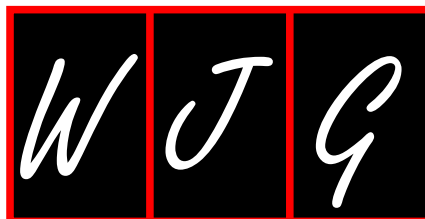
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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Mitochondrial DNA alterations and mitochondrial dysfunction in the progression of hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is ranked third in mortality among cancer-related diseases. Mitochondria are intracellular organelles that are responsible for energy metabolism and cellular homeostasis, and mitochondrial dysfunction has been regarded as a hallmark of cancer. Over the past decades, several types of mitochondrial DNA (mtDNA) alterations have been identified in human cancers, including HCC. However, the role of these mtDNA alterations in cancer progression is unclear. In this review, we summarize the recent findings on the somatic mtDNA alterations identified in HCC and their relationships with the clinicopathological features of

HCC. Recent advances in understanding the potential roles of somatic mtDNA alterations in the progression of HCC are also discussed. We suggest that somatic mtDNA mutations and a decrease in the mtDNA copy number are common events in HCC and that a mitochondrial dysfunction-activated signaling cascade may play an important role in the progression of HCC. Elucidation of the retrograde signaling pathways in HCC and the quest for strategies to block some of these pathways will be instrumental for the development of novel treatments for this and other malignancies.

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**Key words:** Hepatocellular carcinoma; Somatic mitochondrial DNA mutations; Mitochondrial dysfunction

**Core tip:** In this review, we summarize the recent findings on the somatic mtDNA alterations identified in hepatocellular carcinoma (HCC) and their relationships with the clinicopathological features of HCC. We suggest that somatic mtDNA mutations and a decrease in the mtDNA copy number are common events in HCC and that a mitochondrial dysfunction-activated signaling cascade may play an important role in the progression of HCC. Elucidation of the retrograde signaling pathways in HCC and the quest for strategies to block some of these pathways will be instrumental for the development of novel treatments for this and other malignancies.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the one of most common cancers worldwide and is ranked third with respect to mortality<sup>[1,2]</sup>. There are approximately 434000 new cases of HCC per year<sup>[3]</sup>. Several risk factors have been suggested to be involved in the development of HCC, including aflatoxin exposure, alcohol consumption, chronic inflammation associated with viral hepatitis and familial tendency<sup>[4-8]</sup>. Moreover, inflammation and oxidative stress have been suggested to contribute to the carcinogenesis of HCC<sup>[9-11]</sup>.

In the 1930s, the German biochemist Warburg<sup>[12]</sup> proposed that tumor cells prefer to utilize glycolysis rather than respiration as a primary energy source, even in the presence of abundant oxygen. This phenomenon was termed “aerobic glycolysis” or the “Warburg effect”. He further proposed that defects in energy metabolism, especially in the mitochondria, are involved in the initiation or progression of cancer<sup>[13]</sup>.

Mitochondria are cytoplasmic organelles that play multiple roles in energy metabolism and cellular homeostasis, including the generation of ATP *via* respiration and oxidative phosphorylation (OXPHOS), the production of reactive oxygen species (ROS), metabolic homeostasis, and the initiation and execution of apoptosis<sup>[14,15]</sup>. These roles are executed by proteins that are encoded by genes in the nucleus and mitochondria. Mitochondrial DNA (mtDNA) is a 16.6-kb, double-stranded circular DNA that contains genes for 22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides that comprise the respiratory enzyme complexes<sup>[16]</sup>. In addition to the coding region, mtDNA contains a non-coding region called the “D-loop”, which is approximately 1.1 kb, encompasses nucleotide position (np) 16024-np 576, and controls the replication and transcription of the mtDNA<sup>[17]</sup>.

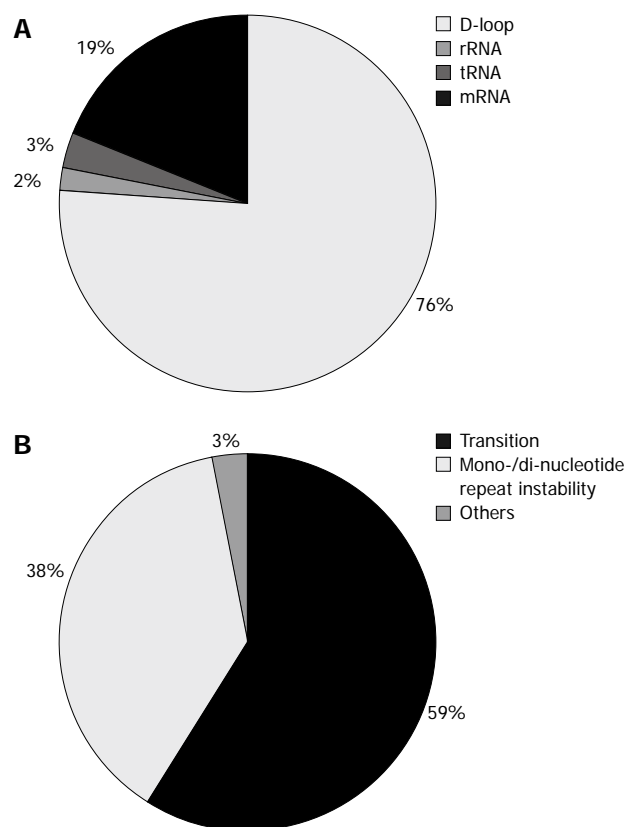
Due to its lack of protective histone proteins, a limited DNA repair system and its spatial proximity to a high level of ROS, mtDNA sustains a 10-fold higher level of damage than that of nuclear DNA (nDNA)<sup>[18-20]</sup>. Somatic mutation and damage to mtDNA can lead to impairment of the OXPHOS system and enhanced ROS generation, which in turn accelerates the occurrence of DNA mutations. This scenario has been proposed to contribute to the initiation and progression of tumors<sup>[21,22]</sup>.

Over the past decade, somatic mtDNA mutations have been identified in several types of cancer, including HCC<sup>[23-27]</sup>. Some of the acquired mtDNA mutations have been suggested to cause mitochondrial dysfunction, increase the production of ROS, and promote tumor growth<sup>[28,29]</sup>.

In this article, we review the recent findings on somatic mtDNA alterations in HCC. In addition, we discuss the potential roles of mtDNA alterations and mitochondrial dysfunction in the progression and metastasis of HCC.

## SOMATIC MITOCHONDRIAL DNA ALTERATIONS IN HCC

Over the past decade, several types of somatic mtDNA



**Figure 1** The location distribution of the identified somatic point mutations (A) and the types of somatic point mutations (B) in the mitochondrial DNA in hepatocellular carcinoma. Data adapted from Yin *et al.*<sup>[29]</sup> and Wong *et al.*<sup>[30]</sup>.

alterations have been identified in human HCC. These mtDNA alterations include point mutations, deletions, insertions and copy number changes.

### Point mutations

Screening for somatic point mutations in the whole mitochondrial genomes of HCC samples<sup>[29,30]</sup> revealed that approximately 52% of HCC patients carry at least one homoplasmic or heteroplasmic point mutation in their tumor tissue mtDNA. Of the identified point mutations, 76% are located in the D-loop region, 2% are located in rRNA genes, 3% are located in tRNA genes and 19% are located in mRNA genes (Figure 1A). The incidence and location distribution of the point mutations are consistent with those observed in other cancer types<sup>[25]</sup>.

The D-loop region is a hot spot for somatic mtDNA mutations in HCC and other cancers. It was reported that the D-loop region of mtDNA, especially the mononucleotide repeat in the np 303-309 poly-C sequence, is the most susceptible site to oxidative damage compared with the other regions of the mtDNA, implying that oxidative damage contributes to point mutations in the D-loop and/or the instability of the mononucleotide or dinucleotide repeats in the mtDNA. However, the unique G-to-T transversion caused by oxidative DNA damage is not detected in HCC<sup>[29,30]</sup>. Among the mtDNA mutations that have been identified in HCC, approximately 59% are transition mutations (G/A-to-A/G or C/T-to-T/C) and



38% are mono- or di-nucleotide instabilities (Figure 1B), suggesting that oxidative damage *per se* is not the major factor responsible for point mutations in HCC. The presence of hepatitis B infection, liver cirrhosis, alcohol abuse or their combination may affect the qualitative changes in the mtDNA in HCC<sup>[30]</sup>.

Because the D-loop region controls the replication and transcription of the mtDNA, mutations in the D-loop region may influence the mtDNA copy number and the expression of the mitochondrial genome<sup>[31]</sup>. It has been shown that the occurrence of point mutations in the D-loop, especially near the replication origin of the heavy-strand (OH) of the mtDNA, affects the mtDNA copy number in HCC<sup>[32]</sup>. In addition, Nishikawa *et al*<sup>[33]</sup> reported that the number of mtDNA mutations in the D-loop region is positively correlated with a poor HCC differentiation grade. These findings suggest that the somatic mutations in the mtDNA D-loop region may affect mitochondrial function by decreasing the mtDNA copy number and/or transcription in HCC, thereby leading to HCC progression.

Among the mutations in the coding region, the non-sense mutation G3842A creates a premature stop codon and the missense mutations T6787C, G7976A, G9267A, and A11708G result in amino acid substitutions in the highly evolutionally conserved regions of the affected mitochondrial genes. Moreover, the base-pair deletion and insertion 11032delA and 12418insA may lead to a frame-shift mutation, and the tRNA mutations T1659C in tRNA<sup>Val</sup> and G5650A in tRNA<sup>Ala</sup> may alter the tRNA structure and were shown to associate with mitochondrial disorders<sup>[29]</sup>. Therefore, these mtDNA point mutations may result in mitochondrial dysfunction in HCC.

### Deletions

Among the large-scale deletions identified in the mtDNA in different cancer types<sup>[32,34-37]</sup>, the 4977-bp deletion is the most common mtDNA deletion in tumors<sup>[23,38-43]</sup>. Consistent with findings in other types of cancer, the incidence of the 4977-bp deletion and its accumulation level are lower in the malignant tissues than the non-tumor tissues of HCC patients<sup>[23,39]</sup>. Moreover, gender and a long-term history of alcohol consumption in HCC patients may affect the accumulation of the 4977-bp-deleted mtDNA<sup>[23]</sup>. Although the role of mtDNA deletion in HCC is unclear, it has been suggested that the observed decrease in mtDNA with a deletion is the result of the tumor cells adapting to a new microenvironment during hepatocarcinogenesis<sup>[25,44]</sup>.

In addition, a 50-bp deletion was previously reported in one HCC patient<sup>[32]</sup>. This deletion is flanked by a 9-bp direct repeat in the D-loop region of the mtDNA. The mtDNA deletion appeared to be homoplasmic in the HCC tissue but was not detected in the corresponding non-tumor liver tissue. The tumor-specific accumulation of this deletion does not seem to be similar to that of the 4977-bp deletion in cancers. Because this deletion

partly truncates the regulatory region of the mtDNA, the mtDNA copy number in the HCC tissue was found to be significantly reduced compared with that in the non-tumor liver tissue<sup>[32]</sup>. This mtDNA deletion may lead to mitochondrial dysfunction *via* mtDNA depletion and/or impairment of the transcription of mitochondrial genes.

### Insertions

Two small insertions (approximately 260 bp and approximately 520 bp) have been identified as a tandem duplication and a tandem triplication and are flanked by two poly-cytosine (poly-C) sequences at np 303-309 and np 568-573 in the D-loop region of the mtDNA in various human cancers, including HCC<sup>[35]</sup>. This tandem duplication or triplication was detected in approximately 4% of HCCs and is highly correlated with the presence of length variation in the poly-C at np 568<sup>[44]</sup>. However, these insertions have also been found in somatic tissues in elderly subjects and, thus, are not specific for cancer tissues.

### Copy number changes

A decrease in the mtDNA copy number is a common event in HCC<sup>[23,32,45,46]</sup>. Over 60% of HCCs have a lower mtDNA copy number than their corresponding non-tumor liver tissues. As mentioned above, it was observed that the reduction in the mtDNA copy number is associated with point mutations located near the replication origin in the D-loop region of the mtDNA<sup>[32]</sup>. Moreover, it was suggested that the decrease in the mtDNA copy number in HCC may be related to or result from the altered expression of genes involved in mitochondrial biogenesis, such as peroxisome proliferator-activated receptor-1 (PPAR-1) and mitochondrial single-stranded DNA binding protein (mtSSB)<sup>[23]</sup>. These results suggest that the mtDNA mutations in the D-loop region and the impairment of mitochondrial biogenesis contribute to the decrease in the mtDNA copy number in HCC<sup>[34]</sup>.

The reduction in the mtDNA copy number seems to be more frequently observed in female patients with HCC compared with male patients with HCC<sup>[23]</sup>. This difference between male and female HCC patients could be a result of clinical manifestation, progression and/or mortality rate<sup>[23]</sup>. Yamada *et al*<sup>[46]</sup> showed that the low mtDNA copy number in HCC is significantly correlated with large tumor size and liver cirrhosis. In addition, HCC patients with a lower mtDNA copy number in their tumors tend to show poorer 5-year survival compared with patients with a higher mtDNA copy number<sup>[46]</sup>. It was also suggested that hepatitis B infection, liver cirrhosis, and alcohol abuse affect quantitative changes in the mtDNA in HCC<sup>[29]</sup>. Recently, it was reported that there is an association between the mtDNA content in the peripheral blood leukocytes and hepatitis B virus-related hepatocellular carcinoma<sup>[47]</sup>, which suggests that the mtDNA copy number in the peripheral blood leukocytes could be used as a predictor of HCC occurrence.

## POTENTIAL ROLES OF MITOCHONDRIAL DNA MUTATIONS AND MITOCHONDRIAL DYSFUNCTION IN HCC PROGRESSION

Several types of somatic mtDNA alterations have been identified in HCC, but the roles of these mtDNA alterations in HCC progression are unclear. Evidence from several lines of research has substantiated the pathological role of mtDNA mutation and mitochondrial dysfunction in HCC.

The majority of the somatic point mutations in the mitochondrial coding region and the decrease in the mtDNA copy number may cause mitochondrial dysfunction in HCC. These findings provide a molecular basis for the Warburg effect. In addition, it has been shown that the low mtDNA copy number in HCC is significantly correlated with large tumor size, liver cirrhosis, and poor 5-year survival<sup>[46]</sup>. Therefore, it is possible that mtDNA mutations and a decrease in the mtDNA copy number and, thereby, mitochondrial dysfunction modify the progression of HCC.

In the human SK-Hep1 hepatoma cell line, mtDNA depletion was demonstrated to induce resistance to oxidative stress and chemotherapeutic agents through an adaptive increase in the expression of manganese superoxide dismutase (MnSOD) and other antioxidant enzymes<sup>[48]</sup>. Moreover, chloramphenicol was found to inhibit mitochondrial protein synthesis in human hepatoma HepG2 cells and to render these cancer cells resistant to mitomycin-induced apoptosis<sup>[49]</sup>. Using similar approaches, respiratory inhibitors and an uncoupler of mitochondrial respiration as well as inhibitors of mtDNA replication or protein synthesis in the mitochondria were found to induce mitochondrial dysfunction and cisplatin resistance in human hepatoma HepG2 cells and to promote cell migration in other hepatoma cells *via* a paracrine signaling pathway<sup>[50]</sup>. It was further demonstrated that the mitochondrial dysfunction-induced upregulation of amphiregulin contributes to the cisplatin resistance and cell migration of hepatoma cells<sup>[50]</sup>. In addition, these treatments also induced changes in the expression of genes that affect the metastatic ability of cancers, including the integrin pathway, the PDGF signaling pathway and the cadherin signaling pathway<sup>[51]</sup>. On the other hand, the overexpression of PGC-1 in HepG2 cells was found to elevate mitochondrial protein expression and to reduce cell mobility *via* increased E-cadherin expression<sup>[52,53]</sup>. These findings support the hypothesis that mtDNA mutations and mitochondrial dysfunction contribute to the malignant progression of HCC.

Mitochondrial dysfunction increases ROS production and  $\text{Ca}^{2+}$  mobilization and reduces ATP generation, which may be involved in the malignant changes induced by mtDNA mutations and mitochondrial dysfunction in HCC. It has been demonstrated that antioxidants and calcium chelators can block mitochondrial dysfunction-induced amphiregulin expression and prevent cisplatin resistance and cell migration<sup>[50]</sup>. In addition, it was re-

cently demonstrated that mitochondrial dysfunction-reduced intracellular ATP content represses the protein expression of hypoxia-inducible factor-1 (HIF-1) through the activation of the AMP-activated protein kinase (AMPK)-mTOR pathways in HepG2 cells<sup>[54]</sup>. HIF-1 is a nuclear transcription factor that plays a crucial role in cancer progression, including angiogenesis, invasion and metastasis<sup>[55]</sup>. These findings suggest that mitochondrial dysfunction regulates nuclear gene expression and phenotypic changes to face the different microenvironments of HCC. Therefore, the activation of retrograde signaling from the mitochondria to the nucleus may play an important role in the malignant progression of HCC.

Consistent findings were observed in other types of cancer. It has been reported that in some cancer cell lines, a pathogenic mtDNA mutation (*e.g.*, the T8993G transversion) promotes tumor growth in nude mice by preventing apoptosis<sup>[56-58]</sup>. Moreover, it was shown that the heteroplasmic 12418insA mutation, which has been identified in HCC<sup>[29]</sup> and other cancers<sup>[24,26,59]</sup>, impairs mitochondrial respiratory function and promotes tumorigenesis by enhancing ROS production<sup>[60]</sup>. In addition, ROS-generating mtDNA mutations have been demonstrated to regulate tumor cell metastasis<sup>[61]</sup>. It was also shown that mtDNA depletion or mitochondrial dysfunction can enhance invasive phenotype changes<sup>[62-64]</sup> or chemo-resistance in some specific types of cancer<sup>[65]</sup>. The underlying mechanisms have been suggested to involve the communication between the mitochondria and the nucleus called “retrograde signaling”<sup>[66,67]</sup>. Several biomolecules have been identified to be involved in this signal transduction, including calcineurin, NFAT, ATF2, Akt, and NF $\kappa$ B/Rel, which then affect the expression of an array of nuclear genes<sup>[68,69]</sup>. The detailed mechanisms by which mtDNA mutations and mitochondrial dysfunction affect HCC progression await further investigation.

Although mtDNA alterations have been identified in HCC, it remains controversial whether mtDNA alterations are correlated with the initiation and progression of HCC. To dissect the role of mtDNA alterations in HCC, a larger sample size of HCC is required in future research. Moreover, some lines of evidence suggest that mitochondrial dysfunction and the dysfunctions caused by mtDNA alterations have the potential to contribute to tumor progression. However, whether a specific mtDNA mutation plays a driving force or is an indirect consequence of HCC progression requires further evaluation. Therefore, it is important to develop a strategy to dissect the role of specific mtDNA mutations in cancer progression and/or to exclude non-causal epiphenomena.

Because the coordination between the nDNA and mtDNA is important for the maintenance of mitochondrial structure and function<sup>[14,17]</sup>, mutations in the nDNA-encoded genes that are responsible for mtDNA integrity and/or mitochondrial function may play an important role in tumorigenesis and cancer progression. For example, it was recently reported that defects in P53<sup>[70]</sup>, mitochondrial DNA polymerase<sup>[71]</sup>, and mitochondrial

deacetylase SIRT3<sup>[72]</sup> may affect mtDNA integrity and promote tumorigenesis. In addition, not only mtDNA mutations but also mitochondrial dysfunction caused by nDNA mutations, oncogenes, and tumor microenvironments (hypoxia and inflammation) are suggested to underlie energy metabolism reprogramming (or the Warburg effect)<sup>[73,74]</sup>. In summary, the interaction between mtDNA and nDNA may play an important role in the initiation and progression of HCC.

## CONCLUSION

Several types of mtDNA alterations, including point mutations, deletions, insertions and copy number changes, have been identified in HCC. Somatic point mutations and deletions are the two most common of these mtDNA alterations in HCC. The low mtDNA copy number in HCC has been shown to be significantly correlated with large tumor size, liver cirrhosis, and poor 5-year survival<sup>[46]</sup>. However, the presence of somatic mtDNA point mutations in HCC does not seem to correlate with the patient's age or sex, the tumor size or grade, hepatitis virus infection, or the patient's survival<sup>[29,30]</sup>. This finding may result from the possibility that mtDNA point mutations do not always play a similar role in HCC progression. In addition, the heteroplasmic or homoplasmic level of the same mtDNA mutation may result in different consequences in tumorigenesis<sup>[60]</sup>. Therefore, the role of the specific mtDNA mutation and its level during HCC progression warrant further study.

The majority of the point mutations in the coding region of the mtDNA and the decrease in the mtDNA copy number likely cause mitochondrial dysfunction in HCC. These findings have provided solid evidence to substantiate the mechanism by which mitochondrial dysfunction is involved in metabolic reprogramming or the "Warburg effect" in cancer. Several lines of evidence have important implications in the pathological role of mtDNA mutation and mitochondrial dysfunction in HCC. Pharmacologic approaches to induce mitochondrial dysfunction can enhance chemo-resistance and promote metastasis, which may contribute to the malignant progression of HCC. Thus, the increased ROS production and Ca<sup>2+</sup> mobilization and the reduced ATP generation induced by mitochondrial dysfunction may be involved in the malignant changes of HCC. However, the detailed mechanism by which mtDNA mutation and mitochondrial dysfunction affect HCC progression remains unclear. Elucidation of the retrograde signaling pathways in HCC and the search for strategies to block these pathways will be important for the development of novel treatments for this and other malignancies.

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## WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

# Prevention of hepatocellular carcinoma in chronic viral hepatitis B and C infection

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## Abstract

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide, with the majority of cases associated with persistent infection from hepatitis B virus (HBV) or hepatitis C virus (HCV). Natural history studies have identified risk factors associated with HCC development among chronic HBV and HCV infection. High-risk infected individuals can now be identified by the usage of risk predictive scores. Vaccination plays a central role in the prevention of HBV-related HCC. Treatment of chronic HBV infection, especially by nucleoside analogue therapy, could also reduce the risk of HBV-related HCC. Concerning HCV infection, besides the advocacy of universal precautions to reduce the rate of infection, pegylated interferon and ribavirin could also reduce the risk of HCV-related HCC among those achieving a sustained virologic response. Recently there has been mounting evidence on the role of chemopreventive agents in reducing HBV- and HCV-related

HCC. The continued advances in the understanding of the molecular pathogenesis of HCC would hold promise in preventing this highly lethal cancer.

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**Key words:** Hepatitis B virus; Hepatitis C virus; Hepatocellular carcinoma; Vaccination; Prevention

**Core tip:** Hepatocellular carcinoma (HCC), with the majority of cases associated with infection from hepatitis B virus (HBV) or hepatitis C virus (HCV), is the most common primary liver tumor. We introduced risk factors and risk predictive scores associated with HCC development among chronic HBV and HCV infection for its early diagnose and prevention. Vaccination plays a central role in the prevention of HBV-related HCC. Treatment of chronic HBV infection, especially by nucleoside analogue therapy, could reduce the risk of HBV-related HCC. Pegylated interferon and ribavirin could reduce the risk of HCV-related HCC. Chemopreventive agents in reducing HBV- and HCV-related HCC were also discussed.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver tumor, and represents the third leading cause of cancer death worldwide. It is the fifth most common cancer in men and seventh in women, accounting for 7% of all cancers<sup>[1]</sup>. Hepatocarcinogenesis

is a multistep process mainly associated with persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV)<sup>[2]</sup>, which affects more than 350 and 170 million individuals respectively worldwide. HCC is highly prevalent in regions endemic for chronic HBV and HCV infection<sup>[3]</sup>.

The incidence of HCC continues to increase worldwide, with a unique geographic, age, and sex distribution. The most important risk factor associated with HCC is liver cirrhosis, which is again predominantly caused by chronic HBV or HCV infection. Primary prevention in the form of HBV vaccination has led to a significant decrease in HBV-related HCC, and the antiviral therapy for chronic HBV and HCV infection also reduce the incidence of HBV- and HCV-related HCC<sup>[4]</sup>.

China has one of the highest carrier rates of HBV in the world, reaching nearly 10% of the general population. The disease burden of HBV infection and HCC is also believed to be among the world's largest, and that of HCV infection is likely to be substantial as well<sup>[5]</sup>.

## RISK PREDICTION

An important component of HCC prevention is the identification of high-risk HBV- and HCV-infected individuals, who will benefit from various chemopreventive therapies discussed below. Several natural history studies have identified important risk factors for HCC among patients with chronic hepatitis B (CHB) and chronic hepatitis C (CHC), with risk predictive scores also designed for practical usage.

### *Risk factors and prediction scores: CHB*

A study evaluating the relationship between serum HBV DNA level and risk of HCC demonstrated that the incidence of HCC among CHB patients increased with serum HBV DNA level. Elevated serum HBV DNA level ( $\geq 2000$  IU/mL) is a strong risk predictor of HCC independent of hepatitis B e antigen (HBeAg)-positivity, serum alanine aminotransferase levels, and liver cirrhosis<sup>[6]</sup>. Subsequent studies also showed patients with moderate levels of serum HBV DNA (60-2000 IU/mL), when compared to individuals not infected with HBV, still had a substantial increased risk of HCC and liver-related death<sup>[7]</sup>.

Besides serum HBV DNA levels, other host- and viral-related factors could also predispose to HCC. A meta-analysis found HBeAg-positive non-cirrhotic patients, when compared to HBeAg-positive cirrhotic patients, had a significantly reduced HCC risk after antiviral therapy<sup>[8]</sup>. HBV genotype also plays a role; HBV genotype C is closely associated with HCC especially in cirrhotic patients aged  $> 50$  years<sup>[9]</sup>. An observation study in Hong Kong also found genotype C HBV infection to be an independent risk factor for HCC development when compared with genotype B<sup>[10]</sup>.

Several clinical scoring systems have been developed for the prediction of HCC in CHB, as depicted in Table

1. These scoring systems are based on the longitudinal follow-up of treatment-naïve CHB patients for 5 years or more. Two common parameters used are age and serum HBV DNA levels. Other parameters used include gender, serum alanine aminotransferase levels, serum albumin, HBeAg status, presence of cirrhosis and presence of core promoter mutations<sup>[11-15]</sup>. Risk prediction is now also possible for CHB patients undergoing nucleoside analogue (NA) therapy. A recent study investigated the risk of HCC among a large population of CHB patients treated with entecavir. Older age and presence of cirrhosis were independently associated with HCC in the entire cohort; advanced age and hypoalbuminemia were associated with HCC in patients without cirrhosis. The risk scores accurately predict which patients with CHB treated with entecavir would have a higher chance of developing HCC<sup>[13]</sup>.

### *Risk factors: CHC*

When compared to CHB, fewer clinical scoring systems have been developed for the prediction of HCV-related HCC. These are as depicted in Table 1. The majority of HCV-related HCC develop in patients with established cirrhosis. In a study investigating prognostic risk factors for HCV-related HCC, among 913 patients followed up for at least 3 years, age, male sex, portal hypertension, hepatic inflammation, and iron storage were significant risk factors for HCV-related HCC<sup>[16]</sup>. In a meta-analysis involving HCV-infected persons, sustained virologic response (SVR) was associated with reduced risk for HCC<sup>[17]</sup>. Even transient virologic control among patients with subsequent relapse after treatment, was associated with a lower risk of the development of HCC<sup>[18]</sup>.

Prediction of HCV-related HCC may be enhanced by the development of related markers. Signal transducer and activator of transcription 1 and phosphatase and tensin homolog are associated with early growth response protein 1 signaling, which potentially promotes angiogenesis, fibrogenesis, and tumorigenesis in HCV-related HCC. This approach has potential for the early diagnosis and possible prevention of HCC. The corresponding serum markers found can help to predict high-risk groups for HCC<sup>[19]</sup>.

### *Host factors*

HCC is more common in HBV carriers with a family history of HCC. In a study of 5238 HBV carriers (553 with HCC and 4685 without HCC), the risk of HCC was significantly higher in those with a family history of HCC, with a multivariate-adjusted rate ratio for HCC of 2.41 compared with HBV carriers without a family history<sup>[20]</sup>. If the carriers had two or more affected family members, the risk was even higher with the ratio increased to 5.55. It is therefore recommended to begin surveillance in adults once a family history of HCC has been identified. A recently published study also included the presence of family history, besides traditional viral-related parameters as a component for risk prediction<sup>[15]</sup>.

**Table 1 Risk factors and prediction scores for hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma**

Risk factors	HBV-related HCC	HCV-related HCC
Increased age	√ <sup>[11-15]</sup>	√ <sup>[16]</sup>
Male gender	√ <sup>[11,12,15]</sup>	√ <sup>[16]</sup>
Increased serum HBV DNA levels	√ <sup>[11,12,14,15]</sup>	
Presence of cirrhosis	√ <sup>[12-14]</sup>	
Increased serum ALT concentration	√ <sup>[11,15]</sup>	
HBsAg positivity	√ <sup>[11,15]</sup>	
Presence of core promoter mutations	√ <sup>[12]</sup>	
Presence of virological remission after 24 mo	√ <sup>[13]</sup>	
Presence of hypoalbuminemia	√ <sup>[13]</sup>	
Decreased serum albumin	√ <sup>[14]</sup>	
Increased serum bilirubin	√ <sup>[14]</sup>	
HBV genotype C	√ <sup>[15]</sup>	
Presence of HBsAg	√ <sup>[15]</sup>	
Family history of HCC	√ <sup>[15]</sup>	
Presence of portal hypertension		√ <sup>[16]</sup>
Presence of hepatic inflammation		√ <sup>[16]</sup>
Increased iron storage levels		√ <sup>[16]</sup>
Presence of sustained virological response		√ <sup>[17]</sup>
Presence of complete viral suppression		√ <sup>[17]</sup>

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase.

## PREVENTION OF HBV-RELATED HCC

HBV infection is the major cause of HCC. Vaccination against HBV is instrumental in the prevention of HCC, and is recommended for all newborns and individuals who are at increased risk for infection. Studies in Taiwan, where universal HBV vaccination was introduced in 1984, have documented a significant decrease in the incidence of HCC in both children and adolescents after the introduction of HBV vaccination as discussed below<sup>[21,22]</sup>.

In patients already chronically infected with HBV, antiviral treatment could prevent disease progression to cirrhosis or HCC. Additionally, periodic surveillance using ultrasonography and serum  $\alpha$ -fetoprotein every 3-6 mo for earlier detection of HCC is also important so that curative treatments (*e.g.*, hepatic resection) can be offered<sup>[23]</sup>.

The antiviral interventions and chemopreventive methods to prevent HBV-related HCC are summarized in Tables 2 and 3 respectively.

### Vaccination

Vaccination plays a central role in HBV prevention strategies worldwide, and a decline in the incidence and prevalence of HBV infection following the introduction of universal HBV vaccination programs has been observed in many countries<sup>[24]</sup>. Control and significant reduction in incidence of new HBV infections as well as HCC have been repeatedly reported in countries in East Asia and Africa<sup>[25]</sup>.

A study of the incidence of HCC in children in

**Table 2 Antiviral interventions for prevention of hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma**

Antiviral interventions	HBV-related HCC	HCV-related HCC
IFN: IFN- $\alpha$	+/- <sup>[28,29]</sup>	√ <sup>[46]</sup>
Pegylated IFN		√ <sup>[47]</sup>
NAs: Lamivudine	√ <sup>[36]</sup>	
Entecavir	√ <sup>[37,38]</sup>	
Ribavirin		√ <sup>[47]</sup>
Vaccination	√ <sup>[21,22]</sup>	
Screening of blood product	√ <sup>[27]</sup>	√ <sup>[27]</sup>

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; IFN: Interferon; NA: Nucleotide analogs.

**Table 3 Chemopreventive agents for hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma**

Chemopreventive agents	HBV-related HCC	HCV-related HCC
Statins	√ <sup>[42]</sup>	
Antidiabetic medications	√ <sup>[42]</sup>	√ <sup>[42]</sup>
Aspirin	√ <sup>[41,53]</sup>	√ <sup>[53]</sup>
Propranolol		√ <sup>[51]</sup>
FASN		√ <sup>[52]</sup>
Dietary agents: Coffee	√ <sup>[54]</sup>	√ <sup>[54]</sup>
Vitamin E	√ <sup>[54]</sup>	√ <sup>[54]</sup>
Vitamin D		√ <sup>[50]</sup>
Fish oil (n-3 PUFA)	√ <sup>[55-57]</sup>	√ <sup>[55-57]</sup>
Phytochemicals: Resveratrol	√ <sup>[43]</sup>	
EGb	√ <sup>[44]</sup>	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PUFA: Polyunsaturated fatty acid; FASN: Fatty acid synthase; EGb: Extract of ginkgo biloba leaf.

Taiwan from 1981 to 1994 showed that the average annual incidence of HCC in children 6-14 years of age declined from 0.70 per 100000 children (between 1981 and 1986), to 0.57 per 100000 (between 1986 and 1990), and to 0.36 per 100000 (between 1990 and 1994). The corresponding rates of mortality from HCC had also decreased. The incidence of HCC in children 6-9 years of age declined from 0.52 per 100000 (for those born between 1974 and 1984) to 0.13 per 100000 (for those born between 1984 and 1986). Since the institution of Taiwan's program of universal HBV vaccination from 1984, the incidence of HCC in children has declined dramatically<sup>[22]</sup>. The risk of developing HCC for vaccinated cohorts was statistically significantly associated with incomplete HBV vaccination. The prevention of HCC by HBV vaccination extends from childhood to early adulthood. Failure to prevent HCC results mostly from unsuccessful control of HBV infection by highly infectious mothers<sup>[21]</sup>.

### Antiviral therapy: Interferon and NAs

DNA integration of hepatitis viruses alters the function of critical genes, promoting malignant transformation of virus-infected liver cells<sup>[26]</sup>. Treatment of CHB infection aims to control viral replication and prevent the development of complications. There are currently seven



drugs available for the treatment of CHB, five NAs and two interferon (IFN)-based therapies. Long-term treatment with NA is often required, and the decision to treat is based on the clinical assessment including the phase of CHB infection and the presence and extent of liver damage<sup>[24]</sup>.

Concerning IFN therapy, a study involving 641 biopsy-proven CHB patients treated with IFN- $\alpha$ 2b were followed up for a median period of 113 months. Although HCC occurred less frequently in biochemical responders than in non-responders, virologic response is not associated with decrease in HCC development. Poor biochemical response, as well as older age and a higher serum AFP level remain independent predisposing factors of HCC development in CHB patients treated with IFN- $\alpha$ <sup>[27]</sup>. In addition, a study about the long-term effects of IFN- $\alpha$  in Chinese patients showed that IFN- $\alpha$  was of no long-term benefit in inducing HBeAg seroconversion or in the prevention of HCC and other cirrhosis-related complications<sup>[28]</sup>.

On the contrary, nearly all the studies showed that NA is able to reduce HCC<sup>[29]</sup>. Many randomized controlled trials showed that lamivudine, one of the earliest oral NAs for antiviral therapy in HBV infection, can reduce disease progression in HBV-related cirrhosis and HCC<sup>[29-34]</sup>. A recent study followed up 293 CHB patients without HCC who were treated with lamivudine for a mean duration of 67.6 mo. In cirrhotic patients, the attainment of maintained viral response (defined as HBV-DNA levels of  $< 4.0$  log copies/mL) during lamivudine treatment was revealed to reduce the risk of HCC development. No significant reduction was observed in the non-cirrhotic group<sup>[35]</sup>.

Entecavir is a potent NA with high genetic barrier to resistance, and prolonged treatment results in regression of fibrosis, hence is currently recommended as first-line antiviral therapy for CHB. In a study of CHB patients with liver cirrhosis, entecavir therapy reduces the risks of hepatic complications, HCC, liver-related and all-cause mortality of CHB patients with liver cirrhosis in 5 years, particularly among those who had sustained viral suppression<sup>[36]</sup>. In another multicentre cohort study, 372 entecavir-treated patients followed up for a mean duration of 114 mo were investigated. Clinical events were defined as development of HCC, hepatic decompensation or death. Virological response to entecavir (HBV DNA  $< 80$  IU/mL) was associated with a lower probability of disease progression in patients with cirrhosis, suggesting that complete viral suppression is essential for NA treatment, especially in patients with cirrhosis<sup>[37]</sup>.

A meta-analysis investigating the effects of IFN or NA on the risk of developing HCC in CHB patients shows that, the reduction in HCC is more significant among patients with early cirrhosis than among non-cirrhotic patients. Five studies ( $n = 2289$ ) compared patients treated by NA with control. The risk of HCC after treatment is reduced by 78%. HBeAg-positive patients have a more significant reduction in HCC risk with treatment.

Patients without cirrhosis benefit more from NA than those with cirrhosis, although resistance to NA blunts the benefit of treatment<sup>[8]</sup>.

In summary, while the evidence of the efficacy of IFN in preventing HBV-related HCC remains conflicting, there is a gradual accumulation of evidence supporting the positive effect of NA on reducing HBV-related HCC.

### Chemoprevention

The observation that anti-platelet therapy inhibits or delays immune-mediated hepatocarcinogenesis suggests that platelets may be one of the key players in the pathogenesis of HBV-associated liver cancer and that immune-mediated necroinflammatory reactions may be an important cause of malignant transformation during chronic hepatitis<sup>[38]</sup>. A prospective study on 300504 patients with chronic liver disease showed that aspirin users had statistically significant reduced risks of incidence of HCC and mortality due to chronic liver disease compared to those who did not use aspirin<sup>[39]</sup>. Further studies are needed to confirm this finding and clarify its underlying mechanism.

A study concerning the association between the use of statins in HBV-infected patients and the risk of HCC shows that statin use may reduce the risk for HCC in HBV-infected patients in a dose-dependent manner<sup>[40]</sup>. This may be related to the effect of statins in reducing fatty change in the liver, and requires future validation studies to confirm the findings.

There are also several investigational drugs which could have potential for chemoprevention against HBV-related HCC. Resveratrol is a natural polyphenol that has beneficial effects across various disease models. In an animal study investigating the efficacy of resveratrol against HBV-related HCC in HBV X protein (HBx) transgenic mice, resveratrol had a pleiotropic effect on HBx transgenic mice in terms of the down-regulation of lipogenesis, the promotion of transient liver regeneration, and the stimulation of antioxidant activity. Furthermore, at later precancerous stages, resveratrol delayed HBx-mediated hepatocarcinogenesis and reduced HCC incidence from 80% to 15%. The potential mechanisms for resveratrol on HCC prevention might be associated with its effects of stimulating the activity of Ampk and SirT1, and downregulating the expression of the lipogenic genes, Srebp1-c and peroxisome proliferator-activated receptor gamma. The decrease in Srebp1-c further downregulates the expression of its target genes, Acc and Fas<sup>[41]</sup>. Several other studies demonstrated resveratrol downregulates cyclin D1 as well as p38 MAP kinase, suppresses Akt and Pak1 expression and activity, and increases ERK activity, suggesting that growth inhibitory activity of resveratrol is associated with the downregulation of cell proliferation and survival pathways, and sensitization to apoptosis<sup>[42]</sup>. Resveratrol also acts as an inhibitor for sirtuins. Overexpression of SIRT1 in cancer tissue has been demonstrated to promote mitotic entry of liver cells, cell growth and proliferation, and inhibit apoptosis related to the PTEN/PI3K/AKT signaling pathway<sup>[43,44]</sup>.

A study in China suggested that extract of Ginkgo Biloba leaf (EGb) could reduce the incidence of the HCC with HBV transgenic mice. The reason may be that EGb could reduce liver HBx, p53, Bcl-2 protein expression in HBV transgenic mice<sup>[45]</sup>. These investigational products would need confirmation in human clinical trials in the future.

## PREVENTION OF HCC RELATED TO HCV

With the commencement of successful vaccination programs against HBV, CHC is now emerging as an important cause of chronic liver diseases. The drive of carcinogenesis during HCV infection is thought to result from the interactions of viral proteins with host cell proteins. Thus, the induction of liver mutation phenotypes through the expression of HCV proteins provides a key mechanism for the development of HCV-associated HCC. With the emerging importance of CHC, mechanisms of HCV-associated hepatocellular carcinogenesis should be clarified to provide insight into advanced therapeutic and preventive approaches to decrease the incidence and mortality of HCC<sup>[46]</sup>.

Strategies aimed at eliminating the virus may provide opportunities for effective prevention of the development of HCC. The first step is to encourage universal precautions to reduce infections transmitted *via* different modalities *e.g.*, iatrogenic routes, sharing of intravenous needles etc and further implementation of universal screening of donated blood products. Concerning therapy for HCV, pegylated IFN plus ribavirin therapy is effective at reducing the risk of HCC in patients with CHC who achieve SVR.

The effects of antiviral therapy and chemopreventive measures in preventing HCC are mentioned in Tables 2 and 3 respectively.

### Antiviral therapy: IFN and ribavirin

Current strategies to reduce HCC incidence in CHC patients include prevention of cirrhosis development by avoiding metabolic, pharmacological, or social factors associated with accelerated progression of liver disease, or through virus eradication by IFN-based treatments. Moreover, a successful antiviral treatment has positive impact on the rate of HCC development in patients who are already cirrhotic<sup>[1]</sup>.

Combination of pegylated IFN and ribavirin therapy is recommended for antiviral therapy worldwide, and is effective in reducing the rate of recurrence of HCV-associated HCC after curative resection or transplantation<sup>[47]</sup>. The pooling of data from the literature suggests a preventive effect of antiviral therapy on HCC development in patients with HCV-related cirrhosis, but the preventive effect is limited to those achieving SVR<sup>[48]</sup>. However, some HCV mutations, such as the amino acid substitution M91L, are associated with treatment failure and a poor prognosis<sup>[47]</sup>.

There is a recent study of the effect of pegylated IFN and ribavirin treatment of CHC on the incidence

of HCC. After a median observation period of 3.6 years, a significantly lower rate of HCC incidence was noted in patients achieving SVR when compared to non-virological responders. A similarly lower rate of HCC incidence was noted among cirrhotic patients achieving SVR (18.9%) when compared to cirrhotic non-virological responders (39.4%)<sup>[18]</sup>.

A meta-analysis study has been performed recently with the data sources from MEDLINE, EMBASE, CINAHL, the Cochrane Library, Web of Science, and the Database of Abstracts of Reviews and Effectiveness from inception through 2012, to systematically review observational studies to determine the association between response to HCV therapy and development of HCC among persons at any stage of fibrosis and those with advanced liver disease. Among HCV-infected persons, there is moderate-quality evidence demonstrating SVR to be associated with reduced risk for HCC; SVR after treatment among HCV-infected persons at any stage of fibrosis is associated with reduced HCC<sup>[17]</sup>.

### Chemoprevention

Vitamin D insufficiency has been associated with the occurrence of various types of cancer. A recent study aimed to determine the relationship between genetic determinants of vitamin D serum levels and the risk of developing HCV-related HCC. The data suggest a relatively weak but functionally relevant role for vitamin D in the prevention of HCV-related hepatocarcinogenesis<sup>[49]</sup>.

Propranolol has antioxidant, anti-inflammatory, anti-angiogenic properties and antitumoral effects and therefore is potentially active in the prevention of HCC. A retrospective long-term observational study suggests that propranolol treatment might decrease HCC occurrence in patients with HCV cirrhosis<sup>[50]</sup>. These findings also need to be verified by prospective clinical trials.

Understanding the interplay between the viral and cellular components of the HCV replication complex could provide new insight for prevention of the progression of HCV-associated HCC. Fatty acid synthase (FASN) is found to interact with NS5B. FASN may thereby serve as a target for the treatment of HCV infection and the prevention of HCV-associated HCC progression<sup>[51]</sup>. Thus, understanding the molecular mechanisms, which are implicated in the development of HCC during the course of HCV infection, may help to design a general therapeutic protocol for the treatment and for its prevention.

## PREVENTION OF HCC RELATED TO HBV AND HCV COINFECTION

HBV and HCV coinfection is not uncommon with an estimated 7-20 million infected individuals worldwide<sup>[52]</sup>. A community-based prospective cohort study evaluating HCC development in HBV and HCV co-infected subjects found the hazard ratios (HRs) of HBV monoinfection, HCV monoinfection, and HBV/HCV coinfection were 17.1, 10.4 and 115.0, respectively. Different geno-

types and multiplicative synergistic effect of HBV and HCV coinfection on HCC risk was observed. Infection with HCV genotype 1 (HR = 29.7) and mixed infection with genotype 1 and 2 (HR = 68.7) significantly elevated HCC risk, much higher than HBV infection. The effect of different HCV genotypes and the multiplicative synergistic effect of HBV/HCV coinfection on HCC risk underline the need for comprehensive identification of hepatitis infection status in order to prevent and control HCC<sup>[53]</sup>.

Pegylated interferon-alpha plus ribavirin should be recommended in patients with dominant HCV replication. However, HBV rebound may occur after elimination of HCV with anti-HBV treatment required. These therapeutic measures may contribute to the prevention of HCC this special group of patients<sup>[52]</sup>.

## OTHER POTENTIAL CHEMOPREVENTIVE METHODS

The use of aspirin, but not nonsteroidal anti-inflammatory drugs, is associated with a decreased risk of HCC and death from chronic liver disease in the National Institutes of Health-AARP Diet and Health Study of patients between the ages of 50 and 71 years<sup>[39]</sup>. However this study does not provide information on the HBV and HCV status of its participants, and would need confirmation by future studies specifically for the HBV- and HCV-infected population.

More recent data have suggested dietary factors, including increased intake of coffee<sup>[54]</sup>, unsaturated fatty acids and fish to be protective against HCC. Subjects with known HBV or HCV status, and subjects who were anti-HCV and/or hepatitis B surface antigen positive were analysed. Consumption of n-3 polyunsaturated fatty acid (PUFA)-rich fish or n-3 PUFAs, particularly eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid, appears to protect against the development of HCC, even among subjects with HBV and/or HCV infection<sup>[55]</sup>, probably through dampening the inflammation in the liver and decreasing formation of tumor necrosis factor (TNF)- $\alpha$ , and through simultaneously inhibition of COX-2 and beta-catenin<sup>[56,57]</sup>. The findings also point to a potential anticancer role for the n-3 PUFA-derived lipid mediators 18-HEPE and 17-HDHA, which can down-regulate the important proinflammatory and proliferative factor TNF- $\alpha$ .

## CONCLUSION

Clinical experts evaluated ten previously identified dimensions of HCC control: clinical education; risk assessment; HBV strategy; HCV strategy; life-style risk factors; national statistics; funding for screening; funding for treatment; political awareness; and public awareness. Of these strategies, the most significant needs in regional efforts to control HCC are political awareness, public awareness, and life-style risk factors<sup>[58]</sup>.

HCC is a challenging malignancy of global importance. As HCC is strongly associated with chronic viral hepatitis, prevention against the infection is crucial for prevention against HCC. Vaccination against HBV in the newborns and early childhood is highly effective to lower infection rates substantially. For HCV, universal precautions when dealing with human blood, education on high-risk behaviours and screening programs for blood donors can reduce infection rates. Although prevention and treatment of CHB and CHC have been improved within the last decades even in high-risk countries, further effective and sustainable reduction of these infections is still needed<sup>[26]</sup>.

Antiviral therapies for CHB and CHC, while important, can only reduce but not completely eliminate HCC. Improvement in identification of infected persons, accessibility of care and affordability of treatment are needed for antiviral therapy to have a major impact on the global incidence of HCC<sup>[59]</sup>. Further advances in our understanding of the molecular pathogenesis of HCC hold promise in improving the diagnosis and treatment of this highly lethal cancer<sup>[4]</sup>.

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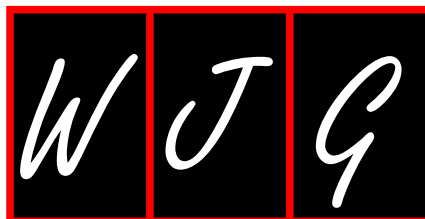
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WJG 20<sup>th</sup> Anniversary Special Issues (1): Hepatocellular carcinoma

## Effects of antiviral therapy on preventing liver tumorigenesis and hepatocellular carcinoma recurrence

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### Abstract

Chronic hepatitis B virus (HBV) infection is the key driving force of liver disease progression, resulting in the development of hepatic dysfunction, cirrhosis and hepatocellular carcinoma (HCC). The primary aim of therapy is to suppress or eliminate HBV replication to reduce the activity of hepatitis, thus reducing the risk of, or slowing the progression of, liver disease. Nucleos(t)ide analogues (Nucs) may result in rapid suppression of HBV replication with normalization of serum transaminases and restore liver function, thus increasing survival in patients with hepatic decompensation. Long-term Nuc therapy may result in histological improvement or reversal of advanced fibrosis and reduction in disease progression, including the development of HCC. The long-term benefits of a finite course of interferon (IFN)- $\alpha$  therapy also include a sustained and cumulative response, as well as hepatitis B surface antigen seroclearance and reduction in the development of cirrhosis and/or HCC. Pegylated IFN and newer Nucs may achieve better long-term outcomes because of improved efficacy and a low risk of drug resistance.

However, treatment outcomes are still far from satisfactory. Understanding the effects of anti-HBV treatment against HCC incidence and recurrence after hepatectomy or liver transplantation is required for further improvement of outcome.

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**Key words:** Hepatocellular carcinoma; Antiviral therapy; Carcinogenesis; Recurrence; Nucleos(t)ide analogues; Interferon; Retrospective study; Clinical trial

**Core tip:** Chronic hepatitis B virus (HBV) infection is the key driving force of hepatocellular carcinoma (HCC). In this review, we discussed the mechanism of HBV induction of HCC and described the current trends in anti-HBV therapy. The associations of anti-HBV therapy with prevention of HCC incidence and recurrence after curative operations were also summarized. Moreover, based on our center's experiences, a standardized antiviral strategy was suggested which greatly benefited those patients who underwent hepatectomy and liver transplantation with regard to better clinical results.

Tan ZM, Sun BC. Effects of antiviral therapy on preventing liver tumorigenesis and hepatocellular carcinoma recurrence. *World J Gastroenterol* 2013; 19(47): 8895-8901 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/8895.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.8895>

### INTRODUCTION

Hepatitis B virus (HBV) is a member of the Hepadnaviridae family, which includes small enveloped DNA viruses. HBV infection affects > 2 billion people worldwide and is a significant cause of liver cirrhosis and hepatocellular

carcinoma (HCC), which increases morbidity and mortality in these patients<sup>[1]</sup>. HBV targets and replicates in hepatocytes, and the risk of developing HCC among HBV carriers is 10 to 100-fold greater compared with that in uninfected people<sup>[2]</sup>. Treatment with antiviral drugs such as nucleos(t)ide analogues (Nucs) or interferon (IFN)- $\alpha$  may result in rapid suppression of HBV replication and thus reduce the progression of fibrosis and the development of HCC<sup>[3]</sup>. Although localized or systemic radiation and chemotherapy have been used to eliminate the tumor mass, surgical resection or liver transplantation are still the most effective treatments, but relapse is common<sup>[4]</sup>. It is widely accepted that comprehensive treatment is required for prevention of HBV-associated HCC development and recurrence. Thus, this review highlights the mechanism of HBV induction of HCC and discusses the current trends in anti-HBV therapy for prevention of HCC and its recurrence.

## HBV INFECTION INDUCES CHRONIC INFLAMMATION AND CANCER TRANSFORMATION

HBV persistently replicates in immortalized hepatocytes *in vitro* without overt cellular damage or death, implying that the viruses are not directly cytopathic, and the pathogenesis of hepatitis is immune mediated<sup>[5,6]</sup>. Liver injury in response to inflammatory hepatitis elicits an inflammatory response in non-parenchymal cells (NPCs), such as myeloid Kupffer cells and hepatic stellate cells. Toll-like receptor-nuclear factor (NF)- $\kappa$ B signaling activation may trigger an innate immune response and inhibit virus replication in HBV-transgenic mice<sup>[7]</sup>. NPCs secrete NF- $\kappa$ B-regulated hepato-mitogens [*e.g.*, tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, and hepatocyte growth factor], which promote compensatory proliferation of quiescent hepatocytes carrying HBV-induced mutations. This process allows for the transmission of genetic alterations to daughter cells, thereby favoring liver neoplastic progression. Alternatively, autocrine secretion of transforming growth factor (TGF)- $\beta$  by hepatocytes induces cell survival and proliferation in the absence of liver damage and independent of NPC-mediated secretion of hepato-mitogens. Increased proliferation is then followed by dysplasia, adenoma, and HCC formation<sup>[8,9]</sup>. In conclusion, NF- $\kappa$ B activation-associated carcinogenesis most likely depends on downstream hepato-mitogen release and death-driven compensatory proliferation<sup>[9,10]</sup>.

HBV X (HBx) protein is encoded by the smallest HBV open reading frame and is 154 amino acids in size, with a molecular weight of approximately 17.5 kDa. HBx can localize to the mitochondria where it acts as an adaptor or kinase activator to influence signal transduction pathways such as: protein kinase C, Janus kinase/signal transducer and activator of transcription (JAK/STAT), phosphoinositide 3-kinase, stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), Ras-Raf-

mitogen-activated protein kinase (Ras-Raf-MAPK), and extracellular signal-regulated kinase (ERK). This may provide a unified mechanism by which HBx exerts many of its pleiotropic activities, including transcription, cell cycle control, and apoptosis<sup>[11-14]</sup>. It is also reported that HBx can activate NF- $\kappa$ B directly, which could be partially *via* upregulated inhibitor of NF- $\kappa$ B kinase activity and the mammalian target of rapamycin (mTOR) pathway<sup>[15]</sup>. As mentioned above, various inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8, which play an important role in the inflammation-carcinogenesis axis of the liver, are NF- $\kappa$ B activation-mediated, and IL-6 is thought to be one of the most important<sup>[16]</sup>. Recently, our group found that IL-22 could also promote HCC *via* STAT3 activation, suggesting that inflammatory cytokines have also attracted considerable attention as mediators of the association between inflammation and hepatocarcinogenesis<sup>[17]</sup>.

Furthermore, HBx could interfere with the anti-tumor immune response *via* other inflammatory cells. Infected intrahepatic natural killer cells are also known to induce cytolytic activity without IFN- $\gamma$  production, suggesting that hepatocellular killing occurs without virus clearance<sup>[18]</sup>. Dendritic cells may be infected with HBV, which will cause defective chronic HBV infection, resulting in poor adaptive immunity<sup>[19]</sup>. CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> regulatory T cells could be induced by HBx-stimulated production of TGF- $\beta$ 1, and their crosstalk with Th17 cells may contribute to an immune tolerance-clearance balance in the liver<sup>[20]</sup>.

## MECHANISMS OF HBV ONCOGENESIS

Increasing evidence suggests that HBV contributes to HCC by directly modulating pathways that may promote the malignant transformation of hepatocytes. Firstly, HBV insertions are associated with host large genetic alterations: deletions, duplications and chromosomal translocations. These events could either induce chromosome changes or act "in cis" on the expression or function of nearby cellular genes that contribute to chromosome instability<sup>[21,22]</sup>. For instance, integration of HBx gene fragments (316-462/262-462 bp) could directly transform human immortalized normal liver L02 cells in studies using a cell model. Further, these integrations could be detected in five of 44 clinical HBV-positive HCC tissues<sup>[23]</sup>. Integration at specific sites in host genes may contribute to a growth advantage in a clonal cell population but subsequent additional mutations will eventually accumulate. Evidence was first provided in two independent HCCs, with retinoic acid receptors and cyclin A being targeted by HBV integration in tumors<sup>[24]</sup>. Recently, more genes involved in cell survival, proliferation and immortalization were also reported as the HBV integration target, such as human telomerase reverse transcriptase (hTERT, a regulator of telomerase), platelet-derived growth factor receptor, calcium signaling-related genes, and ribosomal protein genes<sup>[25]</sup>.

Although it is suggested that upregulated expression of HBx and HBV S proteins is associated with hepatocarcinogenesis in transgenic mouse models, the exact mechanism remains unclear<sup>[26]</sup>. It is worth noting that hepatocytes in cirrhotic livers display decreased proliferation rates with a dominant replicative senescence phenotype characterized by critically shortened telomeres and permanent cell cycle arrest<sup>[1]</sup>. However, during hepatocyte proliferation, low or absent telomerase activity in cirrhotic liver is associated with upregulated HBx or pre-S2 protein<sup>[27]</sup>. In a study of 55 HCC and 17 chronic hepatitis patients, hTERT was positive in 81% of HCCs, and the mean telomere length in HCC was significantly shorter compared with that in chronic hepatitis<sup>[28]</sup>.

HBx is also suggested to have the ability to induce direct chromosomal instability by interfering with the mitotic checkpoints<sup>[29,30]</sup>. HBx induces epigenetic changes, including DNA methylation aberration, histone modification and miRNA expression. Jiang *et al.*<sup>[31]</sup> reported that increased miR-22 is associated with HCC development in male patients. Xu *et al.*<sup>[32]</sup> also suggested that suppression of miR-148a upon HBx activation can enhance tumorigenesis. Moreover, HBx binds and inactivates p53, and interacts with DNA damage-binding protein 1 (DDB1, the DNA repair protein), which may affect repair functions and allow the accumulation of genetic changes, and also confer resistance against nucleolar stress and anti-cancer drugs<sup>[33]</sup>.

## CURRENT OPINION IN ANTIVIRAL THERAPY

Despite dramatic improvements in the treatment of patients against HBV over the past decade, treatment of chronic HBV infection is currently based on two different strategies: (1) IFN- $\alpha$  or thymosin- $\alpha$ 1 (T-a1) aimed at inducing a sustained antiviral response; and (2) oral anti-HBV Nucs to achieve long-term complete suppression of HBV replication<sup>[34]</sup>.

The first strategy is typically used in patients with less advanced liver disease, with high alanine aminotransferase (ALT) and not too high HBV DNA replication. It is particularly successful in younger patients and in those infected with HBV genotype A or B. Since the first introduction of IFN- $\alpha$  in 1976, the long-term benefit of IFN therapy has included a sustained and cumulative immune response. T-a1 is an immunomodulator that triggers maturational events in lymphocytes and T-cell function. It can promote reconstitution of immune defects and promote disease remission and cessation of HBV replication in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B, without significant side effects<sup>[35,36]</sup>. Eighteen patients with HBeAg-positive and serum HBV DNA-positive chronic hepatitis B received 6 mo of treatment with 1.6 mg subcutaneous T-a1 twice weekly<sup>[37]</sup>. They achieved better HBV loss and seroconversion than 30 patients receiving 6 mo of 3-5 MU subcutaneous IFN- $\alpha$  (injection daily for 15 d, then three times weekly).

The results of this trial indicate that T-a1 is of potential interest in patients with anti-HBe- and HBV DNA-positive chronic hepatitis B.

The introduction of 12-kDa linear polyethylene glycol (PEG) for IFN- $\alpha$ 2b and 40-kDa branched PEG for IFN- $\alpha$ 2a has allowed weekly rather than daily or three times weekly injection<sup>[34]</sup>. This has had a significant impact on the tolerability and ease of use. In addition, for patients with HBeAg-positive chronic hepatitis B, Pegylated IFN (PEG IFN)- $\alpha$ 2a offers superior efficacy over lamivudine, on the basis of HBeAg seroconversion, HBV DNA suppression, and hepatitis B surface antigen (HBsAg) seroconversion<sup>[37]</sup>. Overall, PEG IFN- $\alpha$  is an ideal treatment strategy in selected patients with HBeAg-negative chronic hepatitis B, because of its well-recognized and predictable safety profile and unique mechanism of antiviral activity leading to long-lasting immune control.

For high HBV DNA levels, Nucs are typically adopted for patients with more advanced liver disease, and for those who have failed or cannot tolerate IFN therapy. However, the main limitation is the development of resistance: for example, after 5 years of therapy with lamivudine (L-nucleoside), 76% of patients developed resistance. Telbivudine, another L-nucleoside, is more potent than lamivudine but resistance still developed in 25% of HBeAg-positive and 11% of HBeAg-negative patients after 2 years. Adefovir, an acyclic phosphonate, is relatively weak, but is effective against lamivudine- and telbivudine-resistant mutations, and it should be used in combination rather than substituted. Resistance to adefovir develops relatively slowly, rising to 29% for HBeAg-negative patients after 5 years, but more rapidly when used alone for lamivudine-resistant HBV. Currently, the two first-line Nucs are entecavir and tenofovir. Entecavir, a cyclopentane (D-nucleoside), is very potent, with 94% of patients having undetectable HBV DNA after 5 years. Resistance develops in only 1.2% of treatment-naïve patients. Tenofovir, another acyclic nucleotide, is more potent with less renal toxicity compared to adefovir. It is effective against lamivudine-resistant mutations when used alone. No resistance to tenofovir has been described after its use for 3 years or longer, often for patients with human immunodeficiency virus/HBV co-infection<sup>[38]</sup>.

In conclusion, for patients with HBeAg-positive chronic hepatitis B, PEG IFN- $\alpha$  offers superior efficacy on the basis of HBeAg seroconversion, HBV DNA suppression, and HBsAg seroconversion. As a result of these features, new therapeutic regimens based on combinations of PEG IFN- $\alpha$  and third-generation Nucs such as entecavir and tenofovir are being developed to increase the rate of HBsAg seroclearance, which remains the ideal endpoint in all HBeAg-negative chronic hepatitis B patients.

## ANTIVIRAL THERAPY SUPPRESSES THE CHRONIC INFLAMMATION-CANCER TRANSITION

A prospective cohort study with 11 years follow-up



showed that HBV DNA concentration  $> 10^4$  copies/mL is an especially strong predictor of risk of developing HCC in individuals aged  $\geq 30$  years, independent of the level of serum ALT<sup>[39]</sup>. It is accepted that anti-HBV therapy can improve the outcome of chronic HBV infection in terms of HCC incidence.

In a randomized controlled trial of 101 male patients in the Taiwan region, cumulative incidence of HCC development was significantly decreased in the IFN- $\alpha$ -treated group (1 of 67 patients) than in the control group (4 of 34 patients), at 1.1-11.5 years after the end of therapy<sup>[40]</sup>. In addition, a retrospective study suggested that natural lymphoblastoid IFN- $\alpha$  (IFN- $\alpha$  nl) and IFN therapy may provide better long-term beneficial effects than placebo in terms of HBV clearance, reduction of HCC, and prolonged survival. HCC was detected in 1.5% of the IFN- $\alpha$  nl group, 3.7% of the IFN- $\alpha$ 2a group and 14.7% of the control group.

As for the long-term benefits of Nucs, in a randomized control trial, HCC occurred in 3.9% of patients treated with lamivudine and 7.4% of those in the placebo group in a total of 651 patients (HR = 0.49,  $P = 0.047$ )<sup>[41]</sup>. A retrospective multicenter study of 377 Japanese patients receiving lamivudine treatment for up to 96 ( $23.1 \pm 19.0$ ) mo showed a marked reduction in the incidence of HCC compared with a historical control group matched for age, sex, liver fibrosis score, albumin level and platelet count (0.4% per year *vs* 2.5% per year,  $P < 0.001$ )<sup>[42]</sup>. In another study of 656 HBeAg-negative patients (54% had chronic hepatitis, 30% had cirrhosis), lamivudine (median 22 mo, range 1-66) was highly effective in reducing viral load in HBeAg-negative patients, and HBV suppression reduced the development of HCC and disease worsening in patients with cirrhosis<sup>[43]</sup>. A Korean study also showed a reduced incidence of HCC in patients with compensated cirrhosis who received lamivudine therapy (4.9%) compared to untreated patients or patients treated with lamivudine who had viral breakthrough (11.8%) or a sub-optimal response (19.4%)<sup>[44]</sup>. In a recent systemic review, Papatheodoridis *et al*<sup>[45]</sup> reviewed 21 studies that included 3881 treated and 534 untreated patients and found that HCC developed less frequently in Nuc-treated patients (2.8% *vs* 6.4%,  $P < 0.003$ ).

In a systematic review of 11 studies of the effect of IFN and Nuc therapy on the outcome of HBV infection over the past 10 years, Sung *et al*<sup>[46]</sup> indicated that IFN- $\alpha$  or Nuc treatment significantly reduced the risk of HCC. Although IFN benefited patients with cirrhosis, Nucs benefited those with non-cirrhosis and HBeAg-positive infection. From the experiences mentioned above, sustained HBV suppression induced by IFN- $\alpha$  and Nuc therapy may be necessary to reduce the development of HCC in HBV-infected patients.

## EFFECT OF ANTIVIRAL THERAPY ON HCC RECURRENCE

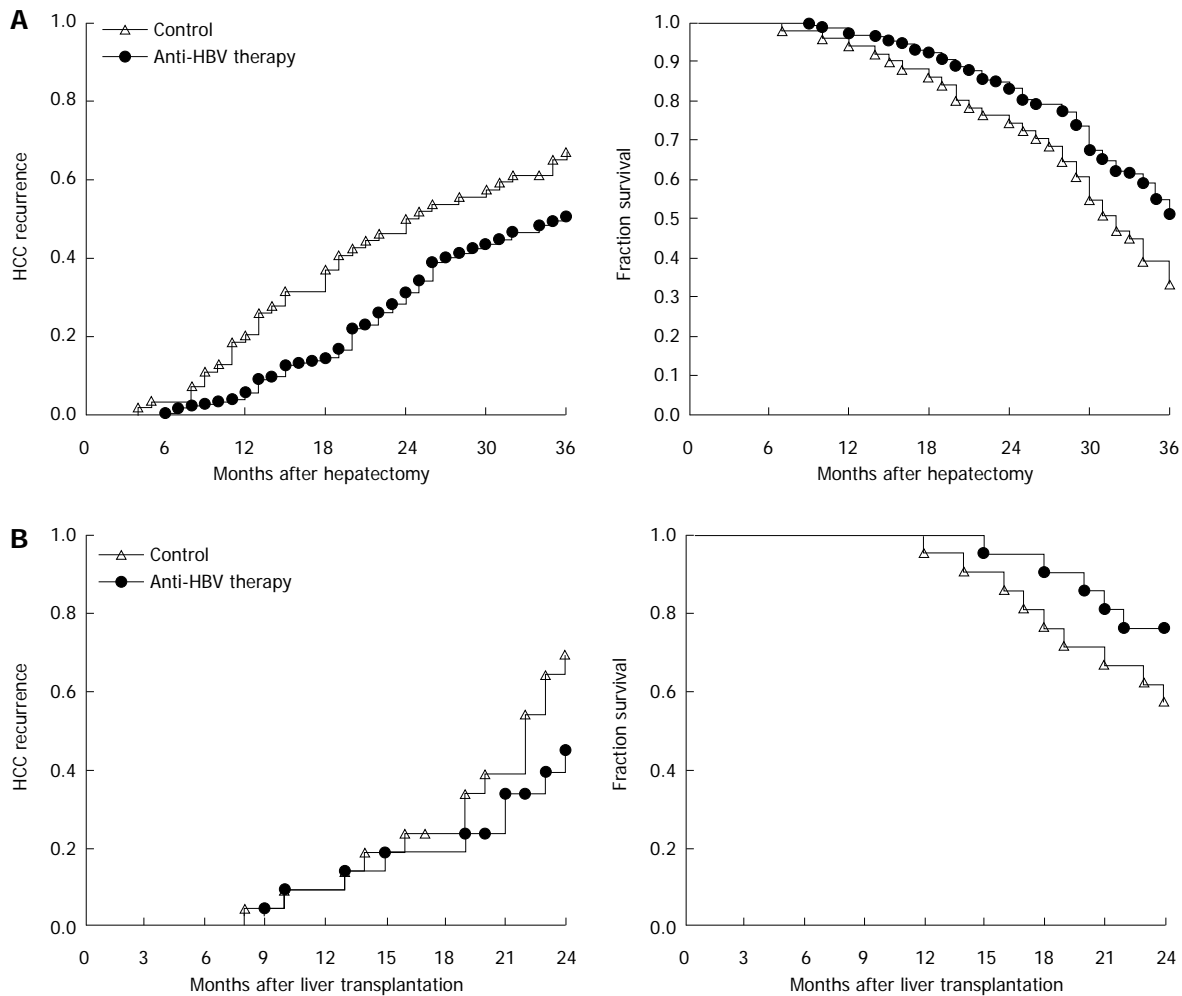
Does anti-HBV therapy decrease the risk of HCC recur-

rence after the most effective methods to reduce tumor burden: partial hepatectomy or liver transplantation? As suggested previously, the 5-year overall survival for all early HCC patients was 58% (transplantation: 63%; resection: 53%)<sup>[47]</sup>. Huang *et al*<sup>[48]</sup> reported that patients with HBV reactivation after liver resection have a higher liver failure rate, lower 3-year disease-free survival rate, and lower overall survival rate than those without reactivation (11.8% *vs* 6.4%,  $P = 0.002$ , 34.1% *vs* 46.0%,  $P = 0.009$ , and 51.6% *vs* 67.2%,  $P < 0.001$ , respectively).

Exploratory subset analysis showed that adjuvant IFN- $\alpha$  had no survival benefit for pTNM stage I / II tumor (5-year survival 90% in both groups;  $P = 0.917$ ) but prevented early recurrence and improved the 5-year survival of patients with stage III / IVA tumor from 24% to 68% ( $P = 0.038$ )<sup>[49]</sup>. Lee *et al*<sup>[50]</sup> also reported that metastasis-associated protein 1-positive HCC recurred post-operatively in 26 of 93 patients (28%), although the PEG IFN group had significantly lower overall cumulative recurrence rates than the control group (7% and 14% *vs* 24% and 34% at 1 and 2 years, respectively;  $P < 0.05$ ). In addition, the 1- and 2-year cumulative survival rates were higher in the PEG IFN group compared with the control group (100% *vs* 93% and 100% *vs* 87%, respectively;  $P < 0.05$ ). In a report of 237 HCC patients after hepatectomy treated with IFN- $\alpha$  or placebo within comparable clinicopathological parameters, the median overall survival was 63.8 mo in the IFN- $\alpha$  group and 38.8 mo in the placebo group ( $P = 0.0003$ ), and the median disease-free survival period was 31.2 *vs* 17.7 mo ( $P = 0.142$ )<sup>[51]</sup>. Chen *et al*<sup>[52]</sup> showed that adjuvant IFN- $\alpha$ 2b treatment was associated with a significantly higher incidence of leukopenia and thrombocytopenia and did not reduce postoperative recurrence of viral hepatitis-related HCC.

Regarding the effect of Nucs on HCC recurrence, Anselmo *et al*<sup>[53]</sup> suggested that hepatitis B immunoglobulin (HBIG) and lamivudine treatment markedly reduced HBV recurrence rate and significantly improved 1- and 3-year recurrence-free survival rates after liver transplantation. Chan *et al*<sup>[54]</sup> also reported that the 1-, 3- and 5-year disease-free survival rates in patients treated with lamivudine or entecavir were 66.5%, 51.4% and 51.4% compared with 48.9%, 33.8% and 33.8%, respectively, in the control group. Kubo *et al*<sup>[55]</sup> reported that the tumor-free survival rate after hepatectomy was significantly higher in the lamivudine than the control group. Recently, multivariate analysis showed that HCC recurrence after transplantation was markedly associated with HBV reinfection. However, HBIG was associated with worse survival as well as HBV reinfection and HCC recurrence ( $P = 0.002$ ,  $P < 0.001$  and  $P < 0.001$ , respectively)<sup>[56]</sup>.

In our center (Liver Transplantation Center, The First Affiliated Hospital of Nanjing Medical University), we suggest that HBsAg-positive patients who have  $> 10^4$  /mL or  $10^3$ - $10^4$  /mL HBV DNA copies with impaired liver function, must take lamivudine after curative surgery. Moreover, for those who have HBV YMDD mutation during initial treatment, entecavir and/or adefovir dipivoxil should be used as the replacement. If drug resistance



**Figure 1** Comparison of hepatocellular carcinoma recurrence and outcome in patients who received anti-hepatitis B virus therapy or placebo after hepatectomy or liver transplantation. A: From September 2009 to May 2010, 224 HCC patients who received partial hepatectomy due to HBV-related HCC were enrolled. HCC recurrence and 3-year overall survival rate in patients with anti-HBV treatment ( $n = 173$ ) and patients without standardized anti-HBV treatment ( $n = 51$ ) were monitored for at least 3 years. Left: log-rank test,  $P = 0.013$ ; right: log-rank test,  $P = 0.006$ ; B: From January 2010 to August 2011, 42 HCC patients within Milan criteria who received liver transplantation were enrolled. HCC recurrence and 2-year overall survival rate in patients with anti-HBV treatment ( $n = 28$ ) and patients without standardized anti-HBV treatment ( $n = 14$ ) are shown. Left: log-rank test,  $P = 0.031$ ; right: log-rank test,  $P = 0.045$ . HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

occurs, tenofovir disoproxil fumarate could be used instead. In our randomized controlled clinical study, we verified that standardized anti-HBV therapy could significantly improve the outcome and decrease the recurrence of patients who underwent partial hepatectomy and liver transplantation (Figure 1).

## CONCLUSION

This literature review describes two different aspects of the tumorigenesis of chronic HBV infection: the direct mechanism by which HBV DNA and its main product HBx induce host DNA instability; and HBV infection-associated liver inflammation and imbalanced immunoregulation. We also briefly introduce the current strategy against HBV infection and show that timely usage of Nucs and immunomodulatory agents can eventually prevent further disease progression, including HCC, in patients with chronic HBV infection. Long-term studies

will probably confirm that new antiviral drugs such as entecavir, tenofovir and telbivudine can offer even more opportunities for reducing disease progression than lamivudine therapy does.

For patients who undergo hepatectomy or liver transplantation as curative treatment for HCC, tumor recurrence must be monitored by ultrasound and  $\alpha$ -fetoprotein assay. More importantly, from our experience, HBV replication should also be monitored because sustained HBV activation or relapse is significantly related to HCC development and recurrence. Standardized anti-HBV treatment can ultimately delay HCC recurrence and benefit survival.

Since PEG-IFN, as the newly introduced IFN, offers a better opportunity to suppress HBV replication in patients who do not have cirrhosis or fibrosis, it should provide promising prospects in reducing HCC development and recurrence. While in many third world countries, lamivudine is still the first-line drug, mass usage of

newly developed IFN could be used more frequently in the future and show better prospects.

In conclusion, developing safe and affordable agents, as well as management strategies to improve sustained or maintained HBV suppression, should be the ultimate goals in the management of chronic HBV infection.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Exploitation of host clock gene machinery by hepatitis viruses B and C

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and the metabolic syndrome. Viruses triggering hepatitis depend tightly on the host cell synthesis machinery for their own replication, survival and spreading. Recent evidences support a link between the circadian clock circuitry and viruses’ biological cycle within host cells. Currently, *in vitro* models for chronobiological studies of cells infected with viruses need to be implemented. The establishment of such *in vitro* models would be helpful to better understand the link between the clock gene machinery and viral replication/viral persistence in order to develop specifically targeted therapeutic regimens. Here we review the recent literature dealing with the interplay between hepatitis B and C viruses and clock genes.

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**Key words:** Hepatitis C virus; Hepatitis B virus; Anti-hepatitis therapy; Clock genes; Chronobiology

**Core tip:** New antiviral strategies have been developed, including the interferon/ribavirin-free therapy, to control hepatitis viruses replication. Although, IFN-free regimens have generated excitement among scientists, for the reason that they are better tolerated, they are not still able to completely eradicate the viruses. Here we underline the circadian relationship between host cell and hosted hepatitis viruses, that has to be taken into account in order to optimize the timing of therapeutic regimens, not only to minimize the pharmacological agents’ toxicity but also to improve the efficacy of treatment modalities through optimized timing of therapeutic regimens, targeting in a better way virus replication.

## Abstract

Many aspects of cellular physiology display circadian (approximately 24-h) rhythms. Dysfunction of the circadian clock molecular circuitry is associated with human health derangements, including neurodegeneration, increased risk of cancer, cardiovascular diseases

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## INTRODUCTION

Viruses are among the most important human carcinogens<sup>[1]</sup>. Numerous mechanisms have been described to be dysregulated by viruses, always focusing on the impairment of the most well known tumor suppressors and/or oncogene proteins and their signaling pathways<sup>[2,3]</sup>.

It has been already established that alteration of the circadian clock molecular circuitry is involved in carcinogenesis. Circadian defects have also been associated with liver diseases, including hepatocellular carcinoma (HCC)<sup>[4,5]</sup>, a condition in which viruses play a role in disease pathogenesis and progression.

Specifically, basic cell functions and processes, such as cell division, proliferation, growth, differentiation, autophagy, apoptosis and metabolism, show time-related fluctuations, and when the period of oscillation is about 24 h the rhythmicity is defined as circadian<sup>[6-10]</sup>. At the cellular level, circadian rhythmicity is driven by a molecular clockwork comprised of a translational-transcriptional feedback loop realized by a set of genes, called core clock genes, coding for proteins that in turn suppress gene expression in a cycle that completes itself each day. Clock genes are transcriptionally activated by the transcription factors circadian locomotor output cycle kaput (CLOCK) and aryl hydrocarbon receptor nuclear translocator-like (ARNTL). The latter two protein heterodimerize and bind to the E-box enhancer elements in the promoters of the Period (*PER* 1, 2 and 3) and Cryptochrome (*CRY* 1 and 2) genes. The *PER* and *CRY* mRNAs are translated into *PER* and *CRY* proteins that form a repression complex, which in turn translocates back into the nucleus, interacts directly with *CLOCK* and *ARNTL* blocking their activity<sup>[4,11-13]</sup>.

Among the processes regulated by the clock gene machinery are pathways of cell metabolism and vesicle trafficking, suggesting the potential role for the circadian clock circuitry in the regulation of viral expression/replication<sup>[14]</sup>. A relationship between circadian dysfunction and tumorigenesis has also been found at both the cellular and the organismal levels, indicating that the circadian clock may impact on the development of cancer<sup>[15-17]</sup>, a disease also influenced by viruses. Recently, scientific evidences support a functional connection between viral expression/replication and circadian dysfunction in the pathogenesis of liver diseases<sup>[14,18-20]</sup>. However, whether the circadian clock directly regulates viral cell cycle in mammalian cells, or whether viruses may play a role in the cycling of mammalian cell clocks is not yet totally clear.

The implication of viral expression/replication and circadian dysfunction in the pathogenesis of liver diseases suggests that a functional connection between these two processes may exist as it has been already showed<sup>[14,18-20]</sup>. Nevertheless, the relationship between circadian cycles

and viral expression/replication is an intriguing area for future study and it has implications for multiple human diseases. The study of new causes which are able to influence the clock genes expression are under investigation as disruption of biological clocks is implicated in a variety of disorders including fatty liver disease, obesity and diabetes<sup>[21,22]</sup>. Exciting data reported the influence of hepatitis B and C viruses on the hepatic clock genes<sup>[18,19]</sup>, demonstrating for the first time that these viruses are able to impair the inner molecular clockwork, presumably to better exploit the host-cell replication machinery. Hepatotropic viruses impair also liver functions, and this effect may be a cause or a consequence of the disruption of the inner cellular biological clock. At the present, the relationship between hepatitis viruses expression/replication and the circadian clock is poorly understood. Here we review the scientific reports addressing the interaction between hepatitis B and C viruses and the molecular clockwork.

## LIVER AND CLOCK GENES

The liver plays an important role in maintaining energy homeostasis within the organism. The major biochemical reactions occurring within the liver are involved in glucose breakdown/genesis, which is strictly linked to fatty acid metabolism (biosynthesis/beta oxidation). All these biochemical reactions and the metabolic networks must be finely coordinated in order to avoid unnecessary interference between the pathways<sup>[21]</sup>. To this end, reactions are separated locally and temporally. Hepatic metabolic functions show rhythmic fluctuations with 24-h periodicity<sup>[23]</sup>, driven by molecular clockworks ticking through translational-transcriptional feedback loops and operated by a set of genes, called clock genes, encoding circadian proteins<sup>[4]</sup>. In the absence of environmental cues, specifically light:dark cycle, it has been demonstrated that rhythmic food intake influences the hepatic circadian oscillator<sup>[23,24]</sup>. Hence, the clock genes oscillations are not phase locked but are flexible to enable adjustment to the changing environments<sup>[23]</sup>.

In the liver, gene expression profiling has shown that transcriptional processes display approximately 24-h rhythmicity and have a crucial role in metabolic processes. Energy and nutrient homeostasis at both cellular and organismal levels is guaranteed by nearly constant adjustments of metabolic gene expression, and the transcriptional networks that regulate glucose and lipid metabolism are sensitive to nutritional status, responding to diverse physiological signals<sup>[25]</sup>. The fractions of cyclic transcripts depending on systemic signals and local oscillators amount to approximately 14% and 86%, respectively. The systemically regulated liver genes include immediate early genes (IEG), which convey rhythmic signals to core clock genes of hepatocyte oscillators and thus are involved in the synchronization of liver clocks, and tissue specific output genes, directly participating in rhythmic liver physiology and metabolism. The IEG

class contains several heat shock protein genes, known to be regulated by heat shock transcription factor 1 (HSF1) and target genes of serum response factor 1 (SRF1), and these immediate early transcription factors (IETFs) act as sensors of blood-borne signals, driving the synchronization of circadian clocks<sup>[26]</sup>. Metabolite sensing is linked to transcriptional responses in hepatocytes by nuclear receptors through switching between co-activator and co-repressor recruitment<sup>[27]</sup>. Nuclear hormone receptors comprise a unique class of transcriptional regulators that are capable of sensing the concentrations of metabolites, including lipids, oxysterols, heme, and bile acids<sup>[28]</sup>. An important role in the control of glucose, lipid, and mitochondrial oxidative metabolism is played by the expression of co-regulators, in particular the PGC-1 $\alpha$ , which is highly responsive to nutritional status and other physiological signals<sup>[29]</sup>. The cross-talk between circadian rhythms and metabolism is operated also by the peroxisome proliferator-activated receptors (PPAR), in particular  $\alpha$  and  $\gamma$ <sup>[30]</sup>. Both factors are already known to be dysregulated by hepatitis B and C viruses. PPAR $\alpha$  regulates transcription of genes involved in lipid and glucose metabolism upon binding of endogenous free fatty acids<sup>[31]</sup>. PPAR $\gamma$  binds eicosanoids deriving from either omega-3 ( $\omega$ -3) or omega-6 ( $\omega$ -6) fatty acids and their oxidized counterparts, is rhythmically expressed, its expression is regulated by PER2 and in turn directly regulates ARNTL transcription<sup>[32]</sup>. The clock gene machinery drives the expression of a large array of enzymes involved in lipid metabolism, controls lipogenesis and regulates triglyceride packaging into chylomicrons (globules that transport dietary lipids) at the level of the intestine, whereas in the liver, clock disruption triggers lipid accumulation<sup>[33-35]</sup>. In liver ARNTL and CLOCK control gene expression of enzymes involved in glucose and lipid homeostasis, as well as in bile acid and apolipoprotein biosynthesis<sup>[36]</sup>. Diurnal oscillation characterizes a number of proteins involved in lipid metabolism [such as hepatic cytochrome P450 cholesterol 7  $\alpha$ -hydroxylase, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, or apolipoprotein AIV] show in both humans and rodents. REV-ERB  $\alpha$  links the clock with the master pathway of hepatic lipid metabolism, is involved in bile acid synthesis and sterol regulatory element-binding protein (SREBP) signaling and SREBPs control both fatty acid and sterol biosynthesis through modulation of rate-limiting enzymes in these pathways<sup>[35]</sup>. Diurnal variations hallmark also glucose metabolism, and the rate-limiting enzymes for gluconeogenesis, glycolysis, glycogenesis and glycogenolysis show circadian variations of activity, determining the circadian rhythmicity of hepatic glucose production and glycogen content. The biological clock drives the circadian regulation of hepatic gluconeogenesis by CRY 1 and CRY2 *via* inhibition of cAMP signaling in response to G protein coupled receptor (GPCR) activation<sup>[37]</sup>, and controls hepatic glycogen synthesis through transcriptional activation of glycogen synthase (GYS2) by CLOCK<sup>[38]</sup>, and the disruption or mutation

of the clock genes CLOCK and ARNTL results in disorders of glucose homeostasis<sup>[39,40]</sup>.

## HEPATITIS B VIRUS AND CLOCK GENES

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family, which causes persistent liver infections<sup>[41]</sup>. With more than 2 billion people being infected worldwide and 400 million suffering from chronic hepatitis B, HBV infection is one of the most significant public health problems. Despite the advance of modern medicine in the development of new antiviral drugs, HBV infection remains a leading cause of liver cirrhosis and cancer<sup>[3]</sup>.

HBV genome is a partial double-stranded DNA that replicates through the reverse transcription of pre-genomic RNA<sup>[42]</sup>. The analysis of the entire sequence of HBV-DNA, constituted by a circular incomplete double-strand DNA molecule, of 3182 bp in length<sup>[43]</sup>, reveals four Open Reading Frames (ORFs), overlapping each other, necessary for transcription and expression of HBV proteins. These ORFs are named: ORF S, ORF C, ORF P and ORF X<sup>[44]</sup> and they encode for four proteins with specific structure and function<sup>[45]</sup>. HBV biology and life cycle were already described<sup>[46]</sup>. The X protein (encoded by ORF X), remains partially explored and its function needs to be established<sup>[47]</sup>. Cultured hepatocytes overexpressing the X-gene, reveal a crucial role of the X protein in trans-activating viral and cellular genes<sup>[48]</sup>. Moreover, some authors associated HBx protein with HCC due to its property of impairing cellular proliferation<sup>[49]</sup>, although the X protein cannot induce infection by itself.

One study reported the ability of the HBx protein in modulating the clock genes in LO2 cells<sup>[19]</sup>. Cultured LO2 cells stably overexpressing the HBx protein displayed higher mRNA and protein levels of the CLOCK gene whilst ARNTL resulted to be decreased as compared to control cells. The authors suggest that the impairment of circadian rhythm of liver cells due to HBx expression may be one of the reasons leading to liver cancer development. It remains to elucidate how HBV impairs the clock gene machinery and to confirm the effect on liver cancer progression due to impairment of the cellular molecular clockwork by HBx.

## HEPATITIS C VIRUS AND CLOCK GENES

Hepatitis C virus (HCV) is a hepatotropic virus belonging to the Flavivirus family. It is estimated that 170 million people worldwide are infected with HCV<sup>[50]</sup>. In the majorities of the cases, HCV infection leads to severe liver diseases and is considered one of the major risk factors for HCC development<sup>[51]</sup>.

HCV genome consists in a positive-stranded RNA of approximately 9.6 kb, coding for a single polyprotein of about 3000 amino acids, processed co- and post-translationally by cellular and viral proteases cleaving it into three structural (core, E1 and E2), seven nonstructural (NS2,

NS3, NS4A, NS4B NS5A and NS5B) mature proteins and an ion channel (p7)<sup>[52]</sup>. Despite the small sequence divergences HCV is classified into six major genotypes (further divided into different subtypes)<sup>[50]</sup>. Overwhelming lines of evidence have indicated that the pathogenicity of HCV and its effect on disease progression and treatment is genotype dependent<sup>[50]</sup>.

We used two different *in vitro* models to investigate the relationship between HCV and clock genes, the OR6 cells harboring HCV replication and the Huh-7 cells expressing the HCV core proteins of genotype 1b or 3a. In both cases it was found that HCV down-regulated the expression of two crucial clock proteins CRY2 and PER2.

CRY2 protein is involved in NF- $\kappa$ B activation and pro-inflammatory processes<sup>[53]</sup>, (see next section for discussion), while the role of PER2 on HCV replication is particularly interesting, as this circadian protein regulates the rhythms of IFN $\gamma$  signaling, critical for innate and adaptive immunity against infection<sup>[54,55]</sup>. Exogenous over-expression of PER2 protein in OR6 cells hampered HCV-RNA replication, and consistently, PER2 overexpression influenced the HCV-dependent altered expression of Interferon stimulated genes (ISG) products (OAS1, Mx1, IRF9). PER2 potentiated the expression of OAS1 which activates RNase L resulting in viral RNA degradation and inhibition of viral replication<sup>[56]</sup>.

Of note, when experiments were performed, cells were synchronized using serum shock procedure, a method previously reported to induce circadian gene expression in mammalian cultured cells<sup>[57]</sup>, before RNA extractions at regular time points over 28 h period. This approach allows assessing differences in the time-related fluctuation of expression.

## CROSS-TALK BETWEEN THE BIOLOGICAL CLOCK, HEPATITIS VIRUSES AND IMMUNITY

Hepatic injury in HCV infection is not only directly induced by viral cytopathic effects, but is principally related to host immune responses. Viral persistence is influenced by dynamic restriction of the host's immune response, and the strength of immune response determines resultant acute viral clearance opposed to chronic persistence, leading to pathogenic mechanisms potentially responsible for HCC onset and progression during chronic hepatitis virus infection. Chronic immune-mediated liver cell injury triggers the development of HCC in the absence of viral transactivation, insertional mutagenesis, and genotoxic chemicals<sup>[58]</sup>. Circadian patterns of immune function have been maintained throughout evolution, are driven by the clock gene machinery, and the magnitude of immune response depends in part on the circadian timing of antigen challenge<sup>[59,60]</sup>. Alterations in the circadian regulation of the immune system may therefore lead to viral persistence or reactivation. The components of the immune system show time related variations with

a period of 24 h. In particular, the levels of leukocyte populations in the blood of humans and rodents are characterized by circadian variations. Natural killer (NK) cells are critical for immune surveillance against viral infections and their function is under tight circadian control. NK cells bear no antigen receptor and therefore belong to the innate immune system, however they share several features with highly differentiated T lymphocytes, such as a high tissue migratory potential and the production of granzyme B and perforin, interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and granular macrophage cell stimulating factor, allowing immediate cytotoxic effector defense in the periphery<sup>[61]</sup>. Circadian expression of negative and positive components of the molecular clock, as well as cytokines and cytolytic factors, are evident in NK cells, and perturbations of daily rhythms caused by external and internal stressors may compromise the first line of defense against infections<sup>[61,62]</sup>. In NK cells, expression of cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) and cytolytic factors (granzyme B and perforin) are highly synchronized, peaking approximately during the middle of the active period in rats, and NK cell cytotoxic activity peaks at similar circadian phases. Similarly, NK cytotoxicity is maximal during periods of wakefulness in humans<sup>[60]</sup>. The clock genes drive circadian rhythmicity of NK cell function. Alterations of the molecular clockwork modify the harmonized expression of NK cell cytolytic factors. In particular, knock-down of Per2 or Arntl in rat-derived RNK16 NK cells changes in a diverse way the expression of genes encoding IFN- $\gamma$ , TNF- $\alpha$ , granzyme B, and perforin<sup>[54]</sup>. Furthermore, knock-down of Per2 or Arntl changes protein levels of granzyme B and perforin, but not of IFN- $\gamma$  and TNF- $\alpha$ <sup>[63,64]</sup>. In addition, distorted rhythms of granzyme B and perforin as well as altered rhythm and low levels of IFN- $\gamma$ , together with changes in the rhythm of Arntl and Per2, were evidenced in Per2 mutant mice<sup>[62]</sup>.

In the human blood, higher counts of total lymphocytes, T lymphocytes and B lymphocytes have been consistently observed in the night time, and when T lymphocyte subsets are considered, CD4<sup>+</sup> (T helper) and CD8<sup>+</sup> (cytotoxic) naive, central memory and effector memory T lymphocytes show peak numbers in the night, while CD4<sup>+</sup> effector T cells show no rhythm and CD8<sup>+</sup> effector T cells show a low amplitude rhythm with a peak in the day<sup>[65,66]</sup>. T and B lymphocytes are involved in the adaptive (*i.e.*, antigen-specific) immune response, whereas granulocytes, monocytes and NK cells mainly belong to the innate (*i.e.*, not antigen-specific) immune system. In rodents higher numbers of total leukocytes and of lymphocytes were reported in the day, while in humans higher levels in the counts of innate immune system cells were reported in the daytime or late day<sup>[67]</sup>. Hence, both nocturnal rodents and diurnal humans show higher lymphocyte counts during the rest period, and peaks of other cell types (granulocytes, neutrophils, monocytes) were found in the day in rats, while highest NK cell numbers were observed at the end of the night,



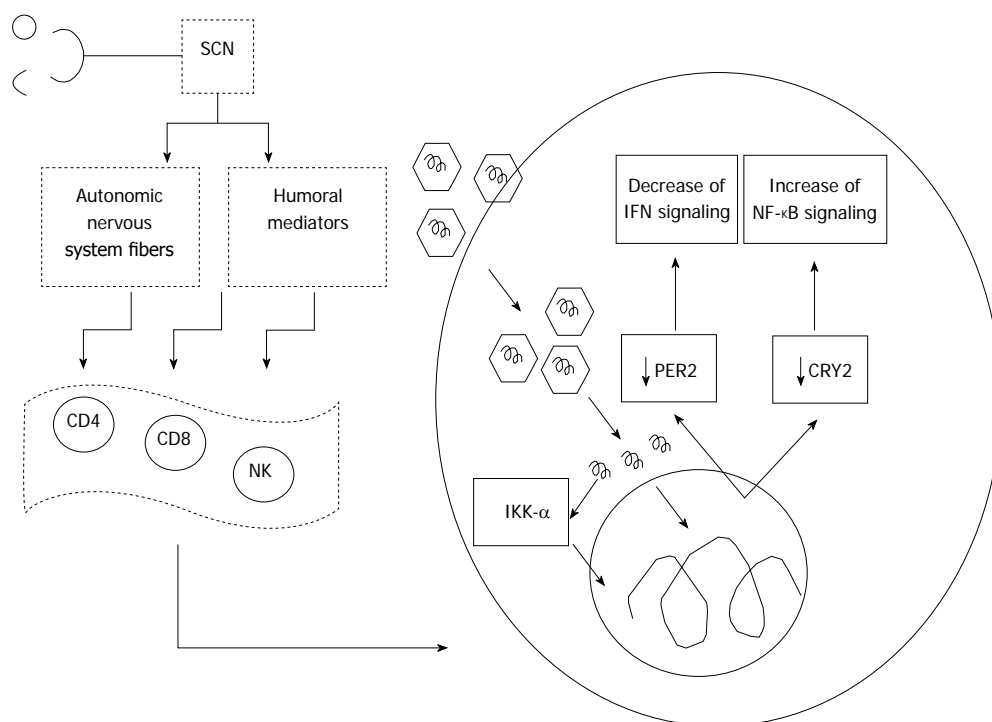


Figure 1 Scheme rendering the interplay between the circadian clock circuitry, the immune system and the alterations induced by hepatitis C virus on the clock gene machinery and downstream signaling pathways. SCN: Suprachiasmatic nuclei.

*i.e.*, at the beginning of the activity period.

Cellular immune rhythms are synchronized by the mammalian central pacemaker located in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus via time dependent changes in the activity of the sympathetic nervous system (SNS), in the release of hormones (growth hormone, prolactin, melatonin, cortisol) and in behavior that is linked to the sleep-wake cycle<sup>[65,68,69]</sup>. The rest period is characterized by peak levels of pro-inflammatory hormones like growth hormone, prolactin (and melatonin in humans) and pro-inflammatory cytokines like interleukin (IL)-1 and TNF- $\alpha$ . Besides, T helper (h) 1 and Th2 responses are likewise highest during sleep<sup>[70]</sup>. During the active period the hypothalamus-pituitary-adrenal axis becomes activated and cortisol suppresses pro-inflammatory cytokine production, CD4<sup>+</sup> T cell numbers and allergic reactions<sup>[71]</sup>. Disruption of this temporal organization of the immune system can lead to immunodeficiency and/or exceeding immune reactions (*e.g.*, low grade systemic inflammation).

Oscillation across the day was observed also for the levels of cytokines and other effector molecules, in particular serum levels and *in vitro* production of IFN- $\gamma$ , tumor necrosis factor TNF- $\alpha$ , IL-1, IL-2, IL-6 and IL-12 were all shown to present a rhythm in humans, with a peak generally observed at night or in the early morning<sup>[60]</sup>. Immune rhythms are influenced by hormone rhythms (*e.g.*, cortisol, melatonin, norepinephrine), and in humans the rhythms of naive, central memory, and effector memory T cell counts are regulated by cortisol, whereas numbers of CD8<sup>+</sup> effector T cells follow changes in endogenous epinephrine<sup>[65,72-74]</sup>.

The presence of biological clocks in immune cells and lymphoid organs drives rhythms in the functions of cells within the immune system, but on the other hand immune responses and mediators influence behavioral and molecular circadian rhythms<sup>[54,62]</sup>. Whether circadian disruption of cellular-mediated immunity or neuroendocrine-immune interaction lead to viral reactivation is unclear.

The cross-talk between the clock and innate immune functions is mediated among other circadian factors by CRY2, which transcriptionally regulates STAT3 and hampers activation of NF- $\kappa$ B signaling by negatively regulating the cAMP-PKA pathway<sup>[53]</sup>. Interestingly, we reported a severe down-regulation of CRY2 in OR6 cells replicating HCV genotype 1b<sup>[18]</sup>, which could induce increase of cytokine production related to NF- $\kappa$ B signaling pathway<sup>[53]</sup>. This mechanism could enhance the effects deriving from direct activation of NF- $\kappa$ B by the HCV core protein, which may bind to the death domain of tumor necrosis factor receptor 1 (TNFR1) and to the cytoplasmic tail of lymphotoxin-beta receptor, with resistance to TNF- $\alpha$ -induced apoptosis, suggesting a mechanism by which HCV may evade the host's immune surveillance leading to viral persistence and possibly to hepatocarcinogenesis<sup>[75]</sup>. On the other hand, HCV infection, and in particular core nonstructural protein (NS)4B and NS5B, reduce TNF- $\alpha$ -induced phosphorylation of I $\kappa$ B kinase (IKK,  $\alpha$ ,  $\beta$  and  $\gamma$ ) and inhibitor of NF- $\kappa$ B (I $\kappa$ B), which are upstream regulators of NF- $\kappa$ B activation. HCV plays a role in immune-mediated liver injury in HCV infection also inhibiting nuclear translocation of NF- $\kappa$ B and expression of NF- $\kappa$ B-dependent anti-apoptotic proteins, such as B-cell lymphoma-extra large (Bcl-xL), X-linked

inhibitor of apoptosis protein (XIAP), and the long form of cellular-FLICE inhibitory protein (c-FLIP)<sup>[76]</sup>. Furthermore, a crucial host factor for HCV is represented by IKK- $\alpha$  (Figure 1). HCV interacts with DEAD box polypeptide 3, X-linked (DDX3X) through its 3' untranslated region, and activates IKK- $\alpha$ , which translocates to the nucleus and induces a CBP/p300-mediated transcriptional program involving sterol regulatory element-binding proteins (SREBPs). HCV infection in this way utilizes a NF- $\kappa$ B-independent and the kinase-mediated nuclear function of IKK- $\alpha$ : making use of this intrinsic innate pathway and taking control of lipogenic genes and lipid metabolism, enhances core-associated lipid droplet formation to facilitate viral assembly, which in turn may contribute to high chronicity rates and the pathological hallmark of steatosis in HCV infection<sup>[77]</sup>.

## CONCLUSION

Up to date only few studies reported the influence of viruses on the clock gene machinery. Further studies are required to investigate the relationship between viruses and the clock genes as they could lead to new therapeutic strategies for future treatment options. Performing cell synchronization may be useful to observe *in vitro* differences in time related patterns of expression<sup>[18]</sup>. Consequently, we recommend a better set-up of the experiments and cell synchronization before investigating the biological clock at the molecular level, considering that single cells in culture are asynchronous and this may conditionate the results.

As for the new therapeutic strategies that can be developed based on the circadian regulation of viral replication, circadian rhythm-based treatments (*i.e.*, chronotherapies), have been employed against several different pathological conditions<sup>[78,79]</sup>. Standard therapy for HCV patients involves administration of IFN- $\alpha$  and ribavirin (a nucleoside analogue)<sup>[50,56]</sup>. Recently, an interferon/ribavirin-free therapy based on newly identified and efficacious protease inhibitors (telaprevir, boceprevir) promisingly entered into the clinic to treat HCV patients<sup>[80]</sup>. In light of these findings, if the new strategies to inhibit viral replication take in consideration the circadian relationship between host cell and hosted viruses, this could not only minimize the pharmacological agents' toxicity but can also improve the efficacy of treatment modalities through optimized timing of therapeutic regimens, targeting in a better way virus replication. As already suggested, administration of nucleoside analogues to inhibit viral DNA replication can be matched to parallel the diurnal peaks<sup>[14]</sup> considering the circadian pattern of host cell proliferation and differentiation.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Hepatitis C virus-related mixed cryoglobulinemia: Is genetics to blame?

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## Abstract

Mixed cryoglobulinemia (MC) is the extrahepatic manifestation most strictly correlated with hepatitis C virus (HCV) infection; it is a benign autoimmune and lymphoproliferative disorder that evolves to lymphoma in 5%-10% of cases. MC is reputed to be a multistep and multifactorial process whose pathogenicity is still poorly understood. It is still unknown why only some chronically infected HCV patients develop MC and only some of these exhibit systemic symptoms (MC syndrome). Several studies have investigated the pathogenetic basis of MC and the most recent ones suggest that the virus is able to trigger such a disorder only in the presence of genetic factors that are still unknown. Here, we try to clarify the complex relationship between HCV-related MC and the host's genetic background. The data that we report are heterogeneous and sometimes even conflicting. Therefore, large, multicenter studies are clearly needed. The identification of a characteristic

genetic signature of cryoglobulinemic patients would be an important step toward a personalized approach in their clinical care. The new wide-ranging genomics technologies will hopefully help to resolve these complex issues.

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**Key words:** Hepatitis C virus; Mixed cryoglobulinemia; Genetics; Viral pathogenetic factors; Host pathogenetic factors

**Core tip:** Mixed cryoglobulinemia (MC) is the extrahepatic manifestation most strictly correlated with hepatitis C virus (HCV) infection; it is a benign autoimmune/lymphoproliferative disorder that evolves to lymphoma in 5%-10% of cases. MC pathogenesis is still poorly understood. Several studies have tried to clarify the pathogenetic basis of MC and have suggested that HCV can trigger such a disorder only in the presence of still-undetermined genetic factors. Here, we attempt to clarify the relationship between HCV-related MC and the host's genetic background. The data that we report are heterogeneous and sometimes conflicting, so large, multicenter studies are clearly needed.

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## INTRODUCTION

Mixed cryoglobulinemia (MC) is the extrahepatic manifestation most strictly correlated with hepatitis C virus

(HCV) infection<sup>[1]</sup>, as well as being an autoimmune and B cell lymphoproliferative disorder that evolves to lymphoma in 5%-10% of patients. Defined as a systemic vasculitis, MC is caused by intravascular immune complexes named cryoglobulins (CGs). The term “mixed” refers to the simultaneous involvement of immunoglobulin G (IgG) and IgM in generating the CGs that can include partially monoclonal (type II MC) or totally polyclonal (type III MC) immunoglobulins. The IgM has rheumatoid factor activity and is produced by clonally expanded autoreactive B cells<sup>[2-5]</sup>.

The pathogenesis of MC is still poorly understood, although it is certain that several subsequent events contribute to disease onset, when they occur in a favorable host genetic substrate<sup>[1,6-8]</sup>. The reasons why only some chronically infected HCV patients develop MC and why only some of these exhibit systemic symptoms, the so-called MC syndrome (MCS), are unknown. One of the most obvious explanations, the genetic factor, has only recently been seriously contemplated, when the impact of this disease on chronic HCV infection and its role in predisposing to lymphoid malignancies has been recognized. Since then, several studies have tried to clarify the complex pathogenesis of MC and the most recent have focused on genetics.

Together with genetic predisposition, epigenetic factors such as the expression of specific miRNAs can be a major contribution to the pathogenesis of HCV-related lymphoproliferative disorders<sup>[9]</sup>. In particular, miR-26b is downregulated in peripheral blood mononuclear cells from HCV-related MC but totally restored after complete virological and clinical response to anti-HCV therapy<sup>[10,11]</sup>. However, this review focuses on the numerous attempts to define the specific genetic background predisposing to development of MC.

We try to clarify this topic by reporting all the attempts to define the genetic basis of HCV-related MC, starting from studies that failed to attribute a direct role in triggering this condition to viral factors, and ending with studies proposing an association between some particular host genetic variants and the development of MC. Other studies have shown a relationship between chronic HCV infection and lymphoma or other autoimmune diseases, which are worth considering for their resemblance to MC.

## MC AND HCV FACTORS

### *Viral genotype and MC*

Since the mid-1990s, several studies have analyzed the relationship between HCV factors, such as genotype and viremia, and MC susceptibility. Although results are often conflicting, most studies conclude that the distribution of viral genotypes in MC patients without clinical manifestations does not significantly differ from those observed in HCV patients with no evidence of lymphoproliferation<sup>[12-14]</sup>. The patients in the cited papers had asymptomatic MC and, as speculated by Sinico *et al.*<sup>[14]</sup>, these studies leave open the possibility that HCV genotype or subtype

could influence progression to symptomatic MC. However, the analysis of 60 MC patients, including 22 with symptoms, reported by Frangeul *et al.*<sup>[15]</sup>, did not show a significant association between MCS and HCV genotype.

### **Specific HCV hypervariable region 1 and 2 mutations and MC**

Some authors have thoroughly investigated the possible role of mutations in the N-terminal hypervariable regions 1 and 2 (HRV1 and HRV2) of the E2 envelope glycoprotein in predisposition to MC.

The initial results about the relationship between E2 mutational pattern and MC pathogenesis suggest an association of particular HVR1 variants (insertion at codon 385 and deletion at codon 384) with type II MC<sup>[16]</sup>. The authors focused on 385 insertions responsible for improved ability of E2 to bind the HCV putative receptor CD81, with consequent higher stimulation of CD81 itself leading to augmented lymphoproliferation<sup>[16]</sup>.

Another attempt, published some years later, did not confirm these data, but correlated different viral mutations with MC (positions 389 and 398 for HVR1 and positions 474, 493 and 497 for HVR2)<sup>[17]</sup>. Conversely, a study published by Rigolet *et al.*<sup>[18]</sup>, after an accurate approach of cloning and sequencing HVR1 regions isolated from HCV-positive MC patients, clearly concluded that any particular motif of E2 coding sequence could be associated with MC. These data were confirmed in a study conducted on a population of 80 MC patients by Bianchetti *et al.*<sup>[7]</sup>. A similar experimental plan and accurate statistical and bioinformatic approaches suggested that MC arose by as-yet-unidentified host rather than virus-specific factors, meaning that attention should be focused on the host.

Convincing proof of the role played by host genetics in determining HCV-related MC onset appeared in an epidemiological study by Pozzato *et al.*<sup>[19]</sup>, which demonstrated that there were ethnic differences in the prevalence of asymptomatic HCV-associated monoclonal B-cell expansion. Based on an observational suspicion of a high prevalence of MC in Italy versus a low prevalence in Japan, the authors investigated 60 Italian and 44 Japanese HCV patients and concluded that there were no differences in the two groups apart from ethnicity. This clearly suggests that HCV is able to induce B-cell expansion only in the presence of unidentified genetic factors.

## MC AND GENETIC FACTORS

### **MC and HLA polymorphisms**

The first studies regarding the host genetic factors conditioning susceptibility to development of MC during chronic HCV infection analyzed human leukocyte antigen (*HLA*) gene cluster variants. *HLA* gene products are responsible for presenting viral antigens to T cells, therefore, it has been speculated that some HLA variants could be implicated in driving the immune response against the virus to produce autoreactive antibodies (the CGS). An early attempt to investigate the genetic predisposition to MC was published even before the discovery

of HCV and HLA class II polymorphisms. Migliorini *et al*<sup>[20]</sup> did not find any association between MC and either class I or class II HLA molecules. Since then, several studies and some controversial data have been published. Ossi *et al*<sup>[21]</sup>, studying 16 MC patients, showed a higher expression of HLA-B51 and B35 antigens, previously correlated with other autoimmune disorders, as well as the presence of HLA-A9 with its A24 split in 50% of the same population.

An almost contemporary study performed in a large cohort of multi-transfused patients, including 116 HCV-positive ones, showed no association between a specific HLA pattern and MC. The authors conclude that the HLA class II DR2 subtype (DRB1\*1601, DQB1\*0502), which is characteristic of multi-transfused patients who maintain HCV negativity after years of blood transfusions, could be considered as a sort of protection against HCV infection<sup>[22]</sup>.

A meticulous study, mostly for the accuracy of the statistical analysis, showed a higher frequency of HLA-B8 and HLA-DR3 in a group of 25 HCV-positive cryo-patients<sup>[23]</sup>. The odds ratio was also calculated and the highest corresponded to the presence of both B8 and DR3, suggesting the existence of an HLA-B8-DR3 haplotype associated with HCV-infected MC patients. These results were partially confirmed in a Chinese study in which HLA-DR3 was significantly associated with the presence of HCV-related cryoglobulinemia that was mostly asymptomatic<sup>[24]</sup>.

The absence of an association between HLA and MC was demonstrated by another Italian group. Analysis of HLA-DRB1 alleles in 46 patients with HCV infection concluded that HLA class II polymorphisms did not distinguish patients with MC from those without MC<sup>[25]</sup>.

Cacoub *et al*<sup>[26]</sup> also evaluated *HLA-DRB1* and *HLA-DQB1* polymorphisms in a cohort of 76 symptomatic or asymptomatic MC patients. Multivariate logistic regression analysis of several features indicated the presence of HLA-DR11 as a positive predictor of MC, together with the already known female sex and age. The same HLA class II alleles were evaluated in another study that focused on the association between particular HLA-DR-DQ combinations and HCV-positive non-Hodgkin's lymphoma (NHL) with and without a background of MC<sup>[27]</sup>. Various HLA II associations have been found for HCV-positive NHL in the presence of MC (higher frequency of DR5-DQ3 HLA) and for HCV-positive and MC-negative NHL (higher frequency of DR1-DQ1), suggesting the presence of alternative pathogenetic processes for similar but different HCV lymphomas.

### MC and cytokine mutations

Alterations in the cytokine/chemokine patterns, also involving proinflammatory and Th1 chemokines, have been demonstrated in MC and other extrahepatic disorders induced by HCV infection<sup>[28]</sup>. These previous studies have investigated genetic variants of this complex class of immune response regulators.

Several studies have shown that interleukin (IL)-10

may be involved in the pathogenesis of lymphoid disorders; moreover, three different mutations in the IL-10 promoter (-1082G→A, -819C→T and -592C→A) were associated with higher IL-10 production. In a study by Persico *et al*<sup>[29]</sup>, conducted on 270 well-characterized patients with NHL and/or HCV-related chronic hepatitis, a high prevalence of IL10-1082GG genotype was significantly associated with NHL in HCV-infected patients.

Polymorphisms of inflammatory chemokines are also significantly correlated with the outcome of HCV infection, because chronic hepatitis itself is closely associated with inflammation.

Recent reports have shown high levels of a B-cell-specific cytokine, namely B-cell-activating factor (BAFF; or B lymphocyte stimulator), in the serum of HCV patients with lymphoproliferative disorders but could not define the mechanisms underlying this phenomenon<sup>[30-33]</sup>. BAFF is a tumor necrosis factor  $\alpha$  family member and a key regulator of B-cell differentiation, survival, and immunoglobulin secretion, and the mutated genotype of its promoter (-871T) is associated with higher BAFF mRNA levels in monocytes<sup>[34,35]</sup>. Two consecutive studies conducted on a well-characterized MC population indicated a significantly higher prevalence of T allele homozygosity in patients with MCS, as well as the presence of the T allele (homozygous TT plus heterozygous TC) compared to HCV carriers without MC<sup>[8,36]</sup>. These results are consistent with the serum BAFF levels found in the different groups. Therefore, the transcriptional activation induced by the BAFF promoter variant could be considered one of the mechanisms contributing to the pathogenesis of HCV-related lymphoproliferative disorders.

### MC and IgG Fc receptors

Two independent studies have evaluated the role of the genetic variability of IgG Fc receptors (FcGRs) in the susceptibility to MC during the course of HCV infection. The FcGRs, present on leukocytes, are responsible for the clearance of immune complexes, phagocytosis, antibody-dependent cellular cytotoxicity, and regulation of the release of inflammatory mediators and B-cell activation, mainly in phagocytes. Their polymorphic variants are associated with reduced affinity for immune complexes, autoimmune diseases, and cancer<sup>[37]</sup>. In the first study, Vassilopoulos *et al*<sup>[38]</sup> analyzed a cohort of HCV patients with different autoimmune/lymphoproliferative disorders, including MC, discriminating between symptomatic and asymptomatic individuals and investigating FcR III A and the *NA1/NA1 FcGR III B* genotypes. They did not find any increased frequency of particular alleles in the autoimmune manifestations group compared to historical controls. In the second study, a more numerous cohort of cryoglobulinemic patients was evaluated. Despite the wider and better characterized MC population, this recent screening of FcGR2A 131R/H, FcGR2B 232 I/T, FcGR3A 176 V/F and FcGR3B NA1/NA2 confirmed the previous results, with the distribution of FcGR genotypes not being significantly different compared to the controls<sup>[8]</sup>. We reported in 21 HCV-MC patients treated



**Table 1 Association between hepatitis C virus-related lymphoproliferative disorders and host genetic factors**

Factors	References
HLA polymorphisms	
HLA-A9	[21]
HLA-B8	[23]
HLA-DR3	[23,24]
HLA-DR11	[26]
HLA-DR5-DQ3	[27]
Cytokine mutations	
IL-10 promoter (-1082GG)	[29]
BAFF promoter (-871T)	[8]
Sporadic associations	
Fibronectin Msp I and HaeIIIb	[40]
CYP27B1	[41]

HLA: Human leukocyte antigen; IL: Interleukin; BAFF: B-Lymphocyte activating factor.

with rituximab (anti-CD20 monoclonal antibody) that the response was strictly related to the F allele homozygosity of FcGR3A, suggesting that this genotype could be involved in response to rituximab therapy.

### Sporadic associations

The role of mutations within Fas and Fas-L genes has been described in mice with an increased prevalence of autoimmune manifestations, therefore, some authors have postulated that such mutations could be related to autoimmune diseases and lymphoproliferation. Results obtained from a small number of patients with Sjögren's syndrome or type II MC do not support such a hypothesis, suggesting that the germline mutations of the Fas receptor and its ligand are probably not involved in the pathogenesis of HCV-related type II MC<sup>[39]</sup>.

A possible relationship between two fibronectin polymorphisms (called *Msp* I and *Hae*IIIb) and type II MC has been investigated, in order to define the risk of lymphoma development. Fabris *et al*<sup>[40]</sup> analyzed 74 patients with MC, including 21 who developed B-cell NHL and 72 with HCV-negative and MC-unrelated NHL. None of the major MC-related clinical manifestations was significantly linked with a particular allele or genotype of the *Msp* I and *Hae*IIIb fibronectin gene polymorphisms. However, the two genetic sites seem to confer an independent increased risk of NHL in MC patients.

As a result of the critical role of vitamin D in the regulation of the immune system, the analysis of the serum vitamin D status in HCV-infected patients with extrahepatic manifestations seems particularly interesting. Terrier *et al*<sup>[41]</sup> found a strong association between low serum levels of vitamin D and the presence of MC and systemic vasculitis in patients with chronic HCV infection. Regarding the B-cell compartment, they observed significant correlations between serum 1,25-dihydroxyvitamin D and the B-cell activation status.

Lange *et al*<sup>[42]</sup> previously found that 1,25-dihydroxyvitamin D serum concentrations were higher in HCV patients with *CYP27B1* AA genotype compared to patients

with *CYP27B1* AC or CC genotype, thus, it is conceivable that MC patients harbor these latter genotypes. Unfortunately, no further studies have been published on this topic but an abstract of Terrier Benjamin *et al*<sup>[43]</sup> reports an exactly opposite association between phenotype and genotype in patients with HCV-related systemic vasculitis.

Recent important advances in the HCV field strongly suggest that the polymorphic variants of the *IL-28B* gene should be analyzed. Indeed, in 2009 several independent studies have shown that single nucleotide polymorphisms near the *IL-28B* coding region are closely associated with HCV clearance. *IL-28B* is involved in innate immunity and a recent study evaluated the influence of these genetic variations in the development of HCV-related MC<sup>[44]</sup>. The allele distribution reported in the study was similar in patients with or without MC, and does not support the hypothesis that these polymorphisms influence the development of MC.

The associations between HCV-related lymphoproliferative disorders and host genetic factors are summarized in Table 1.

## CONCLUSION

It is clear from the reports described in this review that the role of genetics in HCV-related MC is a current and compelling research topic. Each patient is genetically unique, which can affect the evolution of chronic HCV infection towards benign lymphoproliferation predisposing to lymphoma. The identification of a characteristic genetic signature of cryoglobulinemic patients could be a step towards personalized approaches in the clinical care of HCV infection, which are useful for targeted follow-up of high-risk individuals. The above data are heterogeneous and sometimes even conflicting, thus, there is a clear need for multicenter studies including large numbers of patients, and the future application of the new genomic and proteomic wide-range technologies will surely assist in this direction.

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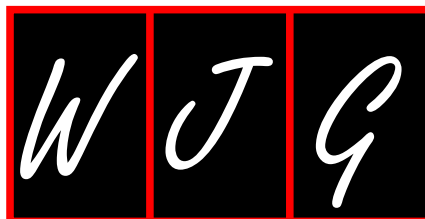


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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Immunological alterations in hepatitis C virus infection

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## Abstract

A higher prevalence of immunological processes has recently been reported in patients with hepatitis C virus (HCV) infection, focusing the attention of physicians and researchers on the close association between HCV and immune disorders. HCV lymphotropism represents the most important step in the pathogenesis of virus-related immunological diseases and experimental, virologic, and clinical evidence has demonstrated a trigger role for HCV both in systemic autoimmune diseases, such as rheumatoid arthritis, Sjögren syndrome, hemolytic anemia and severe thrombocytopenia, and in organ-specific autoimmune diseases, such as autoimmune hepatitis, thyroid disorders and diabetes. This review will outline the principal aspects of such HCV-induced immunological alterations, focusing on the prevalence of these less characterized HCV extrahepatic manifestations.

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**Key words:** Hepatitis C virus; Immune disorders; Cytopenia; Extrahepatic manifestation; Autoantibody

**Core tip:** Hepatitis C virus (HCV)-infected lymphoid tissue of the host represents a site for the persistence

of HCV infection which exerts a chronic stimulus to the immune system, facilitating clonal B-lymphocyte expansion and consequent wide autoantibody production, including cryo- and non-cryo-precipitable immune complexes which may lead to organ- and non-organ-specific immunological alterations. This review outlines the principal aspects of such HCV-induced immunological alterations, focusing on the prevalence of these less characterized HCV extrahepatic manifestations.

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## INTRODUCTION

Autoimmunity and viral infections are closely related, and the hepatitis C virus (HCV), is recognized as one of the viruses most often associated with autoimmune features. For this reason HCV is not only associated with chronic hepatic inflammation but also an array of extrahepatic complications. In the majority of these associated extrahepatic manifestations, the pathogenic mechanism appears to be immunologically driven, with many having features of autoimmunity. HCV infection has been associated with both organ-specific [thyroiditis, diabetes, autoimmune hepatitis (AIH)] and systemic autoimmune diseases and this association has generated growing interest in recent years since it is often observed in patients with chronic HCV infection.

## PATHOGENESIS OF HCV RELATED IMMUNE DISORDERS

HCV lymphotropism represents the most relevant step in the pathogenesis of virus-related immunological dis-

orders<sup>[1]</sup>. Indeed, infected lymphoid tissue of the host represents a site for the persistence of the HCV infection<sup>[2-6]</sup>. HCV exerts a chronic stimulus to the immune system, facilitating the clonal B-lymphocyte expansion and consequent wide autoantibody production, including cryo- and non-cryoprecipitable immune complexes<sup>[3,7-9]</sup> which may lead to organ- and non-organ-specific immunological alterations<sup>[3,7,8,10]</sup>. The first step is translocation, demonstrated in a high percentage of HCV-infected patients, with consequent Bcl-2 proto-oncogene activation, antiapoptotic activity and prolonged survival of lymphocytes<sup>[3,7,9,10]</sup>. Besides, the identification of HCV envelope protein E2 able to bind the CD81 molecule expressed on both hepatocytes and B-lymphocytes seems to be crucial for HCV-driven autoimmunity<sup>[3,7,9,10]</sup>.

Dysregulation of cytokine networks skewing regulatory T-cells to a Th2 phenotype, which may be associated with enhanced humoral immune responses and autoantibody production has also been related to the expansion of autoantibody-producing B-cells and chronic lymphoproliferation in HCV infection<sup>[11]</sup>. HCV infections induce a massive chemokine and cytokine burst and therefore recruit leukocytes to the site of infection with the goal to stop viral spread. This excitation of the human defense system could stimulate a potentially self-reactive lymphocytes inducing autoimmunity in susceptible individuals<sup>[11]</sup>.

Many studies have linked Th1 immune response with HCV infection<sup>[12]</sup>, mixed cryoglobulinemia (MC)<sup>[13]</sup> and organ specific autoimmune disorders<sup>[14]</sup>. These findings suggest that a possible common immunological Th1 pattern could be the pathophysiological base of the association of autoimmunity related HCV infections.

Several studies have shown an increased expression of interferon-gamma (IFN- $\gamma$ ), and IFN- $\gamma$  inducible chemokines (C-X-C motif chemokine 10 - CXCL10), in hepatocytes and in lymphocytes of HCV-infected patients<sup>[12,15,16]</sup>, which are directly related to the degree of inflammation and an increase in circulating levels of IFN- $\gamma$  and CXCL10<sup>[17,18]</sup>.

Furthermore, it has been shown that NS5A and core proteins, alone or by a synergistic effect with Th1 cytokines [IFN- $\gamma$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )], are capable of upregulating CXCL10 and monokine induced by gamma interferon (MIG) gene expression and secretion in cultured human hepatocyte derived cells. These data suggest that CXCL10 produced by HCV-infected hepatocytes could play a key role regulating T-cell trafficking into a Th1-type inflammatory site by recruiting Th1 lymphocytes, that secrete IFN- $\gamma$  and TNF- $\alpha$ , with a synergistic effect on CXCL10 secretion by hepatocytes, thus perpetuating the immune cascade<sup>[19]</sup>.

## HCV AND SYSTEMIC AUTOIMMUNE DISEASES

### *Mixed cryoglobulinemia*

MC is the most well documented extrahepatic manifesta-

tion of HCV infection<sup>[2,20]</sup>. MC, which is defined by documenting cryoprecipitates in serum (Ig precipitates from serum at temperatures under 37 °C and dissolves upon re-warming), is characterized by the presence of circulating immunocomplexes produced by a benign proliferation of B-cells. MC represents the link between HCV and various autoimmune and lymphoproliferative disorders. Although serum cryoglobulins (CGs) are frequently present in patients with chronic HCV<sup>[3-5,21,22]</sup>, in many of them CGs are present at low levels and symptoms are often absent or very mild. Only about 5% of HCV-infected subjects have clinically overt MC syndrome.

### *HCV-related arthritis*

Chronic oligo-polyarthritis during chronic HCV infection is often associated with MC but can also represent an independent entity. Indeed, it is not rare to observe a simple association between HCV infection and classical rheumatoid arthritis (RA) that can co-exist by chance or can be related to the ability of HCV to act as a trigger of the immune disease in individuals genetically predisposed to RA.

A polyarthritis, which is often non-erosive and rarely progressive, and involves small joints is the most common kind of arthritis associated with HCV chronic infection without the coexistence of cryoglobulinemia. Instead, 40%-80% of HCV-infected patients with MC<sup>[23]</sup> are reported to have a bilateral and symmetric arthralgia, which is non-deforming and includes mainly the knees and hands, and, more seldom, the elbows and ankles. Rheumatoid factor (RF) activity is found in 70%-80% of MC patients but is not correlated with the presence of articular disease, as patients chronically infected with HCV in the absence of HCV-MC or RF may have prominent articular symptoms. Usually there is no evidence of joint destruction, and antibodies to cyclic citrullinated peptide, which are highly specific to rheumatoid arthritis, are absent<sup>[24]</sup>. These evidences suggest that HCV infection should be considered in the differential diagnosis of patients with atypical arthritis.

### *Sjögren syndrome*

Another autoimmune condition associated with HCV is a chronic lymphocytic sialoadenitis similar to sialoadenitis associated with idiopathic Sjögren syndrome (SS), which has been reported in approximately 50% of patients with HCV infection<sup>[25]</sup>.

Some authors have distinguished the HCV-related sicca syndrome from Sjögren's syndrome based on several differences, including absence of anti-SSA and anti-SSB antibodies, pericapillary and non pericanalary lymphocytic infiltration, lack of glandular canal damage, high prevalence of mixed cryoglobulinemia (50%), hypocomplementemia (51%), and systemic vasculitic manifestations (58%)<sup>[25-28]</sup>. Moreover, the lymphocytic type of the infiltrate in the minor salivary gland shows a predominance of CD8 lymphocytes which is not observed in primary SS<sup>[29]</sup>. Although the possible etiopathogenetic



role of HCV in SS remains a controversial issue<sup>[27]</sup>, the explanation for this extrahepatic manifestation could be a cross reactivity between the HCV envelope and host salivary tissue which lead to an immune reaction directed against salivary glands<sup>[26]</sup>. The correct classification of patients with sialoadenitis related to HCV chronic infection have important clinical, prognostic and therapeutic implications since it may evolve into a B cell malignant lymphoma, especially in the presence of MC<sup>[10,30]</sup>.

### **HCV related cardiac disorders**

Several observations suggest that HCV infection is an important cause of a variety of otherwise unexplained heart diseases. Indeed, it was reported that (+) or (-) chain HCV-RNAs can be detected in the biopsied myocardial tissue or in the autopsied heart suggesting that HCV might proliferate in the myocardium<sup>[31]</sup>, resulting in induction of cardiomyopathy. Frustaci *et al.*<sup>[32]</sup> have shown that HCV replicates in myocardial tissue of patients with myocarditis, and that HCV infection may contribute to the development of an autoimmune myocarditis, frequently associated with myocardial antibodies and responsive to immunosuppressive therapy. In 2000, Matsu-mori suggested that some specific HCV clones with high affinity for the heart can develop and cause cardiomyopathy<sup>[33]</sup> and in 2006, in a large study involving more than 1000 patients, the same group identified anti-HCV antibodies, HCV RNA, NT-proBNP, and cardiac troponin I and T in sera stored for up to 17 years, and found the anti-HCV antibodies were more prevalent in patients with myocarditis than in the general US population<sup>[34]</sup>. These results suggest that in regions where its prevalence is high, HCV infection may be an important cause of myocarditis and heart failure. Moreover, the same authors concluded that NT-proBNP is a more sensitive marker of myocardial injury than cardiac troponins in patients with heart failure from HCV myocarditis. More recently, other studies confirmed that NT-proBNP is a sensitive biomarker for identifying patients with heart failure caused by HCV-related myocarditis<sup>[35,36]</sup>. Antonelli *et al.*<sup>[36]</sup> assessed serum NTproBNP in 50 HCV-positive patients and in 50 sex- and age-matched controls. HCV patients showed significantly higher mean NT-proBNP level than controls<sup>[35]</sup>. This result was confirmed by the same group in another study where TNF- $\alpha$  was also found to be higher in HCV+ patients with respect to controls, suggesting the presence of subclinical cardiac dysfunction<sup>[36]</sup>.

## **AUTOIMMUNE CYTOPENIAS IN PATIENT WITH HCV INFECTION**

Hemolytic anemia and severe thrombocytopenia were the most frequent cytopenias observed in patients with HCV infection. The different types of immune-mediated cytopenias may be severe and clinically significant.

### **Hemolytic anemia**

Although autoimmune hemolytic anemia (AHA) has frequently been reported in association with HCV in the setting of interferon (IFN) treatment<sup>[37,38]</sup>, it has also been observed as an isolated extrahepatic manifestation. The existence of AHA in patients with chronic hepatitis was first described in 1951, when Hyman *et al.*<sup>[39]</sup> described AHA in 3 patients with chronic liver involvement. In 1973, Panush *et al.*<sup>[40]</sup> described a patient with chronic active hepatitis who presented with AHA with a positive Coombs test, who responded to treatment with steroids. In 1982, Portell *et al.*<sup>[41]</sup> reported 5 patients with chronic hepatopathy (3 with active chronic hepatitis and 2 with cirrhosis) and a positive Coombs AHA, with positive ANA in 4 and sicca syndrome in 1. In 2001, 2 cases of HCV infection associated with Coombs-positive AHA, in the absence of treatment with IFN, were reported by Srinivasan<sup>[42]</sup> and Chao *et al.*<sup>[43]</sup>, respectively. In 2003, Ramos-Casals *et al.*<sup>[44]</sup> presented the largest series of cases of HCV-related AHA not treated with antiviral therapy. Seventeen HCV patients, mostly women with a mean age of 56 years, presented a high level of association with autoimmune diseases, with cryoglobulinemia as the most frequent immunologic marker. Most patients had a history of liver cirrhosis and even if they had a good response to corticosteroids, the prognosis was poor (56% mortality).

### **HCV-associated immune thrombocytopenic purpura**

Although thrombocytopenia during the course of chronic liver disease is usually attributed to hypersplenism, an autoimmune mechanism has been suggested as playing a role in some patients with HCV infection. This hypothesis is based on the observation of a greater prevalence of thrombocytopenia and antiplatelet antibodies in HCV patients compared with HBV patients<sup>[45]</sup>, and of the frequency of HCV infection seen among patients initially diagnosed with idiopathic thrombocytopenic purpura (ITP)<sup>[46-48]</sup>. The pathophysiology of infection-related ITP involves diverse immunologic pathways as well as non-immune mechanisms that accelerate platelet destruction and/or decrease platelet production.

High affinity binding of HCV to the platelet membrane with subsequent binding of anti-HCV antibody might lead to phagocytosis of platelets<sup>[49]</sup>. Dysregulation of the host immune system triggering the production of autoantibodies against platelet glycoproteins has also been postulated<sup>[45,50]</sup>. However there have been conflicting data on the presence of specific antibodies in platelets in patients with HCV-related ITP<sup>[45,50-52]</sup>.

Thrombocytopenia in HCV patients may be present even in the absence of clinically evident liver disease or splenomegaly and may be mistakenly diagnosed as primary chronic immune thrombocytopenic purpura (CITP)<sup>[48,53]</sup>. Six cross-sectional studies have reported serologic evidence of HCV infection in 20% of patients with a clinical diagnosis of CITP<sup>[48,53-57]</sup>, and in the largest series published to date, HCV antibodies were identified in 30% of

250 patients fulfilling the American Society of Hematology criteria for CTP<sup>[54]</sup>. There were significant differences in the demographic characteristics of patients with HCV-positive compared with patients with HCV-negative CTP. Patients positive for HCV were older and the incidence was distributed equally between the sexes compared with the female predominance in HCV-negative CTP.

## ORGAN-SPECIFIC AUTOIMMUNE DISEASES

### *Thyroid disorders and HCV*

Autoimmune thyroid involvement and hypothyroidism were significantly more frequent in patients with chronic hepatitis C (CHC) than in comparison groups such as patients with viral hepatitis B or D<sup>[58-60]</sup> or normal subjects<sup>[61,62]</sup>. The most frequent thyroid disorder in this setting is the presence of circulating anti-thyroid antibodies which is more commonly reported in female subjects<sup>[58]</sup>. The prevalence of abnormally high levels of anti-thyroid antibodies observed in these patients ranges from 2% to 48%<sup>[58,61,63,64]</sup>, with heterogeneous geographic distribution<sup>[65]</sup>. These discrepancies are related to variable genetic predisposition and environmental co-factors, such as iodine intake or other infectious agents<sup>[66]</sup>. The evidence of a subclinical hypothyroidism was observed in 2%-9% of patients with chronic HCV infection, particularly in those patients with MC<sup>[59,60,62,63,67]</sup>, and these patients seem to be susceptible to Hashimoto's autoimmune thyroiditis and Grave's disease when treated with interferon.

Antonelli *et al.*<sup>[21]</sup> in 2004 analyzed 630 consecutive patients affected by CHC compared with a large control group of subjects from iodine-deficient and sufficient areas and with 86 patients with chronic hepatitis B. They demonstrated that patients with CHC were more likely to have hypothyroidism, anti-thyroglobulin and anti-thyroid peroxidase antibodies than any of the other groups. The same group evaluated thyroid function, the presence of thyroid autoantibodies, thyroid nodules and thyroid cancer, in 93 HCV + MC consecutive patients matched by sex and age to 93 patients with CHC without MC and 93 healthy (HCV-negative) controls. Subclinical hypothyroidism and thyroid autoimmunity were significantly more frequent in HCV + MC patients than in HCV-negative controls. Moreover, serum thyroid peroxidase antibodies were also significantly more frequent in HCV + MC patients than in CHC patients. Finally, the prevalence of thyroid nodules was not significantly different in the three groups<sup>[68]</sup>. In conclusion, pooling all data about HCV-positive patients (with CHC or HCVAb positivity) and using as control healthy subjects and HBV-infected patients, there was a significant increase in the prevalence of both thyroid autoimmune disorders (OR = 1.6; 95%CI: 1.4-1.9) and hypothyroidism (OR = 2.9; 95%CI: 2.0-4.1)<sup>[69]</sup>.

Some authors have reported that patients with chronic HCV have a higher prevalence of papillary thyroid carcinoma<sup>[70,71]</sup>. In 2002, the prevalence of thyroid cancer in

a series of 94 HCV-related mixed cryoglobulinemic patients was investigated<sup>[70]</sup>. A control group was obtained from a sample of the general population (2401 subjects) who had undergone thyroid ultrasonography. The prevalence of thyroid nodules was higher, although not significantly so, in control subjects than in MC patients but 2 patients with papillary thyroid cancer were found in the MC series, while no case was observed among controls.

A more recent study<sup>[71]</sup> prospectively investigated the prevalence and features of thyroid cancer in 308 patients with CHC in comparison with 2 large sex- and age-matched control groups from the general population with different iodine intake. Thyroid status was assessed by measurement of circulating thyroid hormones and auto-antibodies, thyroid ultrasonography, and, when indicated, fine-needle aspiration cytology. The authors have found that circulating thyrotropin, anti-thyroglobulin, and anti-thyroperoxidase antibodies levels, and the prevalence of hypothyroidism were significantly higher in HCV patients and 6 cases of papillary thyroid cancer were detected among HCV patients, whereas only 1 case was observed in controls, suggesting a high prevalence of thyroid papillary cancer in HCV patients. Because of this high prevalence of thyroid disorders, the guidelines on management of CHC recommend investigation of thyroid function, including free T4 and TSH in all patients, and since interferon-based therapy could exacerbate thyroid dysfunction, thyroid function tests should be fully evaluated prior to initiating HCV treatment.

### *Diabetes mellitus and HCV*

Data from the literature have shown a higher incidence of type 2 diabetes mellitus with chronic HCV when compared with patients with other liver disorders<sup>[72-74]</sup>. In a large study<sup>[75]</sup> involving 229 consecutively recruited MC-HCV patients compared with 217 sex- and age-matched controls without HCV infection, the prevalence of type 2 diabetes was significantly higher in MC-HCV patients than in controls. Moreover, MC-HCV diabetic patients more often had non-organ-specific autoantibodies than non-diabetic MC-HCV patients.

Another study conducted in 2005 by the same group<sup>[22]</sup>, established the prevalence and clinical phenotype of type 2 diabetes in a large series of non-cirrhotic HCV patients. The prevalence of type 2 diabetes was significantly higher in HCV patients compared with control subjects or non-cirrhotic HBV patients. Moreover, type 2 diabetic HCV patients had a significantly lower BMI than type 2 diabetic control subjects and significantly higher BMI than non-diabetic HCV patients. In contrast, no association with diabetes mellitus type 1 has been identified<sup>[22,72,73,76-78]</sup>. The association between chronic HCV and diabetes mellitus seems to be independent of the severity of the liver disease and is associated with insulin-resistance, but not with the presence of pancreatic anti-insulin antibodies<sup>[79]</sup>. In contrast, interferon treatment of HCV has been associated with the appearance of diabetes mellitus type 1 and development of anti-

pancreas autoimmunity<sup>[80-82]</sup>.

### AIH and HCV infection

Finally, an intriguing, still controversial aspect is the possible etiopathogenetic role of HCV in AIH<sup>[3,6,8,9,65]</sup>. Patients with AIH may present with mixed cryoglobulins, HCV infection, and extrahepatic manifestations such as thyroiditis, sicca syndrome, and arthritis<sup>[6]</sup>, while patients with HCV infection show one or more non-organ-specific auto-antibodies. The antigenic target specificity of HCV-related autoantibodies shows only quantitative differences compared with those associated with "primary" AIH<sup>[8]</sup>.

In clinical practice, the search for serum autoantibodies should be limited to cases for whom treatment with IFN is planned. An exception may be cases where clinical data (female gender, young age), high biochemical activity (transaminase-globulins) and histological aspects (interfaces hepatitis) of liver disease may suggest the presence of AIH with superimposed HCV infection.

The heterogeneous geographical distribution of HCV-associated AIH<sup>[65]</sup> suggests a possible involvement of various pathogenetic co-factors; among these, HCV might trigger a particular AIH clinico-serological subset, which is prevalent in specific geographical areas.

## CONCLUSION

In the case of patients with chronic HCV infection, the possible existence of extrahepatic manifestations should be taken into account and an accurate analysis of clinical and anamnestic data is recommended. Some patients may display the entire complex spectrum of HCV-related disorders which could be mild for many years and progress, generally during a long follow-up, to more severe systemic manifestations. In the last few years, very consistent data have been accumulated through different *in vivo* and *in vitro* models, suggesting that a more accurate characterization of the modalities and consequences at the molecular level of HCV infection of lymphatic cells may be of great importance in the future for the clarification of the pathogenesis of several pathological manifestations of HCV.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Interleukin 28B polymorphisms as predictor of response in hepatitis C virus genotype 2 and 3 infected patients

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## Abstract

Single nucleotide polymorphisms near the interleukin 28B (*IL-28B*) gene have been identified as strong predictors of both spontaneous or Peg-interferon (Peg-IFN) and ribavirin (RBV) induced clearance of hepatitis C virus (HCV). Several studies have shown that, in patients with genotype 1 (GT-1), rs12979860 C/C and rs8099917 T/T substitutions are associated with a more than two-fold increase in sustained virological response rate to Peg-IFN and RBV treatment. Although new treatment regimens based on combination of DAA with or without IFN are in the approval phase, until combination regimens with a backbone of Peg-IFN will be used, we can expect that IL28B holds its importance. The clinical relevance of IL28B genotyping in treatment of patients infected with HCV genotype 2 (GT-2) and 3 (GT-3) remains controversial. Therefore, after a careful examination of the available literature, we analyzed the impact of IL28B in GT-2 and -3. Simple size of the studies and GT-2 and GT-3 proportion were discussed. An algorithm for the practical use of IL28B in these patients was suggested at the aim of optimizing treatment.

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**Key words:** Hepatitis C virus; Genotype 3; Interleukin

28B; Liver cirrhosis

**Core tip:** The clinical relevance of interleukin 28B genotyping in patients with hepatitis C virus genotype 2 and 3 infection is debated. In this critical analysis of studies performed so far, it was shown that this genetic tool may help in optimizing treatment of genotype 3 patients, whilst it plays a marginal role in genotype 2 infected patients management.

Mangia A, Mottola L, Santoro R. Interleukin 28B polymorphisms as predictor of response in hepatitis C virus genotype 2 and 3 infected patients. *World J Gastroenterol* 2013; 19(47): 8924-8928 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/8924.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.8924>

## INTRODUCTION

In patients with hepatitis C virus (HCV) genotype 1 (GT-1), the standard treatment based on dual combination of Pegylated interferon (Peg-IFN) and ribavirin (RBV) has been replaced by the triple combination regimen including a protease inhibitor; on the contrary, in patients with HCV genotype 2 (GT-2) and genotype 3 (GT-3), it continues to represent the standard of care<sup>[1,2]</sup>.

Interleukin28B (*IL-28B*) genotype is a strong predictor of response to IFN-based treatment in GT-1<sup>[3-6]</sup>, but at a first glance, genetic analyses so far conducted in GT-2 and -3 patients provided ambiguous results<sup>[7-14]</sup>. The studies published so far may have bias but we should also bear in mind that identification of response predictors assumes a different relevance in different HCV genotypes. Indeed, high rates of sustained virologic response (SVR) achieved in GT-2 infected patients make response predictors of marginal interest and limit their use to the identification of patients who may take

**Table 1** Prevalence and impact of interleukin 28B rs12979860 in studies combining genotype 2 and genotype 3

Study	No. of patients	Prevalence of <i>IL-28B</i> CC genotype	Treatment duration (wk)	RVR in <i>IL-28B</i> CC genotype	SVR in <i>IL-28B</i> CC genotype
Mangia <i>et al</i> <sup>[10]</sup>	268	41%	12-24	59%	79%
Sarrazin <i>et al</i> <sup>[11]</sup>	267 <sup>1</sup>	43%	24-48	51%	47%
Lindh <i>et al</i> <sup>[13]</sup>	341	44%	12-24	67%	70%
Bitetto <i>et al</i> <sup>[19]</sup>	101	37%	24	na	78%

<sup>1</sup>Follow-up information not available in all patients. RVR: Rapid virological response; SVR: Sustained virological response; IL: Interleukin.

profit from a treatment of short duration whilst. On the contrary, in GT-3 patients, the unsatisfactory rate of SVR reported even with IFN-free regimens, induce a continuous search of predictors of response<sup>[15]</sup>.

A detailed examination of the studies on IL28B in GT-2 and GT-3 patients suggests that there are valid explanations for the contrasting results on SVR association. In our opinion, the analysis of genetic predictors performed in mixed cohorts, incorporating GT-2 or -3 in different proportions may be the first responsible of these contrasting results<sup>[10,11]</sup>. Additional confounders are the different treatment regimens of the patients included in these studies, heterogeneous in terms of duration and intensity<sup>[11,14]</sup>, and, more importantly, the different populations of patients evaluated in these retrospective genetic analyses that combines naïve and previous treatment-experienced<sup>[16]</sup>.

Finally, the use of either rs8099917 SNP located 7.6 kb upstream the *IL-28B* gene or rs12979860, located 3.2 kb upstream the open reading frame of IL28B gene may be an additional source of confusion. Indeed, these two SNPs showed similar distribution in Caucasian patients but different frequency and, consequently, lower strength of association in races other than Caucasian.

To overcome some of these issues, two meta-analyses on the role of IL28B in GT-2 and 3 have been recently published<sup>[17,18]</sup>. However, as happened with (the) single studies, these meta-analyses reached contrasting results. In the study by Chen *et al*<sup>[17]</sup> no association between SVR and *IL-28B* CC was found in the subgroup of GT-2 and 3, although it was shown that TT at rs8099917 SNP is associated with a favorable response in GT-2 Asian subjects. In the second meta-analysis, the Authors reached the conclusion that the favorable *IL-28B* CC genotype is a statistically significant predictor of SVR and rapid virological response (RVR) in Caucasian patients treated with Peg-IFN and ribavirin for 24 wk, with the exception of Asian patients with GT-2 achieving higher rates of RVR when carrying the favorable *IL-28B* genotype<sup>[18]</sup>.

## DETAILED ANALYSIS OF THE STUDIES

Data summarizing the results of the studies on rs1297860 in HCV mono-infected patients are reported in Table 1. Studies including GT-2 and -3 lumped together<sup>[10,11,13,19]</sup> and studies investigating cohorts of patients with GT-3 alone were separately analyzed<sup>[12,14,20,21]</sup>. Results by genotype were provided by a large study from our group investigating 710 patients<sup>[21]</sup>. Another study focused on viral kinetics of IL28B polymorphisms by GT-1 *vs* GT-2 and -3<sup>[20]</sup>.

### Studies combining GT-2 and GT-3

The results of the largest studies on IL28B treatment response prediction in GT-2 and GT-3 are here analyzed. Combined results for GT-2 and GT-3 together are generally provided. Stored DNA samples from 268 Caucasian patients enrolled in a multicenter controlled trial from Italy were tested for rs12979860. Patients were randomized to Peg-IFN and RBV for standard (24 wk) or variable (12/24 wk) treatment duration on the basis of RVR. Two hundred and thirteen patients were GT-2 and 55 GT-3 infected<sup>[10]</sup>. *IL-28B* CC-type was present in 37% of patients. Rates of SVR were 82% in patients with CC-type, 75% in CT and 58% in TT. The CC-type resulted an independent predictor of SVR, but the predictive role was largely driven by the capability of predicting SVR among patients without RVR. Like in GT-1 (22), among the 165 (61%) patients with HCV RNA undetectable at week 4, *IL-28B* genotype was not predictive of SVR<sup>[10]</sup>.

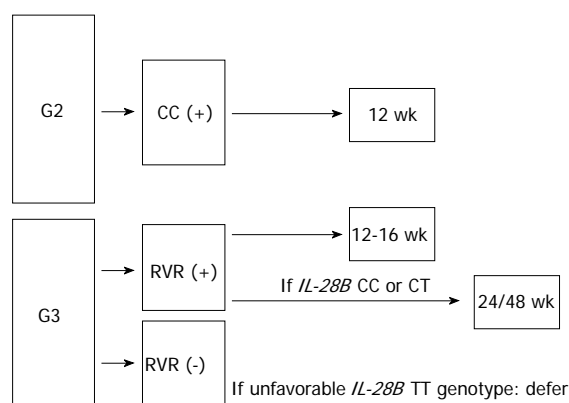
These findings reinforce the concept that a week 4 undetectable HCV RNA is the strongest predictor of SVR to Peg-IFN and RBV treatment; at the same time, they suggest that the clinical relevance of *IL-28B* genotype for GT-2 and -3 is far from being borderline, as it can help in selecting patients that may be interferon insensitive at baseline.

The results reported by Sarrazin *et al*<sup>[11]</sup> regarding 267 patients (GT-2 = 77, GT-3 = 190), among which only 205 received treatment, are apparently in contrast with our conclusions. The Authors showed an association between *IL-28B* CC-type and SVR in the subgroup of patients with RVR. No association was observed in patients without RVR<sup>[11]</sup>. However, despite a lower rate of RVR in observed in this study than in others (40%), only 11 patients without RVR were analyzed (CC = 3, CT = 4 TT = 3). Therefore, a type II error cannot be ruled out. Moreover, if we analyze CC *vs* CT plus TT patients who completed the treatment, we can observe a trend toward a statistically significant association with SVR in the subgroup of patients without RVR ( $P = 0.08$ ).

If we consider the results of the previously reported studies on GT-2 and -3 in comparison with those attained in patients with G1 infection<sup>[22]</sup>, we could hypothesize that the lower the rate of RVR, the stronger the association between SVR and CC-type (Figure 1).

Similar considerations apply to the study by Lindh *et al*<sup>[13]</sup>. In this study, 341 White patients with GT-2 and





**Figure 1** Algorithm for clinical application of interleukin 28B genotyping in hepatitis C virus GT-2 and GT-3 infected patients. RVR: Rapid virological response; IL: Interleukin.

-3 were evaluated. The RVR rate was 61%; 134 patients did not achieve RVR. Patients were subdivided according to a treatment duration of 12 or 24 wk ( $n = 166$  and  $n = 175$ , respectively). When patients were considered overall, a significant association between IL28B and RVR was shown. However, association between CC and SVR was significant for patients treated for 24 wk ( $P = 0.02$ ), but not for those receiving a short course of treatment with Peg-IFN alpha-2a and fixed 800 mg doses of RBV. Among patients without RVR, CC genotype was represented in 35%, CT in 47% and TT in 15%. In the subgroup of patients without RVR treated for 24 wk, SVR rates were higher for patients with CC as compared to CT and TT (74% *vs* 59% and 29%, respectively).

The study by Bitetto *et al.*<sup>[19]</sup> evaluated 101 patients with GT-2 and -3 as part of a larger cohort of patients with different HCV genotypes. In this study no association with either RVR or SVR was demonstrated among GT-2 and -3. In particular, 78% of IL28B CC infected with GT-2 and GT-3 combined together and 88% of IL28B CT/TT achieved viral clearance after treatment.

### Studies evaluating GT-3 separately

Two studies focused on GT-3 only; Scherzer *et al.*<sup>[12]</sup>, evaluated a small cohort of 71 patients, while Moghaddam *et al.*<sup>[14]</sup> studied 281 patients (Table 2)<sup>[20]</sup>. Data on 475 GT-3 were separately available also in the study by Fattovich *et al.*<sup>[20]</sup> and in our prospective cohort of 710 patients with GT-2 and GT-3<sup>[21]</sup>. These studies are analysed below.

Scherzer *et al.*<sup>[12]</sup> investigated both rs12979860 and rs8099917 SNPs in a cohort of patients originally randomized 1:1 to 800 or 400 mg of RBV in combination with Peg-IFN alpha-2a. The results of this study might be limited by the small sample size, moreover the low dosages of RBV (used) may impact the generalizability of the conclusions. In the final analysis, only patients who completed the treatment were considered, they were 37 and 31 in each arm, respectively. A CC genotype was identified in 38% of patients, but no association with SVR was observed. Indeed, 19/25 (76%) CC and 34/44 CT and TT combined together (77.3%) achieved SVR<sup>[12]</sup>. As shown in patients with genotype 1 infection<sup>[22]</sup>, higher

**Table 2** Prevalence and impact of interleukin 28B rs12979860 in studies analyzing genotype 3 separately

Study	No. of patients	Prevalence of IL-28B CC Genotype	Treatment duration (wk)	RVR in IL-28B CC genotype	SVR in IL-28B CC genotype
Scherzer <i>et al.</i> <sup>[12]</sup>	71	38%	2	78%	76%
Moghaddam <i>et al.</i> <sup>[14]</sup>	281	46%	14-24	84%	77%
Fattovich <i>et al.</i> <sup>[20]</sup>	55	51%	24	86%	87%
Mangia <i>et al.</i> <sup>[21]</sup>	470	41%	12-24	80%	84%

RVR: Rapid virological response; SVR: Sustained virological response; IL: Interleukin.

levels of baseline HCV RNA were associated in this study with CC genotype as compared to CT or TT<sup>[12]</sup>.

A larger study investigating IL28B in GT-3 has been published by Moghaddam *et al.*<sup>[14]</sup>. The Authors evaluated both rs12979860 and rs8099917 SNPs in DNA extracted from plasma of 281 GT-3 patients representing 51% of patients enrolled into two previous clinical trials, a non-randomised and a randomised one<sup>[23,24]</sup>. Authors demonstrated rates of SVR comparable between CC, CT and TT (77% *vs* 81% and 96%), whereas a statistically significant association between RVR and favorable genotypes was shown. Indeed, 84% of CC-type, 62% of CT and 56% of TT achieved RVR (OR = 3.3, 95%CI: 1.9-5.8). Genotyping rs8099917 SNP, the results were not different (OR = 2.7, 95%CI: 1.6-4.7). In this study, the exclusion of a number of patients who did not fit the inclusion criteria may have represented a bias. Moreover, the association analysis between the different host genotypes and SVR should have been adjusted for confounders, as for example an uneven distribution of patients with favourable genotypes across the different treatment arms. Strikingly, in this study the frequency of CC genotype was 8%-9% higher than in other studies<sup>[10]</sup>.

Negative results were reported also by Fattovich *et al.*<sup>[20]</sup> in a retrospective cohort study on Italian patients. This study offered the possibility to a separate analysis of GT-2 and GT-3 but the overall number was not higher than 159 including 104 GT-2 and 55 GT-3. No association with RVR was reported in 24 of 28 patients with IL28CC and GT-3 in comparison to 20 of 27 CT/TT ( $P = 0.31$ ). Similarly, the results were not different for 20 of 23 GT-3 CC and 28 of 32 subjects with CT/TT who achieved SVR ( $P = 0.79$ ).

The Write study with IL28B available in 93.7% of 710 GT-2 and GT-3 patients is the largest series prospectively evaluating for IL28B. Results of this study including 475 GT-3 showed that while within GT-2 no association between IL28B CC and SVR (90.3% for CC *vs* 82.0% for non CC,  $P = 0.15$ ) can be observed, within GT-3, the association between IL-28B CC and SVR is highly significant (84% *vs* 60%,  $P < 0.001$ ).

These results demonstrate that when the sample size is adequate, the association between IL28B and RVR or

SVR can be appreciated. Therefore, it may be rational to evaluate *IL-28B* genotyping in patients with GT-3, unless further evidence suggest otherwise. Despite the occurrence of side effects or poor tolerability, patients who bear a favorable *IL-28B* genotype should not discontinue treatment. At the same time, an unfavorable *IL-28B* genotype in patients with GT-3 infection may suggest to defer treatment in waiting for more efficacious drugs.

## DISCUSSION

After a careful analysis of the available data, a few aspects deserve consideration. Sample size is one of the most relevant issues in genetic studies as the power of the single study is influenced by the effect size and by the frequency of the minor allele<sup>[25]</sup>. The effect size of IL28B is large, yet the frequency of the minor allele for SNP rs1297860 ranges between 8% and 16% across the studies evaluating GT-2 and -3<sup>[10,12]</sup>. Although with such variability, it might be difficult to establish a minimum sample size valid across the studies, the risk for many of them to be underpowered is not negligible<sup>[26]</sup>. With the assumption of a 0.37 frequency of CC-type and an expected rate of SVR of 0.68 in CT patients, more than 520 patients are required to detect an odds ratio of at least 1.8. Therefore, study's conclusions should be based on studies with large sample size.

A further consideration owes to be made, the role of predictive factors is not absolute, but it depends on the efficacy of treatment. With about 80% of SVR attained in patients with GT-2 treated with P/R combination it is easy to understand that the sensitivity of the *IL-28B* genotype for the prediction of SVR in patients with GT-2 is limited and it is easy to understand that combining together GT-2 and GT-3 the sensitivity of the *IL-28B* genotype for the prediction of SVR is no higher than 40%-47%<sup>[10,11]</sup>. The lower rate of SVR in patients with GT-3 only put things in a different context suggesting that the combination of unfavorable IL28B and advanced fibrosis may represent a good reason to defer treatment based on Peg-IFN backbone due to the expectancy of a very poor response. Waiting for alternative treatment may be in these case a more reasonable choice.

Based on these considerations, we have imagined an algorithm for the management of patients with chronic GT-2 and GT-3 infection including IL28B and on treatment response (Figure 1). Our proposal is to perform *IL-28B* genotyping in patients with GT-3 at the aim of encouraging them to treatment, when undecided, to establish the duration of treatment and to decide not to treat those with very poor likelihood of SVR.

In conclusion, in easy to treat GT-2 patients IL28B may be considered as an additional not essential predictor of shortened treatment duration, while in GT-3, genotyping of *IL-28B* polymorphisms may be used to convince skeptical patients, to maintain on treatment those who are at risk of withdrawing because of side effects and to defer treatment in patients with low likelihood of response.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Post-translational modifications of hepatitis C viral proteins and their biological significance

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## Abstract

Replication of hepatitis C virus (HCV) depends on the interaction of viral proteins with various host cellular proteins and signalling pathways. Similar to cellular proteins, post-translational modifications (PTMs) of HCV proteins are essential for proper protein function and regulation, thus, directly affecting viral life cycle and the generation of infectious virus particles. Cleavage of the HCV polyprotein by cellular and viral proteases into more than 10 proteins represents an early protein modification step after translation of the HCV positive-stranded RNA genome. The key modifications include the regulated intramembranous proteolytic cleavage of core protein, disulfide bond formation of core, glycosylation of HCV envelope proteins E1 and E2, methylation of nonstructural protein 3 (NS3), biotinylation of NS4A, ubiquitination of NS5B and phosphorylation of core and NS5B. Other modifications like ubiquitination of core and palmitoylation of core and NS4B proteins have been reported as well. For some modifications such as phosphorylation of NS3 and NS5A and acetylation of

NS3, we have limited understanding of their effects on HCV replication and pathogenesis while the impact of other modifications is far from clear. In this review, we summarize the available information on PTMs of HCV proteins and discuss their relevance to HCV replication and pathogenesis.

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**Key words:** Hepatitis C virus; Hepatitis C virus proteins; Post-translational modifications of proteins; Hepatitis C virus replication; Hepatitis C virus pathogenesis

**Core tip:** Post-translational modifications (PTMs) are an important step in protein maturation and associated with protein function, activity and/or protein life span. PTMs of viral proteins are often essential for regulation of processes involved in viral infections and can be crucial for infectious virion production. Moreover, identification of PTM sites in viral proteins is particularly useful for the development of antiviral drugs. This overview on PTMs of hepatitis C virus (HCV) proteins discusses how PTMs affect HCV replication and virus-induced pathogenesis.

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## INTRODUCTION

Hepatitis C virus (HCV), a member of the genus *Hepacivirus* within the family *Flaviviridae*, is able to establish chronic infection in humans, which eventually leads to liver cirrhosis, hepatocellular carcinoma (HCC) and liver



failure<sup>[1,2]</sup>. Approximately 3% of the world population are infected with HCV. However, no effective vaccine has been developed and the current antiviral treatments have some limitations<sup>[3,4]</sup>. In order to develop efficient antiviral therapies, a complete understanding of viral pathogenesis and virus-host interactions is fundamental. Like other positive-stranded RNA viruses, HCV hijacks the host cell's translation machinery for producing viral proteins<sup>[5]</sup>. Thereby, post-translational modifications (PTMs) of virus encoded proteins occur as a rather natural step during the cell's general protein synthesis process, but concurrently encompass impact on viral replication and infectivity. In this review, we will start with a discussion on the proteolytic cleavage of HCV polyprotein, and give an overview on PTMs of HCV proteins and discuss their influence on viral replication and pathogenesis.

## PROTEOLYTIC CLEAVAGE OF HCV POLYPROTEIN

HCV genome consists of a 5'-untranslated region (UTR), a large open reading frame (ORF) encoding a polyprotein precursor of about 3000 amino acids and a 3'-UTR<sup>[6]</sup>. Proteolytic processing of the HCV polyprotein giving rise to single viral proteins represents an initial step in viral protein modification. There are nine defined proteolytic cleavage sites within the HCV polyprotein precursor, resulting in the generation of at least ten non-overlapping proteins, including structural proteins core, E1 and E2, and nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Additional viral protein products might be produced by alternative ORFs discovered within the HCV genome<sup>[7-10]</sup>.

Proteolytic processing of the polyprotein precursor occurs co- and post-translationally involving cellular as well as viral proteases<sup>[11]</sup>. The structural proteins are cleaved off the polyprotein precursor by host cellular signal peptidase (SP) located in the endoplasmic reticulum (ER) of the host cell, while the nonstructural proteins are released from the polyprotein precursor by viral proteases NS2-3 and NS3-4A<sup>[9]</sup>. The core protein is found to be additionally cleaved inside the ER membrane by host cellular signal peptide peptidase (SPP), thus yielding the mature core variant<sup>[12]</sup>. This step leads to the release of core from the ER and its trafficking to lipid droplets (LDs) which are believed to serve as a platform for HCV particle assembly<sup>[13]</sup>. E2, p7 and NS2 are first generated as an E2-p7-NS2 precursor protein. Remarkably, the E2-p7-NS2 precursor is proteolytically processed at the p7-NS2 junction efficiently whereas the E2-p7 junction gets cleaved less frequently, hence resulting in the presence of E2 and p7 proteins as well as the non-cleaved E2-p7 variant in infected cells<sup>[14]</sup>. The NS2-3 autoprotease cleaves at the NS2-NS3 junction and the NS3-4A protease cleaves at the sites between NS3 and NS4A, NS4A and NS4B, NS4B and NS5A, and NS5A and NS5B. Proteolytic processing by the NS3-4A complex follows a certain order that the cleavage first happens *in cis* at the NS3-NS4A

junction, then rapidly *in trans* at NS5A-NS5B followed by proteolysis at NS4A-NS4B, and finally at NS4B-NS5A. NS3 to NS5B mainly function in HCV genome replication<sup>[8,15]</sup>. Proteolytic processing releases structural and nonstructural HCV viral proteins that take part in different stages of HCV life cycle.

## POST-TRANSLATIONAL MODIFICATIONS OF HCV PROTEINS

### Core protein

HCV core protein is the most conserved viral protein among different HCV genotypes. It constitutes the viral nucleocapsid that encapsidates the viral RNA genome, and is essential for virus particle assembly<sup>[16,17]</sup>. HCV core also possesses several regulatory functions, such as cellular transcription, virus-induced transformation, signal transduction, steatosis and HCC. Moreover, core is significantly involved in virus-mediated pathogenesis. It is able to modulate apoptosis and cell growth, but also up-regulates reactive oxygen species (ROS) production and has a possible immunoregulatory role<sup>[16,18]</sup>.

The complete core protein is composed of three domains: an N-terminal hydrophilic domain that is essential for RNA binding and homo-oligomerization, a C-terminal hydrophobic domain that associates with LDs and is involved in proper folding, and a hydrophobic signal sequence tail that can target E1 to the ER membrane<sup>[16,19-21]</sup>. Unlike other HCV proteins, core protein liberation from HCV polyprotein precursor needs sequential proteolytic processing. Following cleavage from HCV polyprotein at the core-E1 junction by host cellular SP, the immature core protein is additionally cut by SPP within its hydrophobic C-terminus to release mature N-terminal amino acids 173-179 core protein and dissociate core from the ER membrane<sup>[16,22]</sup>. The exact C-terminus of mature core has not been identified yet in mammalian cells, even though it was reported to be Phe<sup>177</sup> or Leu<sup>179</sup> in insect cells<sup>[23]</sup>. This further processing by SPP relies on previously correct cleavage by SP and the sequential processing controls viral protein production rate<sup>[24]</sup>. Only the mature form of core can attach to LDs and interact with NS5A that transports HCV genome RNA to core<sup>[25,26]</sup>. Therefore, core maturation by SPP cleavage plays a critical role in virus assembly and regulation of HCV life cycle.

It has been demonstrated that disulfide bonds in nucleocapsid proteins of viruses with icosahedral structure play a role in virus assembly and capsid structure stabilization<sup>[27]</sup>. Since HCV virion is packaged into a similar spherical structure, its nucleocapsid might resemble the same organization<sup>[28]</sup>. Mutation analysis discovered that mature core formed a dimeric membrane protein which was linked by disulfide bond at Cys<sup>128</sup>. This disulfide bond formation stabilizes capsid structure and strengthens the interaction between core and membranes, and is critical for virus assembly and virion production. However, the disulfide bond in core has no effect on HCV RNA rep-

lication, the association with LDs or other functions of core<sup>[27]</sup>. Because of the low mutation rate of Cys<sup>128</sup>, drugs targeting Cys<sup>128</sup> disulfide bond formation may be considered as a candidate to inhibit HCV virion formation.

Phosphorylation is a common type of PTMs, which is also observed in HCV core protein. The phosphorylation of core by protein kinases A (PKA) at Ser<sup>53</sup> and Ser<sup>116</sup> and by protein kinases C (PKC) at Ser<sup>53</sup> and Ser<sup>99</sup> was reported both *in vitro* and in Huh-7 and HepG2 cells. However, Ser<sup>99</sup> and Ser<sup>116</sup> are the major and predominant sites with low phosphorylation efficiency<sup>[29,30]</sup>. The phosphorylation at these two sites is critical for inhibition of HBV gene expression and replication in Huh-7 cells, but the detailed *trans*-suppression mechanism of HCV core remains unclear. The same study showed that only truncated core is phosphorylated by PKC suggesting structural conformation might be a prerequisite for phosphorylation<sup>[30]</sup>. The phosphorylation at Ser<sup>116</sup> by PKA is shown to be responsible for the repressive activity of core on cyclin-dependent kinase inhibitor (CKI) p21 promoter<sup>[31]</sup>. Reduced p21 may interfere with p53 driven repair mechanism in cell cycle, which may facilitate tumorigenesis. Another role of phosphorylation of core might be involved in modulating nuclear localization of core, although controversial results have been reported<sup>[29,30]</sup>. Nuclear core is involved in regulating host gene transcription<sup>[32]</sup>.

Moreover, HCV core also undergoes several other types of PTMs. For example, the ubiquitination of core protein by E3 ubiquitin ligase E6AP preferentially at N-terminal lysine residues induces the degradation of core in the cytoplasm by the ubiquitin-proteasome pathway, which could control HCV virion production and have an antiviral effect. The interaction region between core and E6AP is located between amino acids 58 to 71 of the core protein, which are highly conserved in all HCV genotypes<sup>[33-35]</sup>. Palmitoylation of core at Cys<sup>172</sup> plays a vital role in targeting the core to smooth ER and ER-associated LDs, but does not affect SPP proteolytic cleavage-induced maturation and LD accumulation. Importantly, it also affects HCV assembly and production<sup>[36]</sup>.

### Envelope glycoproteins E1 and E2

HCV envelope glycoproteins E1 and E2 play an important role in virus entry and immune evasion<sup>[37]</sup>. In infected cells, E1 and E2 are either found as noncovalent heterodimers, which are mainly localized to the ER, or as disulfide-linked aggregates, which were originally thought to represent misfolded protein complexes<sup>[38-41]</sup>. Heterodimers and oligomers of E1 and E2 are also found in infectious virus particles, whose structure is stabilized by disulfide bonds<sup>[38,42,43]</sup>.

Both E1 and E2 proteins consist of a large N-terminal ectodomain and a C-terminal hydrophobic transmembrane anchor. PTMs of HCV envelope proteins include the attachment of glycans and the formation of disulfide bridges<sup>[44,45]</sup>. Glycans attached to HCV envelope proteins were shown to modulate virus entry by modifying their receptor binding affinity or fusion activities. They are

also involved in protein folding and play a key role in immune evasion by masking potential antigenic sites from binding of neutralizing antibodies<sup>[44,46,47]</sup>. Because glycosylation sites within HCV glycoproteins are rather highly conserved, glycosylation mutants are considered as immunogens to induce a potent antibody response against HCV<sup>[48]</sup>.

There are four to five N-glycosylation sites in E1 and up to 11 N-glycosylation sites in E2<sup>[44,47,49,50]</sup>. N-linked glycosylation occurs at asparagine residues and the consensus sequence is Asn-X-Ser/Thr<sup>[44,51]</sup>. Mass spectrometric analysis of E2 revealed that this protein is mainly modified by high-mannose type oligosaccharides and more complex glycan types are observed for just two glycosylation sites within E2<sup>[52]</sup>. E1 is believed to be modified only by high-mannose type oligosaccharides since a restricted localization of E1/E2 heterodimers to the ER is confirmed by immunofluorescence<sup>[49]</sup>. However, more complex type glycosylations generally occur in the cis-Golgi compartment, where indeed a small population of E2 protein has been detected by immunofluorescence<sup>[53]</sup>. On the other hand, the attachment of complex glycans can happen during the release of viral particles via the exocytotic pathway, which involves the Golgi apparatus. Interestingly, due to the differences in the assembly process, more mature glycoproteins containing complex type glycans could have been observed with HCV pseudoparticles (HCVpp) compared to cell culture-derived HCV particles (HCVcc)<sup>[38]</sup>. HCVpp is found to assemble in post-Golgi compartments<sup>[54]</sup>, while HCVcc assembly takes place in ER-derived compartments<sup>[55]</sup>. HCVpp glycoproteins might also be more accessible to Golgi glycosyltransferases than HCVcc glycoproteins, which are components of high-order virion complexes<sup>[38]</sup>. Differences in the glycosylation pattern of HCVpp and HCVcc might be relevant for studying HCV immune evasion strategies.

Furthermore, the carbohydrate composition of envelope glycoproteins vary to some extent depending on the cell line the virus infected<sup>[56]</sup>. Changes in the glycosylation pattern of HCV glycoproteins have a major impact on virus particle assembly, entry and immunogenicity<sup>[44,50]</sup>, thus affecting virus pathogenesis and virulence. Mutations of glycosylation sites N1 and N4 in HCV glycoprotein E1 (E1N1, E1N4) as well as N8 and N10 in HCV glycoprotein E2 (E2N8, E2N10) strongly interfere with the incorporation of both envelope proteins into HCVpp, suggesting the importance of these sites for protein folding and E1/E2 heterodimerization<sup>[42,44]</sup>. Additionally, mutation of glycosylation site E2N2 or E2N4 leads to the decreased infectivity of HCVpp, confirming a role of both glycans in virus entry<sup>[44]</sup>. Moreover, glycans at positions E2N1, E2N6 and E2N11 are shown to decrease the binding affinity of E2 to the cluster of differentiation 81 (CD81) receptor and to reduce the sensitivity of pseudotyped HCV particles to antibody neutralization, hence contributing to humoral immune evasion<sup>[47]</sup>. These findings are supported and extended by studies with HCVcc glycosylation mutants<sup>[42]</sup>. Apparently, glycosylation sites

E2N1, E2N2, E2N4 and E2N6 seem to surround the CD81 receptor binding site within E2, therefore “protecting” this site from recognition by neutralizing antibodies. Helle *et al.*<sup>[42]</sup> provided structural evidence for glycans attached to HCV envelope proteins to modulate the humoral immune response.

Besides N-glycosylation, little information is available on O-glycosylation of HCV E1 or E2. Supposedly, there is one potential O-glycosylation site within E1 and four potential O-glycosylation sites within E2<sup>[48]</sup>. So far, 3 O-linked glycosylation sites in E2 have been shown to be important for HCV entry, with two of them apparently decreasing E2 binding affinity to CD81 receptor<sup>[48]</sup>.

Virion-associated HCV glycoproteins are assembled into large oligomeric protein complexes which are stabilized by disulfide bonds<sup>[38,42]</sup>. These complexes are able to bind conformation-sensitive neutralizing antibodies and recombinant CD81<sup>[38]</sup>, and therefore can be considered functionally significant rather than the result of a misfolding event.

Proper folding of glycoprotein E1 is dependent on E2 coexpression and *vice versa*<sup>[57,58]</sup>. E1 and E2 consist of eight and 18 highly conserved cysteine residues, respectively<sup>[43]</sup>. Structural information is mainly available for HCV E2 protein, where nine intramolecular disulfide bonds have been identified<sup>[59]</sup>. Because of the difficulties in expressing E1 in the absence of E2, disulfide arrangement of cysteine residues in E1 has not been determined<sup>[43]</sup>. Beside their apparent impact on virus particle structure and infectivity, it is conceivable that disulfide-linked glycoprotein oligomers may play an active role in HCV budding by assisting protein-protein interactions<sup>[38]</sup>. Furthermore, it is possible that the presence of disulfide bridges in HCV envelope proteins could be responsible for the lack of sensitivity of HCVcc to low-pH treatment<sup>[60]</sup>. This suggests their direct influence on virus internalization by affecting the presentation of HCV fusion peptide<sup>[38]</sup>. Additionally, the impact of disulfide rearrangement and the oxidation state of cysteine residues in E1 and E2 glycoproteins on HCV entry and membrane fusion was confirmed by Fraser *et al.*<sup>[43]</sup>. Here the presence of free thiol groups has been shown to be essential for HCV infectivity.

Altogether, PTMs of HCV glycoproteins by glycosylation and disulfide bond formation have a strong impact on several steps in viral life cycle, more specifically entry, fusion of viral membrane with the host cell's endosomal membrane and budding.

## p7

HCV p7 represents a small integral membrane protein which is able to oligomerize and form proton channels within the HCV particle envelope. The precise role of p7 in HCV life cycle has not been determined, even though it has been shown to be essential for infection, but not for viral replication<sup>[61,62]</sup>. Due to incomplete or delayed proteolytic processing, the generation of a p7 species linked to the E2 glycoprotein has been observed. The role

of E2-p7 precursor during HCV infection is not known so far. However, it is speculated that E2-p7 might be involved in regulating the production of native p7 and formation of ion channel complexes<sup>[63]</sup>. The optimal cleavage at the E2-p7 junction is shown to be important for virus production probably due to the increased NS2-associated virus assembly complex formation in close proximity of LDs. It also enhances NS2 interaction with NS3 and E2, but does not affect HCV genome replication<sup>[64]</sup>.

Structural analysis revealed that HCV p7 protein consists of two membrane-spanning  $\alpha$ -helices connected by a short cytoplasmic loop<sup>[65]</sup>. PTMs of p7 have not been demonstrated.

## NS2

HCV NS2 is a transmembrane protein. Together with the N-terminal domain of NS3, NS2 forms the NS2-3 autoprotease. The NS2-3 cysteine autoprotease is a zinc-dependent metalloprotease that cleaves the HCV polyprotein at the NS2-NS3 junction. After its self-cleavage from NS3, NS2 is quickly degraded<sup>[66,67]</sup>. Like p7, NS2 is known to be essential for virus assembly. Even though NS2 is part of the HCV replication complex, which is composed of NS2, NS3, NS4A, NS4B, NS5A and NS5B, NS2 is not essential for virus replication<sup>[62,68,69]</sup>. The interaction of NS2 with E1, E2, NS3 and NS5A results in co-localization of these viral proteins to dot-like structures near LDs, which are the sites for virus particle assembly<sup>[64,70]</sup>. Moreover, proper cleavage at the NS2-NS3 junction is important for an active HCV replication complex formation, but is not required for NS3 protease activity<sup>[71,72]</sup>. Other functions linked to NS2 include the inhibition of apoptosis and modulation of host cellular gene transcription<sup>[73-76]</sup>.

The highly hydrophobic N-terminus of NS2 consists of three trans-membrane segments which form the protein membrane binding domain<sup>[77]</sup>. No attachment of fatty acids or prenyl groups by modifications typically involved in membrane targeting, like farnesylation, myristoylation, palmitoylation or prenylation<sup>[78]</sup>, has been associated with membrane anchoring of NS2 so far. Though NS2 is located to the ER membrane, the protein is not glycosylated<sup>[79]</sup>. The protease activity of NS2 is located within its C-terminal domain, which is able to homodimerize and thus creates two composite active sites<sup>[80]</sup>. Regarding the role of NS2 in HCV particle formation, the overall structural integrity rather than the protease activity of NS2 itself appears to be crucial<sup>[81,82]</sup>.

The C-terminal globular domain of NS2 facing towards the cytoplasm of the infected cell was shown to be modified by phosphorylation. Phosphorylation of NS2 is presumably mediated by host cellular casein kinase 2 (CK2)<sup>[79]</sup>. NS2 is a short-lived protein that is rapidly degraded by the proteasome. Proteasome-mediated degradation of NS2 is regulated in an ubiquitin-independent manner by phosphorylation within its C-terminal domain. Ser<sup>168</sup> as part of a CK2 consensus recognition site (Ser/Thr-X-X-Glu) is shown to be vital for NS2 degra-



dation. It is highly conserved between all HCV genotypes and single point mutation of Ser<sup>168</sup> confers resistance to NS2 degradation<sup>[79]</sup>. Therefore, phosphorylation of NS2 is strongly connected to its abundance within the host cell and can have a strong impact on HCV pathogenesis, more particularly on assembly and virion production.

### NS3-4A complex

HCV NS3-4A is a noncovalent complex composed of the serine protease NS3 and its cofactor NS4A<sup>[83]</sup>. The NS3-4A mediated cleavage releases NS3, NS4A, NS4B, NS5A and NS5B from the HCV polyprotein in a specific order. The NS3-4A protease complex also has three identified cellular targets so far, including mitochondrial antiviral signaling protein (MAVS), T-cell protein tyrosine phosphatase (TC-PTP) and toll/IL-1 receptor homology domain-containing adaptor inducing IFN- $\beta$  (TRIF), which may be involved in the development of persistent infection and HCC<sup>[10,15]</sup>. Therefore, the NS3-4A protease is a prime target for antiviral drug design. For example, the two recently approved direct-acting antivirals (DAAs), telaprevir and boceprevir, are oral NS3-4A protease inhibitors<sup>[4,84]</sup>.

NS3 protein consists of an N-terminal serine protease domain with its catalytic triad composed of His<sup>57</sup>, Asp<sup>81</sup> and Ser<sup>139</sup>, and a C-terminal RNA helicase/NTPase domain. The NS3 helicase/NTPase couples NTP hydrolysis to unwind extensive RNA secondary structure and is important for RNA replication and virus assembly<sup>[85,86]</sup>.

The two domains of NS3 can function independently from each other, and the reason for their physical linkage remains unclear<sup>[83,85]</sup>. The intracellular NS3 protease shows structure homology with extracellular serine proteases, but does not possess disulfide bonds to stabilize its structure as extracellular serine proteases<sup>[87]</sup>. A Zn<sup>2+</sup> ion together with its binding site formed by Cys<sup>97</sup>, Cys<sup>99</sup>, Cys<sup>145</sup> and His<sup>149</sup> stabilizes NS3 protease, activates NS3 hydrolysis and facilitates NS2 processing at the NS2-NS3 junction. Binding of NS4A further stabilizes NS3 by restructuring the N-terminus of NS3 protease through interaction with the central hydrophobic portion of NS4A, increases catalytic efficiency by influencing the spatial configuration of the catalytic triad and directs the cellular membrane localization because of the high hydrophobicity of the N-terminal transmembrane  $\alpha$ -helix of NS4A. In addition, the C-terminal acidic portion of NS4A plays a role in regulating HCV genome replication and virus assembly by interacting with other viral proteins in the replication complex<sup>[15,87-89]</sup>. NS4A also regulates HCV replication by modulating NS5A hyperphosphorylation<sup>[90]</sup>.

Liefhebber *et al.*<sup>[91]</sup> has shown that NS3 might get phosphorylated in subgenomic HCV replicon cells through phosphospecific staining and dephosphorylation assay. However, phosphorylation efficiency is low and the phosphorylation sites are hard to identify. In addition, N-terminal acetylation of NS3 was identified by this research group through mass spectrometric analysis. The role of NS3 phosphorylation and acetylation in HCV life

cycle needs to be further investigated.

It has been reported that protein arginine methyltransferases (PRMTs) can irreversibly and post-translationally methylate arginine residues in the arginine-glycine (RG)-rich region of many RNA-binding proteins<sup>[92,93]</sup>. Since NS3 protein can bind to RNA through its RNA helicase domain and contains seven RG motifs including two RG motifs in the helicase domain, it is a potential methylation target for PRMTs. Full-length NS3 and NS3 helicase domain are shown to be methylated at Arg<sup>1493</sup> in the <sup>1486</sup>Gln-Arg-Arg-Gly-Arg-Thr-Gly-Arg-Gly<sup>1494</sup> motif by PRMT1, but no methylation is found in NS3 protease domain<sup>[94]</sup>. Mutation analysis has demonstrated that Arg<sup>1490</sup> and Arg<sup>1493</sup> are determinants for the helicase activity<sup>[95]</sup>. Methylation of NS3 at Arg<sup>1493</sup> inactivates the helicase by inhibiting unwinding of double-stranded DNA<sup>[96]</sup>. The reason that arginine methylation is involved in protein-nucleic acid interaction is that methyl modification may affect the binding affinity, protein stability, transcription and signal transduction<sup>[92]</sup>. Negative regulation of PRMT1 by protein phosphatase 2A (PP2A) increases NS3 helicase activity and enhances HCV RNA replication, therefore PP2A is considered a potential target for HCV drug development<sup>[96]</sup>.

The cofactor activity of NS4A is mediated by its central region and especially the hydrophobic Ile<sup>25</sup> and Ile<sup>29</sup> residues, since an I25A/I29A double mutant cannot form a complex with NS3<sup>[97]</sup>. To reactivate NS4A cofactor activity, the double mutant requires biotinylation at the N-terminus by biotin-aminohexanoic acid (ahx). However, the N-terminal biotin fusion alone without ahx or C-terminal biotin-ahx fusion cannot restore NS4A cofactor activity. On the other hand, N-biotinylation of wild-type NS4A by biotin-ahx can dramatically promote cofactor activity. Based on these data and the crystal structure, it is predicted that N-biotinylation by biotin-ahx resembles a hydrophobic environment that enhances the stabilization of NS3-4A complex and C-biotinylation may sterically interfere with the substrate binding pocket<sup>[98]</sup>.

### NS4B

HCV NS4B is relatively poorly understood compared to other HCV proteins. The liberation of NS4B happens at last during HCV polyprotein precursor processing in a strictly defined position<sup>[99]</sup>. It is a highly hydrophobic integral membrane protein that induces the formation of the membranous web around ER membrane where HCV genome replication takes place and functions by anchoring the HCV replication complex through an unknown mechanism<sup>[100]</sup>. It has been reported that NS4B can interact with other viral proteins such as NS5A, binds viral RNA and has NTPase activity. It is involved in RNA replication, virus assembly and release<sup>[101,102]</sup>. The multifunctional NS4B is also shown to activate ER stress pathways, contributes to steatosis by altering lipid metabolism and escape from innate immune system by inhibiting interferon<sup>[99]</sup>. Moreover, its anti-apoptosis function might be associated with HCC development<sup>[103]</sup>.



There are two amphipathic helices (AH1 and AH2) located in the N-terminal region of NS4B with their hydrophobic sides facing the cytoplasmic side of ER membrane. AH2 is a membrane interacting domain that is essential for membrane trafficking, HCV genome replication and protein oligomerization. NS4B oligomerization is critical for replication complex formation<sup>[104-106]</sup>. The highly hydrophobic central core region of NS4B contains four transmembrane domains and the highly conserved C-terminus is a membrane binding domain that consists of two  $\alpha$ -helical elements and plays a role in NS4B self-interaction, thus being important for replication complex formation<sup>[107,108]</sup>.

There are three common lipid modifications of protein located in lipid raft, including palmitoylation, N-terminal myristoylation and palmitoylation, and glycosylphosphatidylinositol modification<sup>[109]</sup>. So far, only palmitoylation is detected in NS4B at Cys<sup>257</sup> and Cys<sup>261</sup> in the C-terminus and these two sites are relatively conserved among HCV genotypes. Site-directed mutagenesis confirmed that Cys<sup>261</sup> palmitoylation is more crucial for protein-protein interaction and replication complex formation. Palmitoylation can enhance the polymerization activity of NS4B through its N-terminus<sup>[110]</sup>.

### NS5A

HCV NS5A is a phosphorylated zinc-metalloprotein without any enzymatic activity, but required for RNA replication and virion morphogenesis<sup>[111]</sup>. However, the precise mechanism of how NS5A functions is not clear. It is demonstrated that NS5A can bind to HCV RNA, other HCV proteins such as NS5B and cellular proteins such as human vesicle-associated membrane-associated protein of 33 kDa (hVAP-33), thus contributing to replication complex formation<sup>[8]</sup>. Several other functions of NS5A include interferon resistance, transcriptional activation and signaling pathway regulation<sup>[112,113]</sup>.

NS5A is composed of three domains. Domain I contains a zinc-binding motif and is the determinant for HCV RNA replication. It is a nucleic acid-binding domain that binds to the 3' G/C rich sequence in HCV RNA. It also functions in LD association. Domain II may play a role in evading innate immune response as well as RNA replication. Domain III participates in virus assembly and core protein interaction<sup>[83,86,114]</sup>. In addition, there is an amphipathic  $\alpha$ -helix in the N-terminal region responsible for ER membrane anchoring<sup>[111]</sup>.

NS5A is a phosphoprotein that exists in two forms, a basally phosphorylated form (56 kDa) and a hyperphosphorylated form (58 kDa), which is conserved among HCV genotypes<sup>[115]</sup>. The basally phosphorylated sites are mainly serine residues and the minority are threonine residues located in the central and C-terminal region. Major hyperphosphorylated sites are identified in a serine-rich region in the central portion of NS5A<sup>[112,115]</sup>. The basally phosphorylated form may be affected by NS2 and NS4A, whereas hyperphosphorylation of NS5A requires

NS3, NS4A and NS4B. Cellular protein kinases in the CMGC kinase family are involved in NS5A phosphorylation, including cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and casein kinase II (CKII)<sup>[90,112,116-118]</sup>. Since the subcellular distributions of both NS5A forms are similar, the degree of phosphorylation does not affect NS5A localization to the ER membrane<sup>[119]</sup>. However, the degradation of NS5A is enhanced by increased degree of phosphorylation<sup>[115]</sup>. Mutation analysis revealed that reduced NS5A hyperphosphorylation promotes HCV RNA replication, whereas reduced basal phosphorylation does not have an effect on HCV RNA replication in a replicon system. This suggested that the ratio of these two NS5A phosphorylation forms may be important for viral RNA replication<sup>[120,121]</sup>. NS5A is also involved in virion production through its interaction with core protein, which requires basal phosphorylation of NS5A<sup>[122]</sup>.

### NS5B

HCV NS5B is a conserved RNA-dependent RNA polymerase (RdRp) that initiates complementary negative-strand RNA synthesis and then synthesizes positive-strand RNA using the newly synthetic negative-strand RNA as template. Due to the lack of proofreading of RdRp, HCV replication is error-prone<sup>[83,86]</sup>. NS5B can interact with other viral proteins such as NS3, NS4A and NS5A, and cellular proteins like hVAP-33, which facilitates the formation of the viral RNA replication complex<sup>[123,124]</sup>. Furthermore, it can form a complex with the retinoblastoma tumor suppressor protein (pRb) and promote pRb degradation in an ubiquitin dependent manner, therefore contributing to HCC development<sup>[125]</sup>.

Like other polymerases, the crystal structure of NS5B reveals that it resembles the configuration of a right hand. The finger, thumb and palm domains compose a unique shape. The active site located in the palm domain has a highly conserved GDD motif. There are four allosteric sites within the thumb and palm domains which serve as targets for antiviral development<sup>[126-128]</sup>. Besides, NS5B is a tail-anchored protein with its C-terminal hydrophobic tail associated to the ER membrane<sup>[86]</sup>.

The function of many cellular enzymes for DNA and RNA metabolism and viral RdRps is often regulated by phosphorylation<sup>[129]</sup>. Hwang *et al.*<sup>[130]</sup> demonstrated that NS5B is a phosphoprotein in insect cells. Kim *et al.*<sup>[129]</sup> discovered that the protein kinase C-related kinase 2 (PRK2) is the specific enzyme for NS5B phosphorylation within the N-terminal finger domain. Knock-down and over-expression of PRK2 demonstrated PRK2 up-regulates HCV RNA replication in HCV subgenomic replicon cells, suggesting that NS5B phosphorylation can enhance HCV replication.

Gao *et al.*<sup>[131]</sup> identified an interaction between ubiquitin-like protein hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton) and NS5B. Since hPLIC1 interacts with both proteasome and

E3 ubiquitin protein ligases E6AP and  $\beta$ TrCP, the ubiquitination modification of NS5B through hPLIC1 binding promotes ubiquitin-dependent proteasome degradation, resulting in decreased level of NS5B. NS5B mainly functions in RNA replication, so decreased NS5B leads to HCV genome RNA reduction<sup>[131,132]</sup>. Although the ubiquitination sites within NS5B and the detailed mechanism of hPLIC1-induced NS5B degradation are still not clear, up-regulating NS5B ubiquitination may represent a target for anti-viral development.

## SUMMARY AND PERSPECTIVES

PTMs of HCV viral proteins include phosphorylation, glycosylation, disulfide bridging, methylation, palmitoylation, acetylation, and ubiquitination. These protein modifications ensure proper protein functions by regulating protein activity, subcellular localization, protein-nucleic acid interaction, and protein-protein interactions. Among the already identified PTMs of HCV proteins, some are essential for HCV virion production such as sequential proteolytic cleavage of core protein, whereas others have regulatory roles in virus replication such as phosphorylation of NS5A and ubiquitination of NS5B. PTM sites and PTM pathways are potential pharmacological targets for antiviral drug development. However, much work remains to be done to unveil the precise PTM sites and the underlying mechanisms.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Hepatitis C virus protease inhibitor-resistance mutations: Our experience and review

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## Abstract

Direct-acting antiviral agents (DAAs) for hepatitis C virus (HCV) infection are one of the major advances in its medical treatment. The HCV protease inhibitors boceprevir and telaprevir were the first approved DAAs in the United States, Europe, and Japan. When combined with peginterferon plus ribavirin, these agents increase sustained virologic response rates to 70%-80% in treatment-naïve patients and previous-treatment relapsers with chronic HCV genotype 1 infection. Without peginterferon plus ribavirin, DAA monotherapies increased DAA-resistance mutations. Several new DAAs for HCV are now in clinical development and are likely to be approved in the near future. However, it has been reported that the use of these drugs also

led to the emergence of DAA-resistance mutations in certain cases. Furthermore, these mutations exhibit cross-resistance to multiple drugs. The prevalence of DAA-resistance mutations in HCV-infected patients who were not treated with DAAs is unknown, and it is as yet uncertain whether such variants are sensitive to DAAs. We performed a population sequence analysis to assess the frequency of such variants in the sera of HCV genotype 1-infected patients not treated with HCV protease inhibitors. Here, we reviewed the literature on resistance variants of HCV protease inhibitors in treatment-naïve patients with chronic HCV genotype 1, as well as our experience.

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**Key words:** Direct-acting antiviral agent; Hepatitis C virus; Protease inhibitor; Resistance mutation; Sequence analysis

**Core tip:** The standard of care for the treatment of hepatitis C virus (HCV) infection was peginterferon plus ribavirin until the recent approval of telaprevir- and boceprevir-containing combination therapies. These HCV protease inhibitors occasionally cause HCV variants with resistance mutations. We reviewed the literature reports of resistance variants of HCV protease inhibitors in treatment-naïve patients with chronic HCV genotype 1, as well as our experience. Even in treatment-naïve patients with chronic HCV genotype 1, naturally occurring HCV protease inhibitor-resistance mutations exist in some cases. The combination of direct-acting antiviral agents against regions other than HCV NS3/4A could eradicate HCV with these resistance variants.

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## INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single-stranded RNA virus, approximately 9600 nt in length, that belongs to the *Flaviridae* family. Globally, HCV infects 170 million people and approximately 120-140 million chronic HCV carriers exist<sup>[1,2]</sup>. HCV infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)<sup>[3,4]</sup>. HCV is classified into six major genotypes and > 100 subtypes<sup>[5]</sup>. HCV genotype 1 (subgenotypes 1a and 1b) is the most common genotype in western countries and Japan<sup>[5]</sup>. Treatment of HCV is complicated by the existence of different HCV genotypes. The standard of care was peginterferon plus ribavirin until the recent approval of telaprevir- and boceprevir-containing combination therapies<sup>[6-14]</sup>. Combination of peginterferon plus ribavirin results in sustained virological response (SVR) in nearly 70%-80% of patients with HCV genotype 2 or 3, but in only approximately 50% of those with HCV genotype 1<sup>[15,16]</sup>. Thus, treatment response is dependent on HCV genotypes and viral loads<sup>[17]</sup>, viral sequence<sup>[18-21]</sup>, host factors such as IL28B genotypes<sup>[22-35]</sup>, drug adherence<sup>[36]</sup>, and adverse events induced by therapeutic drugs<sup>[36]</sup>.

Pharmaceutical companies are actively investigating and developing direct-acting anti-viral agents (DAAs) against HCV, which directly target specific HCV proteins such as NS3/4A protease<sup>[6-14]</sup>, NS5A protein<sup>[37-39]</sup>, and NS5B polymerase<sup>[40]</sup>, which are important for HCV replication in hepatocytes. Two first-generation HCV protease inhibitors, boceprevir and telaprevir, were approved in combination with peginterferon plus ribavirin for treatment of chronic HCV genotype 1 in 2011<sup>[6-14]</sup>. Both protease inhibitors combined with peginterferon plus ribavirin increased SVR rates up to 70%-80% in treatment-naïve patients and previous-treatment relapsers with chronic HCV genotype 1 infection<sup>[6-14]</sup>. Next-generation HCV protease inhibitors will be available in clinics in the near future (Table 1)<sup>[41]</sup>. For example, simeprevir<sup>[42,43]</sup>, faldaprevir<sup>[44,45]</sup>, and vaniprevir<sup>[46-48]</sup> are currently in phase 3 trials. HCV protease inhibitors primarily are specific agents for HCV genotype 1. However, studies have demonstrated that simeprevir is fairly active against most HCV genotypes with the exception of HCV genotype 3a<sup>[42]</sup>, and recently, in a phase 2 trial, the novel protease inhibitor MK-5172 showed even broader activity across HCV genotypes compared to simeprevir<sup>[49]</sup>.

The low fidelity of HCV NS5B polymerase, high replication rate, and strong selective pressures on this virus lead to emergence of viral quasiespecies. The quasiespecies nature exists in a mixed population of viruses, with the fittest viruses being the predominant viral populations, as observed by sequence analysis<sup>[50,51]</sup>. In addition, new

populations with every potential substitution are likely created and lost each day, some of which convey various degrees of resistance to DAAs<sup>[52-54]</sup>. Due to the high sequence diversity of HCV, naturally occurring pre-existing resistance mutations have been found at a low prevalence in HCV-infected, treatment-naïve patients<sup>[55,56]</sup>.

In a previous study<sup>[56]</sup>, 9% of the HCV genotype 1a-infected patients who were not treated with HCV protease inhibitors had at least one pre-existing dominant protease inhibitor-resistant variant, as observed by population sequencing. In another report<sup>[57]</sup>, although the number of patients was small, the prevalence of protease inhibitor-resistance mutations was high (28%) in 53 genotype 1a samples, while no mutations were found in only 5 patients infected with HCV genotype 1b. In HCV genotype 1b treatment-naïve patients, the percentage of naturally occurring pre-existing resistance mutations appears to be lower<sup>[58]</sup>. Recently, next generation sequencing technology, with a detection limit as low as < 0.1%, have shown the ability to detect most resistance variants (including high resistance variants, *i.e.*, 155, 156 and 168) in patients infected with protease inhibitor-untreated HCV genotype 1<sup>[59]</sup>. Thus, the prevalence of naturally occurring pre-existing resistance mutations in patients infected with HCV genotype 1 who were not treated with HCV protease inhibitors remains unclear.

## NATURALLY OCCURRING PREEXISTING RESISTANCE MUTATIONS IN PATIENTS INFECTED WITH HCV GENOTYPE 1B

We estimated a 98%-99% prevalence of HCV subgenotype 1b among patients infected with HCV genotype 1 in Japan<sup>[60]</sup>. Sera from 88 Japanese patients infected with HCV genotype 1b who were not treated with HCV protease inhibitors were examined. Some patients had been included in a previous study<sup>[60]</sup>. We investigated the naturally occurring pre-existing resistance mutations in these patients by using a direct-sequencing method. The study protocol was approved by the Ethics Committee of Chiba University School of Medicine.

The clinical background of the 88 patients is shown in Table 2. All but one patient had high viral loads. Population sequencing of the HCV NS3 region was performed in these 88 patients, and then the amino acid sequences were compared to the HCV NS3 amino acid sequence corresponding to the HCV genotype 1b Con1 strain. The prevalence of pre-existing variations in the HCV genotype 1b samples was 39% (34/88). Among these mutations, the resistance mutations T54S and D168N were found in 6.8% (6/88) and 1.1% (1/88) of the patients, respectively. Other resistance mutations Q80L, V170Y, V170N and V170L were found in 22% (19/88), 4.5% (4/88), 3.4% (3/88) and 1.1% (1/88) of the patients, respectively. In 5.7% (5/88) of the patients, more than one mutation was identified: four patients had T54S and Q80L, and one patient had T54S, Q80L and



**Table 1 Overview of representative clinical trials of hepatitis C virus NS3/4A protease inhibitors**

Name of drug (other name)	G	Trial phase	Features of clinical trials (ClinicalTrials.gov Identifier)
Telaprevir (VX-950)	1	FDA approved	Telaprevir, PEG-IFN alpha-2a, RBV
	1b	3	Telaprevir, Daclatasvir (NS5A inhibitor), PEG-IFN alpha-2a, RBV (COMMAND-3) (NCT01492426)
	1	3	Telaprevir, PEG-IFN lambda-1a, RBV (NCT01598090)
	4	2	Telaprevir, PEG-IFN alpha-2a, RBV (NCT0050801)
Boceprevir (SCH 503034)	1	FDA approved	Boceprevir, PEG-IFN alpha-2a, RBV
	1	3	Simeprevir, PEG-IFN alpha-2a, RBV (NCT01290731)
Simeprevir (TMC435)	1b/4	2	Simeprevir, IDEX719 (NS5A inhibitor), RBV (NCT01852604)
Faldaprevir (BI201335)	1	3	Faldaprevir, PEG-IFN alpha-2a, RBV
	1a	2	Faldaprevir, PPI-668 (NS5A inhibitor), BI207127 (non-nucleoside NS5B inhibitor), (+ RBV) (NCT01859962)
	1b	2	Faldaprevir, BI207127, RBV (NCT01858961)
Danoprevir (ITMN-191)	1	2	Danoprevir, PEG-IFN alpha-2a, RBV (NCT00963885)
	1/4	2	Danoprevir, Ritonavir, PEG-IFN alpha-2a, RBV (NCT01220947)
	1	2	Danoprevir, Ritonavir, RO5024048 (NS5B inhibitor), RBV, (± PEG-IFN alpha-2a) (NCT01331850)
Vaniprevir (MK-7900)	1	3	Vaniprevir, PEG-IFN alpha-2b, RBV (NCT01405937)
Asunaprevir (BMS-650032)	1	3	Asunaprevir, Daclatasvir (NCT01497834)
	1	2	Asunaprevir, PEG-IFN lambda, RBV (NCT01309932)
	1/4	2	Asunaprevir, PEG-IFN alpha-2a, RBV (NCT01030432)
	1a/1b/4	2	Asunaprevir, Daclatasvir, BMS-791325 (NS5B inhibitor) (NCT01455090)

Data from <http://www.clinicaltrials.gov> accessed on September 8, 2013. FDA: United States Food and Drug Administration; G: Genotype; HCV: Hepatitis C virus; PEG-IFN: Peginterferon; RBV: Ribavirin.

**Table 2 Clinical characteristics of hepatitis C virus genotype 1b-infected patients in sequence analysis study of the hepatitis C virus NS3 region**

No. of patients (men/women)	88 (43/45)
Age (yr)	55 ± 14
HCV RNA levels (low/high)	1/87
ALT (IU/L)	67 ± 44
WBC (x 10 <sup>3</sup> /mL)	5.2 ± 1.5
Hemoglobin (g/dL)	14 ± 1.2
Platelet counts (x 10 <sup>4</sup> /mL)	20 ± 18
IL28B rs8099917, TT/TG/GG/unknown	45/29/0/14

HCV RNA levels, low: less than 5 log IU/mL; HCV RNA levels, high: equal to or more than 5 log IU/mL; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; WBC: White blood cell.

V170N (Table 3). We did not identify high resistance variants at 155 and 156 in protease inhibitor-untreated HCV genotype 1b-infected patients (Table 3). Suzuki *et al.*<sup>[58]</sup> reported that amino acid substitutions conferring resistance to protease inhibitors (V36A, T54S, Q80H, and D168E) were detected in 15 of 307 (4.9%) patients infected with HCV genotype 1b who had not received DAAs previously, and T54S (3.3%) predominated over V36A (0.3%), Q80R (0.7%) and D168E (0.7%), similar to our results. Leggewie *et al.*<sup>[61]</sup> measured the prevalence of natural resistance polymorphisms in 38 acutely human immunodeficiency virus (HIV)-HCV co-infected treatment-naïve patients by using direct and deep sequencing. They found that 26% of patients (10/38) had a majority variant resistance mutation (in order of frequency: Q80K-16%, V36M-5%, T54S-3%, V55A-3% and D168A-3%). Low-frequency mutations were detected in all samples.

## RESISTANCE MUTATIONS AND VIROLOGIC FAILURE

Despite extensive efforts to develop more potent next-generation protease inhibitors, the long-term efficacy of this drug class is challenged by the rapid emergence of resistance<sup>[62,63]</sup>, which could result in treatment failures such as viral breakthrough or relapse. Our identified mutations associated with resistance to protease inhibitors are shown in Figure 1. In the Protease Inhibitor for Viral Evaluation (PROVE) 1 and 2 clinical trials<sup>[8,9]</sup> of telaprevir in combination with peginterferon plus ribavirin, viral breakthrough occurred in approximately 7% of patients with HCV genotype 1a infection, compared with about 2% of those with HCV genotype 1b infection; approximately 10% of patients with either subgenotype 1a or 1b suffered a relapse after cessation of HCV protease inhibitor-treatment. In both ADVANCE and Illustrating the Effects of Combination Therapy with Telaprevir (ILLUMINATE) trials<sup>[11,13]</sup>, about 60% of patients treated with telaprevir-based triple therapy achieved an extended rapid virologic response (eRVR), with no virus detected at weeks 4 and 12.

HCV variants associated with on-treatment virologic failure or relapse were evaluated by using site-directed mutagenesis in HCV replicon assay<sup>[62,64]</sup>. Variants V36A/M, T54A/S, R155K/T, and A156S conferred lower levels of *in vitro* resistance to telaprevir (three- to 25-fold increase in telaprevir IC<sub>50</sub>), and A156V/T and V36M + R155K variants conferred higher levels of *in vitro* resistance to telaprevir (> 25-fold increase in telaprevir IC<sub>50</sub>). HCV replicon variants generated from patient-derived

**Table 3** Naturally occurring pre-existing resistance amino acid mutations in the hepatitis C virus NS3 regions of 28 protease inhibitor-naïve patients infected with hepatitis C virus genotype 1

Patient No.	V36	T54	V55	Q80	R155	A156	D168	V170Y/N/L		
	A/M	S	A	L	K/T/Q	S/T/V	N	Y	N	L
31		S		L						
95				L						
15				L						
17				L						
24				L						
26		S		L						
29		S								
81		S		L						
61								Y		
72								Y		
11				L						
12				L						
2								Y		
53				L						
55				L						
66				L						
84				L						
85				L						
110				L						
112								Y		
114		S		L						
100				L						
101									N	
107							N			
111									N	
99										L
92				L						
97		S		L					N	

sequences showed similar results. The *in vitro* replication capacity of telaprevir-resistant variants was lower than that of wild-type virus in the HCV genotype 1b Con1 replicon system<sup>[64-67]</sup>. When telaprevir-resistant variants were tested for cross-resistance against representative protease inhibitors in the HCV replicon system, HCV replicons with single substitutions at position 155 or 156 and double variants with substitutions at residues 36 and 155 showed cross-resistance to all protease inhibitors tested with a wide range of sensitivities. All telaprevir-resistant variants studied remained fully sensitive to interferon- $\alpha$ , ribavirin, and representative HCV nucleoside and non-nucleoside polymerase inhibitors in the replicon system. There are limited clinical data regarding re-treating patients who have failed an HCV NS3-4A protease inhibitor-based therapy such as telaprevir monotherapy, suggesting that re-treatment with triple therapy might be useful for certain patients.

In the boceprevir Serine Protease Inhibitor Therapy 2 (SPRINT-2) trial<sup>[6]</sup>, patients showing a decrease in HCV viral load  $\geq 1$  log<sub>10</sub> IU/mL during the four-week lead-in period of peginterferon plus ribavirin therapy had very low rates of emergence of boceprevir-resistant mutants (4%-6%) during subsequent triple therapy, whereas those with a  $< 1$  log<sub>10</sub> IU/mL decrease in HCV RNA had higher rates (40%-52%) of boceprevir-resistance-associated variants (genotypic mutations of the protease

conferring reduced sensitivity to boceprevir). The majority of boceprevir-treated subjects not achieving SVR had one or more specific treatment-emergent NS3 amino acid substitutions, most of which were previously shown to reduce the anti-HCV activity of boceprevir. These substitutions included V36A, V36M, T54A, T54S, V55A, V107I, R155K, A156S, A156T, A156V, V158I, D168N, I/V170A, and I/V170T. Detection of these substitutions was most common among subjects who experienced virologic breakthrough or incomplete virologic response<sup>[68]</sup>.

## COMBINATIONS OF DAAS FOR HCV STRAINS WITH RESISTANCE MUTATIONS

Protease inhibitors are used in combination with peginterferon plus ribavirin because monotherapy with protease inhibitors results in the early emergence of drug-resistance mutations<sup>[62,63]</sup>. As peginterferon plus ribavirin treatment is frequently associated with serious adverse events, an interferon-free DAA combination therapy such as protease inhibitors with an NS5A inhibitor and/or NS5B inhibitor would offer an ideal treatment option for patients with chronic HCV infection. However, combinations of DAA-resistant variants both in a single target protein and across multiple targets have been reported following failure of single and combination DAA regimens<sup>[55,69-71]</sup>. HCV population sequences of the complete HCV NS3 and 4A regions obtained from 2,111 HCV subgenotype 1a and HCV subgenotype 1b DAA-naïve patients were analyzed by Bartels *et al.*<sup>[72]</sup>. It was reported that the strongest association was the combination of variants at NS3 V55, with lower-level resistance to boceprevir, and NS3 T54, with lower-level resistance to boceprevir and telaprevir<sup>[73]</sup>. The complete HCV NS3 study dataset showed that 69% (33/48) of patients with HCV NS3 V55I also had T54S. An association was also observed between HCV NS3 positions 54 and 155, with 17% (3/18) of the patients with the HCV NS3 T54S substitution also having R155K. The HCV NS3 T54S and R155K combination appeared in boceprevir and telaprevir trials. The study<sup>[73]</sup> also reported that treatment-naïve patients with viral populations containing the telaprevir-resistant variants HCV NS3 V36M, T54S or R155K at baseline achieved a 74% SVR rate with DAAs, similar to that (76%) in patients without resistant variants detected prior to treatment. Further studies are needed to confirm these findings.

## DIFFERENCES IN RESISTANCE MUTATION SELECTION BETWEEN HCV GENOTYPE 1A AND HCV GENOTYPE 1B

It is possible that a different pattern of nucleotide changes is required for the resistance amino acid mutations between HCV genotypes 1a and 1b<sup>[67]</sup>. Substitutions at

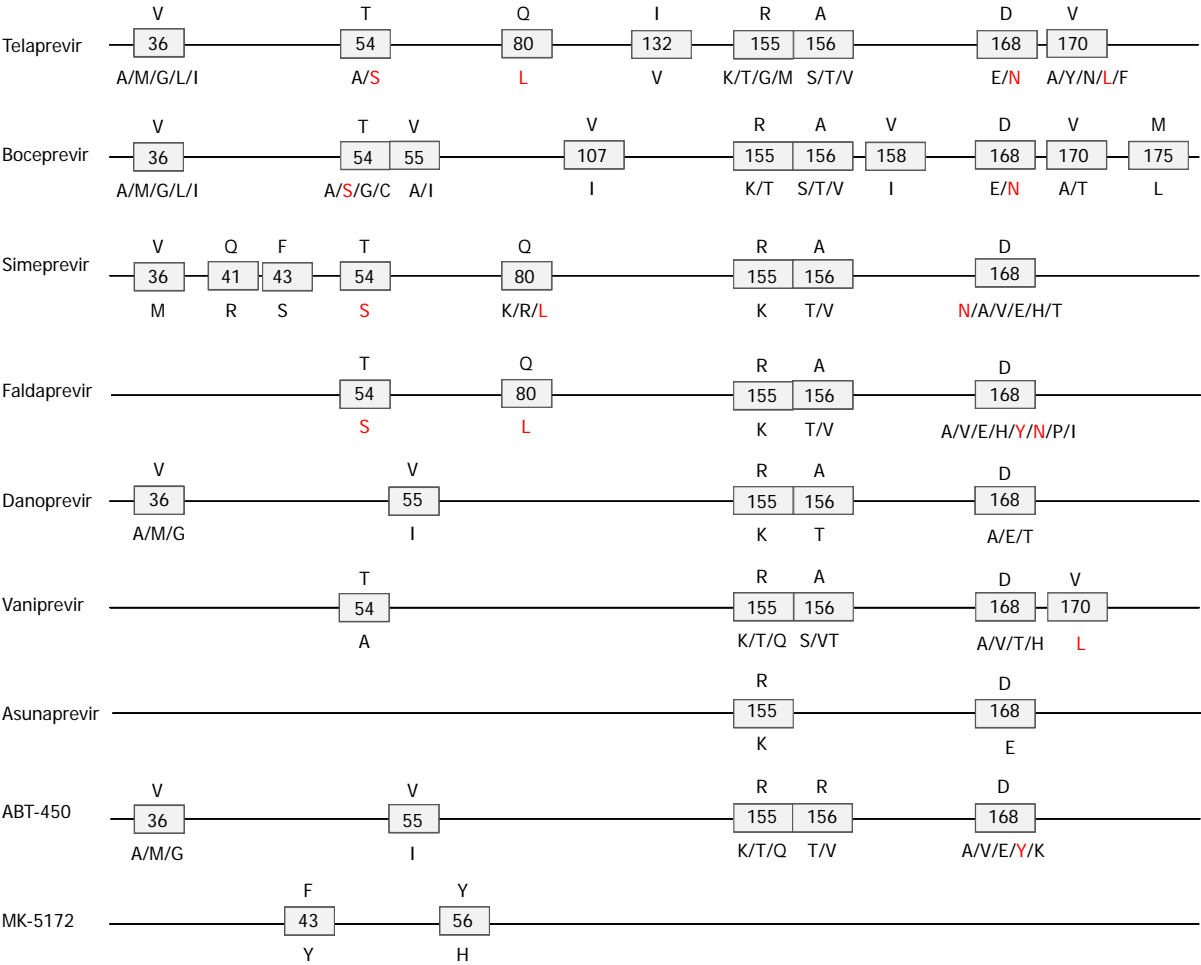


Figure 1 Mutations in hepatitis C virus NS3/4A serine protease that impact susceptibility to hepatitis C virus drugs approved by the United States Food and Drug Administration and investigated in phase 2 or 3 clinical trials. The numbers indicate the positions of the amino acids of the hepatitis C virus genotype 1 Con 1 strain. The amino acids above and below the numbers indicate wild-type amino acids and their substitutions, respectively. The red color indicates the mutations detected among the population using sequencing in the present study.

**Table 4** Nucleotide changes were required for amino acid substitutions at position 155 of hepatitis C virus NS3 among hepatitis C virus genotype 1 samples

Amino acid at position 155	HCV genotype 1a	HCV genotype 1b
R	AGG	CGG
K	AAG	AAG
T	ACG	ACG
S	AGC	AGC
I	ATC	ATC

Bold-faced nucleotides nucleotides were required for amino acid substitutions at position 155. HCV: Hepatitis C virus.

A156 (A156S, A156T or A156V) require only a one-nucleotide change in HCV genotype 1a and HCV genotype 1b. In contrast, substitutions at R155 with K, T, S, M or I require a two-nucleotide substitution in HCV genotype 1b isolates. However, R155K/T/S/M/I substitutions require a one-nucleotide substitution in HCV genotype 1a isolates. The need for a two-nucleotide change for substitution R155 in HCV genotype 1b could be one of the reasons that HCV genotype 1a is more resistant to pro-

tease inhibitors than HCV genotype 1b (Table 4). In the ELECTRON study<sup>[74]</sup> of NS5B inhibitor sofosbuvir, no differential resistance was observed between genotypes 1a and 1b despite 89% of the subjects being in the HCV genotype 1a population, suggesting that combination DAAs targeting other HCV regions with next-generation HCV protease inhibitors could overcome the challenges of resistance mutations. In the near future, although mutation analysis was previously performed with population sequencing using Sanger methods, ultra-deep sequencing technology should provide new information<sup>[60,75-78]</sup>. Ligand bioactive conformation also plays a critical role in the design of HCV NS3 protease inhibitors and may allow for a large variety of HCV protease drug candidates to be designed<sup>[79]</sup>.

## CONCLUSION

In summary, we reviewed the literature reports of resistance variants of HCV protease inhibitors in treatment naïve patients with chronic HCV genotype 1, as well as our experience. Even in treatment-naïve patients with

chronic HCV genotype 1, naturally occurring HCV protease inhibitor-resistance mutations exist in some. Monotherapy with HCV protease inhibitors should be absolutely avoided. Regarding HCV protease inhibitor-resistance mutations, attention should also be paid to DAA-treatment-experienced patients, who previously used HCV protease inhibitor-monotherapies or combination therapies with HCV protease inhibitors. HCV genotype 1a is more resistant to protease inhibitors than HCV genotype 1b, and it is easier for HCV genotype 1a strains to be resistant to the currently available HCV protease inhibitors. At present, patients should be treated according to the recommendations of several HCV clinical practice guidelines<sup>[80-86]</sup>. However, it may also be possible that the combination of DAAs against regions other than HCV NS3/4A could eradicate HCV with these resistance variants.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Prospects for nucleic acid-based therapeutics against hepatitis C virus

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## Abstract

In this review, we discuss recent advances in nucleic acid-based therapeutic technologies that target hepatitis C virus (HCV) infection. Because the HCV genome is present exclusively in RNA form during replication, various nucleic acid-based therapeutic approaches targeting the HCV genome, such as ribozymes, aptamers, siRNAs, and antisense oligonucleotides, have been suggested as potential tools against HCV. Nucleic acids are potentially immunogenic and typically require a delivery tool to be utilized as therapeutics. These limitations have hampered the clinical development of nucleic acid-based therapeutics. However, despite these limitations, nucleic acid-based therapeutics has clinical value due to their great specificity, easy and large-scale synthesis with chemical methods, and pharmaceutical flexibility. Moreover, nucleic acid therapeutics are expected to broaden the range of targetable molecules essential for the HCV replication cycle, and therefore they may prove to be more effective than existing therapeutics, such as interferon- $\alpha$  and ribavirin combination therapy. This review focuses on the current status and future

prospects of ribozymes, aptamers, siRNAs, and antisense oligonucleotides as therapeutic reagents against HCV.

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**Key words:** Hepatitis C virus; Nucleic acid-based therapeutics; Ribozyme; Aptamer; siRNA; Antisense oligonucleotide

**Core tip:** Nucleic acids have emerged as new anti-hepatitis C virus (HCV) agents due to their great specificity, chemical synthesizability, pharmaceutical amenability, and broad targeting ability. Clinical applications of nucleic acids have been delayed due to their potential immunogenicity and toxicity, low efficacy, possible off-target effects, and lack of efficient delivery vehicles. However, recent advances in delivery carriers and chemical modification methods have improved the efficacy and bioavailability of nucleic acid-based agents. Hence, nucleic acids may be attractive anti-HCV options. In this report, the current status and future prospects of ribozymes, aptamers, siRNAs, and antisense oligonucleotides as anti-HCV regimens will be discussed.

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## INTRODUCTION

Hepatitis C Virus (HCV) infection is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular



carcinoma<sup>[1,2]</sup>. Nearly 170 million people are chronically infected worldwide by HCV, and approximately 27% of all cases of liver cirrhosis and approximately 25% of hepatocellular carcinoma cases may be related to HCV infection<sup>[3]</sup>. Given this obvious therapeutic need, international efforts to develop new antiviral drugs and vaccines that are effective against all HCV genotypes have been prompted. However, HCV has seven major genotypes with numerous subtypes<sup>[4]</sup> and exists as a variable quasispecies because HCV NS5B displays an error-prone RNA-dependent RNA polymerase activity that lacks proofreading functions<sup>[5]</sup>. Unfortunately, this high variability in HCV genomic RNA hampers the development of prophylactic and therapeutic vaccines and antiviral drugs<sup>[5,6]</sup>. Until recently, the usual treatment option for HCV infection has been a combination of pegylated interferon- $\alpha$  (PEG-IFN $\alpha$ ) and ribavirin. This treatment clears infections by genotypes 2 and 3 in up to approximately 85% of cases. However, in infections with genotype 1, approximately 45% of cases are able to support a sustained viral response after the combination treatment<sup>[7]</sup>. Moreover, this treatment is associated with many side effects including flu-like symptoms, severe depression and hemolytic anemia<sup>[8]</sup>. Recent approval of two direct-acting antivirals (DAA) targeting the HCV NS3 protease, telaprevir (VX-950) and boceprevir, gives hope for the treatment of HCV infection. However, these drugs, given in combination with PEG-IFN $\alpha$  and ribavirin, are prone to selecting for drug-resistant viruses<sup>[9,10]</sup>. Therefore, DAAs that are more specific, effective, and safer are required. Over the last three decades, nucleic acids have been developed as potential antiviral therapeutic agents. Nucleic acid-based drugs are theoretically capable of targeting many types of molecules such as DNA, RNA, protein, lipid and even small molecules<sup>[11]</sup>. This property could overcome the limitations of the current therapeutics, which target only a limited number of proteins. Nucleic acid-based agents bind to target molecules through sequence complementarity (antisense oligonucleotide, siRNA, ribozyme, and antimiR) or on the basis of three dimensional structure (aptamer) (Figure 1 and Table 1)<sup>[12]</sup>. For example, aptamers bind to target molecules and function as decoys and/or inhibitors, whereas siRNAs and miRNAs make use of the RNA-induced gene silencing complex (RISC), which induces target RNA cleavage or translation inhibition<sup>[13]</sup>. AntimiRs block miRNA activity and thus induce expression of miRNA target genes<sup>[14]</sup>. Antisense oligonucleotides bind to complementary RNAs and suppress access to the cellular machinery, thereby inhibiting expression or function of the targeted RNAs<sup>[12]</sup>. Ribozymes are catalytic RNAs that cleave target RNAs (for example, hairpin ribozyme and hammerhead ribozyme) or selectively replace target RNAs with desirable RNAs (*trans*-splicing ribozyme)<sup>[15]</sup>. These variable modes of action provide many opportunities and options for the treatment of intractable diseases including genetic disorders, cancers, and infectious diseases. Despite their great potential, only a few nucleic acid-based therapeutics have

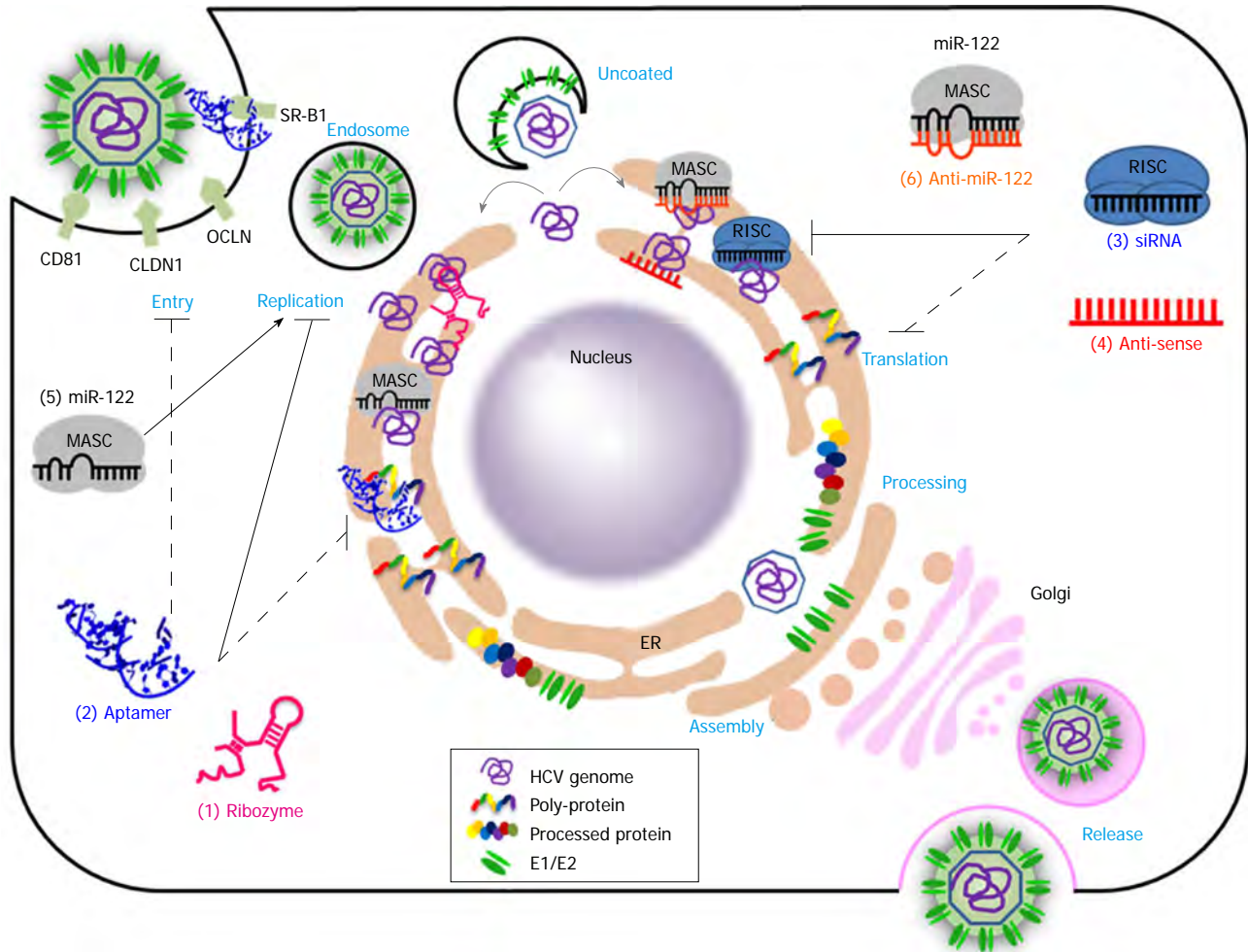
been approved; these include fomivirsen (an antisense oligonucleotide drug for the treatment of cytomegalovirus retinitis in patients with AIDS), pegaptanib (an aptamer for combating wet age-related macular degeneration), and mipomersen (an antisense oligonucleotide drug for the treatment of homozygous familial hypercholesterolemia)<sup>[16-18]</sup>. The problems involved in the application of RNA therapeutic agents include potential immunogenicity, inherent unstable nature, and the requirement for a delivery tool<sup>[11]</sup>. However, recent technological advances, such as the improvement of synthetic delivery carriers and the chemical modifications of nucleic acids, may help to overcome these obstacles. Recently, a phase II clinical trial with SPC3649 (formerly Miravirsen), an LNA-modified antimiR-122, was completed for the treatment of HCV<sup>[19]</sup>. Many other nucleic acid-based anti-HCV therapeutics are in the pre-clinical and clinical stage. In this review, we summarize the current status of nucleic acid-based therapeutics that target the HCV RNA genome or HCV-encoded proteins. Moreover, we summarize their mechanisms of action and discuss the prospects for their future application to the treatment of HCV infections.

## RIBOZYMES

A ribozyme is a catalytic RNA that cleaves or reprograms a target RNA sequence specifically, thus inhibiting the target RNA's expression or inducing new therapeutic gene expression only when the target RNA exists<sup>[20]</sup>. Since HCV has an RNA genome that replicates and exists exclusively in the cytoplasm, ribozymes are an attractive therapeutic option for HCV RNA clearance in infected cells. As HCV NS5B is an error-prone RNA-dependent RNA polymerase that lacks proofreading functions, viral replication is accompanied by the occurrence of mutations<sup>[5]</sup>. Therefore, sequence-specific therapeutics may induce escape mutant viruses. To overcome this obstacle, most ribozymes against the HCV genome have been designed to specifically target the HCV 5'- or 3'-untranslated regions (UTRs), which are highly conserved among all HCV genotypes and are essential for viral replication<sup>[21]</sup>. Promising *in vitro* results were obtained in the 1990s, using ribozymes directed against the HCV 5'- and 3'-UTRs<sup>[22-25]</sup>. Typically, naturally occurring ribozymes can be categorized into two groups depending on their mechanism of action: cleaving ribozymes (RNase P, hairpin ribozyme, and hammerhead ribozyme) and splicing ribozymes (group I and II introns)<sup>[20]</sup>. For therapeutic tools against HCV infection, researchers have modified and optimized these naturally occurring ribozymes or have engineered synthetic ribozymes to target the HCV 5'- or 3'-UTRs.

### Cleaving ribozymes

Cleaving ribozymes are divided into two subgroups according to their natural traits: self-cleaving or *trans*-cleaving<sup>[20]</sup>. Hairpin, hepatitis delta virus and hammerhead ribozymes are all naturally self-cleaving ribozymes required



**Figure 1** Overview of hepatitis C virus life cycle and antiviral target. The hepatitis C virus (HCV) life cycle includes entry, un-coating, replication, translation, processing of poly-proteins, assembly, and release. HCV has an RNA genome which replicates and is translated in the cytoplasm. Various nucleic acid-based therapeutics target viral or host factors during the HCV infection as follows: (1) Ribozymes cleave or reprogram HCV RNA, sequence-specifically, thus inhibiting HCV RNA expression or inducing new therapeutic gene expression; (2) Aptamers can target to receptors (CD81, CLDN1, OCLN, SR-B1), which are needed for HCV entry, or to HCV regulatory proteins. Therefore, aptamers function as decoys during HCV entry or during replication to inhibit the viral life cycle; (3) siRNAs target the HCV genome as well as host factors, and can cleave and/or suppress translation of target RNA, sequence-specifically, through the RNAi induced silencing complex (RISC); (4) Antisense oligonucleotides induce inhibition of HCV gene expression through RNase H-dependent degradation of hybridized HCV RNA or by blocking access to cellular machinery necessary for the HCV translation; (5) MiR-122 is a host factor which regulates HCV replication. MiR-122 is incorporated into microRNA associated stabilizing complex (MASC) and increases HCV replication through binding to the HCV 5' IRES; and (6) Anti-miR-122 down-regulates miR-122, inhibiting HCV replication. Lines represent the following: inhibition of replication (solid line), translation (short dashed line) and entry (long dashed line). An arrow indicates augmentation (black arrow) or the direction (gray arrow) of replication. Nucleic acid-based therapeutic molecules are shown as follows: ribozyme (pink), aptamer (blue), siRNA (sky blue), anti-sense (red) and anti-miR-122 (orange). CD81: Cluster of differentiation 81; CLDN1: Claudin-1; OCLN: Occludin; SR-B1: Scavenger receptor B1; ER: Endoplasmic reticulum.

for the replication process of RNA genomes. RNase P is an essential enzyme in the biosynthesis of tRNAs that specifically cleaves the pre-tRNAs, releasing 5'-sequences and mature tRNAs. Except for the plant chloroplast and Trypanosomatid enzymes, all known RNase P enzymes are ribonucleoproteins that contain an RNA subunit essential for the catalysis<sup>[26]</sup>.

### Hairpin ribozymes

Hairpin ribozymes consist of four helical domains and five loops. The cleavage site is flanked by the two substrate-binding sequences formed between the target RNA and the ribozyme, allowing the design of *trans*-acting ribozymes for target RNA sequence-specific cleavage<sup>[27]</sup>.

The first effort to utilize engineered *trans*-cleaving hairpin ribozymes occurred in the 1990s<sup>[24]</sup>. *Trans*-cleaving hairpin ribozymes targeting HCV RNA 5'-UTR and capsid gene regions were generated and shown to inhibit the expression of a cotransfected reporter gene containing the HCV RNA target sequences. However, these ribozymes were not tested using an HCV cell culture replication system, such as the subgenomic replicon or the virus particle-producing JFH-1 system<sup>[6]</sup>, due to the unavailability of those systems at that time. Therefore, the effects of these ribozymes on HCV replication are unknown. Recently, other hairpin ribozymes targeting the HCV 5'- or 3'-UTRs were reported<sup>[28]</sup>. In Huh-7 cells that stably express subgenomic HCV construct, 1389/hyg-ubi/NS3-3' 5.1, the

**Table 1** Nucleic acid-based anti-hepatitis C virus therapeutics

Class	Mode of action	Target	Status
Hairpin ribozyme	Cleave target RNAs	5'-UTR <sup>[24,28]</sup> , 3'-UTR <sup>[28]</sup> , and core region <sup>[24]</sup>	Tested in <i>in vitro</i> <sup>[24]</sup> and in cell culture model <sup>[28]</sup>
HDV ribozyme		5'-UTR <sup>[30]</sup>	Tested in <i>in vitro</i>
Hammerhead ribozyme (Hepatozyme)		5'-UTR <sup>[33-35]</sup>	Completion of phase II
DNAzyme		5'-UTR <sup>[41]</sup> , core and NS5B region <sup>[39,40]</sup>	Tested in <i>in vitro</i> and in cell culture model
RNase P		5'-UTR <sup>[45-47]</sup>	
Splicing ribozyme	Selectively replace target RNAs with desirable RNAs	5'-UTR <sup>[51]</sup>	
Allosteric ribozyme	Inhibit HCV replication <sup>[54]</sup> or cleave HCV RNA <sup>[31]</sup> through recognizing ligands	miR-122 <sup>[54]</sup> and 5'-UTR <sup>[31]</sup>	
Aptamer	Bind to target molecule and function as decoys and/or inhibitors	NS3 <sup>[68-70]</sup> NS5B <sup>[75-78]</sup> E1E2 <sup>[79]</sup> Viral RNA <sup>[80,81]</sup>	
RNAi	Target RNA cleavage or translation inhibition	5'-UTR and 3'-UTR <sup>[93-96,100-102]</sup> Protein coding regions <sup>[103-109]</sup>	
Antisense oligonucleotide	Bind to complementary RNAs and suppress the access to cellular machinery, thereby inhibiting expression or function of the targeted RNAs	5'-UTR <sup>[134-137]</sup>	Completion of Phase II
AntimiR	Block miRNA activity	miR-122 <sup>[139,138]</sup>	Completion of Phase II

inhibitory efficacy of these ribozymes on HCV replication was minimal (approximately 30%-40%). However, the inhibitory effects of these ribozymes were increased when combined with an HCV 3'-UTR targeting ribozyme (rather than HCV 5'-UTR targeting) and an HCV 5'-UTR targeting siRNA. This approach reduced HCV RNA and NS5B protein levels by 80%-90%. This result offers a promising combinatorial strategy for silencing HCV replication.

### Hepatitis delta virus ribozyme

Among the different ribozymes, the hepatitis delta virus (HDV) ribozyme is the only catalytic cleaving RNA enzyme that has been discovered in humans<sup>[29]</sup>. The HDV ribozyme appears to be well adapted to the human cell environment, and hence, is a potential candidate for the development of anti-HCV therapeutics. The HDV ribozyme has been modified and designed to be able to cleave any specific RNA targets in *trans* possessing a complementary sequence to the recognition sequence of the ribozyme<sup>[20]</sup>. Unfortunately, the HDV ribozyme has not been developed extensively as an anti-HCV reagent; thus, its catalytic activity has been tested only in an *in vitro* trans-cleavage assay using the HCV 5'-UTR as a substrate<sup>[30]</sup>. Recently, an HDV ribozyme possessing a specific on/off adapter (SOFA), named SOFA-HDV ribozyme, was reported<sup>[31]</sup>. This SOFA-HDV ribozyme is discussed further in the allosteric ribozyme section.

### Hammerhead ribozyme

The hammerhead ribozyme is one of the smallest ribozymes and likely the most widely studied ribozyme<sup>[26]</sup>. Due to its small size, specificity, and catalytic efficacy, the hammerhead ribozyme is the most commonly used ribozyme as a therapeutic agent for human diseases. Many attempts have been made to develop hammerhead ribozymes that

can efficiently cleave the HCV RNA genome and inhibit HCV translation and replication<sup>[22,23,32]</sup>. For example, a nuclease-resistant synthetic ribozyme with modified nucleotides and phosphorothioate linkages that target the HCV 5'-UTR was developed by Ribozyme Pharmaceuticals (RPI) in collaboration with Eli Lilly. This hammerhead ribozyme, named Hepatozyme<sup>TM</sup>, successfully inhibited viral replication in cell culture with a chimeric HCV-poliovirus in a dose-dependent manner, and this effect was potentiated by interferon<sup>[33,34]</sup>. In a phase I trial, Hepatozyme, administered either by subcutaneous injection or intravenously, was found to be safe<sup>[35]</sup>. A subsequent phase II trial assessing the safety and efficacy of this ribozyme in combination with IFN $\alpha$  has been initiated. While a reduction of HCV RNA in serum was observed in some patients, RPI opted to discontinue the development of this drug because of toxicology findings in primates<sup>[36]</sup>.

### Deoxyribozyme

Unfortunately, ribozymes have the disadvantages of being both short lived and prone to losing their biological activity when they encounter alternative base substitutions<sup>[37]</sup>. Deoxyribozyme (DNAzymes) can be an effective alternative because of their small size (30-40 bases), ease of synthesis, and increased resistance to chemical or nuclease degradation<sup>[38]</sup>. DNAzymes have been shown to efficiently cleave target RNAs at purine-pyrimidine junctions *in vitro*. Similar to the RNA-based ribozymes, DNAzymes were usually engineered to target highly conserved sequences in the HCV core and/or the NS5B protein coding region<sup>[39,40]</sup> and 5'-UTR<sup>[41]</sup>. Lee *et al.*<sup>[42]</sup> constructed a pool of 10-23 DNAzymes that possessed randomized annealing arm sequences and then selected the most available site for DNAzyme cleavage. All reported DNAzymes targeting the HCV genome cleaved the tar-



get RNA and thus inhibited translation and replication of HCV in the cell culture system. However, the effects were minimal or not superior compared with those produced with RNA-based ribozymes. DNAzymes are yet in their infancy as therapeutics, and further improvements are needed.

### Ribonuclease P

Ribonuclease P (RNase P) is a ubiquitous endoribonuclease and is one of the most abundant and efficient enzymes in the cell. RNase P is a ribonucleoprotein complex that specifically cleaves pre-tRNAs, releasing 5'-sequences and mature tRNAs<sup>[43]</sup>. RNase P requires a short complementary oligonucleotide called an external guide sequence for its activity to recognize and cleave target RNA<sup>[44]</sup>. As an anti-HCV therapeutic, RNase P has been shown to display cleavage activity against the HCV 5'-UTR *in vitro*, but has not yet been extensively studied in a cell culture system<sup>[45-47]</sup>. Therefore, further evidences of its efficacy and safety in cell culture systems are needed to develop RNase P as an anti-HCV drug.

### Splicing ribozymes

The self-splicing group I intron from *Tetrahymena thermophila* has been previously demonstrated to *trans*-splice an exon attached to its 3' end onto a separate 5' exon RNA not only *in vitro*<sup>[48]</sup> but also in *Escherichia coli*<sup>[49]</sup> and mammalian cells<sup>[50]</sup>. A promising advantage of *trans*-splicing group I intron is the cleavage of target RNA and the simultaneous induction of new therapeutic gene expression only when target RNA exists. Thus, *trans*-splicing ribozymes could potentially be used for the selective induction of new antiviral gene activities only in HCV-infected cells while simultaneously destroying the viral RNAs. Our group developed a *trans*-splicing group I ribozyme targeting the HCV 5'-UTR with the diphtheria toxin A (DTA) gene as a 3' exon<sup>[51]</sup>. This *trans*-splicing ribozyme specifically cleaved the HCV 5'-UTR and ligated DTA RNA to the cleaved HCV 5'-UTR, thus inducing HCV RNA-specific cell death. To further improve the anti-HCV activities and the safety of the *trans*-splicing ribozyme, a more careful selection of an antiviral gene as 3' exon, such as interferon instead of DTA, may be required as DTA can cause extensive death of HCV-infected hepatocytes.

### Allosteric ribozymes

An allosteric ribozyme is a ribozyme whose activity can be specifically regulated by ligands. Commonly, ribozymes recognize only a 7-15 nucleotide long target RNA, and thus, the possibility of nonspecific off-target side effects is significant. To overcome this limitation, specific sensing ligands, such as RNAs, proteins or small molecules, can be tagged to the ribozyme to specifically regulate its activity. The SOFA (a specific on/off adapter)-HDV ribozyme and aptazyme have been suggested as representative allosteric ribozymes for HCV therapeutics. The SOFA-HDV ribozyme can switch its

cleavage activity from off to on solely in the presence of the desired RNA ligand. The SOFA module is composed of three domains: a blocker, a biosensor, and a stabilizer<sup>[52]</sup>. The blocker sequence inhibits the cleavage activity of the ribozyme by binding *in cis*. The biosensor must bind its complementary sequence on the substrate to unlock the SOFA module. This binding induces the folding of the catalytic core of the HDV ribozyme into the on conformation. Both the blocker and the biosensor increase the substrate specificity of the ribozyme's cleavage by several orders of magnitude, compared with the wild-type HDV ribozyme<sup>[53]</sup>. Lévesque *et al.*<sup>[31]</sup> attempted to develop a SOFA-HDV ribozyme to target HCV. They screened and identified the most active SOFA-HDV ribozyme against HCV RNA strands of both polarities. Unfortunately, the inhibition of HCV replication through targeting of the HCV replicon system with the SOFA-HDV ribozymes was not very effective, even though the SOFA-HDV ribozymes were active in an *in vitro* cleavage assay. Further elucidation of the reasons why SOFA-HDV ribozymes were not active in the cell culture HCV model is needed to optimize their activities against HCV. An aptazyme is composed of three independent modules: aptamer, communication module, and ribozyme. An aptamer binding to its ligand results in conformational change in the communication module, which can induce the on or off status of ribozyme activity. Recently, our group developed a specific aptazyme that can silence miR-122 activity only in HCV-infected cells<sup>[54]</sup>. MiR-122 is a positive regulator of HCV translation and replication. Functional sequestration of miR-122 effectively reduces the abundance of viral RNA, implicating miR-122 as a potential target for anti-HCV therapeutics<sup>[19]</sup>. However, miR-122 can also regulate the expression of a large number of genes involved in cellular physiological functions such as lipid metabolism and tumor suppression<sup>[55-60]</sup>. To overcome any possible nonspecific side effects due to miR-122 silencing in the normal liver, we created a hammerhead ribozyme-based aptazyme that can release anti-miR-122 through self-cleavage activity, depending on the presence of the HCV NS5B protein<sup>[54]</sup>. This HCV NS5B-dependent anti-miR-122 releasing aptazyme specifically inhibited miR-122 function only in the HCV-infected cells. Moreover, this aptazyme more efficaciously hampered HCV replication than the miRNA silencing approach did, as it contains an aptamer domain that can specifically bind and sequester the HCV NS5B protein. Through the combination of selective miR-122 silencing and specific sequestering of HCV NS5B, this aptazyme approach could be a promising anti-HCV therapeutic treatment.

## APTAMERS

Aptamers are small structured single-stranded nucleic acid sequences that have emerged as attractive and feasible alternatives to small molecule and antibody-based therapy, due to their great specificity, high affinity, easy and large-scale synthesis with a chemical method, phar-



maceutically flexibility, and poor immunogenicity<sup>[61,62]</sup>. Aptamers can be evolved using systematic evolution of ligands by exponential enrichment, an iterative selection method, and can bind target proteins with high affinity and specificity<sup>[63,64]</sup> through formation of well-defined complementary three-dimensional structures<sup>[65]</sup>. The first aptamer drug, known as pegaptanib (Macugen), was approved for the treatment of wet age-related macular degeneration by the United States FDA<sup>[18]</sup>. Other aptamer drug candidates now in the clinical development phase include transcription factor decoys and aptamers against thrombin, factor IXa, and nucleolin<sup>[62]</sup>. Establishment of a robust HCV cell culture system<sup>[66,67]</sup> has allowed the identification and biochemical characterization of two viral enzymes, NS3-4A and NS5B, that are major targets for antiviral therapeutics. NS3-4A and NS5B are essential proteins for the HCV replication cycle, and therefore, most of the aptamers have been developed against these two viral proteins to clear HCV infection.

### NS3 targeting aptamers

The HCV NS3 is a multifunctional protein with three known enzymatic activities. The serine protease activity (in conjunction with cofactor NS4A) is present within the first 180 N-terminal amino acids, while the nucleoside triphosphatase (NTPase) and helicase activities are in the carboxy-terminal region<sup>[6]</sup>. These three activities are important to HCV replication. Most DAAs, including the FDA-approved VX-950 and boceprevir, target NS3 protease activity, as DAAs targeting the NS3 helicase domain have met with limited success<sup>[6]</sup>. In contrast, aptamers have been developed to target not only the protease domain<sup>[68]</sup> but also the helicase<sup>[69,70]</sup> and NTPase domains. Moreover, simultaneous targeting of protease and helicase activities through conjugation of protease and helicase aptamers is possible<sup>[71]</sup>. So far, among the NS3 aptamers, only the helicase-specific aptamer developed by our group has been tested for its ability to inhibit HCV replication in an HCV cell culture system<sup>[70]</sup>. As the NS3 region of the HCV genome may be a hot spot for mutations that are not deleterious to HCV replication, and due to the similarity of the NS3 helicase to cellular RNA helicases<sup>[72,73]</sup>, a more careful examination is required to develop NS3 protease and helicase targeting aptamers as anti-HCV drugs.

### NS5B targeting aptamers

The RNA dependent RNA polymerase (RdRp) NS5B is the key enzyme in HCV RNA replication. Due to its essential role in the HCV life cycle, the NS5B protein is an attractive target for the development of specific anti-HCV drugs. Many nucleoside analogues and nonnucleoside inhibitors have been shown to inhibit RdRp activity *in vitro*, as well as in the replicon cell culture system<sup>[74]</sup>. Jones *et al.*<sup>[75]</sup> developed a DNA aptamer against the HCV genotype 3a NS5B protein. They confirmed that selected DNA aptamers specifically inhibited the NS5B polymerase activity of genotype 3a, but not of genotypes

1a and 1b. The therapeutic effectiveness of such aptamers should be carefully considered, as the most prevalent HCV genotype throughout the world is genotype 1. Moreover, their inhibitory effects against HCV should be carefully tested using the genotype 3a cell culture system that has been recently developed<sup>[76]</sup>. Bellecave *et al.*<sup>[77]</sup> also reported DNA aptamers against HCV NS5B, and their aptamers inhibited HCV JFH-1 replication and viral particle formation in the cell culture system. However, those aptamers were not examined with regard to cell toxicity profiles, distribution in animals, or side effects during long-term treatment. Therefore, concerns about safety and the possibility of escape mutant virus appearance during repeated treatment cannot be excluded with these DNA aptamers. Recently, our group reported two types of RNA aptamers against the HCV NS5B protein composed of 2'-hydroxyl ribonucleotides (2'-OH) or 2'-fluoro pyrimidine ribonucleotides (2'-F)<sup>[78]</sup>. Both aptamers avidly bound to the HCV NS5B replicases of genotypes 1b and 2a and efficiently inhibited HCV replication of both genotypes in cells without inducing the generation of escape mutant viruses, innate immunity, or cellular toxicity. In addition, therapeutically amenable quantities of 2'-F aptamer conjugated with galactose-PEG moiety were efficiently distributed in the mouse liver tissue. These results suggest that RNA aptamers against HCV NS5B have a potential as a new therapeutic tool and are a potentially feasible alternative or additive to the current HCV therapeutics.

### Viral RNA or HCV structural protein targeting aptamers

In addition to aptamers against the HCV regulatory proteins, a DNA aptamer targeting the HCV E1E2 structural protein was recently reported<sup>[79]</sup>. The DNA aptamer exerted its antiviral effects through inhibition of virus binding to the host cell receptors and thus inhibited the viral life cycle. Other aptamers have been reported that target viral RNA to inhibit either HCV translation<sup>[80]</sup> or replication<sup>[81]</sup>. Efficacy of the aptamers was confirmed in the HCV cell culture system. However, issues about cell toxicity profiles, distribution in animals, escape mutant appearance or side effects during the long-term treatment were not addressed.

## RNA INTERFERENCE

RNA interference (RNAi) is a sequence-specific cellular post-transcriptional gene silencing (PTGS) pathway that regulates gene expression and is considered as a defense mechanism against invading viral pathogens and transposable elements in multiple organisms from worms to plants to mammals<sup>[82,83]</sup>. RNAi is initiated by double-stranded RNA (dsRNA) that is processed in the cytoplasm by the RNase III enzyme Dicer to form 21-22 nucleotide (nt)-long small interfering RNA (siRNA) with 5' phosphate groups and two nt 3' overhangs<sup>[84,85]</sup>. siRNA is then incorporated into an Argonaut-containing RISC (RNA-induced silencing complex), which unwinds the

siRNA into the sense (passenger) strand and the anti-sense (guide) strand. The passenger strand is then cleaved and removed, while the guide strand brings RISC to the mRNA, which has a sequence that is complementary to the guide strand<sup>[86,87]</sup>. The degree of complementarity between the target mRNA and the guide strand determines the extent to which RISC silences the expression of the target mRNA. If there is perfect complementarity of the guide strand with the target mRNA, RISC mediates site-specific cleavage that degrades the target mRNA. In contrast, partial complementarity represses translation of the target mRNA<sup>[88,89]</sup>. RNAi in mammalian cells was first described in 2001<sup>[90]</sup>, and triggering of the RNAi pathway with synthetic (exogenous) siRNA has become the most powerful and essential tool for drug development against various human diseases such as viral infections, tumors, and metabolic disorders, due to its high knockdown efficacy and sequence specificity<sup>[91,92]</sup>. Because HCV has a single positive-stranded RNA genome that replicates in the cytoplasm, RNAi is an attractive therapeutic option for the treatment of HCV infection. Many attempts have been made to target HCV RNA with siRNA or with short hairpin RNA (shRNA) as an RNAi trigger.

#### RNAi against HCV 5'- and 3'-UTR sequences

Because siRNAs display high sequence specificity (up to a single nucleotide resolution), any mismatches between the siRNA and target RNA affect the activity of siRNA<sup>[91,92]</sup>. The 5'- and 3'-UTRs are the most highly conserved regions of HCV RNA and are also essential for HCV translation and replication. Therefore, both 5'- and 3'-UTRs are ideal regions for targeting with siRNAs<sup>[93,94]</sup>. Several groups have reported potent siRNA activity against HCV 5'-UTR in the subgenomic replicon system<sup>[93-95]</sup>. These reports demonstrated that siRNA targeting the HCV 5'-UTR resulted in 80%-90% inhibition of HCV. Prabhu *et al.*<sup>[96]</sup> showed that siRNA that targets the highly conserved stem loop II region of the HCV IRES efficiently inhibited translation and replication of infectious full-length clones of HCV 1a and 1b strains. Moreover, this siRNA effectively mediated degradation of the HCV IRES RNA and inhibited GFP expression that was controlled by the IRES sequences of six different HCV genotypes. Compared with synthetic 21-22 nt siRNAs, expressed shRNAs can induce long term stable knockdown of their target RNAs as long as transcription of the shRNAs occurs<sup>[97,98]</sup>. Moreover, shRNAs can act as substrates for Dicer, which increases the incorporation rates of siRNAs into RISC. This process enhances RNAi potency and efficacy<sup>[99]</sup>. For these reasons, two groups have utilized HCV 5'-UTR-targeting vector-derived shRNAs instead of 21-22 nt siRNA<sup>[100,101]</sup>. In both cases, the shRNAs inhibited replication and decreased titers of HCV genotypes 1a and 2a. Ray *et al.*<sup>[102]</sup> also reported that shRNA targeting the 5'-UTR suppressed the replication of different HCV genotypes in the replicon cell culture systems.

#### RNAi against HCV coding regions

Because HCV RNA replicates in the cytoplasm, and its genome acts like mRNA, any region of the HCV genome is theoretically targetable with RNAi. A number of groups have demonstrated siRNAs or shRNAs that target the protein coding regions of HCV. Three different groups have shown that siRNA against the HCV core region reduced HCV RNA and protein expression<sup>[103-106]</sup>. Ansar *et al.*<sup>[106]</sup> showed that siRNAs against the HCV core region showed a 70% reduction in viral titers, while siRNAs against E1 and E2 caused viral titers to drop by as much as 93% in HCV-infected liver cells. Moreover, Kim *et al.*<sup>[105]</sup> demonstrated that siRNAs against the NS3, NS4A, and NS4B regions of HCV effectively inhibited HCV replication and translation. Ali Ashfaq *et al.*<sup>[107]</sup> showed an 88% reduction in HCV replication with siRNA directed against HCV NS3 and a greater than 90% inhibition with siRNAs directed against the NS4B and NS5B regions. Two other studies also demonstrated that siRNAs against HCV coding regions significantly inhibited HCV RNA replication<sup>[108,109]</sup>. For example, Wilson *et al.*<sup>[109]</sup> showed that siRNAs against the NS5A and NS5B regions dramatically reduced HCV replicon RNA levels by up to 99% and 94%, respectively.

#### RNAi against host factors

Host genes that modulate HCV infection and replication have been identified<sup>[110-113]</sup>, and, unlike HCV itself, these genes are not prone to mutations. Therefore, these genes could be important targets for anti-HCV therapeutics. Several studies have shown that siRNAs against HCV entry receptors, such as CD81, SRBI, Claudin I, or occludin, markedly decreased the susceptibility of human hepatoma cells to HCV infection<sup>[114-116]</sup>. In addition, cellular proteins with enzymatic functions have also been targeted by siRNA as an anti-HCV therapy<sup>[117-120]</sup>. Importantly, a combination of siRNAs directed against cellular HCV cofactors and the HCV genome had more pronounced effects on suppressing HCV replication than either treatment alone<sup>[116,118,121]</sup>. The instances of siRNAs targeting cellular factors for antiviral therapy against HCV has been more extensively reviewed in the literature<sup>[122,123]</sup>.

#### RNAi with multiple siRNA

Because HCV has an error prone RNA-dependent RNA polymerase, the occurrence of drug-resistant escape mutant viruses is one of the major concerns for the development of antiviral therapies against HCV. Because RNAi has a high sequence specificity, prolonged treatment with siRNA could result in the appearance of escape mutant viruses that cannot be targetable by the siRNA. Wilson *et al.*<sup>[124]</sup> reported that continuous treatment with one siRNA to an HCV replicon could induce the emergence of multiple point mutations within the target sequence region. One strategy to prevent the formation of escape mutant viruses is to use multiple siRNAs targeting multiple regions of the HCV genome combined with siRNAs

against cellular HCV cofactors<sup>[116,118,121]</sup>. Long shRNA can be processed by the host cell machinery into two or more siRNAs. A vector that directs expression of three shRNAs targeting the 5'-UTR and two NS5B regions of the HCV genome showed sequence-specific antiviral activity in the HCV replicon and in infectious virus systems<sup>[125,126]</sup>. When using a mutant virus with a genome containing an escape mutation against one siRNA, the remaining two siRNAs that could target the mutant virus displayed fully active and effective anti-HCV effects. Yang *et al.*<sup>[127]</sup> designed a vector-derived shRNA that could be processed into multiple siRNAs, using the endogenous miRNA-17-92 cluster as scaffolds. These authors did this because a previous study had demonstrated that overexpression of exogenously introduced shRNA competed with endogenous miRNA and thus led to saturation of the endogenous miRNA pathway, resulting in serious toxicity in mouse liver, and in some instances, death<sup>[128]</sup>. This vector-derived shRNA consisted of five siRNAs targeted against HCV RNA; three target sequences in the 5'-UTR and two others in the core and NS5B regions of HCV. This vector-derived shRNA inhibited HCV RNA replication and translation up to between 93%-98% in the infectious virus systems without inducing toxicity.

#### Current limitations and future prospects of RNAi

RNAi-based antiviral therapeutics has a number of advantages. However, some limitations exist, such as the inherently unstable nature of RNA, the requirement of a delivery vehicle, off-target effects, potential immunogenicity, and toxicity resulting from interference with the endogenous miRNA machinery<sup>[11,119,128]</sup>. These barriers may be overcome with improved chemical modification of siRNAs and synthetic and viral delivery tools. Recent advances in chemical modification methods have increased the stability and efficiency and reduced the off-target effects, immunogenicity, and toxicity of siRNAs. The properties of chemically modified siRNAs have been extensively described in recent reviews<sup>[11,12]</sup>. Delivery methods are also important to consider when contemplating the use of an siRNA as an antiviral therapy. As vector-derived shRNAs are difficult to modify chemically, many researchers have manipulated the shRNA structure<sup>[127]</sup> and expression strategies by using tissue specific or inducible promoter to improve their usefulness as antivirals<sup>[129]</sup>. To test siRNA as an anti-HCV therapeutic in animal models, viral delivery systems have been employed<sup>[125-127,129]</sup>. Sakamoto *et al.*<sup>[130]</sup> used adenovirus to deliver an shRNA expression vector into the livers of transgenic mice that could be induced to express HCV structural proteins by the Cre/loxP switching system. These authors showed that intravenous injection of the adenovirus expressing shRNA resulted in the specific suppression of virus protein synthesis in the liver. In other studies, adeno-associated virus (AAV) was used as a delivery vehicle<sup>[126,127]</sup>. Suh *et al.*<sup>[126]</sup> described an AAV serotype 8-based viral vector that expresses three shRNAs simultaneously. A single intravenous injection

of AAV8 expressing the shRNAs showed comprehensive transduction into hepatocytes in a nonhuman primate model. In addition to viral delivery systems, Chandra *et al.*<sup>[131]</sup> have demonstrated the efficacy of a nanosome (lipid nanoparticles)-based siRNA delivery system. Multiple siRNAs directed against the 5'-UTR of HCV and encapsulated into nanosomes efficiently inhibited HCV replication in a liver tumor-xenotransplanted mouse model. Recent advances in nanobiotechnology will increase the available repertoire of synthetic delivery carriers for siRNAs directed against HCV RNA.

#### ANTISENSE OLIGONUCLEOTIDE

Antisense oligonucleotide (ASO) refers to a short DNA or RNA molecule that is designed to base pair with a specific target gene sequence in a sequence-specific manner. Most ASOs are synthetic single-stranded DNA or modified derivatives. Therefore, sequence-specific hybridization of ASOs to the target mRNA induces inhibition of target gene expression through RNase H-dependent degradation of the hybridized mRNA or through steric hindrance that blocks the access of the cellular machinery necessary for mRNA processing or translation<sup>[12,132]</sup>. Various modifications have improved the efficacy of ASOs through enhancement of nuclease resistance, increase in tissue half-life, affinity, and potency, and reduction of non-sequence-specific toxicity<sup>[133]</sup>. To improve resistance against nuclease degradation, a phosphorothioate-modified backbone was used (first-generation ASO). In addition, to further enhance nuclease resistance and increase binding affinity, 2'-O-Methyl (2'-OME) and 2'-O-Methoxyethyl (2'-MOE) modifications were developed (second-generation ASO). Peptide nucleic acid (PNA), locked nucleic acid (LNA), and phosphoramidate morpholino oligomer (PMO) have been recently developed as third-generation ASOs to further improve target affinity, nuclease resistance, biostability, and pharmacokinetics<sup>[132,133]</sup>. The United States FDA has approved ASOs Fomivirsen (ISIS 2922: Isis Pharmaceuticals) and, more recently, Mipomersen (ISIS301012: Isis Pharmaceuticals) for the treatment of cytomegalovirus retinitis in patients with AIDS and in patients with homozygous familial hypercholesterolemia, respectively<sup>[16,17]</sup>. In addition, a number of other ASOs are also undergoing clinical trials<sup>[133]</sup>. Several ASOs have been reported to inhibit HCV replication and translation. A phase II clinical trial with ISIS 14803 (Isis Pharmaceuticals), a phosphorothioate oligodeoxynucleotide against the HCV 5'-UTR IRES, was completed in 2007, although results were not announced<sup>[134,135]</sup>. McCaffrey *et al.*<sup>[136]</sup> demonstrated that morpholino phosphoramidate antisense oligonucleotides (morpholinos) complementary to the HCV 5'-UTR specifically inhibited HCV IRES-dependent luciferase translation by up to 95% for at least 6 d in mouse liver. Moreover, an adenoviral vector-expressing an RNA ASO has been reported to block HCV replication in the HCV replicon and in the infectious HCV JFH-1 cell culture



system by up to 40% and 76%, respectively<sup>[137]</sup>. Recently, a very promising ASO against HCV was reported with LNA-modified Miravirsin (SPC3649; Santaris Pharmaceuticals), which is directed against microRNA 122 (miR-122)<sup>[57]</sup>. MiR-122 has been reported to promote HCV replication through an increase in either stability or translation of HCV RNA by interacting with the 5'-UTR of the viral genome<sup>[138-140]</sup>. Therefore, silencing of miR-122 is a new plausible approach for anti-HCV therapeutics. LNA-modified ASO (SPC3649) caused a long-lasting suppression of HCV viremia in chronically HCV infected chimpanzees<sup>[141]</sup>. Moreover, a phase II clinical trial showed that SPC3649 treatment resulted in a dose-dependent prolonged reduction of up to 2-3 logs of HCV RNA in patients chronically infected with HCV genotype 1<sup>[19]</sup>. More studies are needed regarding the long-term suppression of miR-122, as miR-122 functions as a tumor suppressor miRNA<sup>[58-60]</sup>, and HCV escape variants resistant to SPC3649 could potentially occur<sup>[142]</sup>.

## CONCLUSION

For almost two decades, major endeavors to develop nucleic acid-based therapeutics against hepatitis C virus have been undertaken. Compounds such as ribozymes, aptamers, siRNAs, and antisense oligonucleotides have been shown to perturb various steps in the HCV life cycle (Figure 1 and Table 1). However, clinical application of these nucleic acid-based therapeutics has been hampered by their low efficiency, off-target effects, toxicity, inefficient delivery, and the lack of cell culture and animal models. These limitations have been gradually overcome with recently improved delivery carriers (viral and synthetic) and chemical modifications of nucleic acids that can ameliorate the efficiency and bioavailability, while also reducing the toxicity and off-target effects<sup>[11,12]</sup>. Moreover, great efforts have been made to establish HCV cell culture systems<sup>[66,67,76]</sup> and small animal models<sup>[143,144]</sup>, which are highly useful for the evaluation of anti-viral efficacy, and thus, for the realization of effective nucleic acid-based anti-HCV drugs in the future.

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## WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

# Antiviral treatment of hepatitis C virus infection and factors affecting efficacy

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## Abstract

Hepatitis C virus (HCV) infection is the leading cause of chronic liver-related diseases, including cirrhosis, liver failure, and hepatocellular carcinoma. Currently, no effective vaccine is available for HCV infection. Polyethylene glycol interferon- $\alpha$  (PegIFN- $\alpha$ ) in combination with ribavirin (RBV) is the standard of care (SOC) for chronic hepatitis C. However, the efficacy of PegIFN- $\alpha$  and RBV combination therapy is less than 50% for genotype 1 HCV, which is the dominant virus in humans. In addition, IFN and RBV have several severe side effects. Therefore, strategies to improve sustained virological response (SVR) rates have been an important focus for clinical physicians. The serine protease inhibitors telaprevir and boceprevir were approved by the United States Food and Drug Administration in 2011. The addition of HCV protease inhibitors to the SOC has significantly improved the efficacy of treatments for HCV infection. Several direct-acting antiviral drugs currently in late-stage clinical trials, both with and without peg-IFN and RBV, have several advantages over the previous SOC, including higher specificity and efficacy, fewer side effects, and the ability to be administered orally, and might be optimal regimens in the future. Factors affect-

ing the efficacy of anti-HCV treatments based on IFN- $\alpha$  include the HCV genotype, baseline viral load, virological response during treatment, host *IL28B* gene polymorphisms and hepatic steatosis. However, determining the effect of the above factors on DAA therapy is necessary. In this review, we summarize the development of anti-HCV agents and assess the main factors affecting the efficacy of antiviral treatments.

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**Key words:** Hepatitis C virus; Treatment; Interferon; Protease inhibitors; IL28B protein; Polymorphisms; Viral load; Genotype; Hepatic steatosis

**Core tip:** Understanding the effectiveness and affecting factors of antiviral regimens are critical for making informed treatment decisions for hepatitis C virus (HCV) infection. In this review, we have summarized the history of anti-HCV agents from interferon to the direct-acting antiviral drugs (DAAs) without polyethylene glycol interferon- $\alpha$  therapies and the affecting factors of antiviral treatment, focusing on investigating the optimal combination of antiviral therapies to achieve higher efficacy and better medication compliance. Although the efficacy of DAAs is significantly improved, many unmet needs and questions remain, such as avoidance of cross-resistance, the remaining high incidence of side effects, the role of IL28B status as well as the management of patients who do not respond to therapy.

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## INTRODUCTION

Hepatitis C virus (HCV) infection, a worldwide public health problem affecting 170 million patients, is likely the cause of chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma<sup>[1]</sup>. Of the patients with chronic HCV infection, 40%-75% still exhibit extrahepatic manifestations including metabolic, hematological, vascular and rheumatological diseases<sup>[2-5]</sup>. Until recently, however, there have been no effective vaccines available. In the early 2000s, polyethylene glycol interferon- $\alpha$  (PegIFN- $\alpha$ ) combined with ribavirin (RBV) became the standard of care (SOC) regimen for HCV, which showed a SVR that was mainly associated with its genotype. For example, patients with genotype 1 achieved a sustained virological response (SVR) of less than 50%. Additionally, this treatment regimen has several side effects, including granulocytopenia, anemia, and depression, and it is associated with a long treatment duration and increased cost. In 2011, the first direct-acting antiviral drugs (DAAs), telaprevir and boceprevir, were approved by the United States Food and Drug Administration (FDA). Combined with PegIFN- $\alpha$  and RBV, these DAAs resulted in a higher SVR rate in patients with HCV genotype 1. Thus, this treatment regimen became the SOC regimen for such patients. Soon afterward, other DAAs in the pre-clinical or pilot phase also achieved good treatment results. Current studies are focusing on investigating the optimal combination of antiviral therapies to achieve higher efficacy, shorter treatment duration, more simple administration, and better medication compliance. In response to an approved DAA, an evaluation of multiple factors (HCV genotype, baseline viral load, virological response during the treatment, and *IL28B* gene polymorphisms) affecting anti-HCV treatment therapy based on IFN is necessary.

## ADVANCES IN ANTIVIRAL TREATMENT

### Interferon

**PegIFN- $\alpha$ :** When administered as a once-a-week injection, PegIFN- $\alpha$  increased the SVR rate and compliance in patients by delaying renal clearance to extend the *in vivo* half-life by cross-linking polyethylene glycol and interferon- $\alpha$ . Currently, treatment combining PegIFN- $\alpha$  and RBV is still the most widely used SOC regimen.

Many clinical studies have compared the SVR rates in patients receiving different PegIFN- $\alpha$  (e.g., IFN- $\alpha$ -2a and IFN- $\alpha$ -2b), dosages, and treatment durations. The results suggested that the patients given a standard dose (180  $\mu$ g) of PegIFN- $\alpha$ -2a had higher SVR rates than those given a weight-based dose (1.5  $\mu$ g/kg) of PegIFN- $\alpha$ -2b<sup>[6-8]</sup>. The IDEAL study, which included 3070 patients with hepatitis C, showed that the SVR rate in patients with HCV genotype 1 infection given different doses of PegIFN- $\alpha$ -2b (1.0 or 1.5  $\mu$ g/kg) was not different from that in patients given PegIFN- $\alpha$ -2a (180  $\mu$ g)<sup>[9]</sup>. In patients with HCV genotype 2/3 infections, those given a standard dose of PegIFN- $\alpha$ -2b (1.5  $\mu$ g/kg) had a higher SVR rate than those given a low dose<sup>[10-12]</sup>. Meanwhile,

the SVR rates in patients receiving a high dose of RBV (1000-1400 mg/d) were higher than those in patients receiving a low dose (800 mg/d) of the PegIFN- $\alpha$ -related treatments<sup>[13,14]</sup>. The difference was especially obvious in the patients with a genotype 1 infection. Some studies investigated the antiviral therapy administered to patients with a genotype 2/3 infection. Although the overall SVR rate decreased after shortening the duration, a 12- or 16-wk treatment period was recommended for patients who achieved rapid virological responses (RVR)<sup>[10,15,16]</sup>.

The IDEAL study results showed that regardless of which PegIFN- $\alpha$  was chosen to treat hepatitis C, the type and frequency of the adverse responses appeared similar (serious adverse responses, approximately 4%; headaches, 46%; myalgia, 40%; neutropenia, 5%; hemoglobin less than 86 g/L, approximately 3%), with a higher incidence of depression but lower incidence of skin rash associated with PegIFN- $\alpha$ -2b compared with PegIFN- $\alpha$ -2a<sup>[9]</sup>.

**Human serum albumin IFN- $\alpha$  fusion:** Albinterferon is a genetic fusion protein used for the treatment of chronic hepatitis C (CHC), which takes advantage of the long half-life of human albumin to provide a new treatment approach that enables albinterferon administration at 2- or 4-wk intervals in individuals with CHC. Studies have demonstrated that the SVR rate resulting from the combined treatment of albinterferon and RBV was equivalent to that resulting from the SOC treatments, and the incidence rates of adverse drug reactions were also similar<sup>[17,18]</sup>. However, albinterferon is associated with the risk of reduced lung function, particularly in patients being treated for more than 6 wk<sup>[19]</sup>.

**PegIFN- $\lambda$ -1a:** IFN- $\lambda$  is a class III interferon and has completely different receptors from those of IFN- $\alpha$  *in vivo*. Its receptors are mainly distributed in the liver, which means that the extrahepatic adverse reaction from IFN- $\lambda$  is significantly reduced compared with that from IFN- $\alpha$ . In recent years, PegIFN- $\lambda$ -1 has been confirmed to have anti-HCV activity and mild adverse reactions<sup>[20]</sup>. One clinical trial assessed the efficacy and safety of PegIFN- $\lambda$ -1a plus RBV compared to the SOC for the treatment of naive patients with HCV genotypes 2/3. The results showed that the curative effects of the two treatments were similar but that the viral load in the PegIFN- $\lambda$ -1a group decreased faster and that the adverse reactions were significantly reduced<sup>[21]</sup>.

### DAAs

**NS3 protease inhibitors:** The unique structure and function of NS3 protease in the HCV life cycle makes it a new target for anti-HCV drug development. In addition to cleaving the polyprotein and generating the NS3, NS4A, NS4B, NS5A, and NS5B proteins, NS3 protease acts as an antagonist of the host innate immune system by cleaving signaling molecules that mediate a cellular antiviral response and resulting in the suppression of interferon production. The two NS3 protease inhibitors

discussed herein are telaprevir and boceprevir.

Telaprevir, as the first approved DAA, has a recommended dose of 750 mg tid, combined with PegIFN- $\alpha$  and RBV treatment (triple therapy), for a duration of 48 wk for naive or previous treatment failure HCV genotype 1 patients. The disadvantage of this medication is the need to ingest it with greasy food, which may cause an incredible weight increase during treatment. Six randomized clinical trials assessed the efficacy of the triple therapy compared with the SOC in naive HCV genotype 1 patients<sup>[22-27]</sup>. All patients were treated with telaprevir, PegIFN- $\alpha$ , and RBV for 8 or 12 wk, followed by the combined therapy of PegIFN- $\alpha$  and RBV. The results showed that the telaprevir triple therapy for 24 wk yielded a higher SVR rate than the SOC<sup>[22,24-26]</sup>. Even when the duration was shortened to 12 wk, the SVR rates were equivalent to those of the SOC<sup>[24]</sup>, but prolonged duration did not improve its efficacy for those who achieved rapid virological response (RVR) and early virological response (EVR)<sup>[26,27]</sup>. The administration frequency of telaprevir (750 mg tid or 1125 mg bid) and PegIFN type ( $\alpha$ -2b or  $\alpha$ -2a) in patients with RVR and EVR had no effect on the SVR rate<sup>[23]</sup>. Among the previous treatment failure patients, the telaprevir triple therapy group had a higher SVR rate than that of the SOC group, but the overall effect was poor, especially for non-responders, with an SVR rate of 29%-33%<sup>[28]</sup>. Common adverse reactions to telaprevir include anemia, rash, nausea, hemorrhoids and itching. Because telaprevir treatment can lead to resistant mutants over the short term, the long-term use of the drug should be limited. Drug resistant mutants have been found to exhibit the following changes: V36A/M, T54A/S, R155K/T, and A156S/T.

Boceprevir is another NS3 protease inhibitor approved at the same time as telaprevir. The recommended dose of boceprevir is 800 mg tid, combined with PegIFN- $\alpha$  and RBV therapy, for a duration of 48 wk for naive or previous treatment failure HCV genotype 1 patients. Unlike telaprevir, boceprevir is started at week 4 of treatment, following a 4-wk lead-in period of treatment with peg-IFN and RBV, and RBV is required to enhance the efficacy of boceprevir<sup>[29]</sup>. Studies showed that the boceprevir triple regimen among naive patients for 48 wk increased the SVR rate associated with the SOC treatment to 16%-37%<sup>[30,31]</sup>, whereas the SVR rates in the previous treatment failure patients were significantly higher (59%-66% *vs* 21%)<sup>[32]</sup>. Boceprevir-related adverse effects include fatigue, anemia, nausea, headache, dry mouth, granulocyte decreases, taste disorders, and thrombocytopenia. The long-term use of this drug can also lead to resistance mutations, including V36A/M, T54A/S, V55A, R155K/T and A156S/T/V.

Simeprevir is a second-generation NS3 protease inhibitor and a competitive reversible macrocyclic, non-covalent inhibitor of NS3/4A protease<sup>[33]</sup>. Phase II clinical trials compared the efficacy of simeprevir, PegIFN- $\alpha$  and RBV with the SOC treatment for naive or previous treatment failure HCV genotype 1 patients. Among the

naive patients, those treated with the triple therapy with different doses of simeprevir (75 or 150 mg) once a day (qd) for 12 or 24 wk and then with PegIFN- $\alpha$  and RBV, for a total treatment course of 24 or 48 wk, obtained a higher SVR ratio compared with that of patients treated with the SOC (74.7%-86.1% *vs* 64.9%). In addition, for the majority of patients, the duration can be shortened to 24 wk<sup>[34]</sup>. The phase II b ASPIRE study demonstrated that simeprevir is a highly potent, efficacious, and well-tolerated once-daily PI for the majority of prior null or partial responders and relapsers compared to IFN-based therapy. Simeprevir has entered a phase III clinical study. The most common adverse reactions are nausea, fatigue and hyperbilirubinemia, which are generally mild and reversible. The resistance mutations include Q8K and R155K.

Faldaprevir is a second-generation HCV NS3/4A protease inhibitor. Phase II clinical trials have compared the efficacy of the SOC with that of the combined treatment with faldaprevir, PegIFN- $\alpha$ , and RBV in treatment-naive or treatment-experienced patients with chronic hepatitis C genotype 1 infection. The SVR rate in the treatment-naive patients who underwent 24-wk triple therapy including faldaprevir 240 mg qd with no lead-in was the highest, at up to 84%, whereas the group receiving the same drug dose with lead-in during the early phase of treatment or receiving a half dose of faldaprevir had a 72% SVR; in contrast, the other group (SOC regimen) had a SVR of only 56%<sup>[35]</sup>. Similar results were obtained for the treatment-experienced patients. The group receiving triple therapy with faldaprevir 240 mg qd for 48 wk with no lead-in had the highest SVR rate (50% in prior partial responders and 35% in prior null responders); the SVR rate in the lead-in treatment group that received the same dose was the lowest<sup>[36]</sup>. The adverse responses of faldaprevir include jaundice, skin changes (*e.g.*, rash), photosensitivity, pruritus, nausea, vomiting, diarrhea, and drying. The incidence of side effects is associated with the dosage. To date, the resistance mutations R155K and D168V/E have been observed.

Danoprevir is another second-generation NS3 protease inhibitor used for the treatment in naive or experienced HCV genotype 1 patients, and it is expected to eliminate the use of IFN-based drugs. One clinical trial compared the efficacy of the SOC with that of the combined treatment with danoprevir, PegIFN- $\alpha$  and RBV in treatment-naive patients with HCV genotype 1 infection<sup>[37]</sup>. The SVR rate in the group given danoprevir 600 mg q12h was the highest at up to 85%, whereas the group receiving the SOC had a SVR rate of 42%. Even when the duration among patients given danoprevir who had an extended rapid virological response (eRVR4-20: HCV RNA < 15 IU/mL during weeks 4-20) was shortened to 24 wk, 96% had an SVR. The INFORM-1 study evaluated the combination of danoprevir and mericitabine. Combination therapy was administered for up to 2 wk, resulting in a reduction in viral load and undetectable HCV RNA levels at the end of dosing in 63% of



treatment-naïve patients<sup>[38]</sup>. Relevant evidence indicates that ritonavir can inhibit the metabolism of danoprevir *in vivo*, reduce the side effects, and improve the SVR rate, providing the possibility for IFN-free combination therapy. The INFORM-SVR study provided SVR data for the combination of mericitabine and danoprevir/ritonavir with or without RBV for 12-24 wk. SVR rates in HCV genotype 1a and genotype 1b patients were 26% and 71% in treatment arms including RBV, respectively, but significantly lower SVR rates were found in all RBV-free treatment groups<sup>[39]</sup>. The adverse reactions of danoprevir mainly include anemia, neutropenia, and rash. The resistance mutations R155K and D168T/E have been observed.

ABT-450 is a potent, specific protease inhibitor of HCV NS3. Ritonavir is used to increase the plasma concentration of ABT-450, prolong its half-life, and reduce the risk of drug resistance, enabling an ABT-450 dose regimen of once daily<sup>[40,41]</sup>. Fifty HCV genotype 1 patients including naïve, prior partial or null responders participated in an open-label, multiple-center phase II ABT-450 clinical trial. In the application of the combined treatment of ABT-333 [non-nucleoside inhibitors (NNI)], RBV, and ritonavir, the curative effects of different doses of ABT-450 over 12 wk were assessed. The results suggested that the SVR rates were higher than 90% in treatment-naïve patients and 47% in prior partial or null responders<sup>[40]</sup>. The common adverse responses of ABT-450 include fatigue, pain, hyperbilirubinemia, and vomiting.

There are many other NS3 protease inhibitors in clinical studies, such as asunaprevir (BMS 650032), vaniprevir (MK-7009), narlaprevir (SCH 900518), VX 985, and MK-5172. Some of these NS3 protease inhibitors are expected to be approved for anti-HCV therapy in the near future.

**NS5A inhibitors:** NS5A is an essential viral component of the membrane-associated HCV replication complex and plays an important role in the formation of HCV infectious particles. Daclatasvir (BMS 790052) was the first-in-class NS5A-specific targeted molecular inhibitor to be developed. Preclinical studies have shown that this NS5A inhibitor has broad genotype antiviral activity, but the associated mechanism is unclear. A phase II study compared the efficacy of the combination of daclatasvir and asunaprevir (two-drug treatment) with or without the addition of PegIFN- $\alpha$  and RBV for the treatment of HCV genotype 1 prior null responders over a 24-wk duration. The results showed that the sustained virological response at post-treatment week 14 (SVR24) of the two-drug treatment was 36% and that the sustained virological response at post-treatment week 12 (SVR12) and SVR24 of the four-drug treatment were 100% and 90%, respectively<sup>[42]</sup>. High virological response rates were obtained in 90 treatment-naïve patients administered the combination of daclatasvir with sofosbuvir, with or without RBV, for 24 wk. In HCV genotype 1 patients, RVR and SVR rates were 100% and 100%, while in HCV

genotype 2 and genotype 3 patients they were 100% and 91%, respectively<sup>[43]</sup>. However, it is notable that all failures were relapses after therapy. Analyses of resistance *in vivo* and *in vitro* showed mutations in the amino acid residues L31V/M and Y93H/N.

Several other NS5A inhibitors have also entered clinical trials, including ABT-267, ledipasvir (GS-5885), ACH-2928, and IDX791. Some of these inhibitors may be approved to become anti-HCV drugs.

**NS5B polymerase inhibitors:** NS5B is an RNA-dependent RNA polymerase (RdRp) in the HCV replication complex that catalyzes the synthesis of positive- and negative-stranded viral RNAs. Because mammals lack RdRp, new drugs to act as HCV NS5B polymerase inhibitors will be highly specific. NS5B enzyme activity can be inhibited by two different types of compounds: nucleoside/nucleotide derivative inhibitors (NIs) and NNIs. NIs can competitively bind to RdRp active sites, whereas NNIs target allosteric enzyme binding sites. Therefore, because both classes of drugs affect RdRp at different sites, cross-resistance is not easily produced.

NIs can simulate natural polymerase nucleotide substrates and act as a terminator that can be incorporated into RNA. The highly conserved HCV RdRp activation center showed that NIs have a similar efficacy on different HCV genotypes, as well as a high barrier to and low incidence of resistance genes.

Sofosbuvir can be used for the treatment of non-genotype 1 HCV infection<sup>[44,45]</sup>. A randomized, double-blind phase II clinical trial showed that treatment with sofosbuvir, PegIFN- $\alpha$ , and RBV for 12 wk, followed by subsequent treatment with PegIFN- $\alpha$  and RBV for 12 or 36 wk, resulted in a SVR12 rate of 90% in HCV genotype 1 patients, which was similar to that in genotype 2/3 patients (92%)<sup>[44]</sup>. Another clinical trial showed that the 12-wk treatment of HCV genotype 1 naïve patients with sofosbuvir, PegIFN- $\alpha$  and RBV was safe and effective. In addition, extended duration did not improve the efficacy, although these results need to be further confirmed by phase III clinical trials<sup>[45]</sup>. It is notable that no viral breakthrough or resistance development during therapy has been described. Because of the absence of cross-resistance with the other DAAs, including NS5A inhibitors, sofosbuvir can be used for salvage therapy.

Mericitabine is a nucleoside analog polymerase inhibitor of HCV. Phase II clinical study data showed that the treatment with mericitabine combined with PegIFN- $\alpha$  and RBV was safe and well tolerated. In the triple regimen for 24 wk, the SVR rate in HCV genotype 1/4 treatment-naïve patients was higher than the SOC group<sup>[46]</sup>. The phase II MATTERHORN study showed that for genotype 1a/1b prior null and partial responders after the combined treatment with ritonavir, danoprevir, mericitabine, PegIFN- $\alpha$  and RBV, the sustained virological response at post-treatment week 4 (SVR4) reached 83% and 100%, respectively. Currently, resistance mutants have not been found.

The design of NNIs involves targeting one of at

least five non-contiguous sites of RdRp allosteric enzymes, resulting in conformational changes that inhibit the enzyme activity, which have limitations on the genotype compared with NIs. A low genetic barrier may soon induce virus mutations. In phase I and II clinical studies, the results showed that BI 207127 and VX-222, regardless of whether they were combined with PegIFN- $\alpha$  treatment, can both improve the genotype 1 HCV infection RVR or EVR rate and demonstrate good tolerance. However, reducing the treatment with PegIFN- $\alpha$  resulted in a relatively high proportion of virological breakthroughs<sup>[47-49]</sup>.

**Cyclosporine - a cyclophilin inhibitor:** Cyclophilins are a family of cell isomerases, including cyclophilins A, B, and C. The importance of human cyclophilins in HCV replication was confirmed by the anti-HCV activity of cyclosporine A. The mechanism of action of cyclosporine A involves NS5A and/or NS5B. Alisporivir (Debio-025) is a derivative of cyclosporine A, which removed the immunosuppressive activity but retained the potent antiviral activity against a wide range of HCV genotypes. All cyclophilin inhibitors have a high barrier to resistance. In vitro studies have shown a lack of significant cross-resistance with NS3/4A or other protease inhibitors. Moreover, there is an additive effect when cyclophilin inhibitors are combined with PEGIFN- $\alpha$ . Thus, in addition to having the advantage of once-daily administration, these agents are promising host-directed antivirals<sup>[50,51]</sup>.

**Supplementation therapy:** *In vitro*, vitamin B<sub>12</sub> acts as a natural inhibitor of HCV replication. A study assessed the effect of vitamin B<sub>12</sub> on the virological response in antiviral therapy-naïve patients with chronic HCV infection. The SVR rate was significantly higher in the SOC plus B<sub>12</sub> group than in the SOC group<sup>[52]</sup>. At present, it is also believed that vitamin D has an anti-HCV activity *in vitro* that is mediated through its active metabolite, calcitriol<sup>[53]</sup>. The SVR of treatment-naïve patients with chronic HCV genotype 1 or 2/3 infection is significantly improved by adding vitamin D to conventional PegIFN- $\alpha$  and ribavirin therapy<sup>[54,55]</sup>. However, given the very small number of available studies, additional studies are needed to assess potential differences in the associations between vitamin B<sub>12</sub>/vitamin D and SVR for HCV.

The hematologic adverse events of PegIFN- $\alpha$  combined with RBV therapy include anemia, thrombocytopenia, and leukopenia, which most frequently lead to drug discontinuation or dose modifications. L-Carnitine is a necessary nutrient factor in energy production and has been proposed as a potential adjuvant treatment to improve anemia, thrombocytopenia, and leukopenia. A study comparing the PEGIFN- $\alpha$  plus RBV plus an L-carnitine group versus the PEGIFN- $\alpha$  plus RBV group observed a significant improvement in SVR for 50% *vs* 25% of patients<sup>[56]</sup>. This finding suggests that L-carnitine supplementation may be useful in patients treated for HCV. Other supplementations including erythropoietin,

zinc and probiotics have been assessed in clinical studies, but the effects of those on SVR are still not clear.

## FACTORS AFFECTING THE EFFICACY OF HCV ANTIVIRAL THERAPY

The main factors influencing the efficacy of HCV antiviral treatments are divided into two categories: viral and host-related. The viral category includes the HCV genotype, baseline viral load, and virological response during treatment, and the host category includes age, gender, race, drinking habits, obesity, degree of liver fibrosis, and *IL28B* gene polymorphisms. In particular, *IL28B* gene polymorphisms are associated with the SVR. With approved DAAs on the market, more clinical treatment choices have been provided. The efficient and reliable prediction of the efficacy is essential to create individual antiviral solutions, improve the efficacy, reduce the side effects, and lower the treatment cost.

### Viral factors

**HCV genotype:** Genotype plays an important role in predicting the response to the SOC treatments and determining the appropriate antiviral treatment. The response of patients with HCV genotype 1/4/5/6 infection is worse than that of patients with genotype 2/3 infection. DAAs are mainly used for the treatment of HCV genotype 1 infection. Although the effects of partial drugs on non-type 1 infection have been evaluated, there have been no sufficient data to clarify the relationship between the genotype and the effect of DAAs. Short-term data from a study on sofosbuvir indicated that the treatment with sofosbuvir combined with the SOC regimen resulted in a SVR12 of 91% in genotype 1 treatment-naïve patients and 92% in patients with genotype 2/3. Another study showed that sofosbuvir combined with RBV resulted in a SVR rate of 84% in genotype 1 treatment-naïve patients and 100% in patients with genotype 2/3<sup>[57]</sup>. Whether the HCV genotype affects the efficacy of DAA treatment remains to be confirmed by further studies.

**Baseline viral load:** Many studies have demonstrated that, regardless of the HCV genotype, a low baseline viral load (before treatment, HCV RNA < 600000-800000 IU/mL) was an independent predictive factor of the SVR<sup>[14,58,59]</sup>. In this range, the impact of the changes in the HCV RNA concentration on the SVR was not linear; when the HCV RNA was lower than 400000 IU/mL, an increase in the amount of virus decreases the SVR rate. However, an HCV RNA concentration higher than 400000 IU/mL results in a relatively stable SVR rate<sup>[51,60]</sup>. In 2011, the European guidelines for the prevention and treatment of hepatitis C suggested that if the baseline viral load was less than 400000-800000 IU/mL, the course of treatment for genotype 1/4 naïve patients who received RVR can be shortened to 24 wk and that for patients with genotype 2/3 may be shortened to 12-16 wk<sup>[52,61]</sup>.

**Virological response during treatment:** Using different patterns of response such as RVR, EVR, and delayed virological response (DVR: not having achieved RVR and EVR but testing negative for HCV RNA before the 24<sup>th</sup> wk) to predict the efficacy, determine the duration, and tailor the program can maximize benefits, rationalize the course of treatment, and minimize the recurrence rate. In the 2011 European guidelines<sup>[61]</sup> for the prevention and treatment of hepatitis C, the following adjustments are made. For the genotype 1/4 patients, if the baseline viral load was low before treatment and RVR was acquired after treatment, the duration could be reduced to 24 wk. If the patient acquired DVR, the duration should be prolonged to 72 wk to reduce the recurrence rate. For the genotype 2/3 patients, if the baseline viral load was low and RVR was acquired, the duration could be shortened to 12-16 wk. For patients who did not acquire RVR and EVR or only acquired DVR or exhibit combined effects from other factors (such as obesity and insulin resistance), as long as the viral load was undetectable at the 24<sup>th</sup> wk, the duration could be extended to 48 or 72 wk. Regardless of the genotype, if the viral load decreased to less than 21log IU/mL at the 12<sup>th</sup> wk and HCV RNA can still be detected at the 24<sup>th</sup> wk, the treatment could be discontinued. RGT principles are also applied to NS3 protease inhibitors. For HCV genotype 1 naive patients, using telaprevir or boceprevir combined with SOC and having acquired RVR and EVR, shortening the duration can be considered, but for patients with liver cirrhosis, a recommended treatment for 48 wk would be appropriate. The simeprevir results show that, according to the RGT principle, the treatment duration in HCV genotype 1 naive patients can be shortened to 24 wk, but further research is needed to confirm this recommendation<sup>[34]</sup>. The existing faldaprevir data show that extending the duration from 24 wk to 48 wk did not increase the SVR rate in HCV genotype 1 naive patients who achieved RVR and EVR, but for the previous treatment failure patients, a 48-wk course should be considered<sup>[35,36]</sup>.

### Host factors

**Polymorphisms of the *IL28B* gene:** In 2009, three genome-wide association studies (GWAS) found that single nucleotide polymorphisms (SNPs) in the *IL-28B* gene, located on chromosome 19, are associated with hepatitis C treatment efficacy<sup>[62-64]</sup>. In patients with HCV type 1 infection, Ge *et al*<sup>[62]</sup> found that rs12979860 (3 kilobases upstream of the *IL28B* gene encoding the type III interferon IFN-13) showed a strong correlation with the treatment response. The SVR rate of SOC in CHC patients carrying the CC genotype was 2-3 times higher than that in patients not carrying the genotype. A Japanese study showed that rs8099917 was correlated with the HCV treatment response and was one of the most important predictors of non-response after the logistic regression analysis<sup>[65]</sup>. The frequency difference in different populations with the rs12979860 CC genotype is very large, with East Asians having the highest frequency of the CC

genotype<sup>[62]</sup>, followed by Europeans, and with Africans having the lowest frequency<sup>[63]</sup>. In a multivariate regression model, the *IL28B* polymorphism was the best predictor of treatment response, being better than the ethnic background, baseline viral load, degree of liver fibrosis, fasting glucose level, BMI, and other predictors<sup>[66]</sup>. Halfon *et al*<sup>[67]</sup> analyzed the predictive values of rs12979860 and rs8099917 in 198 patients with HCV genotype 1 with respect to their response to treatment and showed that rs12979860 seemed to be sufficient for clinical decisions. EASL guidelines showed that *IL28B* polymorphisms can be used to predict treatment response but have a low predictive value<sup>[61]</sup>. In contrast, AASLD argues that for determining the treatment regimen (SOC regimen combined with or without DAA), the *IL28B* polymorphism is a very strong predictor<sup>[68]</sup>.

The predictive value of *IL28B* polymorphisms is not only limited to SOC regimen but has also been demonstrated in a study from Japan in patients receiving triple therapy with telaprevir. The study showed that rs12979860 and rs8099917 were associated with SVR, and the univariate and multivariate analyses confirmed that rs8099917 can be used as an independent predictor of the SVR<sup>[69]</sup>. Similar results were also found in other studies on the SOC treatments combined with DAAs<sup>[70-73]</sup>. An IFN-free study of mericitabine as a monotherapy or in combination with danoprevir showed that the rs12979860 CC genotype was related to faster and earlier viral decline<sup>[74]</sup>.

Thus, the *IL28B* gene has a better predictive value with respect to not only the SOC but also DAAs. However, further research is still needed to confirm these observations.

**Hepatic steatosis and other negative predictors:** The value of steatosis as a negative predictor of response to anti-HCV therapy was confirmed in two large clinical trials. In one study, 574 HCV patients treated with the SOC were evaluated, and the results showed that the presence of steatosis reduces the likelihood of achieving EVR and SVR in genotype-1 infected patients<sup>[75]</sup>. In another study, 231 HCV patients treated with the SOC were evaluated<sup>[76]</sup>. The results showed that steatosis negatively affected SVR in HCV genotype non-3-infected patients. In the last year, new data showing that steatosis is also an independent predictor of relapse in genotype 3 have been published<sup>[77]</sup>. Steatosis has been associated with significantly higher rates of relapse, irrespective of viral load, in patients infected with HCV genotype 3 who had a rapid virological response (RVR)<sup>[78]</sup>. Several studies<sup>[59,78]</sup> have shown that RVR consistently remains an important determinant of SVR in patients with HCV genotype 2 or 3. Recent studies have confirmed that RVR is a good indicator for SVR in genotype 2, but not in genotype 3, in which steatosis is a predictor of relapse. This suggests that the underlying pathogenic mechanisms of steatosis differ between genotype 3 and other genotypes and may influence response to IFN-based therapy. These data sug-



gest that new therapeutic strategies are necessary for this subgroup of HCV genotype 3<sup>[59,78]</sup>.

Other adverse predictive factors affecting the efficacy of HCV treatment include liver cirrhosis<sup>[79]</sup>, age  $\geq 40$  years old<sup>[80]</sup>, insulin resistance<sup>[81,82]</sup> and metabolic syndrome<sup>[83,84]</sup>. In patients with these factors, either the treatment duration may need to be extended or the dose may need to be increased.

## CONCLUSION

PegIFN- $\alpha$  combined with RBV is currently the most classic and widely used standard treatment; however, its limited efficacy and significant side effects, as well as the absence of an HCV vaccine, promoted the development of new drugs. In recent years, the development of HCV antiviral drugs has progressed. Two HCV NS3 protease inhibitors, telaprevir and boceprevir, were approved by the United States FDA in 2011, and their combined treatment with the SOC not only significantly improved the SVR rate in HCV naive patients but also showed good efficacy in patients with previous treatment failure. Many other HCV NS3 protease inhibitors, NS5A inhibitors, and NS5B RdRp inhibitors are in the final stage of clinical trials and are likely to soon be approved as anti-HCV drugs. DAAs have shown a trend toward a gradual replacement of the SOC scheme. Although the efficacy of DAAs is significantly improved, the incidence of treatment-related side effects appears to be high, and because of the direct-acting antiviral effect, resistance mutations appear to be more likely to appear. Therefore, the implementation of personalized treatment approaches is very important. The application of many HCV antiviral drugs provides clinicians with more effective treatment choices for CHC. Host genetic factors guide individualized treatment strategies and aid in determining the best treatment plan for each patient. Polymorphisms in the *IL28B* gene have been used in clinical practice to help determine anti-HCV treatment strategies. Genetic markers need further verification, which can be performed in the preclinical testing stage. At the same time, accurately predicting the success of treatment and the progression of the disease will enhance the treatment compliance of patients, which will aid in maximizing the treatment effect.

Although DAAs show good potential, it is difficult to completely overcome the associated drug toxicity and occurrence of drug resistance; thus, not all patients can be cured by antiviral therapy. Therefore, determining how to prevent infection with HCV is an important research direction. Over the years, HCV vaccine development strategies are mostly based on the viral genome, unable to overcome HCV high variability, and starting from the human genome to explore other ways to prevent HCV infection may open up a new era in infection prevention

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## Extra-intestinal and long term consequences of *Giardia duodenalis* infections

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### Abstract

Giardiasis is the most common waterborne parasitic infection of the human intestine worldwide. The etiological agent, *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*), is a flagellated, binucleated protozoan parasite which infects a wide array of mammalian hosts. Human giardiasis is a true cosmopolitan pathogen, with highest prevalence in developing countries. Giardiasis can present with a broad range of clinical manifestations from asymptomatic, to acute or chronic diarrheal disease associated with abdominal pain and nausea. Most infections are self-limiting, although re-infection and chronic infection can occur. Recent evidence indicating that *Giardia* may cause chronic post-infectious gastrointestinal complications have made it a topic of intense research. The causes of the post-infectious clinical manifestations due to *Giardia*, even after complete elimination of the parasite, remain obscure. This review

offers a state-of-the-art discussion on the long-term consequences of *Giardia* infections, from extra-intestinal manifestations, growth and cognitive deficiencies, to post-infectious irritable bowel syndrome. The discussion also sheds light on some of the novel mechanisms recently implicated in the production of these post-infectious manifestations.

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**Key words:** Giardiasis; Inflammatory disorders; Extra-intestinal manifestations of enteritis; Failure to thrive; Post-infectious irritable bowel syndrome

**Core tip:** This review offers a state-of-the-art discussion on the long-term consequences of *Giardia* infections, the most common waterborne parasitic infection of the human intestine worldwide, from extra-intestinal manifestations, growth and cognitive deficiencies, to post-infectious irritable bowel syndrome. The discussion also sheds light on some of the novel mechanisms recently implicated in the production of these post-infectious manifestations.

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### INTRODUCTION

*Giardia duodenalis* (*G. duodenalis*) (syn. *Giardia lamblia*, *Giardia intestinalis*) is an intestinal flagellated protozoan parasite of the upper small intestine. Very common worldwide, *Giardia* was recently included in the World Health Organisation's Neglected Disease Initiative<sup>[1,2]</sup>. *Giardia* is

**Table 1** Main pathophysiological effects of *Giardia duodenalis* and their mechanisms

<i>Giardia</i> -induced pathophysiological responses	Mechanisms involved or hypothesized to be involved	Selected references
Intestinal epithelial cell apoptosis	Induction of pro-apoptotic factors: Caspase-3, 8 and 9, Inhibition of anti-apoptotic factors: poly (ADP-ribose) polymerase (PARP) cleavage	[10,18,19,21,23]
Halt of enterocyte cell-cycle progression	Nutrient competition (arginine), up-regulation of cell-cycle genes	[25]
Intestinal barrier dysfunction	Disruption of claudin-1 and alpha-actinin by unknown mechanisms, caspase-3 mediated disruption of zonula-occludens (ZO)-1, myosin light chain kinase-mediated disruption of F-actin, and ZO-1	[10,17,19,21,26,27,29,30]
Small intestinal hypermotility	Adaptive immunity, neuronal nitric oxide, mast cell degranulation	[118-120]
Diffuse shortening of brush border microvilli	CD8 <sup>+</sup> lymphocytes - mediated <i>via</i> parasite secretory/excretory products	[13,16,21,31]
Crypt hyperplasia	Alteration villus/crypt ratio	[21,62,99]
Microbiota composition	Microbiota from infected host may become pathogenic	[14,33]
Increased mucus production	Increased mucus secretion in response to the parasite	[66]
Brush border enzyme activity deficiencies	Loss of surface area (microvilli and villi)	[16,21,98,121,122]
Disaccharidases deficiencies	Loss of surface area (microvilli and villi)	[10,16,99,122]
Electrolyte/nutrient/water malabsorption	Loss of surface area (microvilli and villi)	[10,19,21,62,99,123]
Anion hypersecretion	Unknown mechanisms	[10,19,99]

PARP : Poly adenosine diphosphate ribose polymerase.

transmitted through the ingestion of cysts in contaminated food or water, or directly *via* the fecal/oral route. Ingestion of cysts results in giardiasis, a disease causing intestinal malabsorption and diarrhea in a wide variety of species including humans. In developing countries, the prevalence of human giardiasis commonly ranges from 20% to 30% of the population, with reports of 100% prevalence in some populations; in developed countries, prevalence ranges from 3% to 7%<sup>[3,4]</sup>. The classification of *G. duodenalis* is a topic of debate and at present, the species is divided into eight distinct genetic assemblages, *i.e.*, assemblages A to H. Only the assemblages A and B are considered to be pathogenic in humans. Although parasites with assemblage A or B can infect non-human mammalian species, other genotypes appear to have a more restricted host range; for example assemblages C and D are commonly found in dogs<sup>[5]</sup>, while assemblage E is common in cattle<sup>[6]</sup>. Ongoing research suggests that giardiasis is often due to anthroponotic spread, but zoonotic transmission can occur<sup>[7-9]</sup>. A striking feature of giardiasis is the spectrum of clinical symptoms that occur in infected individuals. The clinical manifestations can range from asymptomatic, to acute or chronic diarrheal disease. When present, the clinical signs of infection may include diarrhea, nausea, weight loss, bloating and abdominal pain<sup>[3,10]</sup>. In giardiasis, the acute pathophysiology occurs without invasion of the small intestinal tissues by the trophozoites, and in the absence of overt inflammatory cell infiltration, with the exception of a modest increase in intraepithelial lymphocytes<sup>[11-13]</sup>. Multiple factors have been proposed to account for the disease variability, including the state of the host immune system, host age and nutritional status, strain genotype, infectious dose and, possibly co-infections<sup>[3,8,10]</sup>. The pathophysiological consequences of *Giardia* infection are clearly multifactorial, and involve both host and parasite factors, as well as immunological and non-immunological mucosal processes. Recent observations suggest a role for disruptions of the host intestinal microbiota during the acute

infection stage in the production of chronic symptoms, and further research is warranted to corroborate these findings<sup>[14]</sup>. The pathophysiology of giardiasis, and key aspects of the host response to *Giardia* remains incompletely understood.

## PATHOPHYSIOLOGY OF GIARDIASIS

Central features of the pathophysiology of giardiasis are briefly outlined below, as these mechanisms may be key to our understanding of the complications discussed further (Table 1). While the *Giardia* genotype has been proposed to play a role in the induction of symptoms, there is currently no consensus concerning the connection between genotype and virulence<sup>[15]</sup>.

After cyst ingestion in contaminated water or food, excystation occurs liberating two or four trophozoites, which adhere to the epithelial surface of the intestine *via* a ventral adhesive disk. This tight attachment between *Giardia* trophozoites and intestinal epithelial cells, as well as the production of yet incompletely characterized parasitic products, culminate in the production of diarrhea. Pathophysiology is believed to involve heightened rates of enterocytes apoptosis, intestinal barrier dysfunction, activation of host lymphocytes, shortening of brush border microvilli with or without coinciding villous atrophy, disaccharidase deficiencies, small intestinal malabsorption, anion hypersecretion and increased intestinal transit rates<sup>[10,13,16-22]</sup>.

As it is the case with other enteropathogens, induction of apoptosis in enterocytes by *Giardia* represents a key component in the pathogenesis of the infection<sup>[3,18,19,22-24]</sup>. Enterocytes apoptosis during giardiasis is caspase-3 and -9 dependent<sup>[18,23]</sup>. While both host and parasite factors may modulate intestinal epithelial cell apoptosis, the products responsible for its activation during giardiasis have yet to be identified. In addition to promoting increased rates of enterocyte apoptosis, *Giardia* trophozoites may also halt enterocyte cell-cycle progression *via*

consumption of arginine, and up-regulation of cell-cycle inhibitory genes<sup>[25]</sup>.

Findings from studies on giardiasis *in vivo* demonstrate that the most severe intestinal permeability and macromolecular uptake coincides with the peak of trophozoites colonization<sup>[17,26-28]</sup>. The effects of the infection on gut barrier function following host parasite clearance require further investigation. *Giardia*-mediated increases in intestinal permeability result from alterations to the apical tight junctional complexes, including disruption of F-actin, zonula-occludens-1, claudin-1 and alpha-actinin, a component of the actomyosin ring that regulates paracellular flow<sup>[19,26,28-30]</sup>. The role of *Giardia* proteinases in these effects is a topic of ongoing research.

*Giardia*-induced diffuse shortening of epithelial brush border microvilli represents a key factor in the production of diarrhoeal disease *via* malabsorption and maldigestion<sup>[13,16,31]</sup>. Whether or not the diffuse loss of microvillous border surface area associated with giardiasis is related to the release of a “toxin” by the parasite, a phenomenon similar to the release of proteases in the bacterial overgrowth syndrome<sup>[32]</sup>, remains poorly understood. Regardless, *G. duodenalis* infection causes microvillous shortening in a lymphocyte-mediated manner which in turns impairs activities of disaccharidases<sup>[13]</sup>.

Bacterial components of the intestinal microbiota from *Giardia*-infected hosts may act as stimulatory factors for protozoan pathogenicity<sup>[33]</sup>. Indeed, micro-organisms isolated from the duodenal microbiota of patients with symptomatic giardiasis can stimulate the pathogenicity of *G. duodenalis* in a gnotobiotic animal model<sup>[33]</sup>. The biological basis of this phenomenon remains unclear.

*Giardia* infections tend to be self-limiting in individuals with competent immune systems. A recent study in Brazilian children suggests that symptoms are less severe during re-infection, consistent with the hypothesis that if previous exposure does not always protect against future infections, it does at least reduce the severity of pathology<sup>[34]</sup>. Patients with common variable immunodeficiency and Bruton's X-linked agammaglobulinemia are prone to chronic giardiasis<sup>[35,36]</sup>, which underscores the necessity of antibodies to fully control giardiasis.

In addition to its acute symptoms, giardiasis may also cause anorexia and failure to thrive. Indeed, *Giardia* infections may have detrimental effects on nutritional status, growth status and cognitive function in humans<sup>[37-41]</sup>. *Giardia* infections may also have detrimental effects on body weight in food-producing animals making this a serious concern for the agricultural industry<sup>[42-45]</sup>.

symptoms, suggesting that this phenomenon is not as uncommon as previously thought<sup>[46]</sup>.

**Ocular pathologies:** The first description of ocular complications in patients with giardiasis was made by Barraquer<sup>[47]</sup>, who reported cases of iridocyclitis, choroiditis, and retinal hemorrhages in patients that presented diarrhea linked to the presence of *Giardia*. More recent observations described a “salt and pepper” degeneration (punctuate areas of normal hyperpigmentation on a light yellow pink-retina) involving the retinal pigmented epithelium in children suffering from giardiasis<sup>[48]</sup>. The same complication was described in children with past giardiasis, indicating that the ocular changes observed did not require the concurrent presence of the parasite in the gut<sup>[49]</sup>. Small children appear to be more susceptible to ocular lesions during giardiasis, and the lesions are thought to be caused by damage to the cells of the retina, accompanied by the release of pigment granules in retinal layers, where they can be seen as blackish dots<sup>[49]</sup>. The mechanisms linking ocular lesions with giardiasis remain obscure, but they exclude the possibility of direct invasion by the parasite. It has been speculated that the pigmented degeneration may result from toxic metabolites produced by the parasites, which has yet to be proven<sup>[48]</sup>. The role of increased intestinal permeability in the ocular complications seen in giardiasis needs to be elucidated.

**Arthritis:** Reactive arthritis is classically seen following infection with enteric pathogens such as *Yersinia* sp., *Salmonella* sp., *Campylobacter jejuni* and *Shigella* sp., but inflammatory arthritis has also been described following enteric infections with other organisms such as *Clostridium difficile*, *Brucella* sp. and *Giardia* sp.<sup>[50]</sup>. The interval between the preceding infection and the manifestation of arthritis is 2 to 4 wk<sup>[50]</sup>. Post-infectious arthritis has a predilection for joints of the lower limbs particularly the knee and ankle<sup>[51]</sup>. Post-infectious reactive arthritis has been classified as a classical spondyloarthropathy associated with human leukocyte antigen (HLA)-B27, an allele of the major histocompatibility complex class I present in 50% of the cases of patients with enteric-infection-related arthritis<sup>[51,52]</sup>. However, inflammatory arthritis following infection with *Clostridium* sp. or *G. duodenalis* does not fit classical spondyloarthropathy, as it fails to show association with HLA-B27<sup>[50]</sup>. Therefore, these are referred to enteric-infection-related-arthritis. Although *G. duodenalis* infections account for a significant proportion of enteric infections worldwide, reports of an association with post-infectious arthritis are relatively few. Little is known of the pathogenesis of arthritis in these conditions. Unlike post-enteric reactive arthritis, these arthritides are characteristically responsive to antibiotic therapies<sup>[52]</sup>. The variable degrees of host immune responses, and the lack of a robust systemic inflammatory response, may account for the infrequency of post-giardiasis arthritis despite the high prevalence rate of the infection<sup>[50]</sup>. Antigens from enteric bacteria have been isolated from the synovial fluid

## LONG-TERM CONSEQUENCES OF GIARDIASIS

### *Extra-intestinal consequences of Giardia infections*

Until recently, the scientific literature rarely reported extra-intestinal manifestations in giardiasis. However, a recent study estimated that 1/3 of the patients infected with this parasite will express long-term extra-intestinal

**Table 2** International reports of post-giardiasis metabolic consequences

Post-giardiasis effects	Country	Selected references
Lower cognitive function	India, Peru, Turkey	[40,67,68,76,77]
Lower intellectual quotient		
Lower social quotient		
Lower weight	Brazil, Columbia, Ecuadora,	[37-39,63,64,66-
Lower height	Guatemala, Iran, Israel,	68,72,77,78,124-
Stunting	Mexico, Rwanda, Turkey,	129]
	United States	
Failure to Thrive	Columbia, Ecuadora,	[64,66,127]
	United States	
Nutrient deficiencies	Iran, Mexico, Tanzania	[38,69,78,81]

of affected joints<sup>[52]</sup>. In a case of *Yersinia pseudotuberculosis* reactive arthritis, evidence of viable bacteria within the joint was demonstrated over a year later<sup>[53]</sup>. Here again, a possible role for increased intestinal permeability in enteric-infection-related-arthritis warrants further investigation.

**Allergies:** Concomitant presence of *G. duodenalis*, cutaneous allergic manifestations, and gastrointestinal symptoms have been described, which may explain why complete symptom resolution can be achieved with metronidazole and corticosteroids<sup>[54]</sup>. Significant anecdotal evidence suggests a causative link between giardiasis and the development of urticaria. In a recent study in children, giardiasis was associated with an increase in total serum immunoglobulin E (IgE) levels, and an enhanced IgE antibody response to common allergens<sup>[55]</sup>. These patients also demonstrated IgE reactivity to cow's milk and *Giardia* antigens. These observations suggests that alteration in antigen uptake from the small intestine during giardiasis, perhaps in association with connective tissue mast cell proliferation, may contribute to the development of allergic disease<sup>[56-58]</sup>. Dysfunction of the intestinal barrier during giardiasis may facilitate the translocation of food macromolecules and in turn prime the host for sensitization<sup>[55]</sup>.

**Muscular complications:** Hypokalemic myopathy has been associated with celiac disease, radiation enteropathy, immunosuppressive drugs, and various infectious diseases. In the patient, this presents as marked proximal muscular weakness in all four limbs and the neck<sup>[59]</sup>. Analyses of muscular biopsies reveal an abnormal size of the muscular fiber due to the presence of numerous rounded atrophic and hypertrophic fibers, proliferation of myonuclei, and necrotic fibers<sup>[60]</sup>. The findings are consistent with impairment of muscle excitability and denervation due to muscle necrosis. Analysis of these fiber components showed that glycogen and lipid levels, as well as the inter-myofibrillar network pattern, are normal<sup>[60]</sup>. Several cases of myopathy following hypokalemia induced by giardiasis have been reported in both immunocompetent and immunocompromised patients<sup>[59,60]</sup>. This suggests

that *G. duodenalis* infections can trigger muscular manifestations independently of the immune status of the host. During giardiasis, potassium loss is closely related to the number of bouts of diarrhea per day<sup>[60]</sup>. Loss of potassium result in hypokalemia which can trigger a severe and transient myopathy<sup>[60]</sup>. In fact, muscular symptoms can improve with increased levels of potassium and recovery from diarrhea<sup>[59]</sup>. However, *G. duodenalis* diarrhea as a cause of myopathy due to hypokalemia is rare. It seems that the duration of symptoms is crucial for development of hypokalemic myopathy<sup>[60]</sup>. Giardiasis-associated hypokalemia occurs more often in elderly people, particularly women, who are hospitalized for giardiasis<sup>[61]</sup>. The causes, and the clinical consequences, of *Giardia*-associated hypokalemia remain unclear. It has been suggested that giardiasis-induced impairment of nutrient and electrolyte absorption may contribute at least in part to hypokalemia and hyponatremia<sup>[62]</sup>.

### "Metabolic" consequences of *Giardia* infection

**Nutritional consequences:** In developing countries of the world, because of infectious diseases and lack of food, 206 million children under 5 years of age suffer from stunting, 50 million from chronic wasting disease, and 167 million are grossly underweight<sup>[63]</sup>. Growth failure, reflected in stunting, wasting and underweight conditions, is assessed by anthropometric indices of height-for-age, weight-for-age, and weight-for-height<sup>[64]</sup>. Optimum health for children has long been linked to physical, socio-cultural, economic and environmental factors. In developing countries, the incidence of giardiasis is often over four times higher than the incidence reported in industrialized countries<sup>[65]</sup>. Children between 6 mo and 5 years of age are the most susceptible<sup>[66]</sup>. In combination with diarrhea, *G. duodenalis* infection can cause iron deficiency anemia, micronutrient deficiencies, protein-energy malnutrition, growth and cognitive retardation, and malabsorption<sup>[63,67]</sup>. Studies conducted on children from Brazil and Peru found that diarrheal disease occurring in the first 2 years of life negatively correlates with cognitive function, verbal fluency, and physical fitness, and may lead to long-term growth faltering<sup>[40,68]</sup>. These studies demonstrate that the effects of early childhood diarrhea are more far-reaching than merely causing dehydration. Diarrhea caused by *Giardia* sp. or *Cryptosporidium* sp. has frequently been associated with stunting and lower cognitive function<sup>[40,68]</sup> (Table 2). Intriguingly, a recent study observed that in a cohort of Tanzanian children infected with *Giardia*, infection had a protective role against diarrhea, and that this protection was lost with multi-nutrient supplementation<sup>[69]</sup>. Research needs to determine whether these interesting findings reflect a negative regulation by *Giardia* sp. of other enteric pathogenic processes that may occur in these children.

**Failure to thrive:** Childhood and adolescence are the period of most rapid skeletal growth. Failure to thrive (FTT) is a term generally used when a child presents



with a rate of weight gain that is significantly below that expected of similar children of the same sex, age and ethnicity. Failure to thrive is a common problem, that may be present at any time during the childhood, but is usually prevalent within the first 1-2 years of life. Long-term sequelae involving all areas of growth, behaviour and development may be seen in children suffering from FTT<sup>[70]</sup>. Causes for FTT usually include: (1) inadequate food intake; (2) reduced absorption or digestion of nutrients or excessive loss of nutrients; and (3) excessive utilisation of energy. There is a strong association between *Giardia* infection and malnutrition, wasting and stunting<sup>[38,63-65,69,71]</sup>. Malabsorption, maldigestion and malnutrition due to giardiasis have been shown to affect anthropomorphic factors as well as the calorie intake during childhood, most commonly in the second year of life in infected children<sup>[37-39,63,72]</sup>. Duration of the infection episodes, and their association with diarrhea, appeared to be the key factors associated with growth disturbance and failure to thrive<sup>[37]</sup>. While several studies have established a strong link between *Giardia* infection during the first two years of life, FTT and development impairment, more research is needed to unravel the mechanisms and the potential implications of polyparasitism in these phenomena.

Malnutrition, a common feature of numerous intestinal diseases, has been associated with an increase in macromolecular uptake due to heightened intestinal permeability<sup>[73]</sup>, two phenomena known to occur during giardiasis<sup>[19,56]</sup>. *Giardia* infection can reduce food intake, and produce steatorrhea, maldigestion and malabsorption of carbohydrates and vitamins (including vitamin A, B3, B5, B6, B12, E, and folacin)<sup>[3,21,64,74]</sup>. Together, these effects may contribute at least in part to failure to thrive in giardiasis (Table 2).

**Stunting:** Growth failure due to malnutrition and chronic infections like giardiasis is associated with increased morbidity and mortality in children from developing countries<sup>[37,63,64]</sup>. More specifically, significant impairments in weight-for-age and weight-for-height scores have been associated with *G. duodenalis* infection during the first two years of life<sup>[72]</sup>. Indeed, the relative odds of low height-for-age may be 7.7 times higher among children with giardiasis<sup>[63]</sup>. In a number of developing countries, diarrhea caused by enteric parasitic Protozoa in early childhood represents predictors of stunting<sup>[64]</sup>. Given the high prevalence of asymptomatic infection in this study population (78.8%), children may appear to have normal weight-for-age and weight-for-height early on, but, present with growth retardation at a later age. This phenomenon is known as “homeorhesis”, and it is probable that the high prevalence of asymptomatic *Giardia* infection among children may play a key role in it. Similarly, *Giardia* infection has been associated with decreased weight gain and impaired feed conversion efficiency in lambs and cattle, illustrating that growth retardation associated with *Giardia* infections also poses an important problem to the

agriculture industry<sup>[42-45]</sup>. Overall, human giardiasis combines with other factors, including low nutritional status, as well as sanitary and socioeconomic conditions, to lead to stunting<sup>[64]</sup>. However, findings from numerous studies, to date, indicate that the well established loss of intestinal surface area, maldigestion, and malabsorption caused by giardiasis may contribute to growth retardation following *Giardia* infection (Table 2).

**Impaired cognitive function:** Cognitive function in children can be affected by environmental and health related factors<sup>[75]</sup>. Risk factors that interfere with cognitive function are especially important during infancy because the first two years of life are an essential period of rapid growth and development, that is marked by rapid brain growth and maturation, by neuronal arborisation, myelination and emergence of brain networks. Thus the development of cognitive function in early life depends on the hierarchical maturation of neocortical association areas, as well as interactions with the environment. Nutrition, infection, and environment, have been found to affect neuroplasticity and to have long lasting effects in developing children<sup>[76]</sup>. Many of the hazards to early brain development are well known, and include head injury, newborn asphyxia, infections of the brain *in utero* and in the first year of life, genetic defects, lead poisoning and malnutrition. Micronutrient deficiencies (*e.g.*, Iodine) and iron deficiencies have also been found to impair cognitive development<sup>[76]</sup>. Studies have attempted to link possible long-term cognitive deficits with severe diarrhea in early childhood<sup>[40,41,68]</sup>. The complex interrelation among malnutrition, diarrheal disease and environmental factors such as socioeconomic status and education make it difficult to determine the unique contribution of either malnutrition or diarrheal disease to cognitive development. However, chronic malnutrition and stunting during infancy secondary to *G. duodenalis* infections, has been associated with poor cognitive function<sup>[40,41,77]</sup>. Moreover, diarrhea during early childhood was also found to impair visual-motor coordination, auditory short-term memory, information processing, and cortical cognitive function<sup>[68,76]</sup>.

Interestingly, poor language cognition and impaired psycho-motor development appear to be associated with *Giardia* sp. more so than with other enteropathogenic parasites such as *Entamoeba histolytica*, *Ascaris lumbricoides*, *Enterobius vermicularis*, or *Trichuris trichiura*<sup>[63]</sup>. These studies have suggested a role for nutrient malabsorption and micronutrient deficiencies, such as zinc, iron or vitamins (A and B-12) in humans as well as in animals<sup>[63,74,78,79]</sup>. Indeed, significantly lower levels of iron and ferritin, known to affect psychomotor development, have been detected in patients with giardiasis<sup>[64]</sup>. Similarly, diarrhea due to giardiasis was linked to poor cognitive function by causing zinc and iron micronutrient deficiencies, as well as defects in the anti-oxidant system, which may all affect neuroplasticity<sup>[76]</sup>. Indeed, perinatal iron deficiency in rats reduces neuronal metabolic activity, specifically targeting

areas of the brain involved in memory processing<sup>[80]</sup>. Zinc supplementation was recently found to reduce the rate of diarrhea caused by giardiasis<sup>[81]</sup>. The complexity of these profound effects on functional impairments requires further investigation. More research is also needed to determine whether and how these effects can be reversed with targeted antimicrobials, with micronutrient and/or oral rehydration, or nutrition therapy<sup>[68]</sup> (Table 2).

**Chronic fatigue syndrome:** Viral, bacterial, as well as parasitic pathogens can trigger chronic fatigue syndrome (CFS), and are responsible for work-related disability reflected in long-term sickness, absence from studies and employment<sup>[82]</sup>. Although the biological basis of CFS is unknown, it is generally thought that post-infectious fatigue develops shortly after acute infection. CFS has been described following Q-fever, Epstein-Barr virus infection, Ross river virus infection, brucellosis, Lyme disease, viral meningitis and Dengue fever<sup>[83]</sup>. Recent studies have reported a high prevalence of post-infectious fatigue following a giardiasis outbreak in Bergen, Norway, in 2004<sup>[15,82-86]</sup>. Fatigue was reported in 41% of the people in Bergen 2 years after the *Giardia* outbreak, compared to 22% in the general population<sup>[82]</sup>. In this population, old age and female gender were a significantly higher risk factor for post-infectious fatigue<sup>[84,87]</sup>.

Although *Giardia* is a non-invasive parasite, post-giardiasis CFS is likely to include immunologic components<sup>[82]</sup>. Studies have implicated differences in activation and function of peripheral blood lymphocytes subsets in post-giardiasis CFS, including altered natural killer-cell levels and lowered CD4:CD8 ratios<sup>[87,88]</sup>. The exact roles of immune factors in co-morbidities associated with gastrointestinal disorders and CFS need to be further explored. Fatigue is a frequent symptom in patients with functional gastrointestinal disorders (FGID), especially irritable bowel syndrome (IBS)<sup>[89]</sup>.

### Chronic gastrointestinal consequences of *Giardia* infections

**FGID:** FGID represent a group of disorders characterized by recurring or chronic gastrointestinal symptoms without an identifiable disease process. IBS and functional dyspepsia (FD) are the best described FGID. Post-infectious-IBS (PI-IBS) has been reported following acute gastroenteritis due to bacteria such as *Salmonella* sp., *Shigella* sp. and *Campylobacter jejuni*<sup>[90,91]</sup>. Recent evidence now indicates that a proportion of patients diagnosed with *Giardia duodenalis* will also develop PI-IBS symptoms in the absence of detectable parasitic loads<sup>[92,93]</sup>.

**Post-infectious irritable bowel syndrome:** IBS is the most common functional gastrointestinal disorder diagnosed by gastroenterologists today. It is characterized by abdominal discomfort and altered bowel habit, with no abnormality on routine diagnostic tests. Several mechanisms have been considered in the pathogenesis of IBS including genetic, psychological and environmental factors as well as intestinal motor and sensory func-

tions associated with brain-gut interactions<sup>[94]</sup>. In some patients, IBS symptoms seem to arise *de novo* following acute gastroenteritis (GE). This PI-IBS denotes the persistence of abdominal discomfort, bloating and diarrhea, despite clearance of the inciting pathogen. Recent meta-analyses demonstrated that the risk of developing IBS increases six-fold after gastrointestinal infection and remains elevated for at least 2-3 years post-infection, and it is estimated that 7%-31% of patients with infectious GE go on to develop PI-IBS<sup>[90,91]</sup>. Higher risk factors include longer duration of symptoms, young age and female gender. The current conceptual framework regarding the pathophysiological mechanisms for PI-IBS suggests that it is associated with increased intestinal permeability and motility, increased numbers of enterochromaffin cells and persistent intestinal inflammation, characterized by increased numbers of T-lymphocytes and mast cells, and increased expression of pro-inflammatory cytokines<sup>[95-97]</sup>. Possible mechanisms for PI-IBS include genetic predisposition, motility dysfunction, such as accelerated colonic transit and smooth muscle hyper-reactivity to acetylcholine, continuous antigenic exposure (bacterial, parasitic or dietary), or molecular mimicry of foreign antigens<sup>[98]</sup>.

Early reports indicated that *Giardia* may cause prolonged symptoms, including secondary lactose intolerance, for several weeks after successful treatment<sup>[99]</sup>. Chronic giardiasis resembles IBS, and symptomatic infection may exacerbate existing IBS<sup>[100]</sup>. *Giardia* infection has been diagnosed in 5%-10% of patients with IBS<sup>[101,102]</sup>, and it was recently demonstrated that *G. duodenalis* may indeed cause IBS and functional dyspepsia<sup>[93]</sup>. High frequency of microscopic duodenal inflammation was found in patients post-giardiasis when the infection lasted 2-4 mo, further supporting the hypothesis that longer duration of infection is a risk factor for PI-IBS<sup>[103]</sup>. Early diagnosis of *Giardia* infection and treatment may shorten the duration of the infection and hence may help reduce the risk for such complications<sup>[83]</sup>.

Interactions between the host and gastrointestinal microbiota may play a key role in the pathogenesis of IBS. Fecal microbiota are altered in patients with IBS, and patients with diarrhea-predominant IBS appear to host more *Proteobacteria*, and fewer *Bacteroidetes* compared to asymptomatic patients<sup>[104,105]</sup>. The mechanisms by which altered fecal flora may induce disease are poorly understood. Abnormalities in short chain fatty acids have been reported in patients with diarrhea-predominant IBS<sup>[105]</sup>. Whether these alterations may result from abnormalities in the host microbiota requires further investigation<sup>[14]</sup>.

Historically, IBS was considered as a psychosomatic disorder, with an emphasis on psychiatric comorbidity<sup>[106,107]</sup>. During the past decades, GE and low grade inflammation as mechanisms underlying gastrointestinal (GI) dysfunction have been involved in IBS symptoms<sup>[106,108]</sup>. It is now well established that there is a relationship between the neural and immunological networks within the gut, and that the central nervous system and the gut are engaged in constant bi-directional communication, often related to as the brain-gut axis (BGA).

**Table 3** Extra-intestinal and long-term consequences of giardiasis

Post-infectious consequences	Speculated mechanisms involved	References
Ocular pathologies	Speculated involvement of toxic metabolites produced by the parasite	[47-49]
Arthritis	Bacterial antigens in synovial fluids possibly due to increased intestinal permeability	[50-52]
Allergies	Alteration in antigen uptake Dysfunction of the intestinal barrier	[54-57]
Hypokalemic myopathy	Loss of potassium related to diarrhea, impaired nutrient and electrolyte absorption	[59-62]
Failure to thrive	Inadequate food intake, Reduced nutrients absorption, excessive utilisation of energy, Steatorrhea, maldigestion, malabsorption	[38,39,63-65,69,71,118]
Stunting	Nutritional status, sanitary, socio-economic conditions, loss of intestinal surface area, maldigestion, malabsorption	[37,63,64,67,77,121]
Impaired cognitive functions	Chronic malnutrition and stunting following <i>G. duodenalis</i> infection Nutrient malabsorption and micronutrient deficiencies	[40,41,63,64,68,76-78]
Chronic fatigue syndrome	Altered natural killer-cell levels Lower ratio CD4:CD8	[15,82-87,89]
Post-infectious irritable bowel syndrome	Microscopic duodenal inflammation Interaction host - gastrointestinal microbiota	[14,84,93,100-105]
Cancer	Increased T-cells and Mast-cells No cause-to-effect established	[113-116]

Among the pathophysiological mechanisms of IBS, disorder of the BGA has been associated<sup>[106,108]</sup>. Recently, more evidence of emerging dysbiosis in IBS patients have been made<sup>[104]</sup>, suggesting an important role of the microbiota-gut-brain axis<sup>[106-111]</sup>. Nevertheless, our understanding of the mechanisms of the bi-directional interactions between microbiota and GI physiology and its association with behavior needs to be explored with focus on the contributions of immunological and neural components to the microbiota-BGA relationship. Insights into the interactions between enteric pathogens, the host epithelia, and the intestinal microflora are needed to improve our understanding of disease processes that may initiate IBS or even exacerbate intestinal inflammation in patients with IBD<sup>[112]</sup>. Studies on giardiasis offer a powerful model to investigate these mechanisms.

### Cancer

A few reports have described *Giardia* trophozoites in the tumoral mass of pancreatic tissue and gallbladder. While *G. duodenalis* trophozoites are generally localized to the proximal small bowel, they may also be identified in the stomach, distal small bowel, or caecum, and studies have reported pancreatic infection with *Giardia*<sup>[113-115]</sup>. Although the relationship between pancreatic giardiasis and pancreatic cancer is presently unknown, the coexistence of these 2 diseases may prompt exploration into mechanisms of carcinogenesis in giardiasis. In another study, following cholecystectomy with liver bed resection and lymph node dissection, intra-operative cytological examination of the patient's bile juice revealed the presence *G. duodenalis* trophozoites, and pathological examination revealed gallbladder cancer<sup>[116]</sup>. However, no cause-to-effect has yet been established between the presence of *Giardia* and the development of cancer.

## CONCLUSION

Infections with *Giardia duodenalis* may remain asymp-

tomatic, or cause acute or chronic diarrheal disease. The observations discussed herein also demonstrate that, in addition to its classical intestinal presentation, giardiasis may cause ocular complications, arthritis, skin allergies or myopathy. Moreover, giardiasis is now a well established cause of failure to thrive, stunting and growth retardation in human and animals, diminished cognitive functions, and chronic fatigue. Finally, *Giardia* may lead to post-infectious functional gastrointestinal disorders such as irritable bowel syndrome and functional dyspepsia. A few cases of *Giardia* trophozoites associated with tumoral masses have also been reported, but cause-to-effect relationships between giardiasis and cancer have yet to be established (Table 3).

Long-term complications of giardiasis may present 2 to 3 years following the infection. In some cases, they may last for a few weeks, and may be eliminated with anti-parasitic treatment, observations that have been reported for example in cases of myopathy and skin allergies. In other cases, long-term consequences may be present for several years, in the form of failure to thrive, stunting, IBS, and chronic fatigue syndrome, in the absence of any parasite.

The mechanisms responsible for post-infectious and extra-intestinal manifestations in giardiasis remain obscure. Both parasitic and host factors have been implicated, indicative of a multifactorial pathogenic process. However, as anti-microbial treatment often leads to recovery, the infection itself represents a key element in these complications. As giardiasis can be asymptomatic, the complex processes leading to extra-intestinal and post-infectious manifestations represent a challenging topic for further research (Table 3).

Given the high prevalence of giardiasis in young children in developing countries, its significant effects on stunting and wasting, and the lasting effects of early childhood diarrhea and malnutrition, giardiasis is of considerable public-health importance. Even though the consequences of giardiasis are variable, school health



programs and health education for children and parents aimed at reducing the prevalence of intestinal parasitic infection in children may have beneficial effects on child growth and development. Improved diagnostic methods, particularly in asymptomatic patients, as well as more-effective treatment and control strategies, are sorely needed to help reduce the detrimental impact of the infection on human societies as well as in the agriculture industry.

In the recent few years, and particularly since the 2004 giardiasis outbreak in Bergen, Norway, *G. duodenalis* infections have been linked to post-infectious IBS and functional dyspepsia *via* mechanisms that are unclear. Findings from several studies indicate roles of specific pro-inflammatory cytokines, and hyperplasia of enterochromaffin cells, mast cells, and lymphocytes, perhaps causing motility dysfunction and visceral hypersensitivity; but much controversy remains on the topic<sup>[117]</sup>. Together, the data strongly suggest that the appearance of post-infectious complications in giardiasis are multifactorial. A number of the post-infectious complications seen after giardiasis are shared with those caused by common bacterial enteropathogens like *C. jejuni*, *E. coli*, or *Salmonella* sp. One area of growing interest in this field is the role played by disruptions of the host microbiota during the acute stage of the infection in the initiation of delayed immune-mediated pathophysiology. Therefore, a better understanding of the mechanisms responsible for the extra-intestinal and post-infectious manifestations of giardiasis will help unravel common pathways leading to these phenomena.

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## Non-microbial approach for *Helicobacter pylori* as faster track to prevent gastric cancer than simple eradication

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### Abstract

Although the International Agency for Research on Cancer declared *Helicobacter pylori* (*H. pylori*) as a definite human carcinogen in 1994, the Japanese Society for Helicobacter Research only recently (February 2013) adopted the position that *H. pylori* infection should be considered as an indication for either amelioration of chronic gastritis or for decreasing gastric cancer mortality. Japanese researchers have found that *H. pylori* eradication halts progressive mucosal damage and that successful eradication in patients with non-atrophic gastritis most likely prevents subsequent development of gastric cancer. However, those who have already developed atrophic gastritis/gastric atrophy retain potential risk factors for gastric cancer. Because chronic perpetuated progression of *H. pylori*-associated gastric inflammation is associated with increased morbidity culminating in gastric carcinogenesis, a non-microbial approach to treatment that provides long-term control of gastric inflammation through nutrients and other interventions may be an effective way to decrease this morbidity. This non-microbial approach might represent

a new form of prerequisite "rescue" therapy that provides a quicker path to the prevention of gastric cancer as compared to simple eradication.

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**Key words:** *Helicobacter pylori*; Gastric cancer; Prevention; Atrophic gastritis; Non-microbial approach

**Core tip:** Gastric cancer is a multi-factorial and multi-step disease associated with various risk factors including environmental and pathogenic microbial chronic inflammation. Pharmaceutical intervention and the eradication strategy can provide rapid relief of acute inflammation but fails to correct the underlying cause of chronic inflammation. A non-microbial approach for modulating *Helicobacter pylori* associated gastric inflammation may be an attractive and rapid alternative to optimize cancer prevention strategies and minimize adverse side effects associated with therapeutic regimens.

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### INTRODUCTION

*Helicobacter pylori* (*H. pylori*), a Gram-negative bacterial pathogen that infects approximately 50% of the world's population, provokes chronic gastric inflammation which is considered a major risk factor for the development of gastric and duodenal ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma<sup>[1]</sup>. In 1994, *H. pylori* was classified as a type I (definite) carcinogen

by the International Agency for Research on Cancer<sup>[2]</sup>. Although the relationship between *H. pylori* and gastric cancer has been acknowledged by diverse forms of clinical evidence, it is still debatable as to whether eradication can lead to the prevention of gastric cancer<sup>[3-5]</sup>. Traditionally, treatment for *H. pylori* has focused primarily on eradicating the bacteria from the stomach using a combination of antibiotics, such as amoxicillin and clarithromycin, with a proton-pump inhibitor<sup>[6-10]</sup>. The eradication rate, however, has been declining due to the increasing prevalence of antibiotic resistance, especially clarithromycin resistance<sup>[11-15]</sup>. This increase in the prevalence of antibiotic resistance has diminished enthusiasm for the use of many popular *H. pylori* eradication therapies. To overcome this decline in the use of first-line treatment options, bismuth-containing quadruple and sequential therapies are emerging as second-line treatments for *H. pylori* infection<sup>[16-21]</sup>. Although newer treatments for eradicating *H. pylori* continue to be introduced, research on even more effective eradication regimens continues to be conducted. Unfortunately, literature from all over the world continues to document increases in *H. pylori* resistance to antibiotics and this major obstacle has prompted the introduction of new drugs and treatment schemes. It is also important to note that although removal or amelioration of gastric inflammation has been implicated in the prevention of gastric carcinogenesis, the persistent gastric inflammation observed in *H. pylori*-associated gastric carcinogenesis is not always amelioration by *H. pylori* eradication alone.

Because gastric cancer is a multi-step and multi-factorial disease, not all individuals infected with *H. pylori* will develop gastric cancer. In fact, the multi-factorial processes associated with the development of gastric cancer can give hope to some susceptible individuals that it may be prevented through the eradication of *H. pylori*. Conversely, in cases where chronic inflammation is caused by other environmental factors such as diet, eradication of *H. pylori* may only delay the development of gastric cancer rather prevent it. Importantly, there is no overt biomarker supporting the rationale of *H. pylori* eradication in clinic, although endoscopic findings might be recommended (Figure 1). Moreover, the nationwide cost associated with eradicating *H. pylori* in order to prevent gastric cancer would be prohibitive and represent a burden to socio-economically challenged people in developing countries. Therefore, the strategy of cancer prevention through chemopreventive agents may be the most efficacious way to reduce the global burden of cancer.

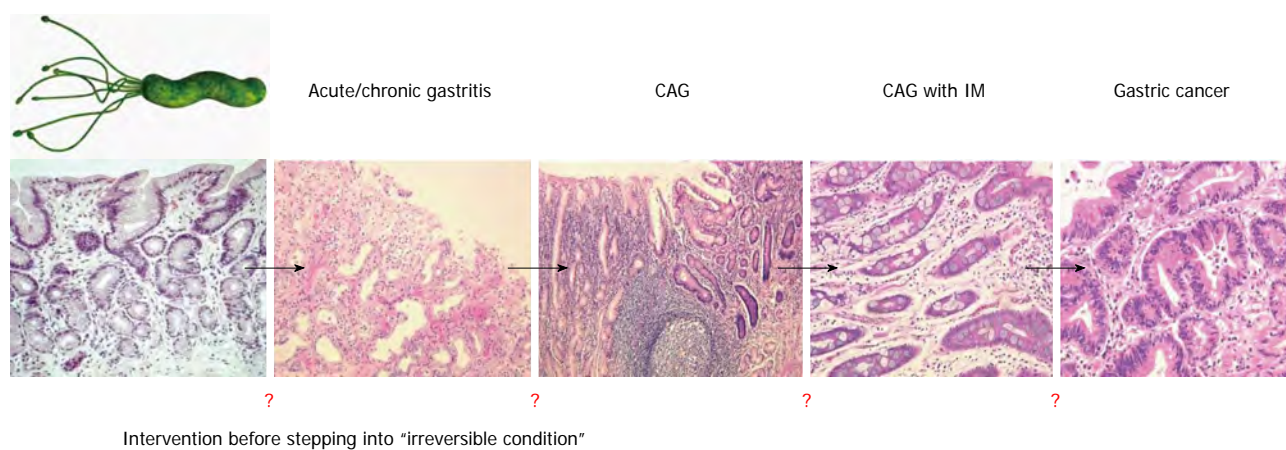
Cancer chemoprevention was established by Dr. M. Sporn in 1976 and was defined as “the use of natural, synthetic, or nontoxic chemical substances to reverse, suppress, delay or prevent carcinogenic progression” by Dr. M. Sporn and Dr. W. K. Hong<sup>[22,23]</sup>. The results of several preclinical and clinical studies have indicated that diverse chemoprevention strategies can decrease gastrointestinal (GI) cancer incidence and mortality rates<sup>[24]</sup>. Essentially, the chemoprevention strategy involves inter-

ventions during all three stages of carcinogenesis, (initiation, promotion, and progression) using chemopreventive agents in order to interfere with tumor promotion or progression and reduce the risk of various cancers. All GI cancers have a unique etiology but share common mechanisms including oxidative stress-induced damage of genomic DNA, modification of cellular proteins and lipids, altered cell signaling, and persistent local tissue inflammation. Therefore, the combination of *H. pylori* eradication, anti-oxidant interventions, interventions to normalize aberrant cell signaling, and anti-inflammatory interventions may be an essential and anticipatory strategy for prevention of gastric cancer. Recently, numerous studies have investigated the potential therapeutic benefits of probiotics, phytochemicals, and antioxidant or vitamin supplementation as chemopreventive agents as well as adjuncts to increase the eradication rates of *H. pylori* infection. In this article, we discuss what is known currently about non-microbial preventive strategies for chronic infection with *H. pylori* which may represent a faster option for cancer prevention *via* enhancement of host adoptive responses as well as removal of inflammation responsible for mutagenesis (Figure 2).

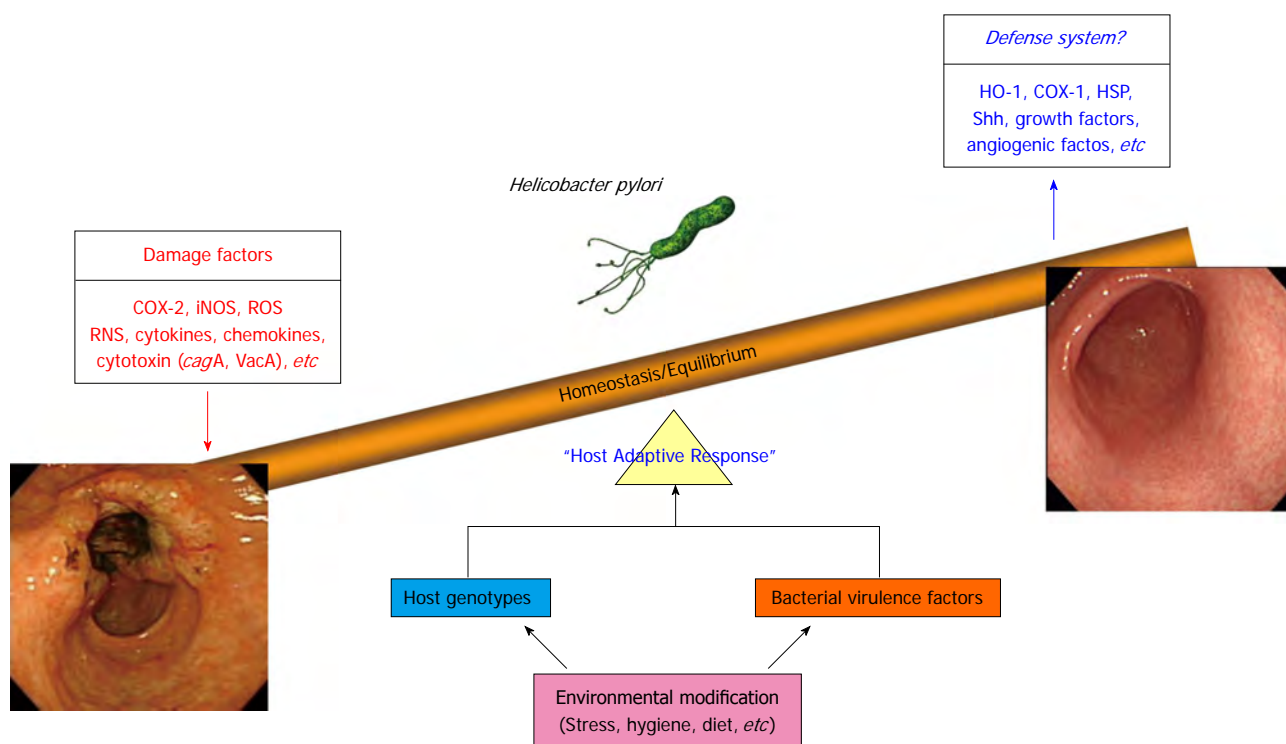
## ANTICIPATING NON-MICROBIAL APPROACHES FOR PREVENTING *H. PYLORI*-ASSOCIATED GASTRIC CARCINOGENESIS

### *Cyclooxygenase and 5-lipoxygenase inhibition*

*H. pylori*-induced inflammatory responses have been associated with high concentrations of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) which is an essential enzyme for the release of arachidonic acid (AA). AA metabolites, prostaglandins (PGs) or hydroxyl fatty acids (HETEs), are key mediators in inflammatory responses and are metabolized by cyclooxygenase (COX) and lipoxygenase (LOX)<sup>[25]</sup>. COX-1 and COX-2 are responsible for the production of inflammatory PGs and 5-LOX increases the release of gastrototoxic leukotrienes (LTs)<sup>[26]</sup>. Conversely, findings demonstrating that inflammatory responses were decreased by inhibiting COX and 5-LOX led research on COX and 5-LOX inhibitors as attractive medications for anti-inflammatory effects<sup>[27-29]</sup>. *H. pylori* infection induces higher levels of COX-2 expression, overexpression of which has been detected in various cancers including gastric cancer<sup>[30-36]</sup>. In this regard, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for preventing cancers as well as reducing pain and inflammation by inhibiting both COX-1 and COX-2 or COX-2 only<sup>[37-39]</sup>. Long-term use of NSAIDs attenuated gastric mucosal chronic inflammation induced by *H. pylori* infection suggesting that NSAIDs may be preventive agents of the gastric carcinogenesis associated with *H. pylori* infection<sup>[40]</sup>. However, the adverse effects associated with the use of NSAIDs may present an obstacle to their use as chemopreventive agents. Traditional NSAIDs non-selectively inhibit both



**Figure 1** Point of no return in *Helicobacter pylori* infection. *Helicobacter pylori* (*H. pylori*) infection is responsible for acute and chronic gastritis, chronic atrophic gastritis, and intestinal metaplasia. The results of a few studies have shown that the eradication of *H. pylori* significantly reverted these gastric pathologies and promoted restoration of gastric function. *H. pylori* is also implicated in several extragastric manifestations including idiopathic thrombocytopenic purpura, iron deficiency anemia, atherosclerosis, and chronic urticaria. Because there are no biomarkers suggestive of a point of no return, the results of several large scale cohort studies continue to provide support for the strategy of *H. pylori* eradication in gastric cancer prevention.



**Figure 2** Host adoptive response through a non-microbial approach as the core defensive mechanism against *Helicobacter pylori* infection, especially gastric cancer prevention. Even though host genotype, environmental risk, and bacterial virulence factor are all implicated in *Helicobacter pylori* (*H. pylori*) infection, a non-microbial approach may provide the fastest means of cancer prevention as well as amelioration of *H. pylori*-associated gastric pathologies.

COX-1 and COX-2 and may cause GI toxicity, as COX-1 is a house keeping enzyme involved in the cytoprotection of gastric mucosa. Though selective COX-2 inhibitors (*coxibs*), such as celecoxib, rofecoxib, and valdecoxib, have been developed to improve the GI safety<sup>[41]</sup>, *coxibs* also carry the risk of thromboembolic or cardiovascular complications<sup>[42-44]</sup>. 5-LOX inhibitors also suppressed *H. pylori*-induced proinflammatory mediators such as interleukin-8 and tumor necrosis factor- $\alpha$  in *H. pylori*-infected gastric

epithelial cells, indicating that 5-LOX inhibitors can be preventive agents against *H. pylori*-associated gastric inflammation and carcinogenesis by inhibiting the 5-LOX signaling pathway and suppressing its expression<sup>[25]</sup>.

### Phytochemicals and phytonutrients

The results of several recent studies have shown that dietary phytochemicals can modulate key molecular signaling cascades by interacting with small molecules in



cancer cells<sup>[45]</sup> and that phytochemicals present in foods can inhibit *H. pylori*-induced inflammation. Therefore, the combination of *H. pylori* eradication and the suppression of *H. pylori*-induced inflammation may represent a promising strategy for gastric cancer prevention. For instance, curcumin (diferuloylmethane), the yellow pigment of turmeric (*Curcuma longa* L.) possesses strong anti-inflammatory activities and has shown diverse suppressive actions against various cancers including gastric cancer. It has been reported that curcumin inhibits *H. pylori*-induced nuclear factor (NF)- $\kappa$ B activation, pro-inflammatory cytokines such as interleukin 8, matrix metalloproteinase-3 and -9, and the *H. pylori*-induced motogenic response<sup>[46,47]</sup>. In addition to these anti-inflammatory and anti-mutagenic actions, curcumin has showed anti-microbial effects in *H. pylori*-infected C57BL/6 mice as well as restorative actions following *H. pylori*-induced gastric damage<sup>[48]</sup>. Furthermore, curcumin inhibited the proliferation and invasion of gastric cancer cells by suppressing PAK1 activity and cyclin D1 expression<sup>[49]</sup>. Collectively, the results of these studies suggest that curcumin has potential as an antimicrobial compound and chemopreventive agent against *H. pylori* infection. The results of one recent study suggested that curcumin may prevent cancer therapy-induced oral mucositis due to its antibacterial and anti-inflammatory kinetics<sup>[50]</sup>. Further research has shown that foods such as broccoli sprouts and oils possess anti-*H. pylori*-associated inflammatory effects mediated by reducing the release of pro-inflammatory cytokines and suppressing the NF- $\kappa$ B pathway<sup>[51,52]</sup>. Additionally, daily intake of sulforaphane-rich broccoli sprouts was associated with anti-*H. pylori* activity and protection of the gastric mucosa against *H. pylori*-induced oxidative stress<sup>[53]</sup>. Broccoli sprouts contain high levels of glucoraphanin, a glucosinolate precursor of the isothiocyanate sulforaphane known to suppress interleukin (IL)-8 *via* the NF- $\kappa$ B pathway<sup>[51,54]</sup>. Because *H. pylori*-induced inflammation has been associated with the expression IL-8, a potent neutrophil-attracting chemokine, *via* activation of the NF- $\kappa$ B pathway<sup>[55,56]</sup>, reduction or disruption of this cascade or levels of this cytokine may be an appropriate strategy to intervene in *H. pylori*-induced inflammation.

### **Omega-3 polyunsaturated fatty acids**

There is growing evidence that the diverse biological roles of n-3 polyunsaturated fatty acids (PUFAs) may contribute to their protective actions against chronic inflammatory disease<sup>[57]</sup>. In bacteria, n-3 PUFAs cause cell lysis, while in other cell types, n-3 PUFAs can be incorporated into membrane phospholipids that can cause a loss of membrane fluidity and may be associated with lipid raft assembly and function<sup>[58]</sup>. These lipid rafts are cholesterol-rich microdomains at the host cell surface and are required for NF- $\kappa$ B-dependent responses to *H. pylori*<sup>[59]</sup>. Recently, the results of several studies have suggested that n-3 PUFAs can be converted into bioactive mediators, including resolvins, that have inflammation-resolving properties *via* counter-regulation of lipid mediators such

as pro-inflammatory LTs and PGs<sup>[52,57]</sup>. Correia *et al*<sup>[60]</sup> conducted experiments that showed that docosahexaenoic acid (DHA) significantly inhibited *H. pylori* growth both *in vitro* and *in vivo* in a dose-dependent manner and decreased mouse gastric mucosa inflammation. These results suggested that DHA could be used as an adjunct agent in *H. pylori* eradication treatment. In contrast, Meier *et al*<sup>[61]</sup> showed that an n-3 PUFA-containing eradication regimen failed to show any benefit when compared to a conventional eradication regimen. Thus, our group investigated the long-term treatment of n-3 PUFAs in an *H. pylori*-infected animal model and found that long-term administration of n-3 PUFAs ameliorated *H. pylori*-induced gastric inflammation, atrophied gastritis, and attenuated the incidence of *H. pylori*-associated gastric carcinogenesis. Kuriki *et al*<sup>[62]</sup> conducted a clinical investigation of the association between gastric cancer risk and the erythrocyte composition of DHA using 179 incident gastric cancer cases and 357 non-cancer controls (matched by age, sex, and season of sample collection). The study authors found that the erythrocyte composition of DHA was negatively associated with the risk of gastric cancer, especially of well-differentiated adenocarcinoma. Detailed, randomized, controlled trials should be conducted to obtain strong evidence for the incorporation of nutraceuticals, including n-3 PUFAs, into the therapeutic armamentarium in near future, as their use as therapeutic agents for GI disorders is moving rapidly into clinical settings and scientific studies are providing mechanisms of action to explain the therapeutic effects.

### **Probiotics and microbiota**

Probiotics such as non-pathogenic microbial feed or food supplements are already being widely studied in the treatment of GI diseases including irritable bowel syndrome, inflammatory bowel disease, severe acute pancreatitis, and chronic liver diseases<sup>[63-66]</sup>. The use of probiotics in the treatment of GI infections is gaining traction as an alternative or complement to antibiotics due to their potential to decrease the use of antibiotics or reduce their side effects<sup>[67]</sup>. Results of clinical trials combining the use of agents for first-line eradication and adjunctive probiotics have been reported to increase the *H. pylori* eradication rate<sup>[68-70]</sup>. Moreover, emerging evidence shows that probiotics attenuate *H. pylori* infection rates and associated inflammation. The results of several *in vitro* studies have shown that *Lactobacillus* can ameliorate *H. pylori*-induced inflammation by modulating cytokine induction, activating suppressor of cytokine signaling (SOCS) expression, and inactivating the JAK2, Smad7 and NF- $\kappa$ B signaling pathways<sup>[71-73]</sup>. Twelve human studies have investigated the efficacy of combinations of antibiotics and probiotics, whereas 16 studies used probiotics alone as an alternative to antibiotics for the treatment of *H. pylori* infection. Most of the studies showed an improvement of *H. pylori* gastritis and decreases in *H. pylori* colonization after probiotic administration. None of the studies, however, could demonstrate complete eradication of *H. pylori* in-



fection by probiotic treatment<sup>[67,74]</sup>. It should be noted, however, that one of the well-documented advantages of probiotic combinations was a reduction in adverse effects induced by *H. pylori* eradication treatment<sup>[75]</sup>. Since long-term intake of products containing probiotic strains may have a favorable effect on *H. pylori* infection in humans, particularly by reducing the risk of developing disorders associated with high degrees of gastric inflammation, it is possible that they contributed ultimately to chemoprevention. Recent advances in high throughput analysis technology have highlighted the importance of probiotics in *H. pylori* infection as well as other GI diseases involving “microbiota” as key controllers of *H. pylori* infection. The human organism is colonized by a large number of microorganisms that play important roles in several biochemical reactions. The microorganisms that colonize the human GI tract are collectively described as *microbiota* and a typical human may carry over  $40 \times 10^3$  bacterial species in the intestinal microbiome<sup>[76]</sup>. The microbiota of the human stomach and its influence on *H. pylori* colonization has been characterized. Most phylotypes belong to the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Fusobacteria*. *Lactobacillus* species are acid-resistant and commensal and their concentrations in the normal human stomach vary between 0 and  $10^3$  mL<sup>-1</sup>. The human microbiome co-evolved with mankind, is part of human physiology, and contributes to homeostasis. Although microbiota–host interactions through metabolic exchange and co-metabolism of substrates, or metabolome–metabolome interactions are still poorly understood, they may be implicated in the etiology of many human diseases including *H. pylori* infection. Therefore, the advantages attributed to probiotics in *H. pylori* infection, such as augmentation of the eradication rate, attenuation of side effects associated with eradication drugs, and some direct anti-inflammatory action, may represent only a small part of their involvement. Extensive investigation of the *microbiota* relevant to *H. pylori* infection will be required to elucidate additional mechanisms and relationships.

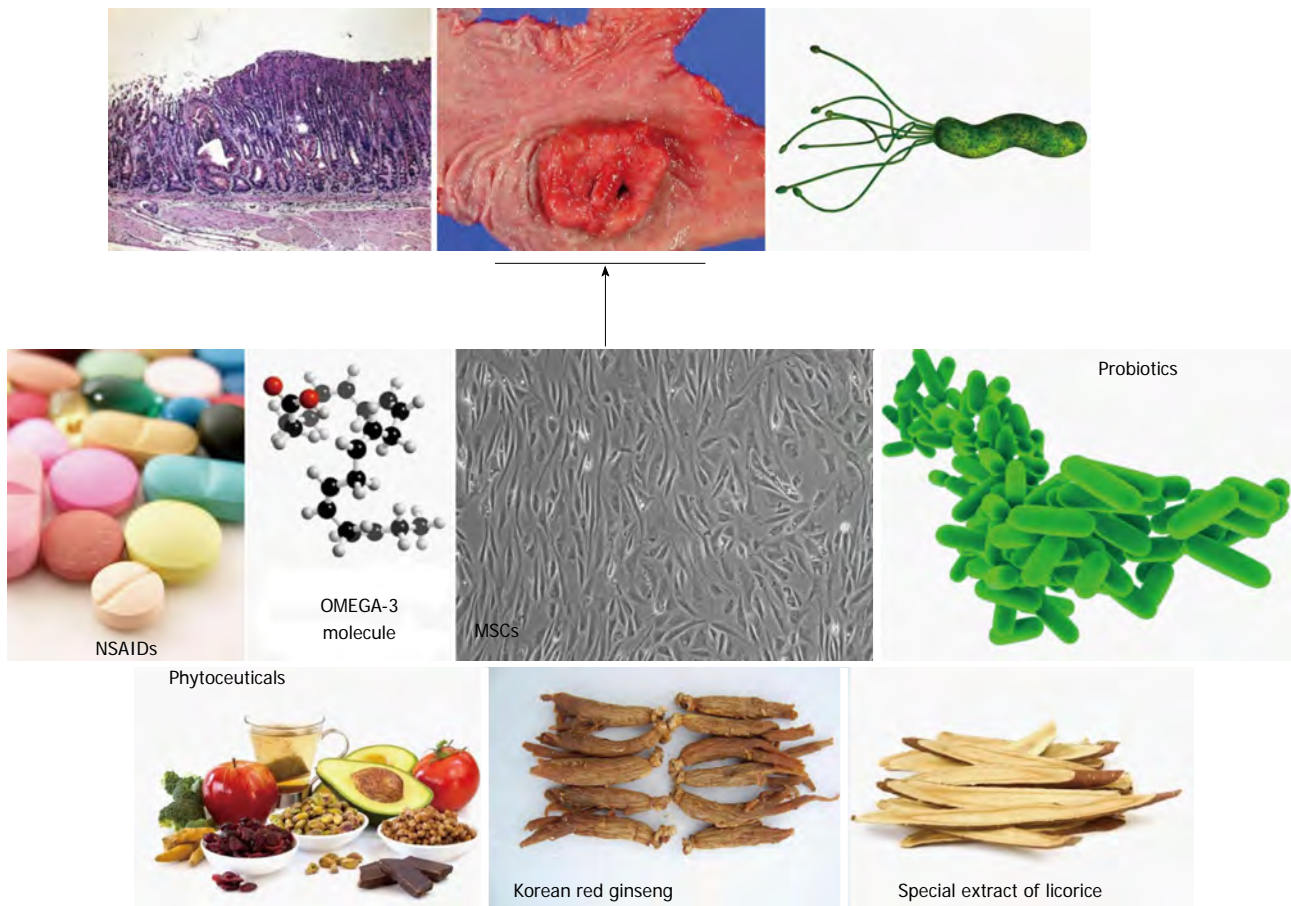
### Mesenchymal stem cells

Although gastric epithelial stem cells have been localized, little is known about their molecular biology. Recent reports described the use of inducible Cre recombinase activity to indelibly label candidate stem cells and their progeny in the distal stomach<sup>[77]</sup>. *H. pylori*-induced chronic inflammation affects differentiation and promotes metaplasias, in which cellular and molecular mechanisms in spasmodic polypeptide-expressing TFF2 pseudopyloric metaplasia predominates. The identification of signaling pathways and events that take place during embryonic development that eventually establish adult stem cells to maintain the specific features and functions of the stomach mucosa have elucidated how gastric epithelial stem cells contribute to either good regeneration, such as healing or rejuvenation, or bad regeneration, such as carcinogenesis. For example, because bone marrow-derived mesenchymal stem cells [BM-Mesenchymal stem

cells (MSCs)] are known to play an important role in *H. pylori*-induced gastric carcinogenesis, Lin *et al.*<sup>[78]</sup> transplanted BM-MSCs into the stomach of mice with a 44 wk mouse-adapted *H. pylori* infection. Study results revealed that transplantation of BM-MSCs into a chronic *H. pylori*-infected mouse led to an immunosuppressive environment such that stem cells fostered an environment compatible with the development of *H. pylori*-induced gastric cancer. Similarly, recent investigations into gastric stem cell or progenitor cell biology have uncovered valuable information for understanding gastric gland renewal and maintenance of homeostasis relevant to *H. pylori* infection. Ding and Zheng<sup>[79]</sup> provided clues for further defining the mechanisms by which gastric cancer may originate and progress. Using Lgr5, villin-promoter, TFF2-mRNA, and Mist, all of which are factors identified as gastric stem/progenitor cell markers, they explored how *H. pylori* or chronic inflammation affected gastric stem cells or their progenitors which give rise to mucus-, acid-, pepsinogen-, and hormone-secreting cell lineages. From their study results, they concluded that *H. pylori* infection induced oncogenic transformation and propagation into tumors based on the tumor microenvironment. In his recent publication, Peek stated that chronic *H. pylori* infection led to DNA damaged stem cells, a condition which could have severe negative consequences<sup>[80]</sup>. In detail, *H. pylori*-infected rodents that developed dysplasia harbored a subset of gastric epithelial cells in which levels of spermidine oxidase (SMO) production and DNA damage were high, but which were resistant to apoptosis, thereby representing a cellular population poised for neoplastic transformation targeted for gastric stem cells. In contrast to the results of these harmful interventions using gastric stem cells in *H. pylori*-associated gastric carcinogenesis, we found that exogenous stem cells could provide options for cancer prevention and intervention, as MSCs were able to rejuvenate atrophic gastritis into non-atrophic condition and significantly ameliorate *H. pylori*-induced gastritis. Because gastric stem cells can have positive or negative effects dependent upon how they are used, further experimentation will be necessary to advance our understanding of stem cell properties in *H. pylori* infection, as well as the potential for rejuvenation of *H. pylori*-infection-associated chronic atrophic gastritis with or without intestinal metaplasia.

### Antioxidants

*H. pylori* leads to chronic inflammation which in turn leads to oxidative stress derived from immune cells and gastric epithelial cells and is one of the main contributors to DNA damage associated with apoptosis and neoplastic transformation<sup>[81]</sup>. Both pathogen and host factors contribute directly to oxidative stress, including *H. pylori* virulence factors, and pathways involving DNA damage and repair, polyamine synthesis and metabolism, and oxidative stress responses. As previously mentioned, polyamine oxidation by SMO causes H<sub>2</sub>O<sub>2</sub> release, DNA damage and apoptosis, and subsequent gastric transfor-



**Figure 3** A non-microbial approach for *Helicobacter pylori*-associated gastritis as well as gastric cancer. Simply removing *Helicobacter pylori* (*H. pylori*) can contribute to gastric cancer prevention in some patients. For example, *H. pylori* eradication suppressed the metachronous occurrence of gastric cancer in patients who underwent endoscopic submucosal dissection, whereas insignificant outcomes were noted in general eradication. Supplementation or treatment with long-term phytochemicals or other agents were proven to be very efficacious in the prevention of *H. pylori*-associated gastric carcinogenesis. These treatment strategies are supported by the clear mechanisms of anti-inflammation, anti-oxidation, and anti-mutagenesis associated with their use.

mation<sup>[82,83]</sup>. Since many studies reporting the potential contribution of oxidative stress and chronic inflammation to *H. pylori*-associated gastric carcinogenesis, antioxidants can provide enough hope for cancer prevention. *H. pylori*-associated inflammation can induce DNA damage due to oxygen radicals by persistent inflammatory cell infiltrations in the gastric mucosa, which may lead to alterations of the gene and result in the development of diffuse-type carcinoma. In order to elucidate the influence of *H. pylori* on changes in inflammation-related DNA damage, Hahm *et al.*<sup>[84]</sup> measured the sequential changes of the 8-hydroxydeoxyguanosine (8-OHdG) content of DNA and changes of two biomarkers, inducible nitric oxide synthase (iNOS) and apoptosis, from human gastric mucosa according to the status of *H. pylori*. The increased levels of oxidative DNA damage, increased occurrences of apoptosis, and increased expressions of iNOS seemed to provide the mechanistic links between *H. pylori* infection and gastric carcinogenesis. In a subsequent study, we treated *H. pylori*-associated chronic atrophic gastritis with an antioxidative drug, rebamipide, and found that it contributed to either augmentation of the eradication rate or a significant decrement of 8-OHdG content<sup>[85]</sup>. Diseases

associated with free radical overproduction are provoked by “blazed reactive oxygen species productions” far beyond the host’s capacity to quench. Free radicals have been implicated in the pathogenesis of diverse GI diseases including gastroesophageal reflux disease, gastritis, enteritis, colitis, and associated cancers, as well as pancreatitis and liver cirrhosis<sup>[86]</sup>. Antioxidants administered in a nutritional way or *via* pills will surely contribute to the amelioration of *H. pylori*-associated gastric carcinogenesis. However, additional proof of concept evidence is required.

## CONCLUSION

Gastric cancer is a multi-factorial and multi-step disease associated with a variety of risk factors including environmental and pathogenic microbial chronic inflammation. In addition to life-style factors, especially diet, infection with the pathogenic microorganism *H. pylori* is a major concern for gastroenterologists because *H. pylori* infection causes chronic atrophic gastritis and peptic ulcer with an inflammatory response. Unfortunately, modern medicine cannot completely prevent gastric cancer

and even eradication of *H. pylori* is problematic due to expense and antibiotic resistance, as well as insufficient evidence supporting a rationale for eradication. However, the Japanese government decided to take on the great challenge of *H. pylori*-associated chronic gastritis by including its eradication in their guideline this year in an attempt to decrease gastric cancer incidence and mortality. Until such time as proof emerges supporting the concept that *H. pylori* eradication is the fastest means of preventing gastric cancer, the attenuation or intervention of *H. pylori*-induced chronic inflammation may be alternative or complementary methods to achieve the prevention of gastric cancer. As shown in this review, the inhibitors of COX and LOX, a number of natural phytochemicals, including curcumin and broccoli sprouts (sulforaphane), oils such as omega-3 PUFAs, probiotics, and stem cells have been shown to have anti-inflammatory and antimicrobial activities by targeting small molecules or regulating signaling cascades (Figure 3). Pharmacotherapy and the eradication strategy can provide rapid relief of acute inflammation but cannot correct the underlying cause of chronic inflammation. However, a non-microbial approach for modulating *H. pylori*-associated gastric inflammation may be an attractive and fast way to optimize cancer preventive strategies and minimize adverse side effects associated with therapeutic regimens.

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## Digestive cancer surgery in the era of sentinel node and epithelial-mesenchymal transition

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**Key words:** Lymph node count; Lymph node ratio; Sentinel node; Tumor budding; Tumor deposits

**Core tip:** We summarize the current knowledge on the assessment of nodal status and nodal staging in digestive carcinomas and highlight the prognostic impact of two epithelial-mesenchymal transition-related phenomena, tumor budding and tumor deposits, that are involved in tumor progression. In light of the biological, prognostic and therapeutic impact of these phenomena, the role of staging and surgical procedures in digestive carcinoma could be reevaluated and redefined.

### Abstract

Lymph node involvement is one of the most important prognostic indicators of carcinoma of the digestive tract. Although the therapeutic impact of lymphadenectomy has not been proven and the number of retrieved nodes cannot be considered a measure of successful cancer surgery, an adequate lymph node count should be guaranteed to accurately assess the N-stage through the number of involved nodes, lymph node ratio, number of negative nodes, ratio of negative to positive nodes, and log odds, *i.e.*, the log of the ratio between the number of positive lymph nodes and the number of negative lymph nodes in digestive carcinomas. As lymphadenectomy is not without complications, sentinel node mapping has been used as the rational procedure to select patients with early digestive carcinoma in whom nodal dissection may be omitted or a more limited nodal dissection may be preferred. However, due to anatomical and technical issues, sentinel node mapping and nodal basin dissection are not yet the standard of care in early digestive cancer. Moreover, in light of the biological, prognostic and therapeutic impact of tumor budding and tumor deposits, two epithelial-mesenchymal transition-related phenomena that are involved in tumor progression, the role of staging and surgical procedures in digestive carcinomas could be redefined.

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### INTRODUCTION

Lymph node involvement is one of the most important prognostic indicators of carcinoma of the digestive tract. In contrast to Eastern countries, in Western countries lymph node involvement is not considered to be a prognostic "governor" and the therapeutic impact of lymphadenectomy is not acknowledged. Recent advances in minimally invasive treatment procedures for cancer have promoted their application for the assessment of lymph node status (positive/negative), *i.e.*, sentinel node mapping and biopsy. In addition, other prognostic factors related to epithelial-mesenchymal transition (EMT) that are involved in tumor progression, such as tumor budding and tumor deposits, have been gaining ground.

Here, we review the current knowledge on these issues and highlight the need for a redefinition of the role of surgical and staging procedures in digestive cancer surgery in light of recent advances in our understanding of the biology of tumor progression.

## NUMBER OF EXAMINED NODES, LYMPH NODE RATIO, LOG ODDS

Several studies have shown an association between the number of excised nodes and overall survival, providing evidence that examination of an insufficient number of lymph nodes (LNs) may have a detrimental effect on survival in patients with gastrointestinal carcinoma<sup>[1,2]</sup>. However, much of this appears to be the effect of stage migration, which impacts the stage-specific survival without affecting overall survival<sup>[3]</sup>. Variations in patient demographics, tumor location and tumor biology raise questions regarding the evidence for a minimum LN harvest<sup>[1,4]</sup>. In gastric cancer, the stage migration effect is most striking when fewer than 10 LNs are assessed, but it is still present with a greater number of examined LNs<sup>[5,6]</sup>. Therefore, although current guidelines support the assessment of a minimum of 16 LNs, examination of more LNs is necessary to reduce the stage migration effect<sup>[1]</sup>. In colorectal cancer, the aim should be to collect as many LNs as possible to improve staging and increase survival. In fact, particularly following neo-adjuvant treatment for rectal cancer, downstaging with fewer LNs implies a positive treatment response and a more favorable prognosis<sup>[4]</sup>.

An association between better postoperative long-term survival and a greater number of dissected nodes has also been reported in patients with several N0 digestive malignancies, including esophageal<sup>[7]</sup>, gastric<sup>[8]</sup>, colorectal<sup>[9]</sup>, and pancreatic carcinomas<sup>[10,11]</sup>. This may be due to a not negligible rate of nodal micrometastasis, and the probability of missing a positive LN decreases as the number of examined LNs increases, *i.e.*, the Will Rogers phenomenon<sup>[7,9,12]</sup>. In patients with node-negative gastric cancer, a prophylactic D2 lymphadenectomy<sup>[8,13]</sup> with almost 16 LNs examined<sup>[12]</sup> seems to be effective, although retrieval of more than 25 nodes has been suggested<sup>[14]</sup>. The removal of at least 18 LNs during an esophagectomy with curative intent results in improved survival in esophageal cancer, particularly in patients with adenocarcinoma<sup>[7]</sup>. In N0 pancreatic carcinoma, examination of more than 10 LNs has been associated with improved survival<sup>[10]</sup>. In stage II (T3-4N0) colorectal cancer, current guidelines consider a number of harvested LNs of less than 12 an indication to perform adjuvant chemotherapy; harvesting of less or more than 12 LNs allows a better prognostic stratification of stage IIa (T3N0) patients for postoperative treatment<sup>[9,15]</sup>. On the basis of statistical considerations, the current recommended goal of 12-15 recovered lymph nodes without evidence of metastatic disease provides approximately 80% negative predictive value for colorectal carcinoma metastasis<sup>[16]</sup>.

However, the clinical significance of micrometastasis [pN1(mi), *i.e.*, tumor cell clusters of  $> 0.2$  mm but  $\leq 2$  mm] and isolated tumor cells [pN0(i), *i.e.*, single tumor cells or small clusters of cells of  $\leq 0.2$  mm at their greatest extent that can be detected by routine hematoxylin and eosin (HE) stains or immunohistochemistry (IHC) or clusters of  $\leq 200$  cells in a single histological cross-section]<sup>[17]</sup> in gastrointestinal carcinoma remains unclear<sup>[18]</sup>. In early and advanced pN0 gastric cancer, the occurrence of nodal micrometastasis was shown to have no impact on prognosis<sup>[19]</sup>; however, other studies showed that LN micrometastasis was one of the most important prognostic factors in multivariate survival analysis of pT1N0<sup>[20]</sup>, and the prognosis was significantly poorer in patients with isolated tumor cells than in those without them<sup>[21]</sup>. A recent systematic review and meta-analysis reported that molecular detection of tumor cells (isolated tumor cells and/or micrometastasis) in regional lymph nodes is associated with an increased risk of disease recurrence and poor survival in patients with N0 colorectal cancer<sup>[22]</sup>.

In N+ digestive carcinomas, lymph node ratio (LNR) is a better prognostic factor than number of metastatic nodes (pN), and it may minimize the stage migration effect<sup>[23-28]</sup> because it is assumed to be constant regardless of the number of examined nodes<sup>[29]</sup>. However, LNR stages can be more accurately differentiated with a large number ( $> 15$ ) of examined nodes<sup>[11,30-32]</sup>. Negative node count has been proposed as a prognostic indicator in patients with gastric cancer based on the assumption that nodal metastasis and micrometastasis cannot be prevented without adequate negative node dissection<sup>[33,34]</sup>. A negative lymph node count has been associated with improved survival in colorectal cancer patients, independent of patient, pathologic and molecular characteristics; however, the beneficial effects of a negative count are stronger in stage I - II patients than in stage III-IV patients<sup>[35]</sup>. Moreover, a straight ratio between negative and positive lymph nodes (RNPL), which provides direct information on nodal metastasis, micrometastasis, and the immune condition of the patient, could be more accurate than LNR for the prognostic evaluation of curatively resected gastric cancer<sup>[36]</sup>. At the same time, the log odds of positive lymph nodes (LODDS), *i.e.*, the log of the ratio between the number of positive LNs and the number of negative LNs, is superior to the pN+ and LNR classifications for prognostic assessment in gastric and colorectal carcinoma<sup>[37,38]</sup>. In effect, LODDS is a function of the number of negative LNs, whereas LNR is a function of the total number of LNs<sup>[39]</sup>. Moreover, LNR is not applicable to pN0 patients, whereas LODDS is a useful lymph node classification for pN0 patients because it can discriminate between subgroups with different survival rates<sup>[38]</sup>. With respect to the pN and LNR classifications, LODDS has shown more power for minimizing the stage migration phenomenon caused by an insufficient number of retrieved nodes<sup>[38,40]</sup>.

The prognostic power of the number of involved nodes in patients with digestive carcinomas is limited.



Furthermore, although the therapeutic impact of lymphadenectomy has not been proven and the number of retrieved nodes cannot be considered a measure of successful cancer surgery, an adequate LN count should be guaranteed to accurately assess the N-stage through the number of involved nodes, LNR, number of negative nodes, ratio of negative to positive nodes, and LODDS in digestive carcinomas<sup>[4,41]</sup>. In fact, in Western countries, D2 lymphadenectomy is gradually becoming the recommended surgical approach for patients with resectable gastric cancer<sup>[11,42,43]</sup>, and total mesorectal excision (TME) is the recommended procedure for extraperitoneal rectal carcinoma. However, because lymphadenectomy is not without complications and institutional screening programs leading to the detection of cancer at an early stage have increased the prevalence rate of clinical N0 tumors, sentinel node (SLN) mapping has been used as the rational procedure to select patients in whom nodal dissection may be omitted or a more limited nodal dissection may be preferred.

### Sentinel node mapping and biopsy

Recent meta-analyses have shown acceptable SLN detection rates and accurate determination of lymph node status in gastric cancer<sup>[44,45]</sup>. However, SLN mapping and nodal basin dissection are not yet the standard of care in early gastric cancer because of several unsolved anatomical (skip metastasis, multidirectional lymphatic drainage patterns) and technical (dye method, radio-colloid method or combination of the dye method and radio-colloid method) issues that may impact the detection rates and false negative rates. Moreover, there is another problem regarding the pathological diagnosis of SLN metastasis, including micrometastasis. Pathologic examination of SLNs has not been standardized in gastric cancers<sup>[46]</sup>. Serial sectioning results in a more accurate evaluation of metastases; however it is time-consuming. HE staining and IHC have been used in combination with serial sections of frozen and paraffin-embedded specimens for the detection of micrometastatic disease in SLNs<sup>[47]</sup>. Occult metastasis in SLN has been detected in 4% of pN0 gastric cancer patients using IHC in the 5- $\mu$ m-thick serial step sections at 85- $\mu$ m intervals of whole formalin-fixed paraffin-embedded tissues of all resected SLN<sup>[48]</sup>. The highly sensitive real-time reverse transcription polymerase chain reaction (RT-PCR) system, which enables rapid analysis to detect the mRNA of CK19, CK20 and carcinoembryonic antigen<sup>[49]</sup>, and the one-step nucleic acid amplification (OSNA) assay<sup>[50]</sup> are promising tools for intraoperative diagnosis of SLN involvement in gastric cancer. In rectal carcinoma, the “*in vivo*” procedure of sentinel node mapping and biopsy entails breaking the mesorectal fascia intraoperatively to search for and dissect the SLNs. However, from a surgical point of view, the preservation of the integrity of the mesorectal fascia

during rectal excision is necessary to minimize the risk of both residual tumor and relapses, and this assumption is the basis of the TME technique. The aim of the currently adopted SLN mapping procedure in colorectal carcinoma is not to avoid extended nodal dissection and therefore related morbidities, but rather to improve the sensitivity of the histopathological evaluation through the selective application of serial step sectioning, immunohistochemistry, and/or RT-PCR techniques, and “*ex vivo*” techniques of sentinel node mapping have been developed for this goal<sup>[51]</sup>. We observed that this *ex vivo* sentinel node procedure is an effective method for improving nodal staging in clinically node-negative colorectal carcinoma by immunohistochemical detection of micrometastasis in SLNs. However, it is not useful for the detection of satellites (*i.e.*, the presence of macroscopic or microscopic tumor deposits in pericorectal adipose tissue), which should be assessed by TNM staging of colorectal cancers<sup>[52]</sup>. Moreover, the “*in vivo*” and “*ex vivo*” procedures are associated with a identification rate of 90% and a sensitivity of less than 70%<sup>[53]</sup>. Advances in imaging technologies could allow a more accurate preoperative detection of SLNs than the current dye- or radio-guided methods. Moreover, new dye-guided intraoperative technologies might revolutionize the SLN mapping procedure in gastrointestinal cancers. Indocyanine green (ICG) infrared or fluorescence imaging may identify a higher number of SLNs than radio-guided methods because the particle size of dyes is smaller than that of radioactive colloids. In gastric cancer, ICG infrared imaging is a useful tool in laparoscopic detection of SLNs. ICG fluorescence imaging is feasible even by preoperative ICG injection at, for instance, 1 or 3 d before surgery; it is also feasible in laparoscopy-assisted gastrectomy *via* a small laparotomy<sup>[47]</sup>. There is only limited experience with the application of ICG fluorescence-guided SLN mapping in colon cancer. The method has been shown as feasible and safe but further analyses in larger series are necessary to determine its definitive role in colon cancer patients<sup>[54]</sup>.

The rationale for performing SLN mapping and biopsy is to determine the N status in tumors in which the N status may impact the prognosis, thus potentially avoiding unnecessary lymphadenectomy. This is possible if the determination of N status is accurate, *i.e.*, when the SLN procedure has acceptable false-negative rates. Actually, in pN0 cases, a greater number of retrieved nodes have a beneficial impact on outcome, and a false-negative rate of SLN determination is common in gastrointestinal carcinomas. Moreover, apart from anatomical, technical, surgical and pathological issues, in light of the latest knowledge about the biology of tumor progression, determination of N status by the sentinel node mapping procedure, leaving out of consideration currently emerging progression-related phenomena, may not be sufficient for prognostic evaluation.

## EPITHELIAL-MESENCHYMAL TRANSITION-RELATED PHENOMENA OF TUMOR PROGRESSION: TUMOR BUDDING AND TUMOR DEPOSITS

Two EMT-related phenomena involved in cancer progression have been recently shown to have prognostic impact: tumor budding (TB), which is the presence of de-differentiated, isolated single cells or small cell clusters (up to five cells) scattered in the stroma at the invasive front of the tumor<sup>[55]</sup>; and the formation of tumor deposits (TDs, satellites), which are macroscopic or microscopic nests or nodules found in the lymph drainage area of a primary carcinoma without evidence of residual lymph nodes in the nodule. TDs may represent discontinuous spread, venous invasion or a totally replaced lymph node<sup>[17]</sup>.

The EMT process allows an epithelial cell to assume a more mesenchymal phenotype with increased migratory capacity, invasiveness, resistance to apoptosis and production of extracellular matrix molecules<sup>[56]</sup>. Loss of E-cadherin, a transmembrane glycoprotein localized in the adherens junction of epithelial cells, is a key event in EMT, enabling tumor cells to migrate, invade and metastasize<sup>[57]</sup>. Interestingly, the first step in a tumor bud's life seems to be its detachment from the main tumor body by loss of membranous expression of the adhesion molecule E-cadherin<sup>[58]</sup>. TB has been observed in gastrointestinal carcinomas including colorectal, esophageal, gastric, ampullary and pancreatic carcinomas<sup>[55,59-65]</sup>. Although the definition of "high-grade budding" (*i.e.*, 10 buds in a 25 × field) by Ueno *et al*<sup>[55]</sup> is the most widely applied, there are no well-defined, evidence-based criteria for quantitative (*i.e.*, optimal cut-off and field diameter) and qualitative assessment of TB<sup>[66]</sup>. In colorectal carcinoma, TB is an independent predictor of tumor progression and outcome, especially in stage II (T1-3 N0) tumors, in which high TB may be used as a high-risk criterion to select patients for adjuvant therapy<sup>[66,67]</sup>. In pancreatic carcinoma, high grade TB has been identified as an independent and highly unfavorable prognostic factor. Moreover, TB is associated with more aggressive phenotypes such as advanced pT classification and lymphatic invasion<sup>[65]</sup>. In esophageal squamous cell carcinoma, TB is a significant prognostic factor for patients who have undergone surgery alone<sup>[61]</sup>, and high grade TB has been reported to be the most important predictor of poor prognosis in patients who received chemotherapy followed by surgery<sup>[62]</sup>. Moreover, tumor buds could be used as a potential target for new therapeutic approaches<sup>[58,63]</sup>.

TDs have been detected in various types of carcinomas other than colorectal carcinoma, including gastric, pancreatic, gallbladder and bile duct carcinomas<sup>[68]</sup>. The latest TNM classification of colorectal carcinoma has categorized TDs as N1c<sup>[17]</sup>. However, the nature of TDs as well as their histopathological definition and prognostic classification regarding primitive tumor (T), regional

nodal (N), or distant metastasis (M) categories are debated<sup>[69-71]</sup>. Several authors support the inclusion of TDs in the staging of gastric cancer<sup>[70-72]</sup>. Snail and Twist are transcriptional repressors of E-cadherin and EMT inducers. In colorectal cancer, overexpression of Twist enhances TD formation, and upregulation of Snail expression contributes to lymph node metastasis through two different molecular pathways, both involving EMT, by repression of the membranous expression of E-cadherin: Twist-EMT-TDs and Snail-EMT-LN metastasis<sup>[73]</sup>. Overexpression of Snail and Twist has been shown in pancreatic carcinoma<sup>[74]</sup>.

Therefore, the occurrence of TB and formation of TDs seem to be the result of different steps in tumor progression promoted by EMT. Although the precise involvement of the EMT process in tumor progression is not well understood, the existence of other progression-related phenomena with biological, prognostic and therapeutic impact between the T, N and M is undeniable. In digestive cancers, the role of staging and surgical procedures could be re-evaluated and redefined from the perspective of the biological, prognostic and therapeutic impact of these tumor progression-related phenomena.

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## Pancreatic trauma: A concise review

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visualized within several hours following trauma as they are time dependent. Delayed diagnoses of traumatic pancreatic injuries are associated with high morbidity and mortality. Imaging plays an important role in diagnosis of pancreatic injuries because early recognition of the disruption of the main pancreatic duct is important. We reviewed our experience with the use of various imaging modalities for diagnosis of blunt pancreatic trauma.

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**Key words:** Pancreas; Trauma; Pancreatitis; Radiology

**Core tip:** The pancreas is a relatively uncommon organ to be injured in abdominal trauma and difficult to diagnose. Pancreatic injuries are usually subtle to identify by different diagnostic imaging modalities and these injuries are often overlooked in cases with extensive multiorgan trauma. They are associated with considerably high morbidity and mortality in cases of delayed diagnosis, incorrect classification of the injury, or delays in treatment. This review provides an overall concise update on pancreatic trauma and highlights the findings of pancreatic trauma on various imaging modalities.

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## Abstract

Traumatic injury to the pancreas is rare and difficult to diagnose. In contrast, traumatic injuries to the liver, spleen and kidney are common and are usually identified with ease by imaging modalities. Pancreatic injuries are usually subtle to identify by different diagnostic imaging modalities, and these injuries are often overlooked in cases with extensive multiorgan trauma. The most evident findings of pancreatic injury are post-traumatic pancreatitis with blood, edema, and soft tissue infiltration of the anterior pararenal space. The alterations of post-traumatic pancreatitis may not be

## INTRODUCTION

The pancreas is a relatively uncommon organ to be injured in trauma, occurring in less than 2% of blunt trauma cases, and this injury is associated with considerably high morbidity and mortality in cases of delayed diagnosis, incorrect classification of the injury, or delays in treat-

ment<sup>[1,2]</sup>. Mortality for pancreatic injuries ranges from 9% to 34%; however, only 5% of the pancreatic injuries are directly related to the fatal outcome. Physical examination is usually not reliable in the setting of acute pancreatic trauma<sup>[3]</sup>. Early and accurate diagnosis can decrease morbidity and mortality, and various imaging modalities play a key role in recognition of pancreatic injuries<sup>[4,5]</sup>.

Knowledge about the mechanisms of pancreatic injury, the presence of coexisting injuries, the time to diagnosis, the presence or absence of major ductal injury, and the roles of various imaging modalities is essential for prompt, early and accurate diagnosis. Early detection of disruption of the main pancreatic duct is of paramount importance because such disruption is the main cause of delayed complications like pseudopancreatic cyst<sup>[6]</sup>. The most common site of traumatic pancreatic injury is at the junction of the body and tail. Significant pancreatic injury may occur in the absence of abnormality on various imaging modalities.

Pancreatic trauma occurs commonly in connection with multiple injuries after motor vehicle accidents in adults and bicycle handlebar injuries in children<sup>[7]</sup>. Conservative management is mainly advocated for pancreatic trauma without ductal injuries. Computed tomography (CT) is routinely used as the first-line imaging modality in acute abdominal trauma cases and is helpful in recognizing injuries to the pancreas and other organs and their associated complications<sup>[8]</sup>. Ultrasonography (US) is useful in cases of pancreatic ascites and pseudocyst formation, which are more likely to occur in cases with traumatic pancreatitis<sup>[3,9]</sup>. Magnetic resonance cholangiopancreatography (MRCP) allows direct imaging of the pancreatic duct and its disruption<sup>[10]</sup>. The purpose of this paper is to review the findings of pancreatic trauma on various imaging modalities.

## ANATOMIC CONSIDERATIONS

The pancreas is a long J-shaped, soft, lobulated retroperitoneal organ. It is situated transversely across the posterior abdominal wall, at the back of the epigastric and left hypochondriac regions at level of lumbar (L1-2) spine (Figure 1). In adults, the pancreas is about 15-20 cm long, 1.0-1.5 cm thick and weighs approximately 90-100 g<sup>[11]</sup>. The main pancreatic duct of Wirsung traverses the entire length of the gland. The superior pancreaticoduodenal artery from the gastroduodenal artery and the inferior pancreaticoduodenal artery from the superior mesenteric artery run in the concave contour of the second part of the duodenum to supply the head of the pancreas. The pancreatic branches of the splenic artery supply the neck, body and tail of the pancreas. The body and neck of the pancreas drain into the splenic vein, whereas the head drains into the superior mesenteric and portal veins. The lymphatic drainage of the pancreas is *via* the splenic, celiac and superior mesenteric lymph nodes. The proximity of many larger vessels such as the inferior vena cava (IVC), portal vein and abdominal aorta makes injuries to the pancreas difficult to manage because of the risk of

exsanguinating hemorrhage, which is a frequent cause of death in patients with a pancreatic injury. The splenic artery and splenic vein run superior and posterior to the body and tail of the pancreas and are relatively easier to expose and control compared to the IVC and portal vein. The vascular anatomy causes problems in repairing the injuries to the head of the pancreas whereas injuries to the body and tail are easier to manage<sup>[11,12]</sup>.

## PATHOPHYSIOLOGY OF INJURY

Injuries to the pancreas most commonly result from penetrating trauma caused by gunshot or stab wounds and occur in approximately 20%-30% of all patients with penetrating traumas. The penetrating injury caused by firearms results in the highest frequency of pancreatic trauma. The relatively protected retroperitoneal location of the pancreas protects it from most instances of blunt abdominal trauma. Blunt trauma to the pancreas is, in most instances, caused by a sudden localized force to the upper abdomen that compresses the pancreas against the vertebral column (*e.g.*, steering wheel injury in a motor vehicle accident in adults and from bicycle handlebar injury or direct blow from a kick or fall in children)<sup>[8]</sup>. Blunt pancreatic injury is more common in children and young adults because they have a thinner or absent mantle of protective fat, which surrounds the pancreas in older adults<sup>[10]</sup>. In order of frequency, injuries to the pancreas involve the body, head and tail. Pancreatic injury is rarely a solitary injury, and in the majority of instances there is at least one coexistent injury; 60% are duodenopancreatic lesions, while 90% involve at least one other abdominal organ<sup>[1]</sup>. Therefore, multiple organ injuries are a red flag suggesting the possibility of coexistent pancreatic injury.

## CLINICAL PRESENTATIONS

Patients with pancreatic trauma present usually with features of acute pancreatitis. The typical clinical triad of pancreatic trauma is upper abdominal pain, leukocytosis, and elevated serum amylase level, that may, however, be absent in adults during the first 24 h and even for several days<sup>[12,13]</sup>. Pancreatic trauma is difficult to recognize because of coexisting injuries to other intra-abdominal organs and its retroperitoneal location, which makes signs and symptoms less marked, and consequently this trauma ends up causing higher morbidity and mortality rates than observed in injuries to other intra-abdominal organs<sup>[14,15]</sup>. Symptoms of injury to other intra-abdominal organs or structures commonly mask or supersede that of pancreatic injury, both early and late in the course of trauma. Therefore, a high degree of suspicion is required to ensure that pancreatic injuries are not overlooked or missed either early or late in their course.

## LABORATORY FINDINGS

Raised amylase in serum or diagnostic peritoneal lavage (DPL) fluid can be useful in diagnosis, but there is

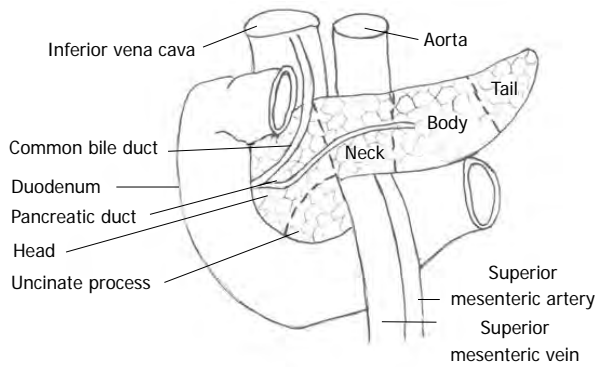


Figure 1 Gross anatomy of the pancreas.

poor correlation between raised amylase and pancreatic trauma because amylase may be elevated in injuries of the salivary gland, in duodenal trauma, hepatic trauma, and injuries to the head and face, and in an intoxicated patient<sup>[16-18]</sup>. A raised amylase level after blunt pancreatic trauma is time dependent, and a persistently elevated or a rising amylase level is a more reliable indicator of pancreatic trauma, but it does not indicate the severity of the injury<sup>[14]</sup>. Amylase detected in DPL fluid is a much more sensitive and specific indicator of pancreatic injury than blood or serum amylase estimations. Serum lipase activity is also not specific for pancreatic injury<sup>[12]</sup>.

## RADIOLOGIC STUDIES

Diagnostic imaging plays an important role in the recognition, evaluation, and follow-up of traumatic pancreatic injuries. The imaging findings in patients with pancreatic trauma are nonspecific and often indistinguishable from those of inflammatory pancreatitis.

### Conventional radiography

A plain X-ray of the abdomen in patients with pancreatic trauma is nonspecific and none of the radiologic abnormalities on plain films can be used for specific diagnostic purposes. Conventional radiography can be valuable in detecting penetrating trauma by visualizing and localizing foreign bodies such as bullet fragments and projectile-induced bony injury, as well as pulmonary parenchymal injury, gastric dilatation and pneumoperitoneum.

Findings are often indistinguishable from those of inflammatory pancreatitis. Pancreatic hemorrhage and edema widen the duodenal sweep with distension of the duodenum. Dissection along the transverse mesocolon results in gaseous distension of the colon, which may terminate abruptly usually at the splenic flexure to produce the “colon-cutoff sign”. A sentinel loop representing localized ileus may be seen in the mid-abdomen.

### US

Although US is easy to perform, portable and cost-effective, pancreatic injuries are difficult to diagnose in spite of technically adequate sonograms<sup>[19]</sup>. However, it is



Figure 2 Ultrasound image. Axial ultrasound image shows localized traumatic enlargement of the pancreas with diffuse edema. Transection of distal body of pancreas communicating with large fluid collection anterior to pancreas (white arrow).

reliable in the follow-up of complications such as pseudocysts. Real-time contrast-enhanced US is an effective technique in emergency imaging, but its role should not be considered as a replacement for CT<sup>[20]</sup>.

US may show localized traumatic enlargement of the pancreas or diffuse edema simulating inflammatory pancreatitis. In trauma patients, peripancreatic fluids may be a sign of pancreatic contusion<sup>[21]</sup>. A traumatic pseudocyst of the pancreas may be detected by US and monitored on serial examinations. Since complications of trauma are most likely to occur from rupture or stenosis of the main pancreatic duct, it is important to try to delineate this structure in all cases of pancreatic injury. Transection throughout the pancreas parenchyma is suggestive of ductal injury (Figure 2).

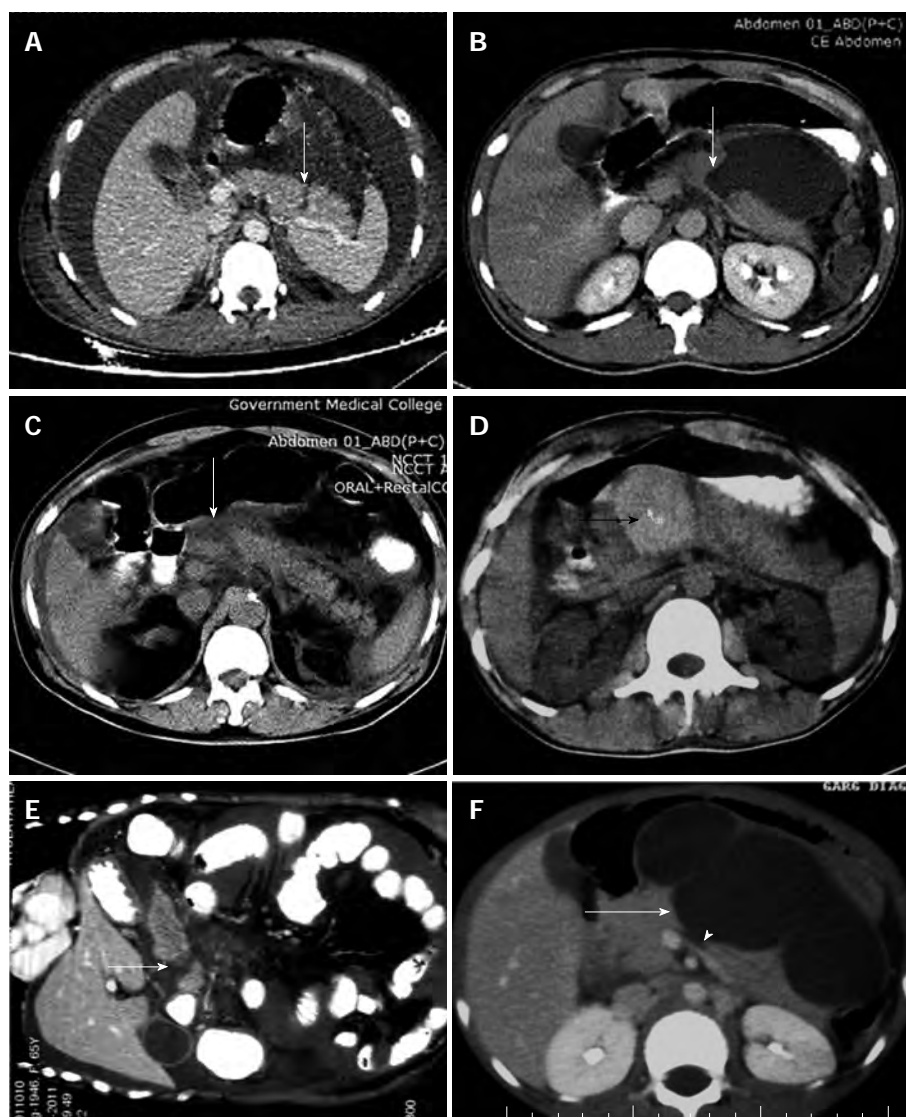
### CT

CT is the simplest and least invasive diagnostic modality currently available for evaluating suspected pancreatic trauma and its complications, because of the subtlety of the US findings. However, this study is only rarely useful in acute penetrating injury. Computed tomography is the radiographic examination of choice for hemodynamically stable patients with abdominal trauma as it provides the safest and most comprehensive means of diagnosis of traumatic pancreatic injury<sup>[10]</sup>.

The pancreas may appear normal in 20%-40% of patients when CT is performed within 12 h after trauma because pancreatic injuries may produce little change in the density which may not be detectable on CT scan<sup>[1,22]</sup>. In addition, there may be minimal separation of lacerated pancreatic fragments (Figure 3A). Currently, multidetector-row CT scanners are used for evaluation of abdominal trauma cases as they are faster to scan, which greatly reduces bowel artifacts and resolves many previous technical problems<sup>[8]</sup>. Lacerations tend to occur at the junction of the body and tail due to shearing injuries with compression against the spine (Figure 3A).

Direct signs of pancreatic injury include laceration, transection, focal pancreatic enlargement and inhomogeneous





**Figure 3** Computed tomography images.

A, B: Axial contrast-enhanced computed tomography shows a heterogeneous appearance of the body and tail of pancreas with a linear laceration (white arrow) across the distal body of the pancreas. There is also fluid in the lesser sac, perihepatic space, perisplenic space and hemoperitoneum. There is free air into chest wall muscles on right side in a case of blunt pancreatic trauma (A), and transection throughout extent of pancreatic parenchyma in proximal body region (suggestive of ductal injury) with a large fluid collection (white arrow) anterior to pancreas communication with the transection in another case of blunt injury to upper abdomen (B); C: Contrast-enhanced computed tomography demonstrating mild diffuse hypodensity of the body of pancreas. Contusions of the head and neck also demonstrated (white arrow) with secondary signs of traumatic pancreatitis, *i.e.*, increased density of the peripancreatic fat, thickening of left anterior pararenal fascia, fluid in the lesser sac and hemoperitoneum; D: Plain axial computed tomography section at the level of pancreas shows a large hyperdense hematoma (black arrow) in proximal body of pancreas suggestive of pancreatic injury. E: Multiplanar reconstruction image of contrast-enhanced computed tomography demonstrating a pancreatic fracture (white arrow) in neck region with separation of pancreatic fragments; F: Contrast-enhanced axial computed tomography scan in a child with bicycle handlebar injury more than a month old shows a large lobulated pseudocyst anterior to pancreas communicating with pancreatic laceration in the neck of pancreas representing ductal injury. There is fluid between posterior pancreas and the splenic vein (arrow heads).

geneous enhancement. Fluid collections like hematoma and pseudocyst are usually seen communicating with the pancreas at the site of laceration or transection (Figure 3B). Secondary signs include peripancreatic fat stranding, peripancreatic fluid collections, fluid between the splenic vein and pancreas, hemorrhage, thickening of the left anterior pararenal fascia and associated injuries to adjacent structures<sup>[10]</sup> (Figure 3C, Table 1).

Contusion appears as focal or diffuse low attenuation areas and laceration is seen as a linear hypodense line perpendicular to the long axis of the pancreas<sup>[6,23,24]</sup>. Pancreatic fracture on CT is diagnosed if there is a clear separation of fragments across the long axis of the pancreas<sup>[25]</sup>. Intrapaneatic hematoma is a very specific sign of pancreatic injury<sup>[26]</sup> (Figure 3D). Fluid between the splenic vein and pancreas is a very non-specific sign but it may suggest pancreatic injury if associated with history of blunt abdominal trauma<sup>[27]</sup>. Pseudocysts are more likely to occur in patients with traumatic pancreatitis<sup>[28]</sup>. The risk of abscess or fistula formation in patients with disruption of the pancreatic duct approaches 25% and 50%, respectively, in comparison with 10% without duct

injuries<sup>[7]</sup>. So it is important that imaging focuses on the integrity of the duct or findings that suggest damage to the pancreatic duct. The accuracy of detecting a major ductal injury by CT has been reported to be as low as 43%<sup>[10,17,29-31]</sup>.

Computed tomography may not always directly demonstrate the ductal disruption; injury to the duct can be suggested based on the degree of parenchymal injury and can only be inferred following visualization of a through and through laceration of the pancreas (Figure 3E). A computed tomography grading scheme has been devised (Table 2), which parallels the surgical classification of Moore<sup>[10,32]</sup>. Grade A injuries with laceration involving < 50% pancreas are usually seen with an intact pancreatic duct by surgical grading, whereas grade B and C injuries correlate with duct disruption, especially when CT shows deep lacerations or pancreatic transection<sup>[32]</sup>. Overestimation on CT can occur in grade C I and C II injuries if merely deep lacerations or “single scan” transections are identified at the pancreatic head. However, urgent endoscopic retrograde cholangiopancreatography (ERCP) may be quite valuable in such patients with strong clinical

**Table 1** Computed tomographic signs of pancreatic injury

Specific signs	Fracture of the pancreas
	Pancreatic laceration
	Focal or diffuse pancreatic enlargement/edema
	Pancreatic hematoma
	Active bleeding/extravasation of intravenous contrast
	Fluid separating the splenic vein from posterior aspect of pancreas
Non-specific signs	Inflammatory changes in peripancreatic fat and mesentery
	Fluid surrounding the superior mesenteric artery
	Thickening of the left anterior renal fascia
	Pancreatic ductal dilatation
	Acute pseudocyst formation/peripancreatic fluid collection
	Fluid in the anterior and posterior pararenal spaces
	Fluid in transverse mesocolon and lesser sac
	Hemorrhage into peripancreatic fat, mesocolon and mesentery
	Extraperitoneal fluid
	Intraperitoneal fluid

**Table 2** Computed tomographic grading of blunt pancreatic injuries

CT grading	CT findings of blunt pancreatic injury
Grade A	Pancreatitis and/or superficial lacerations at any site
Grade B	
B I	Deep laceration at distal pancreas
B II	Transections at distal pancreas
Grade C	
C I	Deep lacerations at proximal pancreas
C II	Transections at proximal pancreas

Reproduced from Wong *et al*<sup>[32]</sup>. CT: Computed tomography.

evidence of pancreatic injury and an equivocal CT scan, to establish the final diagnosis<sup>[10,32]</sup>. A patient with a post-traumatic pseudocyst should be considered to have a ductal leak until proven otherwise<sup>[1]</sup> (Figure 3F).

### MRCP

Since the outcome of pancreatic trauma patients largely depends upon the integrity of the pancreatic duct, evaluation of the duct is essential. In the past, ERCP was the only method available for evaluating pancreatic duct integrity. More recently, MRCP has emerged as an attractive alternative non-invasive diagnostic tool for direct imaging of the pancreatic duct and it is being used more frequently to assess injury to the ductal components<sup>[33]</sup>. Dynamic secretin-stimulated (DSS) MRCP is a variation on standard MRCP and may compete with ERCP in diagnostic accuracy. Like ERCP, DSS MRCP provides dynamic information as to whether there is continuing leakage from an injured main pancreatic duct. The advantages of DSS MRCP include it being noninvasive, faster and more readily available than ERCP, and it can illustrate the entire pancreatic parenchymal and ductal anatomy, as well as pathologic fluid collections and ductal disruptions<sup>[34]</sup>. The main pancreatic duct (MPD) can be identified by MRCP within the pancreatic head

**Table 3** Classification of pancreatic injuries by endoscopic retrograde cholangiopancreatography

Grade	Description
I	Normal main pancreatic duct on ERCP
II a	Injury to branches of main pancreatic duct on ERCP with contrast extravasation inside the parenchyma
II b	Injury to branches of main pancreatic duct on ERCP with contrast extravasation into the retroperitoneal space
III a	Injury to the main pancreatic duct on ERCP at the body or tail of the pancreas
III b	Injury to the main pancreatic duct on ERCP at the head the pancreas

Reproduced from Takishima *et al*<sup>[38]</sup>. ERCP: Endoscopic retrograde cholangiopancreatography.

in up to 97% of cases and within the pancreatic tail in up to 83%<sup>[35]</sup>. In addition, MRCP may demonstrate abnormalities not visible at ERCP, such as fluid collections upstream of the site of duct transection (Figure 4A), and is helpful in assessing parenchymal injury<sup>[36]</sup>. For assessing the parenchyma, fat-suppressed T1- and T2-weighted sequences are performed. Magnetic resonance pancreatograms are acquired by using heavily T2-weighted breath-hold or non-breath-hold sequences. Fast spin-echo (two-dimensional or three-dimensional) and rapid acquisition with relaxation enhancement sequences performed in the coronal and axial planes are usually sufficient<sup>[10]</sup>.

### ERCP

ERCP is increasingly being used to help in both early and in delayed diagnosis of pancreatic ductal injuries in patients with strong clinical evidence of pancreatic injury and an equivocal CT scan. Endoscopic retrograde cholangiopancreatography is the most accurate investigation for diagnosing the site and extent of ductal injury by demonstrating extravasation or a cutoff, especially in patients with delayed presentations<sup>[37]</sup>. It can be performed preoperatively, intraoperatively or postoperatively in patients with pancreatic injury. Although ERCP is the most useful procedure for the diagnosis of pancreatic ductal injury in stable patients, surgery should be considered in hemodynamically unstable patients. A classification of pancreatic injuries (Table 3) has been devised according to the findings on ERCP<sup>[38]</sup>. Although MRCP (Figure 4B) has become the noninvasive imaging method of choice when evaluating for pancreatic duct injury, ERCP remains important because of its potential to direct image-guided therapy (Figure 5). Endoscopic retrograde cholangiopancreatography in selected patients allows non-operative treatment in the absence of ductal injury and earlier operative treatment or primary therapy as stent placement in the presence of ductal injury<sup>[39]</sup>. It also aids the treatment of late complications of pancreatic duct injuries such as pseudocysts and pancreatic fistulae. Both endoscopic transpapillary and transmural drainage are effective options for managing delayed local complications of pancreatic trauma. The endoscopist must be skilled

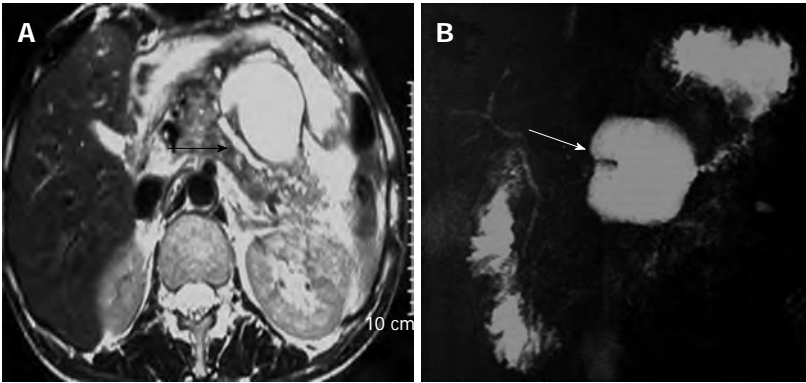


Figure 4 Magnetic resonance images. T2 weighted axial image (A) and magnetic resonance cholangiopancreatography (B) in a case of traumatic pancreatitis show heterogenous signal intensity of pancreas with peripancreatic stranding. Main pancreatic duct is dilated in the body and tail region (black arrow). A lobulated pseudopancreatic cyst is seen in lesser sac anterior aspect of body of pancreas (white arrow) demonstrated in magnetic resonance cholangiopancreatography.



Figure 5 Endoscopic retrograde cholangiopancreatography image. Another case of traumatic pancreatitis. Fluoroscopic image showing main pancreatic duct disruptions during endoscopic retrograde cholangiopancreatography with multiple contrast filled outpouching is seen, suggestive of pseudocysts (white arrow). Multiple contrast filled tracts are also visualized (black arrowhead). Few tracts are seen in retroperitoneum and one of the tracts is reaching into mediastinum (black arrow). Endoscope is visible.

and experienced in its use as this procedure has potential complications that can limit its usefulness in patients with pancreatic trauma.

### COMPLICATIONS OF PANCREATIC TRAUMA

Early diagnosis and treatment are associated with better overall outcomes in traumatic pancreatic injury patients. Mortality associated with pancreatic injuries approximates 20% and results primarily from hemorrhage caused by injuries to other intra-abdominal organs and from sepsis<sup>[40,41]</sup>. There is an increase in infectious complications in patients who have pancreatic wounds co-associated with injury to small and large intestine. Blunt pancreatic injuries without ductal leak usually resolve with mere conservative management. On the other hand, damage to the ductal system, if inadequately treated or untreated, can result in prolonged morbidity. Complications of traumatic pancreatic injury are manifold and range from minor pancreatitis to death<sup>[40,42]</sup>. Fistula formation is the most frequently observed complication. Traumatic pancreatitis, pseudocyst formation, abscesses and duct stricture are

Table 4 American Association for the surgery of trauma classification of pancreatic trauma

Grade	Injury	Description
I	Hematoma	Minor contusion without ductal injury
	Laceration	Superficial laceration without ductal injury
II	Hematoma	Major contusion without ductal injury or tissue loss
	Laceration	Major laceration without ductal injury or tissue loss
III	Laceration	Distal transection or pancreatic parenchymal injury with ductal injury
IV	Laceration	Proximal transection or pancreatic parenchymal injury involving the ampulla
V	Laceration	Massive disruption of the pancreatic head

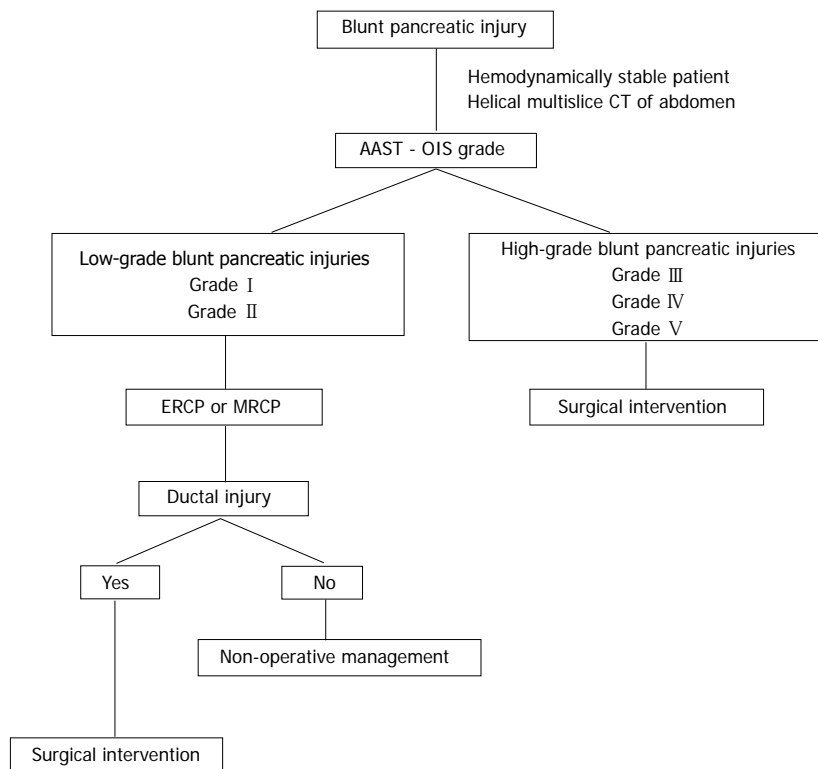
Reproduced from Campbell *et al*<sup>[42]</sup>.

common complications. Other less frequent complications include peritonitis, intestinal obstruction, gastro-intestinal bleeding, endocrine or exocrine insufficiency, splenic artery pseudoaneurysm formation or rupture and splenic vein thrombosis<sup>[6,24]</sup>.

### CLASSIFICATION AND GRADING OF PANCREATIC INJURIES

Pancreatic injuries are classified and graded according to the damage to the pancreatic parenchyma and the ductal system. Grading of pancreatic injuries enables an exact description of injuries, can influence management, and allows a comparison of outcomes and effective quality control of treatment<sup>[12]</sup>. There are several classification systems of traumatic pancreatic injuries<sup>[32,38]</sup> (Tables 2 and 3) but the pancreatic organ injury scale (OIS) proposed by the American Association for the Surgery of Trauma (AAST) fulfills most of these criteria and at present is the universally accepted classification scheme<sup>[43]</sup>. This OIS scale involves five grades, which concedes the significance of more complex injuries to the pancreas, and particularly those injuries affecting the pancreatic duct and the pancreatic head (Table 4). This classification scheme can also be correlated with other organ injury scales, as well as integrated into more complex scoring systems, such as injury severity score or trauma score - injury severity score from which probability of survival of an individual case is determined.





**Figure 6** Management algorithm for traumatic pancreatic injury patients. Reproduced from Ilahi *et al.*<sup>[44]</sup>. ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

## MANAGEMENT OF PANCREATIC INJURIES

Many patients with pancreatic injuries have multiple associated injuries including vascular and other intra-abdominal organs injury; priority must be given to stabilizing the patient before any definitive management of the pancreatic injury. The initial priorities include control of hemorrhage and spillage of intestinal contents. The decision regarding therapeutic approach of the traumatic pancreatic injury, either with a conservative approach or a surgical approach, depends upon the integrity of the MPD, extent of pancreatic parenchymal damage, anatomical location of the injury, stability of the patient and degree of associated organ damage (Figure 6)<sup>[44]</sup>. In patients with an isolated pancreatic contusion or superficial lacerations without ductal disruption, conservative management may be warranted. Treatment of traumatic pancreatitis consists of bowel rest, nasogastric suction, and nutritional support<sup>[29]</sup>. ERCP-guided stent placement to the MPD injury has been indicated in select cases<sup>[45]</sup>. Endoscopic transpapillary drainage has been successfully used to heal duct disruptions in the early phase of pancreatic trauma and in the delayed phase to treat the complications of pancreatic duct injuries. However, in patients with major ductal injury in blunt pancreatic trauma cases, morbidity and mortality greatly increase unless surgery is undertaken within the first 24 h. By using the pancreatic OIS grading system of the AAST to help to guide the appropriate surgical management, the morbidity and mortality in blunt pancreatic injury are decreased<sup>[46]</sup>. Grades I and II are treated with non-operative management techniques or simple drainage, whereas

grade III or higher injuries often require resection with possible reconstruction and/or drainage procedures<sup>[47]</sup>. There are a number of alternative procedures that can be used for the management of high-grade blunt pancreatic injury, such as duodenal diversion, pyloric exclusion, the Whipple procedure or simple drainage, with the choice dependent on the patient's hemodynamic status and the presence or absence of associated duodenal injury<sup>[48,49]</sup>. Sometimes, the decision to perform a pancreaticoduodenectomy is unavoidable in select cases. If the patient is hemodynamically unstable, pancreaticoduodenectomy should be performed as a two-step procedure. After the initial damage control surgery, anastomoses are completed at a second surgery when the patient is stable<sup>[50]</sup>.

The standard of care in penetrating injuries is a surgical approach depending upon the location of the injury and associated abdominal injuries. Damage control surgery in hemodynamically unstable patients with massive injury to the pancreas and associated intra-abdominal organs reduces morbidity and mortality.

## CONCLUSION

Pancreatic injury is uncommon and usually difficult to diagnose. Because of the subtlety of the ultrasound findings, computed tomography is the preferred method for evaluating suspected pancreatic trauma; however, pancreatic duct injury may not be detected on computed tomography scan except when there is through and through laceration. In select situations, including minor injuries, a conservative approach may be successful. With modern imaging modalities and expertise in endoscopic retrograde cholangiopancreatography, isolated pancreatic



duct injury can be successfully managed. A surgical approach is appropriate with major pancreatic injury that necessitates urgent surgical intervention.

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## Clinically detected gastroenteropancreatic neuroendocrine tumors are on the rise: Epidemiological changes in Germany

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a particularly substantial database the epidemiological data from the federal states of Mecklenburg-Western Pomerania, Saxony, Brandenburg and Thuringia, covering a population of more than 10.8 million people, were analyzed. Survival probabilities were calculated using life table analysis. In addition, GEP-NET patients were evaluated for one or more second (non-GEP-NET) primary malignancies.

**RESULTS:** A total of 2821 GEP neuroendocrine neoplasms were identified in the two registries. The overall incidence increased significantly between 1976 and 2006 from 0.31 (per 100.000 inhabitants per year) to 2.27 for men and from 0.57 to 2.38 for women. In the later period studied (2004-2006), the small intestine was the most common site. Neuroendocrine (NE) neoplasms of the small intestine showed the largest absolute increase in incidence, while rectal NE neoplasms exhibited the greatest relative increase. Only the incidence of appendiceal NET in women showed little change between 1976 and 2006. Overall survival of patients varied for sex, tumor site and the two periods studied but improved significantly over time. Interestingly, about 20% of the GEP-NET patients developed one or more second malignancies. Their most common location was the gastrointestinal tract. GEP-NET patients without second malignancies fared better than those with one or more of them.

**CONCLUSION:** The number of detected GEP-NET increased about 5-fold in Germany between 1976 and 2006. At the same time, their anatomic distribution changed, and the survival of GEP-NET patients improved significantly. Second malignancies are common and influence the overall survival of GEP-NET patients. Thus, GEP-NET warrant our attention as well as intensive research on their tumorigenesis.

### Abstract

**AIM:** To study the epidemiologic changes of gastroenteropancreatic neuroendocrine tumors (GEP-NET) in Germany, we analyzed two time periods 1976-1988 and 1998-2006.

**METHODS:** We evaluated epidemiological data of GEP-NET from the former East German National Cancer Registry (DDR Krebsregister, 1976-1988) and its successor, the Joint Cancer Registry (GKR, 1998-2006), which was founded after German reunification. Due to

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**Key words:** Neuroendocrine; Tumor; Epidemiology; Gastrinoma; Insulinoma; Endoscopy; German history; Reunification; Second malignancy

**Core tip:** Modern endoscopic and radiological tumor imaging have been implicated in the rise of the incidence of detected neuroendocrine tumors (NET) in Western countries. The particularities of German history, which resulted in two German states with two different health care systems from 1949-1989, allowed to study the epidemiological changes of NET in Germany on the background of two health care systems in 1949-1989. The number of detected gastroenteropancreatic-NET increased about 5-fold between 1976 and 2006. Most likely, the general availability of endoscopy after German reunification contributed to the major rise in frequency of detected rectal, gastric and duodenal NET in the new federal states of reunified Germany.

Scherübl H, Streller B, Stabenow R, Herbst H, Höpfner M, Schwertner C, Steinberg J, Eick J, Ring W, Tiwari K, Zappe SM. Clinically detected gastroenteropancreatic neuroendocrine tumors are on the rise: Epidemiological changes in Germany. *World J Gastroenterol* 2013; 19(47): 9012-9019 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9012.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9012>

## INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (GEP-NET) are infrequent, constituting only 1%-2% of all neoplasms. Most commonly they present as indolent, slow-growing tumors<sup>[1-4]</sup>. Their anatomic distribution reflects that of the neuroendocrine cells from which they derive: up to 65% in the gastrointestinal tract, about 25% in the bronchopulmonary system, and the remaining 10% at other sites<sup>[1,5]</sup>.

In Western countries, pancreatic neuroendocrine tumors are diagnosed in 0.5-1 per 100000 inhabitants and represent 1%-2% of all clinically manifest pancreatic neoplasms<sup>[6-8]</sup>. GEP-NET occur in approximately 2.0-2.5 per 100000, carcinoid syndrome being most frequently associated with NET of the jejunum and ileum<sup>[9-13]</sup>.

Previous and current WHO classifications distinguish between well-differentiated and poorly differentiated neoplasms. All well-differentiated neoplasms, whether benign or metastatic, are now called neuroendocrine (NE) and are graded as G1 (Ki-67  $\leq$  2%) or G2 (Ki-67: 3%-20%). The poorly differentiated neoplasms are called neuroendocrine carcinomas and are graded as G3 (Ki-67 > 20%). The term "carcinoid" is now synonymous with G1 well-differentiated neuroendocrine tumor.

Population-based data from the Third National Cancer Survey and the United States Surveillance Epidemiology and End Results (SEER) Program, covering 10%-14% of the United States population, show a steady increase in the incidence of NET throughout the 35-year

period between 1969 and 2004<sup>[14-17]</sup>. The overall incidence of GEP-NET increased two- to three-fold during this period, and there were significant changes in the anatomic distribution. Thus, the proportion of GEP-NET located in the appendix decreased from 43% to 4% with corresponding increases in the stomach (from 2% to 9%), small intestine (from 31% to 42%), and rectum (from 15% to 27%)<sup>[8,16]</sup>. The observed changes may in part reflect the increased number of asymptomatic GEP-NET incidentally identified thanks to increased availability of modern endoscopic and radiological imaging<sup>[3,8,18]</sup>.

There have been few studies on GEP-NET epidemiology in Germany<sup>[17]</sup>, and no comprehensive and comparative epidemiological studies have as yet been published on GEP-NET at various locations.

Therefore we evaluated epidemiological data from the former East German National Cancer Registry (DDR Krebsregister) for 1976-1988 and from its successor, the Joint Cancer Registry (GKR) for 1998-2006. After German reunification, the East German registry was renamed and thus became the GKR of the new federal states of Germany and Berlin. After an interruption of several years, it continued to collect epidemiological data. Thanks to a particularly substantial database we analyzed epidemiological data from the federal states of Mecklenburg-Western Pomerania, Saxony, Brandenburg and Thuringia, covering a population of over 10.8 million people.

## MATERIALS AND METHODS

The official population statistics for Germany as reported by the German government were used to estimate the incidence rate of GEP-NET. Absolute numbers were used to estimate the crude incidence rate both in former East Germany and - after German reunification - in the new federal states, including Berlin. Since other federal states, including Berlin, had a less extensive database, their data were not used in the analysis presented here.

The study included all persons (living in Mecklenburg-Western Pomerania, Saxony, Brandenburg or Thuringia) diagnosed with GEP-NET between 1976 and 1988 or between 1998 and 2006. Mortality data from 1976 to 1990 and from 1998 to 2009 were used. We included all patients with NE tumors at the following tumor sites according to ICD10: C15-C25, D37.1-D37.5 (esophagus, stomach, small intestine, large intestine, appendix, rectosigmoid, rectum, anus and pancreas), C26.0, C26.8-9, D37.7, D37.9 (unspecified location in the digestive tract) and an NE morphology code according to ICD-O-3: 8150-8153, 8155-8157, 8240-8246, 8249 or 8574.

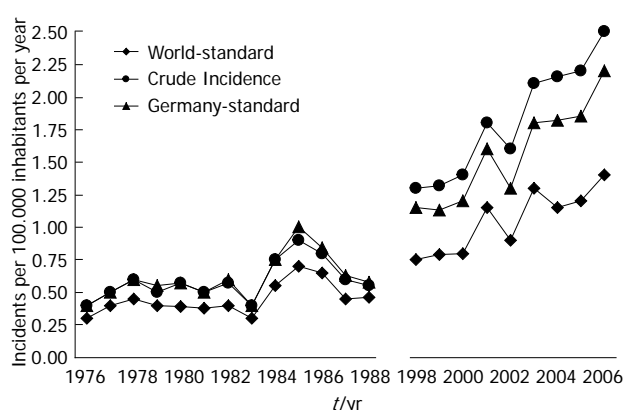
Annual incidence rates were calculated for the periods 1976-1988 and 1998-2006. Incidence rates are presented for each tumor site, sex and age group. Trends in incidence are presented as overall change throughout the two periods and between two representative 2-year periods, *i.e.*, 1976-1978 and 2004-2006. The absolute number of GEP-NET reported per 100.000 inhabitants per year is



**Table 1** Incidence of gastroenteropancreatic neuroendocrine tumors according to anatomic site, sex, and the time periods 1976-1988 and 1998-2006

Period	Stomach (M/F) <sup>1</sup>	Small intestine (M/F) <sup>1</sup>	Pancreas (M/F) <sup>1</sup>	Appendix (M/F) <sup>1</sup>	Colon (M/F) <sup>1</sup>	Rectum (M/F) <sup>1</sup>
1976-1978	0.02/0.00	0.11/0.15	0.03/0.08	0.20/0.35	0.04/0.05	0.01/0.01
1979-1981	0.04/0.03	0.10/0.11	0.07/0.014	0.15/0.34	0.04/0.07	0.01/0.03
1982-1984	0.04/0.04	0.16/0.12	0.11/0.08	0.15/0.35	0.04/0.07	0.03/0.02
1985-1988	0.05/0.04	0.20/0.18	0.10/0.10	0.24/0.35	0.08/0.15	0.03/0.07
1989-1997						
1998-2000	0.16/0.12	0.39/0.31	0.22/0.18	0.13/0.28	0.19/0.19	0.09/0.10
2001-2003	0.18/0.18	0.44/0.40	0.25/0.25	0.23/0.46	0.22/0.23	0.16/0.15
2004-2006	0.27/0.23	0.51/0.52	0.25/0.25	0.31/0.39	0.20/0.28	0.26/0.24

<sup>1</sup>Male/female (M/F) patients. Incidence rates were age-adjusted to the German standard population of 1987. The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for the years 1976-1988 or from its successor, the Joint Cancer Registry for the time period 1998-2006. No valid data are available for the period between 1989 and 1997.



**Figure 1** The incidence of gastroenteropancreatic neuroendocrine tumors is shown over time (1976-1988 and 1998-2006). It is presented either as crude incidence or as the number of tumors (per 100.000) age-adjusted to the 1966 world standard population (World standard) or to the 1987 German standard population (German standard). The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for 1976-1988 or from its successor, the Joint Cancer Registry, for 1998-2006.

referred to as the “crude incidence rate”.

Age-adjusted incidence rates were calculated using the World Standard Population published in 1966<sup>[5]</sup> and the 1987 German standard population.

Survival probabilities were calculated using life table analysis. Since data were available only on the time but not the cause of death, only the overall survival of the registered GEP-NET patients could be determined. Tumor-specific survival could not be analyzed. The Wilcoxon-Gehan test was used to determine significance when comparing the survival rates.

In addition, all GEP-NET patients were evaluated for one or more second primary malignancies. Second (non-GEP-NET) neoplasms were analyzed for location of the second primary.

## RESULTS

### Frequency

From 1976 to 1988 and from 1998 to 2006, a total of 2821 cases of GEP-NET were registered in Mecklen-

burg-Western Pomerania, Saxony, Brandenburg and Thuringia - 1001 cases from 1976 to 1988 and 1820 cases from 1998 to 2006. The total patient population comprised 1291 men (45.8%) and 1530 women (54.2%).

In 2006, a total of 10837539 persons lived in Mecklenburg-Western Pomerania, Saxony, Brandenburg and Thuringia - 5329539 men (49.2%) and 5508000 women (50.8%).

### Incidence rates

The crude incidence rate of GEP-NET (per year and 100.000 population) rose from 0.45 in 1976 to 2.53 in 2006, which corresponds to a 462% increase. The incidence rate increased by 342% when age-adjusted to the 1966 world population and by 270% when adjusted to the 1987 population of Germany (for details, Figure 1).

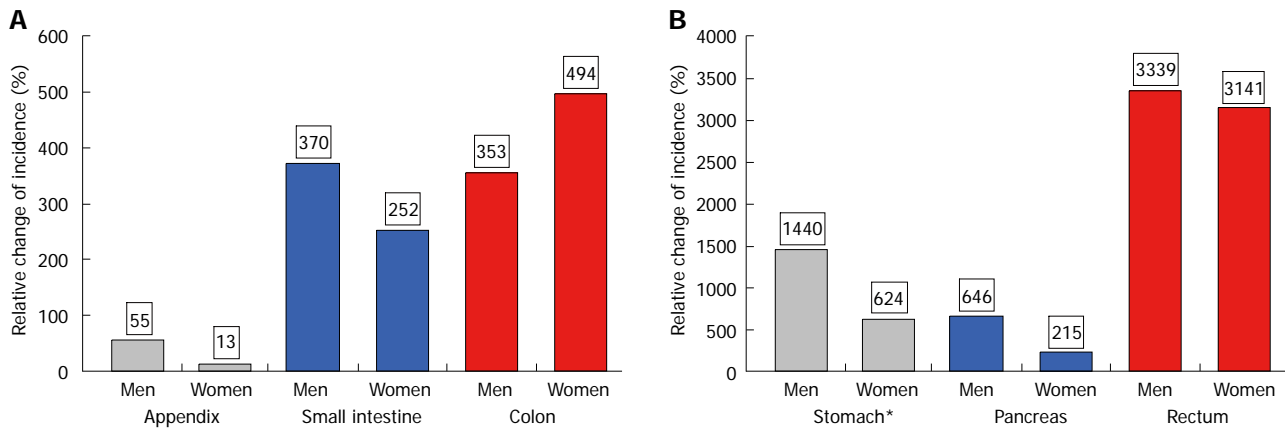
The crude incidence rate increased more prominently in men (from 0.31 in 1976 to 2.7 in 2006) than in women (from 0.57 in 1976 to 2.38 in 2006).

### Tumor site

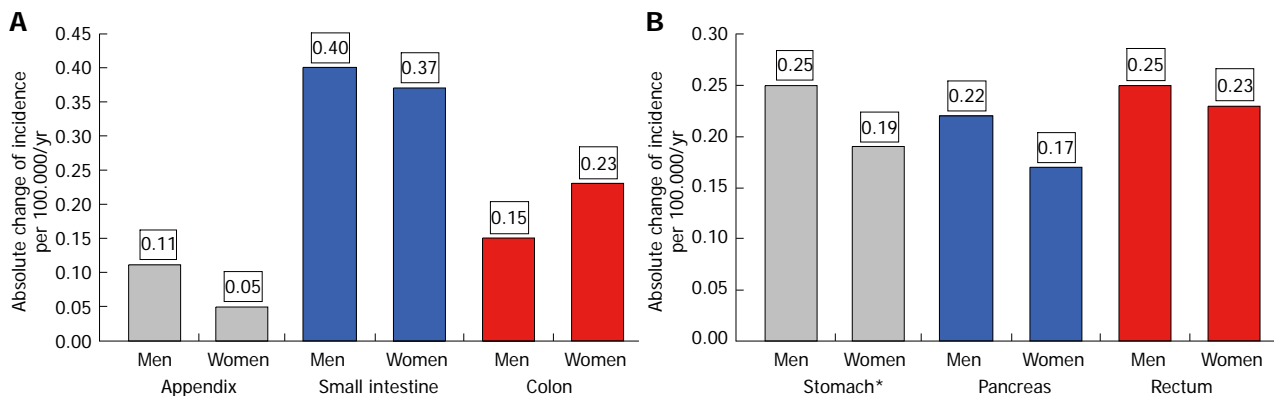
The age-adjusted (population of Germany in 1987) incidence is given in Table 1 for the periods 1976-1988 and 1998-2006. Both age-adjusted and crude incidence rates increased at all tumor sites. When comparing representative time intervals such as 1976-1978 and 2004-2006, the largest increment in absolute numbers was found for the small intestine; the absolute increase in crude incidence was 0.56 in men and 0.48 in women. Focusing on relative changes revealed the largest increase for rectal NE neoplasms; the crude incidence rose by about 6000% in men and by more than 2700% in women. In contrast, hardly any change was observed for appendiceal NE neoplasms in the female population between 1976 and 2006 (see Figures 2 and 3).

### Survival

Overall survival increased significantly between the time periods 1976-1988 and 1998-2006 ( $P < 0.001$ ). The 1-, 5- and 10-year overall survival rates were 59%, 50% and 47% for the earlier period and 79%, 63% and 50% for the later period (Figure 4).



**Figure 2** Relative changes in the incidence of gastroenteropancreatic neuroendocrine tumor are depicted according to tumor site and sex. A: Changes were calculated for the reference periods 1976-1978 and 2004-2006\*; the intervals 1979-1981 and 2004-2006 were chosen for the stomach, since no gastric NE neoplasms were registered in women before 1979; B: Changes in incidence are presented as relative increments age-adjusted to the 1987 German standard population. The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for the years 1976-1978 or from its successor, the Joint Cancer Registry, for the time period 2004-2006. NE: Neuroendocrine.



**Figure 3** Absolute changes in the incidence of gastroenteropancreatic neuroendocrine tumor are shown according to tumor site and sex. A: Changes were calculated for the reference periods 1976-1978 and 2004-2006\*; the periods 1979-1981 and 2004-2006 were chosen for the stomach, since no registered gastric NE neoplasms were registered in women before 1979; B: Changes in incidence are presented as absolute increments age-adjusted to the 1987 German standard population. The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for the years 1976-1978 or from its successor, the Joint Cancer Registry, for the time period 2004-2006. NE: Neuroendocrine.

Overall survival differed significantly ( $P < 0.001$ ) between men and women. In the earlier period (1976-1988), 51% of men and 64% of women were alive after 1 year; 43% of men and 55% of women stayed alive after 5 years, and 41% of men and 51% of women were alive after 10 years. In the later period (1998-2006), 1-year survival was 75% for men and 83% for women; 5-year survival had increased to 57% for men and 68% for women, and 10-year survival was 42% for men and 58% for women. Overall survival differed not only for sex but also for the primary tumor site. Table 2 shows significant differences in survival for various anatomic locations of the primary as well as for the two time periods (1976-1988 and 1998-2006).

### Second primary neoplasms

Of the 2821 NE tumor patients diagnosed between 1976-1988 and 1998-2006, 472 developed 533 second malignancies (Figure 5). The 533 second malignancies

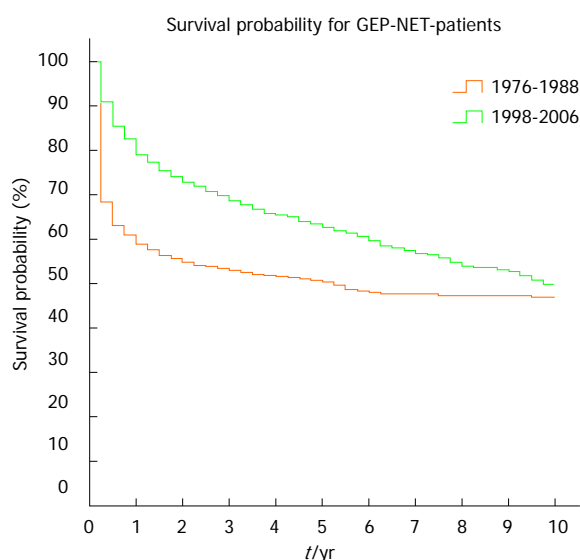
were diagnosed as pre-existing, synchronous or metachronous malignancies during the same time intervals as the GEP-NET (1976-1988 or 1998-2006); 16.7% of the GEP-NET patients suffered from second neoplasms.

GEP-NET patients without second malignancies fared better than those with one or more of them. An analysis of all 2821 GEP-NET patients showed significantly better 5- and 10-year overall survival for those without than for those with one or more second malignancies (5-year survival of 60% *vs* 53%; 10-year survival of 52% *vs* 42%,  $P < 0.05$ ).

The main locations of second malignancies were the digestive tract (28%), the female genital organs (12%), the skin (12%), the breasts (7%) and the male genital organs (7%).

## DISCUSSION

Analysis of DDR Krebsregister for the period 1976-1988



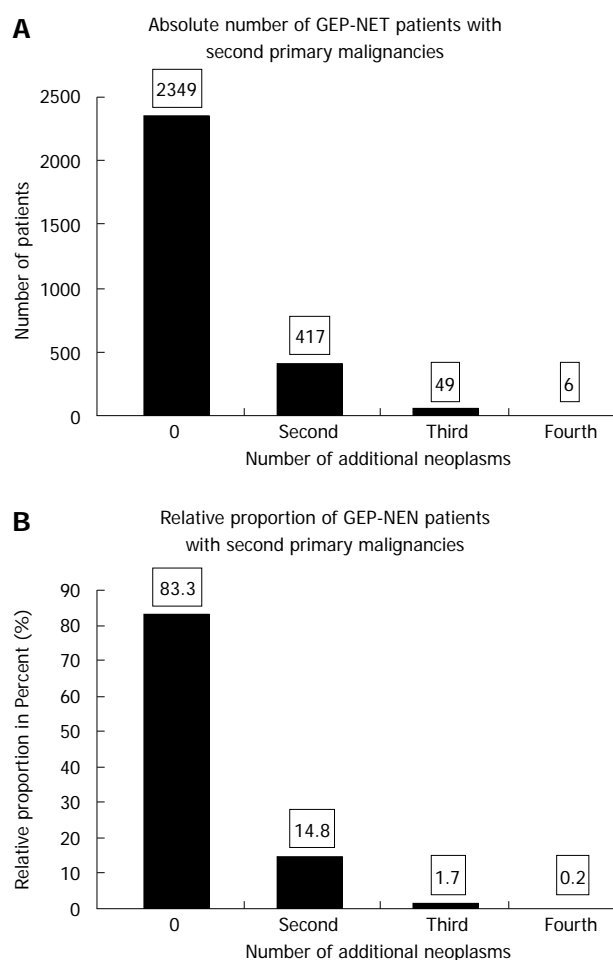
**Figure 4** Overall survival of patients with gastroenteropancreatic neuroendocrine tumor. Two periods of time are compared (1976-1988 and 1998-2006). GEP-NET: Gastroenteropancreatic neuroendocrine tumor.

and its successor, the GKR, for the period 1998-2006 revealed major increases in the incidence of detected GEP-NET in Germany. Our findings are in line with a recent report from the United States American SEER registry<sup>[8,16]</sup>. Recent epidemiological data from England and Norway also showed increases<sup>[8,14]</sup>. The current incidence of GEP-NET in Germany compares well with the incidence rates found in the United States and Australia as well as in several other European countries, *i.e.*, 1.33 to 3.8 per 100,000<sup>[8,14,16]</sup>. Despite these similarities, there are a number of important differences.

The nomenclature and classifications of NE neoplasms have not been uniform in the last 35 years<sup>[17]</sup>. Thus the low incidence rates from 1976-1988 may in part reflect differences between the classification and nomenclature used by East German pathologists (of the DDR Krebsregister) and those applied elsewhere. On the other hand, recent improvements in the general awareness and immunohistological diagnosis of NE neoplasms may have contributed to increased incidences in the last 20 years.

Moreover, colorectal cancer screening and the general availability of high-resolution endoscopy and radiological imaging may well have facilitated the detection of early asymptomatic GEP-NET. The hypothesis is supported by studies demonstrating that the incidence and anatomic distribution differ significantly between tumors detected post mortem and those diagnosed clinically<sup>[1,7,8]</sup>. These studies suggest that most small ( $\leq 1$  cm) GEP-NET remain asymptomatic and were generally not diagnosed in the era before the widespread availability of high-resolution endoscopy and computed tomography (CT) imaging.

The question arises whether the increased detection of early (asymptomatic) NET disease has contributed to recent epidemiological trends. In recent years, localized



**Figure 5** Second malignancies occurred in 472 of 2821 patients with gastroenteropancreatic neuroendocrine tumor. A: The absolute number; B: The percentage of patients with one or more second (non-gastroenteropancreatic neuroendocrine tumor, non-GEP-NET) neoplasms. 0 = No second neoplasm. GEP-NET: Gastroenteropancreatic neuroendocrine tumor.

NET constitute by far the largest subgroup in the SEER registry and are largely responsible for the increased incidence of GEP-NET<sup>[3,18]</sup>. Consistent with this notion, Japanese, South Korean and Polish endoscopy screening programs detected rectal NET in 50-70 of 100,000 persons screened<sup>[19-21]</sup>. The vast majority of rectal NE neoplasms detected by screening are 1 cm or smaller in diameter. Comparison with historical registries shows that screening is associated with a shift to smaller-sized rectal NET<sup>[3,18]</sup>. A national program of endoscopic colorectal cancer screening was introduced in Germany in October 2002. Screening colonoscopy is now offered free of charge to any person aged 55 years or older. Both colonoscopy and esophagogastroduodenoscopy are now readily available in Germany. In former East Germany (up to 1989), on the other hand, gastrointestinal endoscopy was available only at 3-4 centers.

The now widespread availability of endoscopy and radiological imaging may well have contributed to the observed increases in gastroduodenal, rectal and pancreatic NET<sup>[18,22-26]</sup>. On the other hand, the incidence of appendiceal NET remained quite stable in the Eastern

**Table 2** 1-, 5- and 10-year overall survival of patients with gastroenteropancreatic neuroendocrine tumors

Overall survival/period	Stomach	Small intestine	Pancreas	Appendix	Colon	Rectum
1-yr/1976-1988	22%	30%	26%	95%	35%	50%
5-yr/1976-1988	11%	18%	11%	92%	16%	37%
10-yr/1976-1988	5%	10%	8%	90%	13%	37%
1-yr/1998-2006	71%	85%	74%	95%	68%	74%
5-yr/1998-2006	53%	68%	52%	86%	48%	65%
10-yr/1998-2006	43%	53%	35%	81%	34%	50%

Data are given for different primary tumor sites and two periods of time (1976-1988 and 1998-2006).

parts of Germany from 1976 to 2006. Even today, they are generally diagnosed postoperatively in patients who undergo appendectomy for suspected appendicitis. Endoscopy and radiological imaging probably do not have much impact on their early detection ( $\leq 1$  cm). Instead, they are found incidentally in one out of 200-300 appendectomy specimens. Appendectomies are among the most common surgical procedures performed in Germany. They account for 135,000 procedures per year. This contributes to the frequent detection of early appendiceal NET<sup>[27]</sup>.

In line with recent reports from England<sup>[28]</sup> and Austria<sup>[27]</sup>, we observed a large increase in the incidence of gastric NET. In the current analysis, however, the nature of the epidemiological data does not enable an examination of underlying causes. Noteworthy is the fact that the incidence rates of both gastric and rectal carcinoids are most likely underestimated in DDR Krebsregister as well as in its successor registry, the GKR of the new federal states, including Berlin. This is due to the fact that only malignant NE neoplasms had to be reported to either registry. Thus, well-differentiated small ( $< 1$ -2 cm) carcinoids of the stomach or rectum were probably not consistently documented in the past. Recent prospective data from Austria identify the stomach and colorectum as the most common sites of GEP-NET<sup>[27]</sup>. The Austrian observation has been confirmed by a retrospective study including 150 consecutive GEP-NET patients diagnosed at the Vivantes Hospitals in Berlin between 2005 and 2009. The stomach was the most frequent site of GEP-NET in the Vivantes Hospitals, closely followed by the small intestine and colorectum (data not shown).

Overall survival of GEP-NET patients has improved significantly in Germany during the last 35 years. This applies to both sexes and all examined anatomic sites except the appendix. In the latter location, overall survival decreased in women and remained unchanged in men. But even in the period 1998-2006, the 5- and 10-year survival reached 86% and 81%. The significant improvement in overall survival of GEP-NET patients can probably be attributed to earlier diagnosis, the greater effectiveness of modern multimodal treatment strategies, and the general increase in life expectancy.

Only a few studies have reported on the frequency of one or more second malignancies in GEP-NET patients. Second primaries were found in 16.7% of our GEP-NET patients. This is consistent with data from

Florida (23.6%), a meta-analysis from 13 studies including more than 5000 GEP-NET patients (17%), and the SEER registry (22.4%)<sup>[15,29-31]</sup>. At 28%, the incidence of these second primaries was highest in the gastrointestinal tract and much lower at 12% in both the female genital tract and the skin. These data on second malignancies are consistent with the observations reported in the studies mentioned above.

GEP-NET patients appear to have an increased risk of second malignancies, although there is an ongoing debate. In their review, Habal *et al.*<sup>[29]</sup> summarize several theories regarding the influence of NET on the emergence and growth of second malignancies. They estimated that the risk of developing a second tumor is twice as high for patients with GEP-NET than for those with other neoplasms<sup>[29]</sup>. Several studies have examined amines, peptides, growth hormones and other compounds secreted by NET for their relation to the formation and growth of neoplasms in the breast or gastrointestinal tract<sup>[31-33]</sup>.

Due to the high prevalence of second neoplasms, screening for other malignancies seems advisable in GEP-NET patients. Remarkably, Zar *et al.*<sup>[34]</sup> observed that many GEP-NET patients died of their second malignancies but not of their GEP-NET. Consistent with the data of Zar *et al.*<sup>[34]</sup>, our GEP-NET patients with one or more second malignancies did not fare as well as those without them.

We conclude that both the frequency of detected GEP-NET and the overall survival of GEP-NET patients have increased significantly in Germany between 1976 and 2006. These epidemiological changes warrant our attention. Future research efforts will focus on the carcinogenesis of GEP-NET.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Neuroendocrine tumors belong to the three malignancies that increase most in frequency in Western countries.

### Research frontiers

The genetic footprints of neuroendocrine neoplasias are about to be unravelled.



## Innovations and breakthroughs

The frequency of second malignancies in neuroendocrine tumors (NET) patients is highlighted in this paper. The power of screening endoscopy in detecting small neuroendocrine tumors (*e.g.*, of the rectum) is evidenced by its availability in former East Germany after German reunification in 1989.

## Applications

The general availability of modern endoscopy enables the (early) detection of small neuroendocrine tumors of the stomach, duodenum and rectum. Detection of small asymptomatic neuroendocrine tumors appears to have contributed to their epidemiological rise.

## Peer review

The authors describe an increase of gastroenteropancreatic-NET (GEP-NET) for the time period 1977-1988 to 1998-2006 by five-fold. However, they quite clearly demonstrated in the discussion section that this increase is mainly due to different reasons: Nomenclature has been changed; improvement of general awareness and immunological diagnoses; availability of the German National Programme of Colorectal Cancer Screening since October 2002; better imaging diagnoses. An important finding of the project is that almost 17 percent of GEP-NEN patients showed second primary malignancies and therefore screening for other malignancies in those patients should be important for the future.

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## Nrf2 and Snail-1 in the prevention of experimental liver fibrosis by caffeine

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### Abstract

**AIM:** To determine the molecular mechanisms involved in experimental hepatic fibrosis prevention by caffeine (CFA).

**METHODS:** Liver fibrosis was induced in Wistar rats by intraperitoneal thioacetamide or bile duct ligation and they were concomitantly treated with CFA (15 mg/kg per day). Fibrosis and inflammatory cell infiltrate were evaluated and classified by Knodell index. Inflammatory infiltrate was quantified by immunohistochemistry (anti-CD11b). Gene expression was analyzed by quantitative reverse transcription-polymerase chain reaction for collagen I (Col-1), connective tissue growth factor (CTGF), transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 (IL-1), IL-6, superoxide dismutase (SOD) and catalase (CAT). Activation of Nrf2 and Snail-1 was analyzed by Western-blot. TNF- $\alpha$  expression was proved by enzyme-linked immunosorbant assay, CAT activity was performed by zymography.

**RESULTS:** CFA treatment diminished fibrosis index in treated animals. The Knodell index showed both lower fibrosis and necroinflammation. Expression of profibrogenic genes *CTGF*, *Col-1* and *TGF- $\beta$ 1* and proinflammatory genes *TNF- $\alpha$* , *IL-6* and *IL-1* was substantially diminished with CFA treatment with less CD11b positive areas. Significantly lower values of transcriptional factor Snail-1 were detected in CFA treated rats compared with cirrhotic rats without treatment; in contrast Nrf2 was increased in the presence of CFA. Expression of SOD and CAT was greater in animals treated with CFA showing a strong correlation between mRNA expression and enzyme activity.

**CONCLUSION:** Our results suggest that CFA inhibits the transcriptional factor Snail-1, down-regulating profibrogenic genes, and activates Nrf2 inducing antioxidant enzymes system, preventing inflammation and fibrosis.

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**Key words:** Liver fibrosis; Caffeine; Thioacetamide; Bile duct ligation; Profibrogenic genes; Proinflammatory cytokines; Antioxidant enzymes

**Core tip:** This paper shows the protective effect of caffeine in the liver to the constant aggressiveness of a hepatotoxic. Here we present evidence not published before of some molecular mechanisms like inhibition of Snail-1 and activation of Nrf2 that could be involved in this beneficial effect down-regulating pro-fibrogenic genes and up-regulating antioxidant molecules.

Gordillo-Bastidas D, Ocegüera-Contreras E, Salazar-Montes A, González-Cuevas J, Hernández-Ortega LD, Armendáriz-Borunda J. Nrf2 and Snail-1 in the prevention of experimental liver fibrosis by caffeine. *World J Gastroenterol* 2013; 19(47): 9020-9033  
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## INTRODUCTION

The liver performs essential functions in the body<sup>[1]</sup>. Hepatic stellate cells (HSC) are key in the fibrogenic process<sup>[2]</sup>. After stimulation of liver damage, HSC undergo a process called “activation”; characterized by synthesis of type I and III collagens<sup>[3-5]</sup>. This state of activation is maintained by growth factors such as transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1)<sup>[6]</sup>, connective tissue growth factor (CTGF)<sup>[7]</sup>, and pro-inflammatory molecules such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin 6 (IL-6)<sup>[8]</sup> and reactive oxygen species (ROS).

Epidemiological studies had associated caffeine (CFA) consumption with protection against development of chronic liver disease or reduction of disease severity<sup>[9-11]</sup>. *In vitro* studies have shown beneficial effects of CFA, that can be useful in preventing HSC activation and perpetuation of this state<sup>[12,13]</sup>. Among CFA effects observed *in vitro* are: inhibition of expression of CTGF<sup>[14-16]</sup>; reduction of pro-inflammatory cytokines expression such as TNF- $\alpha$ , IL-1 and IL-6 by mechanisms not yet defined<sup>[8]</sup>, and CFA antioxidant effect<sup>[17-21]</sup>.

On the other hand, a potent natural antioxidant, quercetin increases the transcriptional and translational activity of the transcriptional factor Nrf2 which has potent antioxidant activity<sup>[22]</sup>.

Activation of HSC is a complex process where the transcriptional factor Snail-1 has an important role. Several authors have reported the overexpression of Snail-1 in pathological conditions associated with extracellular matrix (ECM) deposition<sup>[23,24]</sup>. Snail-1 expression has been shown in cholangiocytes and hepatocytes of fibrotic livers<sup>[14,16]</sup>, and recently, Snail-1 has been published as a central transcription factor on the activation of HSC demonstrating its essential role in regulating the liver fibrosis process<sup>[25]</sup>.

According to our findings, CFA-mediated molecular mechanisms comprise in part down-regulation of pro-fibrogenic genes, diminishing of inflammatory cell infiltrate, down-regulation of pro-inflammatory cytokines, and up-regulation of antioxidant enzymes. Our results suggest that these events could be mediated, at least in part, by Nrf2 activation and inhibition of Snail-1 which are key factors in the development of this process.

## MATERIALS AND METHODS

### Materials

CFA was acquired from Sigma Aldrich Co., (St Louis Missouri). Thioacetamide (TAA) was purchased from Merck Company, (Darmstadt, Germany). CD11b antibody was obtained from Biolegend (San Diego, CA, United States). Biotinylated secondary antibody and avidin-conjugated

peroxidase were obtained from Vector Laboratories (Burlingame, CA, United States).

DuoSet enzyme-linked immunosorbant assay (ELISA) Development kit was acquired from R and D Systems, (Minneapolis, United States). Primers and probes to design real time polymerase chain reaction (PCR) were acquired from Applied Biosystems (Hammonton, NJ, United States). Poly vinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules CA, United States). Nrf2, Snail-1 and secondary antibodies were purchased from Avcam Inc (Cambridge MA, United States).

### Animals and experimental design

Wistar rats used in this study were obtained from Charles Rivers (Boston, MA, United States) and housed according to the Animal Care protocol established by University of Guadalajara. Thirty male Wistar rats, weighing 250-280 g were divided into three groups (10 rats in each group) as follows: (1) healthy ( $n = 10$ ); (2) TAA ( $n = 10$ ), rats with intraperitoneal TAA to develop liver fibrosis; and (3) bile duct ligation (BDL) ( $n = 20$ ), rats that underwent a laparotomy and BDL. Finally 5 rats of each group were treated with CFA and other 5 rats received vehicle only (fibrotic rats).

### CFA administration in TAA-intoxicated and BDL rats

Two *in vivo* models were intended to assess fibrosis prevention *via* CFA administration, TAA and BDL. TAA-induced fibrosis was achieved using a dose of 200 mg/kg administered intraperitoneally 3 times a week for 7 wk, as described previously<sup>[26-28]</sup>. BDL-induced fibrosis was achieved under general anesthesia and laparotomy was made, the common bile duct was localized, doubly ligated and cut between these two ligatures<sup>[29]</sup>. CFA administration was carried out concomitantly with BDL and TAA intoxication regimen once a day with a dose of 15 mg/kg by the orogastric route. Rats sacrifice was performed at the seventh week for the TAA model, and at the fourth week for the BDL model. Representative liver sections were excised and either fixed with 4% buffered paraformaldehyde for histological examination, or frozen for RNA and protein extraction.

### Biochemical assays

Blood was obtained from animals immediately before sacrifice, and serum transaminases, alanine transaminase (ALT) and aspartate transaminase (AST), were determined in automated Vitros DT 60 equipment (Johnson and Johnson, New Jersey, United States).

### Histological examination of liver sections

For histological studies, livers were removed and fixed by immersion in 4% paraformaldehyde diluted in PBS, dehydrated in graded ethylic alcohol, and embedded in paraffin.

**Assessment of liver inflammatory activity and fibrosis:** The Modified Histological Activity Index of Knodell was used to grade the severity of the necroinflammatory



process (0-18 scale) and fibrosis (0-6 scale), and was performed blindly by two experienced pathologists<sup>[30-32]</sup>. Additionally, liver fibrosis was also quantitatively assessed by Masson's trichromic staining in 4- $\mu$ m liver sections by light microscopy as described previously<sup>[33,34]</sup> using a computer-assisted morphometric analyzer (Image-ProPlus 6.0; Media Cybernetics, Inc., Bethesda, MD, United States) by analyzing ten random fields per slide and calculating the ratio of connective tissue to the whole liver area, expressed as fibrosis percentage.

**Immunohistochemical determination of CD11b:** Hepatic tissue sections were deparaffinized and rehydrated with xylene and decreasing graded ethanol. Slides were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 30 min, followed by incubation with polyclonal anti-rat against purified CD11b/c (Biolegend, Cat. No. 201801, San Diego, CA, United States) diluted in PBS (1:100).

The primary antibody was incubated at 4 °C overnight, followed by incubation with biotinylated secondary antibody (Vectastain, Universal Quick Kit, Cat. No. PK-8800). Secondary antibodies were complexed individually with avidin-conjugated peroxidase Vectastain ABC-Elite reagent (Vector Laboratories, Burlingame, CA, United States) and resulting peroxidase activity was detected with 3,30-diaminobenzidine in sections that were briefly counterstained with hematoxylin. Positive areas were analyzed in 20 random fields of pericentral, mid-zonal and periportal areas. Counting was carried out using automated software (Image-Pro plus Analyzer, Qwin-Leica, United States). Results were expressed as a percentage of the positive area.

#### ELISA assay for TNF- $\alpha$

Liver tissue was homogenized with Polytron (Janke Kunkel IKA-WERK, Staufen im Breisgau, Germany) and centrifugated at 4 °C for 4 min at 12000 g in lysis buffer with protease inhibitors [50 mmol/L Tris (hydroxymethyl) aminomethane-HCl buffer, pH 7.4, containing 0.02% sodium azide, 150 mmol/L NaCl, 0.1% Tween-20, 150 mmol/L NaCl, 10 g/mL aprotinin, 5 g/mL pepstatin, 5 g/mL leupeptin, 1 mmol/L phenyl-methylsulfonyl fluoride and 25 g/mL E64]<sup>[35]</sup>.

Protein concentration of cleared tissue lysates were determined by Bradford method. After quantitation samples were stored at -80 °C until analysis.

We used the kit DuoSet for ELISA for rat TNF- $\alpha$ /TNFSF1A (DuoSet ELISA Development kit, rat TNF- $\alpha$  Cat. No. DY510, R and D Systems, Minneapolis, United States), following the protocol provided by the manufacturer. Finally, the reaction was stopped and the optical density of each well was determined at 450 nm.

#### Quantitative real-time reverse transcriptase-PCR

RNA was isolated from the liver from different groups of rats with Trizol reagent (Invitrogen, Carlsbad, CA, United States)<sup>[36]</sup>. Retrotranscription using 2 g of total RNA was achieved using moloney-murine leukemia virus

reverse transcriptase (Invitrogen). Then, 2  $\mu$ L of cDNAs were subjected to real-time PCR using a Rotor Gene Thermocycler under the following conditions: 2 min at 50 °C, 5 min at 94 °C, and 45 cycles of 30 s at 94 °C and 40 s at 60 °C. Specific primers and probes designed to align in collagen  $\alpha$ 1 (I), CTGF, TGF- $\beta$ 1, TNF- $\alpha$ , IL-1, IL-6, superoxide dismutase (SOD) and catalase (CAT) rat RNAs were acquired from Applied Biosystems (Hammonton, NJ, United States). Gene amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Relative quantification by the  $2^{-\Delta\Delta CT}$  method was realized by comparing to control groups as an internal calibrator<sup>[37,38]</sup>. Expression gene levels are shown as expression relative units.

#### Catalase activity

Reported CAT activity was determined according to the zymographic method by Gennady P Manchenko (1994). The method is based on the starch-iodine reaction. Thio-sulfate in the staining solution is inactivated by hydrogen peroxide except at the sites of CAT activity, where hydrogen peroxide is destroyed enzymatically. The iodide is oxidized by hydrogen peroxide to iodine which forms a chromophore with the starch and sites of CAT localization remain achromatic<sup>[29]</sup>.

#### Western-blot assays

Western blot assays of tissue homogenates were performed to analyze the activation of Nrf-2 and Snail-1. Proteins were extracted from 100 mg of liver tissue using lysis buffer (50 mmol/L Tris-HCl pH 8.0, 150 nmol/L NaCl, 0.02% NaN<sub>3</sub>). After centrifugation at 13000 rpm/5 min/4 °C, supernatant was collected and quantified by Bradford assay. Briefly, 30  $\mu$ g of total proteins were separated by 10% sodium dodecyl sulfate polysulfate polyacrylamide gel electrophoresis under reducing conditions and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules CA, United States). Blocking was carried out using 3% dry milk for 2 h; primary antibody dilution was 1:500 for GAPDH (loading control) and 1:800 for Nrf2 and Snail-1 antibodies. (Abcam Biotechnology, Santa Cruz CA, United States). Antibody binding was revealed with a secondary anti-antibody diluted 1:5000-1:6000 using BM Chemiluminescence kit (Roche Diagnostics, Indianapolis IN, United States). Densitometric analysis was realized with a Kodak 1D 3.5 Image analyzer (Eastman Kodak Co., Rochester NY) GAPDH was used as a cell fractionation control.

#### Statistical analysis

Normally distributed data were analyzed using *t* test, where statistical significance was  $P < 0.05$ . Data are shown as the mean  $\pm$  SD. For real-time PCR experiments, results are shown as the  $2^{-\Delta\Delta CT}$  value (mean  $\pm$  SD), where the standard deviation was calculated as:  $s = [S(\text{GAPDH})^2 + S(\text{target gene})^2]^{1/2}$ , according to user bulletin 2 from Applied Biosystems.

**Table 1** Weight at the beginning of the treatment and after caffeine treatment, serum markers enzymes in bile duct ligation and thioacetamide-intoxicated rats

Group	Healthy	TAA	TAA + CFA	BDL	BDL + CFA
Rats weight at the beginning of the treatment (g)	296.0 ± 9.73	284.0 ± 22.1	282.2 ± 9.1	280.1 ± 18.3	275.0 ± 19.0
Rats weight after CFA treatment (g)	321.0 ± 8.5	223.1 ± 14.0 <sup>b</sup>	253.7 ± 5.7	219.4 ± 15.3	221.0 ± 17.5
AST (U/L)	226.0 ± 61.0	379.7 ± 179.8	318.2 ± 144.3	576.7 ± 70.0	329.5 ± 41.4 <sup>c</sup>
ALT (U/L)	63.0 ± 1.0	132.7 ± 7.5	98.2 ± 28.7	214.7 ± 37.0	76.0 ± 10.6 <sup>d</sup>

Treatment duration: 7 wk for thioacetamide (TAA) and 4 wk for bile duct ligation (BDL). <sup>b</sup>*P* < 0.01 vs TAA group; <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 vs BDL group. Data are shown as the mean ± SD (*n* = 10). CFA: Caffeine; AST: Aspartate transaminase; ALT: Alanine transaminase.

## RESULTS

### CFA prevents weight loss in TAA-intoxicated rats

Basal weight and weight at the end of treatment were registered in all groups. As shown in Table 1, CFA prevented weight loss of rats in the TAA model, which suggested that CFA had an effect in improving the nutritional status of rats measured solely by weight.

### CFA dosed groups had less hepatocellular damage

AST and ALT levels were higher in TAA-intoxicated (1.7- and 2.1-fold respectively) and BDL (2.6- and 3.4-fold respectively) groups compared with the healthy rats group. The BDL + CFA group showed lower levels in AST compared to the BDL group (1.8-fold) (*P* < 0.05). The TAA + CFA group only showed a tendency to lower levels. Similarly, ALT levels in the BDL + CFA group were lower (2.8-fold) when compared against the BDL group (*P* < 0.01) (Table 1).

### CFA treatment reduced both BDL and TAA-induced liver fibrosis

To test the antifibrogenic effect of CFA, morphological analysis of liver sections stained with Masson's was performed. Looking at the histology of the healthy group, we observed a normal morphology, with scarce ECM and hepatocytes arranged in a radial pattern. Histology of TAA and BDL groups showed an altered morphology, with thick collagen bundles, much more noticeable in the BDL group. In contrast, the treated groups TAA + CFA and BDL + CFA showed lower ECM content (Figure 1A). Quantification of ECM demonstrated a potent antifibrogenic effect of CFA. In the TAA + CFA group, fibrosis was lower by 80% compared to the TAA group (*P* < 0.0001). Likewise, in the BDL + CFA group fibrosis was lower by 38% compared to the BDL group (*P* < 0.0001) (Figure 1B).

The Knodell score indicated lower fibrosis in the TAA + CFA and BDL + CFA groups (3 ± 0.5 and 4 ± 0.5 points respectively) compared with the TAA and BDL cirrhotic groups (6 ± 0 and 6 ± 0 points respectively) (both *P* < 0.05) (Figure 1C).

### Fewer inflammatory cells infiltrate in CFA groups

It was noted that CFA groups had a low amount of inflammatory infiltrate. TAA and BDL groups had a large number of inflammatory cells, especially the BDL group.

In contrast, both cirrhotic groups treated with CFA had a lower amount of inflammatory infiltrate, more evident in the TAA + CFA group (Figure 1D). The Knodell score resulted in lower necroinflammation in the treated groups, TAA + CFA and BDL + CFA (8 ± 0.5 and 9 ± 0.5 points), compared with the cirrhotic groups, TAA and BDL (16 ± 0 and 18 ± 0 points) (both *P* < 0.001) (Figure 1E).

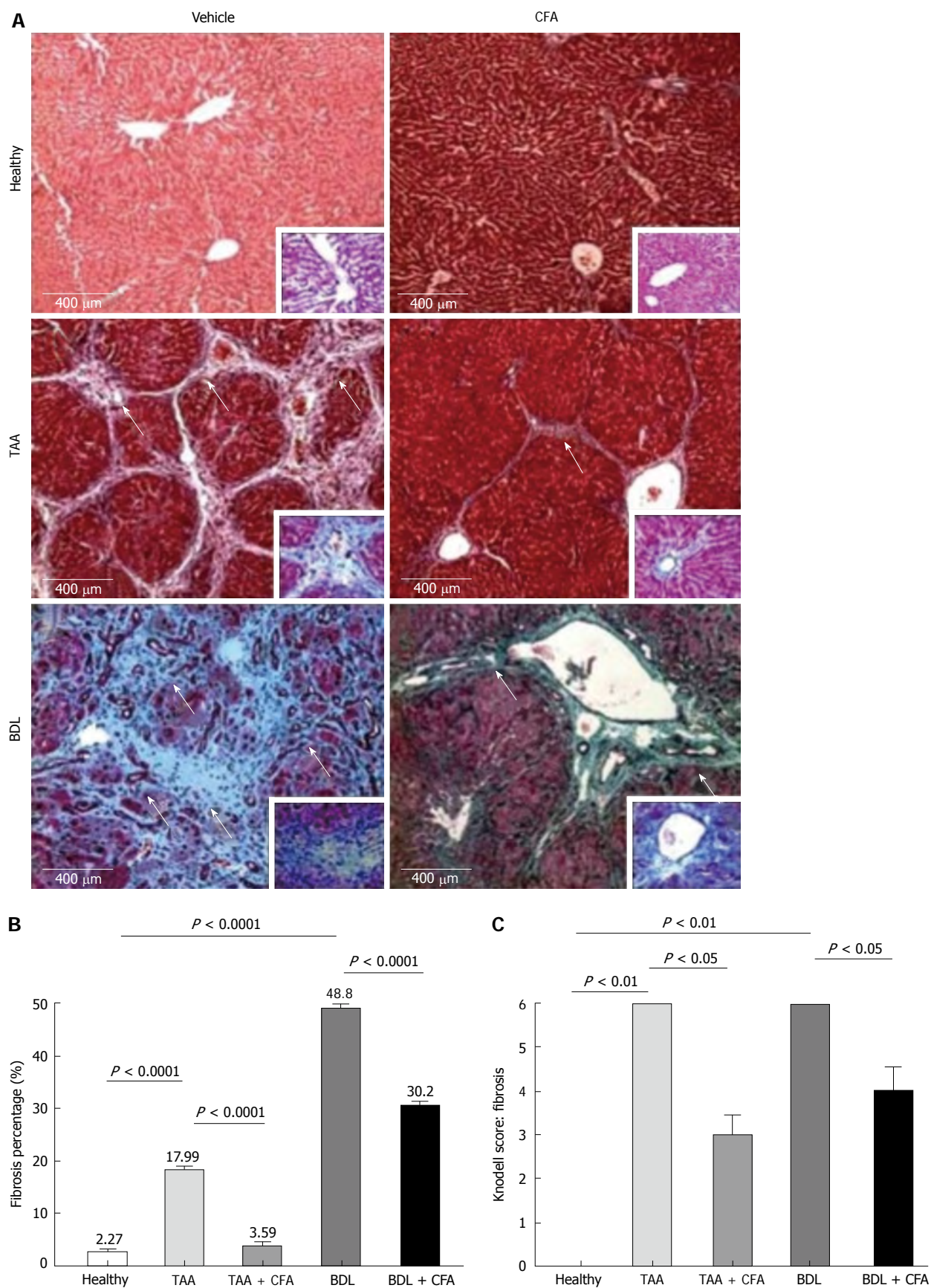
### Fibrogenic genes expression decrease with CFA treatment even in the continuous presence of liver fibrosis inducers

In addition to the histologic analysis we analyzed expression of the fibrogenic genes.

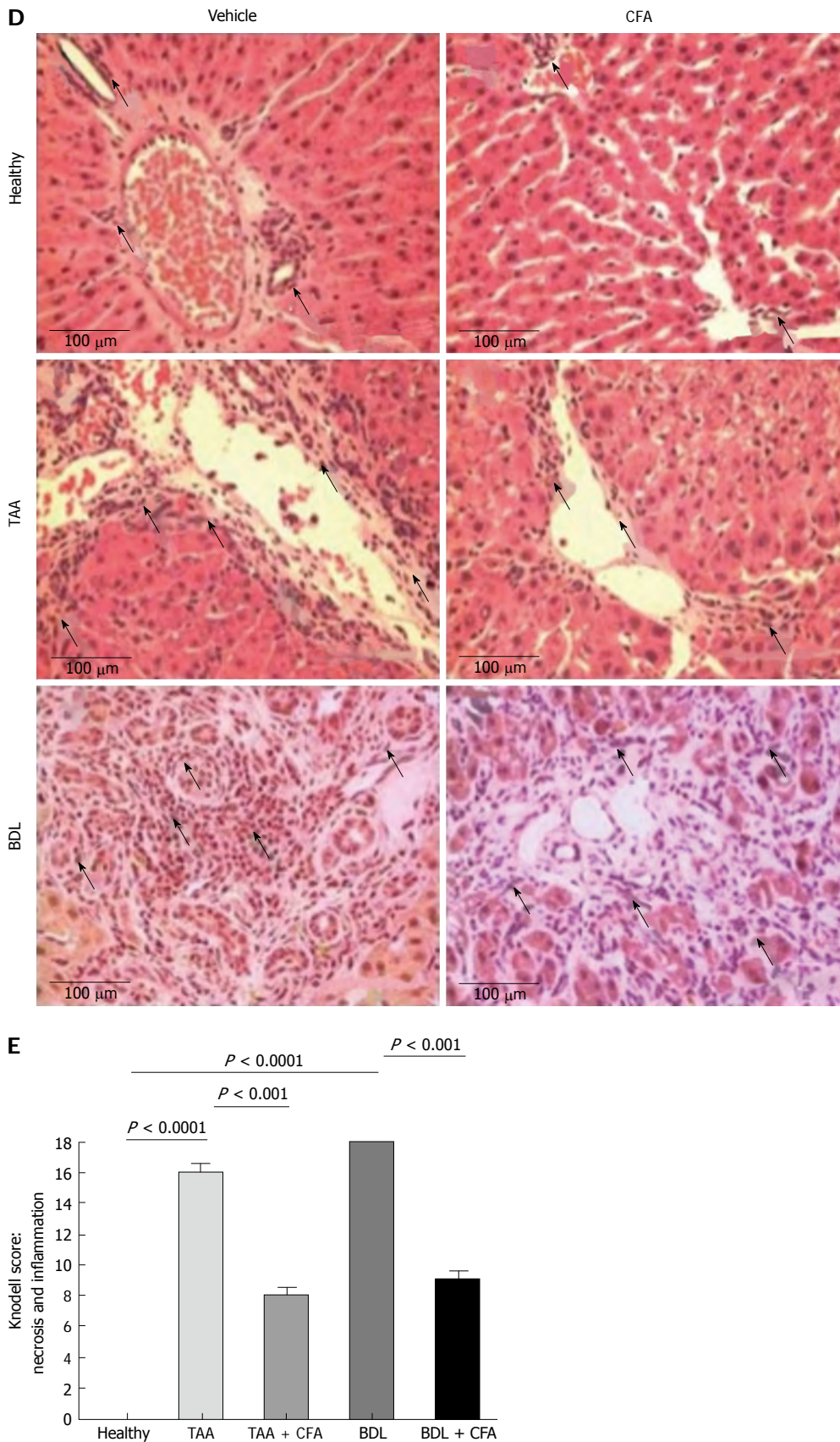
As expected, cirrhotic groups showed an increase in fibrogenic genes expression. In the TAA group there was a 5.3-fold increase for CTGF (*P* < 0.01), a 10.5-fold increase for collagen I (Col-1) (*P* < 0.01) and a 4.3-fold increase for TGF-α1 (*P* < 0.05). In the BDL group fibrogenic genes expression also showed an increase; this increase was 11.6-fold for CTGF (*P* < 0.01), 21.5-fold for Col-1 and 3.5-fold for TGF-α1 (*P* < 0.05), compared with healthy rat group levels (Figure 2A-C). Treatment with CFA also induced a lower expression of fibrogenic genes; in the TAA + CFA group this was 3.5-fold lower for CTGF (*P* < 0.01), 3.5-fold lower for Col-1 (*P* < 0.05) and 3.1-fold lower for TGF-β1 (*P* < 0.01) compared with the TAA group. In the BDL+CFA group the reduction in gene expression was 5.0-fold lower for CTGF (*P* < 0.01), 3.0-fold lower for Col-1 (*P* < 0.01), and 1.5-fold lower for TGF-β1, indicating only a declining trend but no statistical significance, compared with BDL group (Figure 2A-C).

### CFA limits pro-inflammatory genes expression in experimental liver fibrosis models

We performed an immunohistochemical determination of CD11b in hepatic tissue sections. We observed that CD11b positive areas in the TAA + CFA versus the TAA group were lower by 65.5% (*P* < 0.01), and the BDL group treated with CFA versus the BDL group were lower by 60.8% (*P* < 0.05) (Figure 3A). In addition to testing the anti-inflammatory effect of CFA at the protein level, we analyzed TNF-α expression by ELISA. Both liver cirrhotic groups showed an increase in TNF-α levels, 560.2 ± 67.8 pg/mL (*P* < 0.0001) for the TAA group,

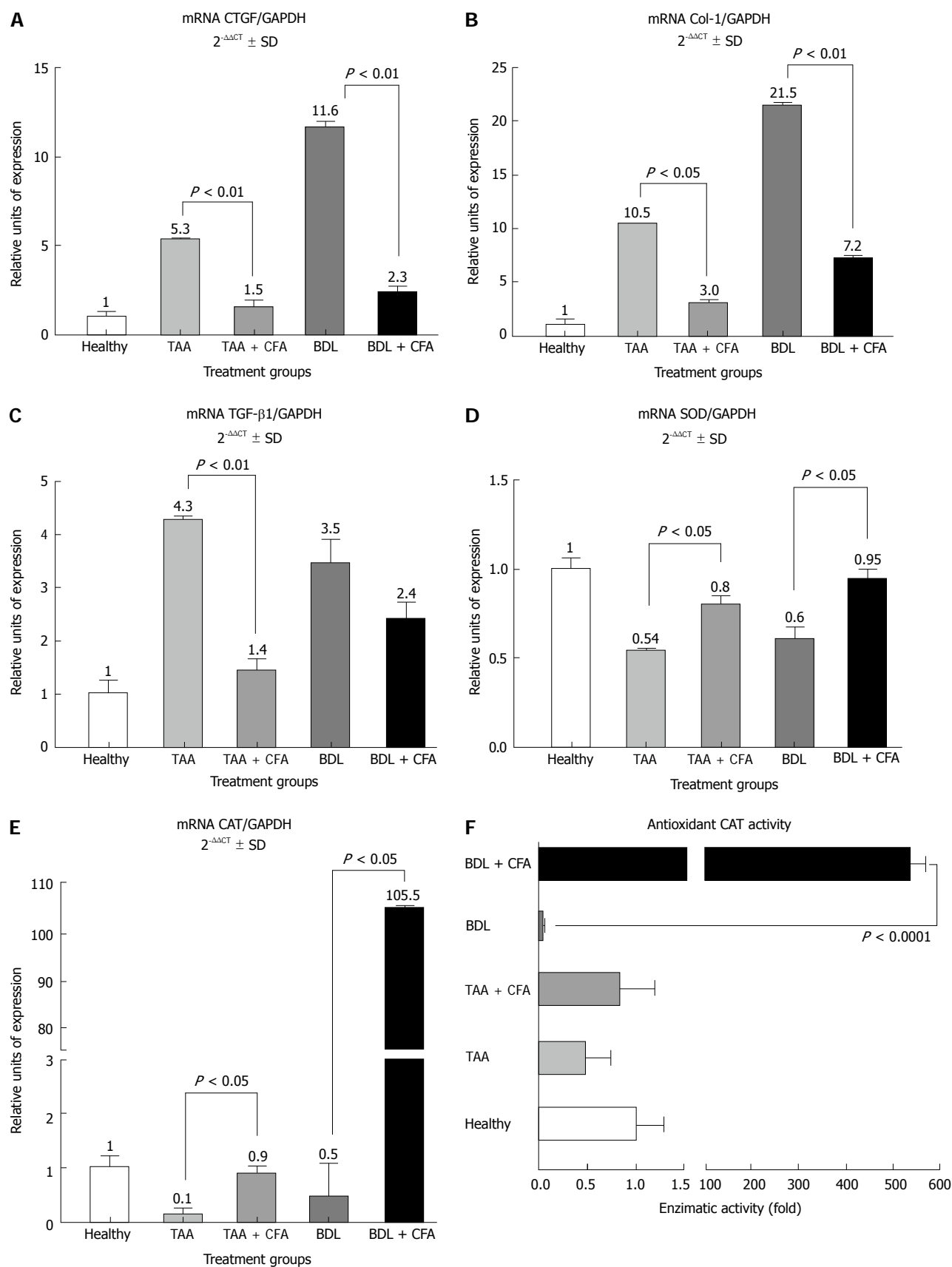


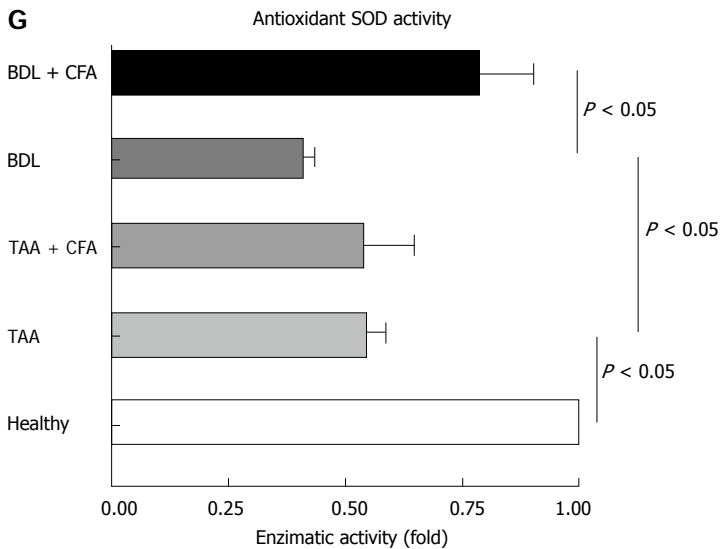




**Figure 1 Macroscopic and histological differences and fibrosis index with caffeine treatment.** A: Liver fibrosis percentage of healthy rats, caffeine (CFA)-treated, thioacetamide (TAA)-intoxicated, TAA-intoxicated treated with CFA, bile duct ligation (BDL) rats, BDL rats treated with CFA. Sections, 4  $\mu$ m thick, stained with Masson's trichrome,  $\times 10$ . White arrows show the extracellular matrix (ECM) (fibrosis); B: Fibrosis quantification. Fibrosis percentages are shown, they were obtained by computer-assisted morphometric analysis (Software Image pro plus 6.3); C: Knodel Index for fibrosis, sections 4  $\mu$ m thick, stained with Masson's trichrome,  $\times 10$ ; D: Inflammatory infiltrate amount. Sections 4  $\mu$ m thick, stained with hematoxylin and eosin,  $\times 40$ . Black arrows show inflammatory cells; E: Knodel Index for fibrosis, sections 4  $\mu$ m thick, stained with hematoxylin and eosin,  $\times 40$ .







**Figure 2 Expression of fibrogenic and antioxidant genes in liver.** Reverse transcription-polymerase chain reactions were performed for connective tissue growth factor (CTGF) (A), collagen I (Col-1) (B), and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) (C), superoxide dismutase (SOD) (D) and catalase (CAT) (E). Gene amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. CAT (F) and SOD antioxidant activity (G) were analyzed by zymography in acrylamide gels. TAA: Thioacetamide; BDL: Bile duct ligation; CFA: Caffeine.

and  $590.3 \pm 71.3$  pg/mL ( $P < 0.0001$ ) for the BDL group, compared with healthy rat group levels ( $140.4 \pm 3.4$  pg/mL). We observed lower levels in the CFA treated groups; for the TAA + CFA group  $313.1 \pm 56.6$  pg/mL ( $P < 0.01$ ), and for the BDL + CFA group  $420.6 \pm 166.1$  pg/mL (Figure 3B).

Then, we analyzed at the molecular level the pro-inflammatory genes expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Both cirrhotic groups showed a significant increase in all proinflammatory genes expression; in TAA group this was 1.8-fold for TNF- $\alpha$  ( $P < 0.05$ ), 1.8-fold for IL-1 ( $P < 0.05$ ) and 32.3-fold for IL-6 ( $P < 0.001$ ). In the BDL group it was 6.6-fold for TNF- $\alpha$  ( $P < 0.001$ ), 3.2-fold for IL-1 $\beta$  ( $P < 0.01$ ) and 128.2-fold for IL-6 ( $P < 0.0001$ ) compared with the healthy rats group (Figure 3C-E). In groups treated with CFA we observed a decrease of expression; in the TAA + CFA group this was 1.6-fold for TNF- $\alpha$  ( $P < 0.05$ ), 9-fold for IL-1 $\beta$  ( $P < 0.01$ ); and 6.1-fold for IL-6 ( $P < 0.05$ ); and in the BDL + CFA group there was a decrease of 9.4-fold for TNF- $\alpha$  ( $P < 0.001$ ), 1.1-fold for IL-1 and 5.1-fold for IL-6 ( $P < 0.001$ ) (Figure 3C-E).

#### ***Antioxidant enzymes gene expression and activity is modified by CFA intake***

It is known that both liver fibrosis models course with an oxidative stress state. Thus, antioxidant enzymes expression levels were analyzed. We noticed that hepatocellular expression of SOD increased 1.5 ( $P < 0.05$ ) and 1.6 ( $P < 0.05$ )-fold in TAA and BDL models, respectively, when they received CFA (Figure 2D). Likewise, CAT enzyme expression was significantly increased, showing an increase of 1.5-fold ( $P < 0.05$ ) in the TAA model, and an increase of 211-fold ( $P < 0.05$ ) in the BDL model (Figure 2E). To explore this last effect we performed an assay to measure SOD and CAT antioxidant activities, where we

found a strong correlation between mRNA expression and enzyme activity; in the BDL + CFA group antioxidant CAT activity was significantly increased (535-fold) ( $P < 0.0001$ ) (Figure 2F) and SOD activity increased twice compared with BDL group (Figure 2G).

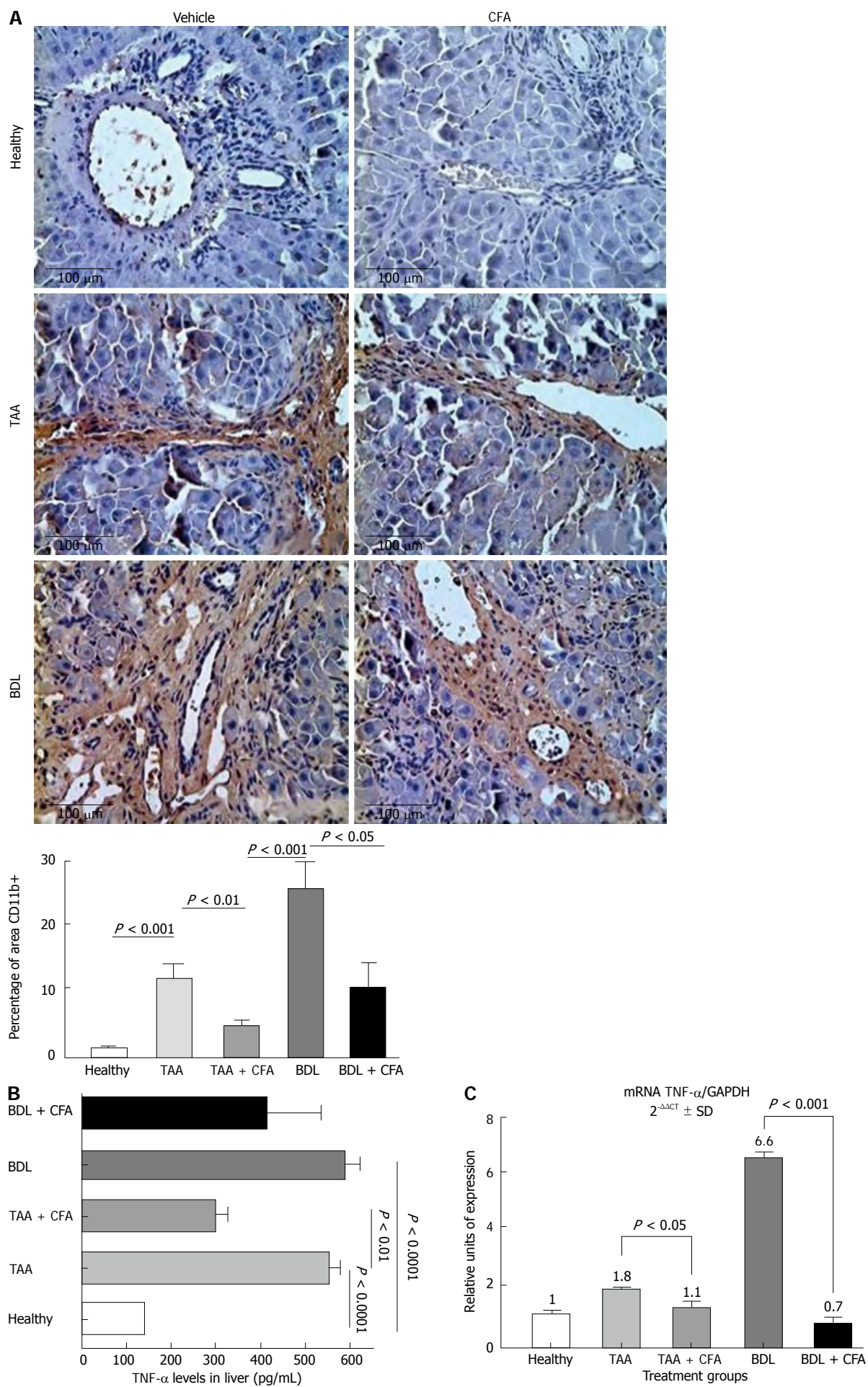
#### ***Activity of Snai-1 and Nrf2 by Western blot***

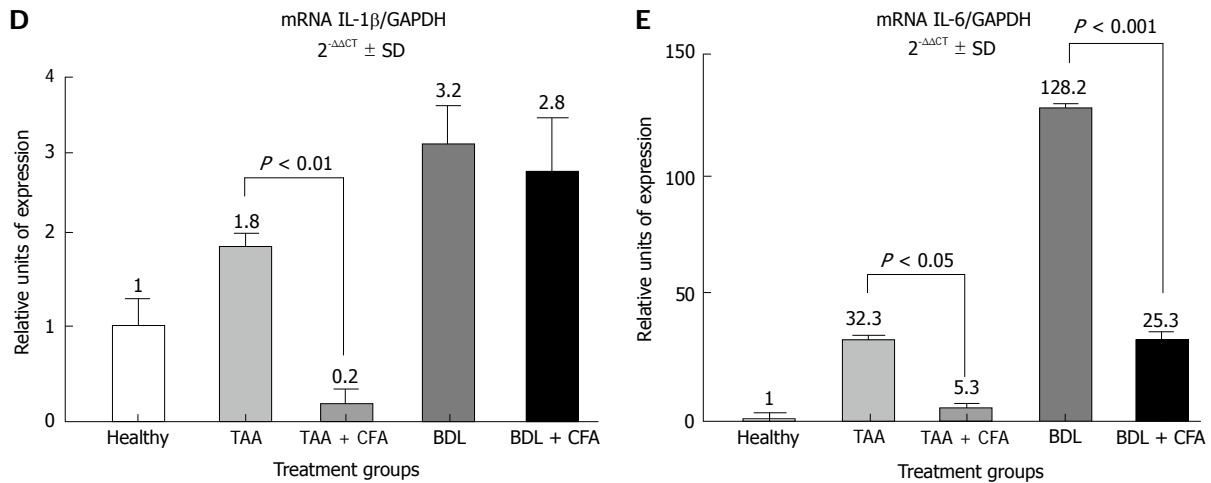
Protein levels of the antioxidant transcription factor Nrf2 were significantly higher in both animal models compared to healthy rats. Treatment with CFA in liver-injured rats increased these levels significantly in both BDL and TAA models (Figure 4A). This increase suggests that Nrf2 could be inducing SOD and CAT expression, thus preventing liver damage.

On the other hand, the pro-fibrogenic transcription factor Snai-1 reduced its protein levels when the animals were treated with CFA in both animal models. These values were 2.33 and 3.25 times higher than healthy animals for BDL and TAA respectively, where animals treated with CFA presented values only of 0.77 and 1.58 times higher with respect to healthy animals (Figure 4B).

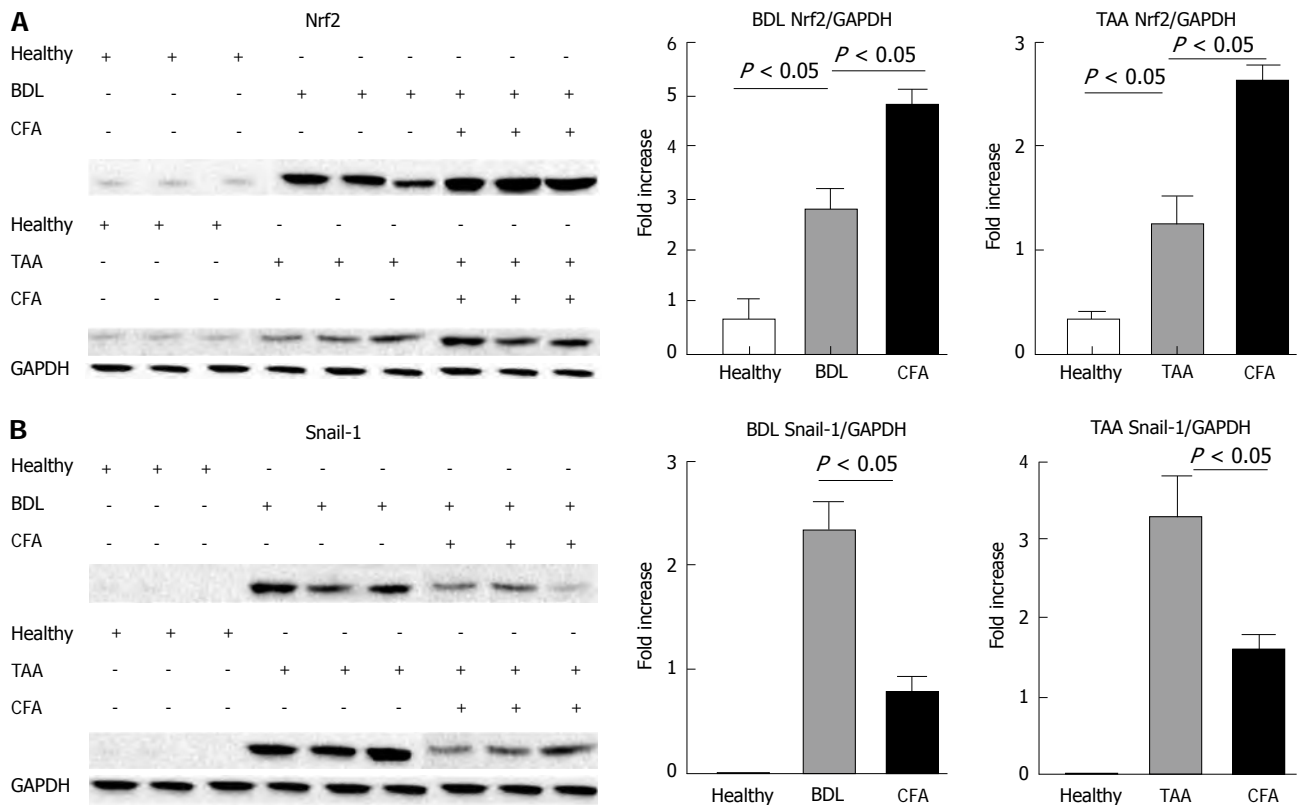
## **DISCUSSION**

There are epidemiological data indicating that consumption of CFA protects against development of chronic liver disease or reduces the severity of the disease<sup>[12-14]</sup>. *In vitro* studies have shown beneficial effects of CFA useful in preventing HSC activation and perpetuation of this state<sup>[15,16]</sup>. Although there is a recent preliminary report describing the effect of coffee on liver fibrosis<sup>[39]</sup>, here we describe a more comprehensible mechanism for CFA action on the most important molecules implicated in liver fibrosis. Our experiments were designed to compare CFA effects in two experimental liver fibrosis models, BDL and chronic TAA intoxication, to test whether the





**Figure 3 Expression of inflammatory genes in liver.** A: Immunohistochemistry for CD11b, sections 4  $\mu$ m thick, stained with Masson's trichrome,  $\times 40$ . Results for CD11b positive area are shown as percentage; B: Tumor necrosis factor alpha (TNF- $\alpha$ ) liver levels in different groups of treatment, performed by enzyme-linked immunosorbent assay; C-E: Reverse transcription-polymerase chain reaction were performed for TNF- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6. Gene amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. TAA: Thioacetamide; BDL: Bile duct ligation. CFA: Caffeine.



**Figure 4 Expression of transcriptional factors.** Western-blot were performed for the transcriptional factor Nrf2 (A) and Snail-1 (B). Densitometric values were normalized against the constitutive protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and represented like fold increase respect to healthy animals. TAA: Thioacetamide; BDL: Bile duct ligation. CFA: Caffeine.

preventive effects of CFA were independent of the etiology of liver damage. Dose was chosen based on positive effects of CFA regarding liver disease in epidemiological studies. These studies suggested a CFA consumption of 274 mg/d approximately in humans (2 cups of coffee)<sup>[12,40-43]</sup>. In our study we used a CFA dose of 15 mg/kg per day, because it is known that rat metabolism is ap-

proximately 10 times more accelerated than humans<sup>[42,43]</sup>. This animal dose is translated to a human dose using the body surface area (calculated with *Du Bois* formula)<sup>[44-47]</sup>, and an adjustment of rat  $k_m$  (9) to human  $k_m$  (41), resulting in a human dose of 2.4 mg/kg (1.5 cups of coffee)<sup>[46]</sup>, and expecting the same beneficial effects observed in the animal model and diminishing the probability of second-



ary effects.

It was observed that TAA-intoxicated rats treated with CFA had a higher body weight indicating overall improvement, probably because CFA reduces liver damage (as seen in histological analysis) and thus prevents the loss of appetite. Liver metabolic functions could be less altered, indicating that BDL and liver damage are the most important factors in weight loss.

In a previous experimental study of acute liver damage induced by a single dose of *D*-galactosamine/lipopolysaccharide, CFA pretreatment correlated with lower levels of AST and ALT. Also, it has been reported in an animal model of liver damage with alcohol that transaminase levels are diminished by the effect of CFA. However these experimental models are different than the ones used in this communication<sup>[48,49]</sup>.

Our results also show that CFA-treated groups had lower levels of AST and ALT. It was observed that levels of both enzymes were similar for both animal models (BDL + CFA and TAA + CFA), and comparing these levels with healthy rats, no significant differences were found. These results suggest that CFA treatment prevents hepatocellular damage resulting in normal levels of these enzymes with prevention of liver fibrosis.

A study in patients with chronic hepatitis C shows that a daily CFA consumption above 308 mg (approximately 2.25 cups of coffee) was significantly associated with reduced liver fibrosis, and the protective association persisted after controlling for age, sex, race, liver disease, body mass index and alcohol intake in all patients<sup>[12]</sup>.

Our results presented in this report are similar; we showed that fibrosis was successfully prevented in the liver of rats treated with CFA, finding a strong effect of CFA on ECM content in rat liver, showing 80% reduction in the TAA + CFA group and 38% reduction in the BDL + CFA group. Both results show that CFA had a powerful preventive effect on the development of fibrosis. The Modified Histological Activity Index of Knodell resulted in significantly lower fibrosis in both treated groups.

In a previous *in vitro* study, it was found that CFA increases intracellular cAMP, resulting in inhibition of CTGF *via* Smads proteosomal degradation<sup>[18]</sup>. CTGF has similar effects to TGF- $\beta$ 1 as ECM production stimulation, chemotaxis, proliferation and integrin expression. Our *in vivo* data shows that CFA has a strong effect on hepatic CTGF expression, resulting in lower expression of profibrogenic and pro-inflammatory genes. TGF- $\beta$ 1 is a major fibrogenic mediator in which expression is increased in inflamed liver and it is considered the principal fibrogenic component<sup>[47]</sup>. It has been suggested that TGF- $\beta$ 1 up-regulates gene expression of connective tissue, and Col-1 in activated HSC<sup>[6,47]</sup>. Results obtained in CFA-treated groups are very interesting, since in both animal models Col-1 expression was significantly lower, a result that correlates with fibrosis percentage shown for each group with CFA.

It has been observed that liver fibrosis process development is accompanied by inflammation, in which pro-

inflammatory cytokines play an important role in the perpetuation of signaling pathways<sup>[47,50]</sup>. Furthermore, one report in alcoholic liver injury shows that CFA decreased serum and tissue inflammatory cytokines levels<sup>[48]</sup>. In this study we found that induction of both liver fibrosis models had a large amount of inflammatory cell infiltrate; in contrast, CFA-treated groups showed decreased number of inflammatory cells, necroinflammation, CD11b positive areas and TNF- $\alpha$  levels. These results at cellular and molecular levels match with serum and tissue inflammatory cytokines levels in other studies about CFA<sup>[48]</sup>.

IL-1 $\beta$  expression was reduced in CFA-treated groups. ECM signaling is of great importance as it serves as a reservoir of various cytokines such as TGF- $\beta$ 1, TNF- $\alpha$ , platelet-derived growth factor (PDGF), IL-6 and IL-1 $\beta$ , protecting these factors for proteolysis and modulating its bioactivity and bioavailability. In this microenvironment, the cytokines might have a key role in the onset of fibrosis, and perpetuating inflammation<sup>[47]</sup>, where CFA treatment could be useful to break this inflammatory circle, as demonstrated in our different experiments.

HSC have an important role in fibrosis and fibrosis development. HSC activation and proliferation, and collagen synthesis are influenced by factors derived from Kupffer cells (TGF- $\beta$ 1, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-4), endothelial cells (PDGF) and hepatocytes (insulin-like growth factor). Also, TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\beta$ 1, IL-6 and IL-4, and PDGF are regulated by NF- $\kappa$ B and this promotes inflammatory signaling pathway perpetuation<sup>[9-11]</sup>. We found that CFA promotes lower levels of pro-inflammatory cytokines expression. These findings could be due to the fact that CFA prevents HSC activation and ECM production.

ROS activate the NF- $\kappa$ B pathway. It is known that both liver fibrosis induction models used here, course with an oxidative stress state. Because of this, we measured antioxidant enzymes gene expression levels to monitor them with CFA treatment<sup>[51]</sup>.

As expected, untreated groups showed lower expression of antioxidant enzymes SOD and CAT, indicating indirectly an oxidative stress state. CFA-treated rats showed higher levels of antioxidant enzymes, especially of CAT in the BDL + CFA group, that could be explained by the type of substrate metabolized (hydrogen peroxide). SOD catalyzes O<sub>2</sub><sup>-</sup> dismutation into O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>. In contrast, CAT catalyzes decomposition of H<sub>2</sub>O<sub>2</sub> into O<sub>2</sub> and H<sub>2</sub>O<sup>[45]</sup>. Considering this, we assume that CAT was much higher in the BDL + CFA group, due to accumulation of H<sub>2</sub>O<sub>2</sub> at 4 wk of treatment by SOD action. To verify this last effect we performed an assay to measure CAT antioxidant activity, where we found a strong correlation between mRNA expression and enzyme activity, especially in the BDL + CFA group; antioxidant CAT activity was significantly increased ( $P < 0.0001$ ) compared with BDL group.

Along with these results, the significant higher expression of transcription factor Nrf2 in the CFA treated groups supports the evidence of the potent antioxidant

effect of CFA acting as an important hepatoprotector agent in the presence of a chronic organ aggression. These results agree with the report by Boettler *et al*<sup>[52]</sup> where they found higher expression of Nrf2 in humans with consumption of coffee with respect to normal diet where Nrf2 expression was reduced. In response to oxidative stress Nrf2 is activated, translocates to the nucleus and binds to the promoter of its target genes such as CAT and SOD inducing their expression. Nrf2 half life is around 13-20 min. In oxidative stress and in the presence of antioxidant molecules like quercetin, the half-life is duplicated<sup>[53]</sup>. Thus, given that CFA is also an antioxidant molecule, we believe the same thing may be taking place, though it would require additional experiments to test this hypothesis. Nguyen *et al*<sup>[54]</sup> have suggested that in oxidative stress, Nrf2 diminishes its degradation accumulating in nucleus increasing its transcriptional activity.

On the other hand, activation of HSC is a complex process where the transcriptional factor Snail-1 has an important role.

In vertebrates Snail-1 is activated by a different signal pathway from ERK2, NF- $\kappa$ B and phosphatidylinositol 3-kinase<sup>[55-57]</sup>. All these pathways have been involved in activation of HSC. Several authors have reported the overexpression of Snail-1 in pathological conditions associated with ECM deposition<sup>[23,24]</sup>. *In vitro* studies showed that Snail-1 is expressed by HSC and its transcription is augmented *in vitro* and *in vivo* in activated HSC compared with quiescent HSC. At the protein level, the nuclear translocation of Snail-1 in activated HSC was observed<sup>[58]</sup>.

Scarpa *et al*<sup>[25]</sup> reported that the use of an adenovector expressing Snail-1 small-interfering (sh) RNA to silence Snail expression in HSC isolated from mouse, dramatically reduced activation-related genes  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and Col-1 and increased quiescence-related gene peroxisome proliferator-activated receptor, evidencing the important role of Snail-1 in HSC activation (Snail-1 transcription factor *Am J Physiol* 2011). However other studies suggest a multiple cell-type origin of cell source for Snail-1 in human liver fibrosis; thus, this fact should be analyzed. Indeed, it was reported that Snail-1 overexpression induces epithelial mesenchymal transition and siRNA against Snail-1 attenuated this epithelial mesenchymal transition. Immunostaining of fibrotic livers from mice treated with CCl<sub>4</sub> revealed the presence of Snail-1<sup>+</sup>,  $\alpha$ -SMA<sup>+</sup> cells as well as Snail-1<sup>+</sup>  $\alpha$ -SMA<sup>-</sup> and Snail-1- $\alpha$ -SMA<sup>+</sup> cells along the fibrotic septa. This staining pattern could be explained by the epithelial mesenchymal transition process where hepatocytes transdifferentiate to mesenchymal cells resulting in new HSC<sup>[59-61]</sup>.

In the same way, Dooley *et al*<sup>[62]</sup> observed a hepatic marker at the border of the inflamed region from human liver Snail-1<sup>+</sup> cells lacking transferrin and they hypothesize that these cells are hepatocytes in a later stage of transition to mesenchymal cells.

In our results, CFA treatment diminished Snail-1 ex-

pression in rats with chronic liver injury suggesting that CFA prevents HSC activation and suggesting its protector effect on fibrosis development. Our results together allow us to propose CFA use in pathologies with early chronic damage before the establishment of fibrosis.

The observed effect of CFA in this work on necrosis of hepatocytes and on HSC activation could be explained by an indirect effect of CFA. This might be taking place through a decrease of oxidative stress in the liver produced principally by Kupffer cells which secrete cytokines activating HSC.

From the very beginning of its administration, CFA neutralizes free radicals and induces antioxidant molecules production which protect hepatocytes from CCl<sub>4</sub> damage; this means there is less hepatocyte death, reflected in there being lower levels of ALT and AST found in CFA treatment groups. TGF- $\beta$  and TNF- $\alpha$  production is decreased rendering a drop in HSC activation, and consequently, less fibrosis. On the other hand, ECM deposition and loss of microvilli on hepatocytes caused by CCl<sub>4</sub> intoxication blocks the free flow of nutrients causing hepatocytes death. It was found in this paper that CFA treatment yields less fibrosis, less block of nutrients and less hepatocyte death. However, a direct effect of CFA on hepatocytes and HSC cannot be ruled out.

## COMMENTS

### Background

Hepatic stellate cells (HSCs) activation is a major hallmark in liver fibrosis, which is perpetuated by growth factors and pro-inflammatory molecules. Caffeine (CFA) modifies these events *in vitro*.

### Research frontiers

CFA inhibits the transcriptional factor Snail-1, down-regulating profibrogenic genes, and activates the Nrf2 inducing antioxidant enzymes system, preventing inflammation and fibrosis.

### Innovations and breakthroughs

CFA treatment diminished Snail-1 expression in rats with chronic liver injury suggesting that CFA prevents HSC activation and provides a protector effect on fibrosis development. Their results together allow the authors to propose CFA use in pathologies with early chronic damage before the establishment of fibrosis.

### Peer review

The present manuscript provides a detailed study on the effect of CFA on experimental liver fibrosis in rats. Overall, this is a very interesting paper. The presented data is throughout of very good quality and the conclusions drawn are supported by sufficient data.

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## Superficial esophageal lesions detected by endoscopic ultrasound enhanced with submucosal edema

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results for detecting lesions of different depth in the esophageal mucosa.

**METHODS:** A canine (Beagle) model was established in which lesions of different depths were created in the esophageal mucosa by thermal burning. Seventy-two hours later, these lesions and adjacent tissue in the esophagus were examined by EUS. EUS findings including infiltrating depth, strength of echogenicity and homogeneity were recorded. Dogs were sacrificed and tissue specimens were obtained. We then compared the EUS findings with the pathology reports.

**RESULTS:** Thermal burns created at different power settings caused lesions of different depth in the esophageal mucosa. When the echo strength was shifted from high, medium, to low echogenicity, an increase in the infiltrating depth of the lesion was noted, which coincided with results of the pathology examination. Obvious submucosal edema visualized by EUS was also detected by pathology. Furthermore, because of the enhancement caused by the submucosal edema, the lesions invading into the submucosa were easily visualized by EUS.

**CONCLUSION:** There is consistency between EUS findings and pathological results of esophageal lesions with different depths. Submucosal edema can serve as an ultrasonic contrast agent.

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**Key words:** Endoscopic ultrasound; Pathology; Lesion; Esophagus; Canine

### Abstract

**AIM:** To determine if there is consistency between endoscopic ultrasound (EUS) findings and pathological

**Core tip:** Nowadays, endoscopic ultrasound (EUS) is an optimal modality to detect early esophageal cancer (EC); however, it is still unknown whether there is correlation between EUS findings and pathological results.

In this animal study, superficial esophageal lesions with different infiltrating depth in dogs were created by thermal burning. There is consistency between EUS imaging and pathology. The accompanied submucosa edema can sever as an ultrasonic contrast agent.

Li JJ, He LJ, Shan HB, Wang TD, Xiong H, Chen LM, Xu GL, Li XH, Huang XX, Luo GY, Li Y, Zhang R. Superficial esophageal lesions detected by endoscopic ultrasound enhanced with submucosal edema. *World J Gastroenterol* 2013; 19(47): 9034-9042 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9034.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9034>

## INTRODUCTION

Treatment options for esophageal cancer (EC) differ according to the depth of the lesion<sup>[1-4]</sup>. According to a recent edition of the American Joint Committee On Cancer (AJCC) and the International Union Against Cancer (UICC) staging system and guidelines, patients with early EC confined solely to the mucosa (substaged as T1a or Tm) are able to undergo endoscopic mucosal resection; however, patients with lesions invading into the submucosa (sub-staged as T1b or Tsm) require esophagectomy<sup>[5,6]</sup>. Therefore, differentiating the depth or stage of the disease is of great importance preoperatively.

Depending on the frequency strength of the probe that is placed into the lumen of the esophagus, three to seven histological layers of the esophageal wall can be discerned<sup>[7-10]</sup>. In the clinic, endoscopic ultrasonography (EUS) is superior to other modalities such as computed tomography (CT) and positron emission tomography (PET) for distinguishing the tissue layers of the esophageal wall, and it has become the method of choice for determining the depth of esophageal lesions<sup>[11-18]</sup>.

However, EUS has several limitations for detecting esophageal lesions. First, the mucosal layers (including squamous epithelium, lamina propria, and muscularis mucosa) have similar echoic characteristics, especially acoustic impedance. Thus, these layers have similar echogenicity, and it is very difficult to distinguish one from another. Second, because ultrasound propagates a similar speed through the different layers of the esophageal wall, it is difficult for EUS to detect minor differences in ultrasonic energy loss (also presenting as echoic gray scale) among the layers<sup>[19,20]</sup>. Other factors that may influence the efficacy of EUS are the size of the lumen in the esophagus (which prevents the ultrasonic probe from pressing close to the mucosa), the motility of the esophagus, and the experience of the endoscopist<sup>[21,22]</sup>. Therefore, the accuracy of EUS for determining the T stage of EC is poor and diverse in the literatures<sup>[5,6]</sup>. In addition, previous reports did not provide information on the accuracy of EUS for identifying the T1 sub-stage of EC, which is an important factor by which physicians determine treatment. Our team tried to sequentially combine

submucosal saline injection (SSI) with EUS to detect the T1 sub-stage of EC, and our preliminary data revealed that this technique is nearly 90% accurate. Therefore, SSI may enhance EUS for early EC diagnosis<sup>[23,24]</sup>.

However, many questions about the efficacy of EUS for EC diagnosis remain<sup>[25,26]</sup>. For example, does the echo in the EUS reflect the actual structure or component of the esophageal wall? Is there consistency between EUS findings and the results of pathological examinations? What are the echoic characteristics of the water/liquid in the submucosa? Can the water/liquid enhance EUS, and if so, how? To answer these questions, we used different doses of thermal burns to create superficial lesions with different infiltrating depth in the esophageal mucosa, and EUS examinations were conducted to detect these lesions in a canine model.

## MATERIALS AND METHODS

### *Animals and anesthesia*

The experimental protocol used in this study was approved by the animal welfare and ethics committee of Sun Yat-sen University Cancer Center (approval number: GZR2012-114). Male adult dogs (10 kg) were provided by the medical animal center in the north campus of Sun Yat-sen University. The flow diagram of this study is shown in Figure 1. The dogs were kept separately with an absolute diet for 8 h and dehydrated for 6 h before the experiment. Dogs were then injected intraperitoneally with 0.03 mg/kg pentobarbital sodium for premedication and then injected peritoneally with 0.03 mg/kg pentobarbital sodium per hour for maintenance.

### *Devices*

EUS examinations were performed using an Olympus GF-UM2000 endoscopy system with a 12 MHz ultrasonic probe (Olympus Co. Ltd., Japan). An Endoscopic Electrosurgical Workstation was purchased from ERBE Co. Ltd., Germany, which included an argon plasma coagulation (APC) system and a high frequency electrocoagulation generator (HFE).

### *Canine model of superficial lesions with different infiltrating depths in the esophagus*

Guided by endoscopy, esophageal lesions of variable infiltration depths were induced in anesthetized dogs using APC (40 W, 1.4 L/min, 2 s each time), short-time HFE (40 W, 2 s each time), medium-time HFE (40 W, 5 s each time), and long-time HFE (40 W, 10 s each time). Superficial round lesions of approximately 1 cm × 1 cm were formed, and the gap between lesions was approximately 5 cm. Seventy-two hours later, the dogs underwent EUS examination. The echoic characteristics of the lesions with different infiltrating depths (including the echogenicity of the lesions, leading and trailing edge, the echogenicity of each layer in the esophageal wall, *etc.*) and submucosal edema were recorded. Then, the dogs were sacrificed, and samples of the normal and abnor-

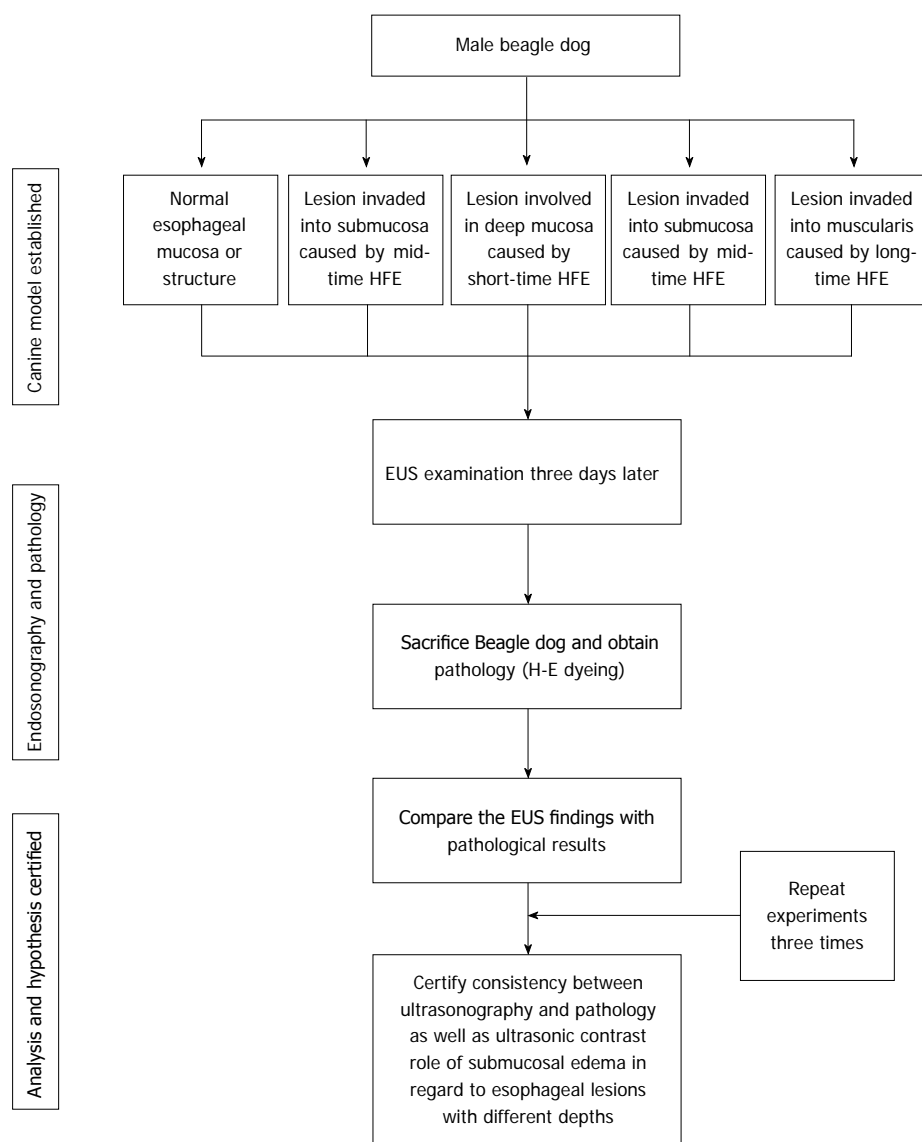


Figure 1 Flow diagram of study protocol. EUS: Endoscopic ultrasonography; HFE: High frequency electro-coagulation generator.

mal esophagus containing superficial lesions with variable infiltrating depths were extracted and stored in 10% formaldehyde solution. The specimens were stained with hematoxylin and eosin (HE). In addition, we focused on submucosal edema and its role as an ultrasonic contrast agent. We compared the EUS findings with the corresponding pathological results to determine whether both were in agreement. Details regarding the creation of this canine are presented in Figure 2. Examinations were performed by an endoscopic expert with over 10 years of experience. Similarly, pathological examinations were performed by an expert with over 10 years of experience. The above experiment was repeated three times.

## RESULTS

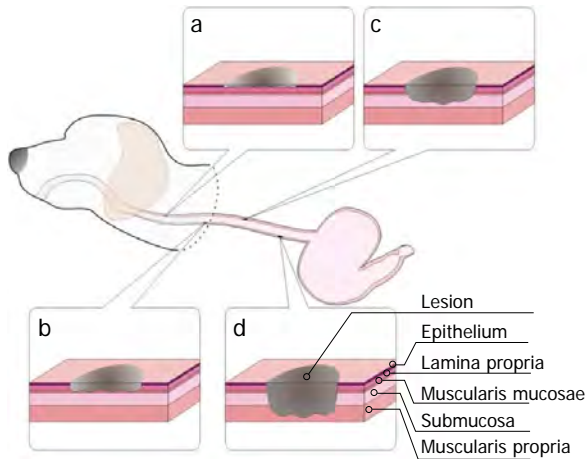
### **Thermal burns caused superficial lesions in the esophagus with different infiltrating depths**

After exposure to APC and HFE at different power levels, lesions in the esophageal mucosa could be observed,

although the clarity was poor due to obvious congestion and edema in the adjacent mucosa. Seventy-two hours later, the edema in the surrounding mucosa decreased, and the lesions became more apparent. However, we only observed lesions in the lumen and could not confirm the exact burn depths by ordinary endoscopy. Therefore, we proceeded with EUS examination.

### **EUS examination of lesions and submucosal edema in the esophagus**

Three layers of the normal esophagus can be visualized using EUS with a 10 MHz ultrasonic probe: the thickest, high echoic belt is revealed as the first layer, which corresponds to the mucosa and submucosa; a thick, low echoic belt corresponding to muscularis propria; and a narrow, high echoic belt that is thought to be the adventitia and other dense connective tissue (Figure 3A). In the canine model, different power levels can cause different burns with variable infiltration depths, as visualized by EUS. APC (Figure 4A), short-time HFE (Figure 4C),



**Figure 2** Schematic diagrams of superficial lesions in the esophagus with different depths in a canine model.

medium-time HFE (Figure 5A), and long-time HFE (Figure 5C) resulted in superficial mucosal injury, deep mucosa injury, injury involving the submucosa, and injury invading into the muscularis propria, respectively. The lesions presented as middle to low echogenicity, which were lower and more asymmetrical echoic compared to the normal mucosa on sonography, particularly in the case of lesions invading into the muscularis propria. Additionally, we found that the high echogenicity of the mucosa was related to the integrity of the squamous epithelium, especially the keratin pearl and intercellular bridge. Once these keratin pearls or intercellular bridges disappeared, the echo of the lesion decreased as shown in Figures 3B, 4B and 5B. Furthermore, a low echoic belt was the evidence of edema in the submucosa of the lesions, except in the lesions invading the muscularis propria. The edema was observed as a low echoic belt with diverse light spots, which separated the mucosa and submucosa, as shown in Figures 4A, C, and 5C. In addition, echo enhancement was observed in the trailing edge of the low echoic belt, confirming that the low echoic belt was water-filled tissue in the submucosa. Therefore, due to the contrast of the low echoic edema, the layer of muscularis propria was displayed as a smooth, middle echo belt. However, in the case of lesions involving the muscularis propria, there was no low echoic belt in the submucosa and no obvious boundary among the layers of the esophagus.

### Pathological results

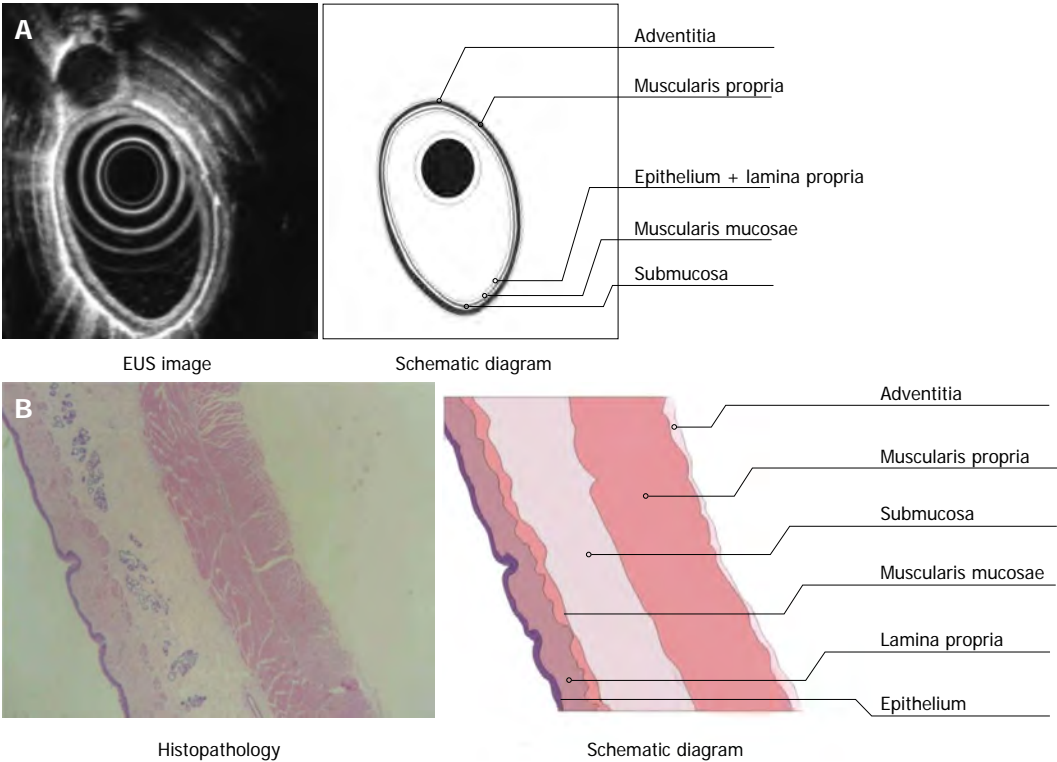
There were, in successive order, squamous epithelium, lamina propria, muscularis mucosa, submucosa, and muscularis propria and adventitia in the normal esophageal wall of dogs. The squamous mucosa was thick, with a distinct keratin pearl, intercellular bridges, and thin lamina propria and muscularis mucosa. The submucosa was also thick, and it was characterized by blood vessels, lymphatic and connective tissues. The muscularis propria contained a ring muscle layer (inner) and a longitudinal

muscle layer (outer), divided by thin connective tissue. The adventitia was composed of fibrous connective tissue (Figure 3B). Tissue sections of the lesions containing superficial mucosa showed partial epithelial degeneration with a complete squamous component and intact muscularis mucosa. Prominent submucosal edema was observed, and there were no obvious inflammatory cells in the submucosa (Figure 4B). Similarly, total degeneration was observed in the mucosa, with a partial squamous cell component and broken muscularis mucosa. Prominent submucosal edema was also found in the lesions containing deep mucosa. There were no obvious inflammatory cells in the submucosa (Figure 4D). In lesions that invaded into the submucosa, the squamous cell structure and muscularis mucosa disappeared with submucosal edema, and inflammatory cells were clearly present in the submucosa (Figure 5B). For lesions that invaded into the muscularis propria, the squamous cell structure and muscularis mucosa disappeared; however, this was not accompanied by submucosal edema and inflammatory cells in the submucosa (Figure 5D).

### Consistency between EUS findings and pathology

In this study, echogenicity reflected characteristics of tissues propagated by ultrasound (Table 1). First, there was a correlation between the echoic belts or layers in the tissue sections. The esophageal mucosa and submucosa of dogs presented as a high echoic belt on sonography with a 10 MHz probe; the second low echoic belt corresponded to the muscularis propria; and the adventitia (mainly composed of dense fibrous connective tissue) was observed as a thin high echoic belt by EUS. Second, the ultrasonic echo decreased with increasing infiltration depth, and the inner echogenicity of the lesion changed from homogeneous to heterogeneous. The echoic changes (high-middle-low) corresponded to gradual changes of the lesions with intact squamous epithelium (complete, incomplete, and totally broken) and the different infiltrating depths of the lesions (located in the mucosa, involving the submucosa, and invading into the muscularis propria). Third, the identification of submucosal edema by sonography and pathology was highly correlated. Submucosal edema was obvious in lesions which were located in the mucosa, whereas the submucosal edema was narrow in lesions involving the submucosa. However, there was no submucosal edema in lesions involving the muscularis propria. With the help of submucosal edema, the infiltrating margin of the lesion was significantly distinguished. Therefore, we were able to easily judge whether a lesion invaded into the submucosa. After long-time HFE thermal burns, a lesion with a low echoic belt extending from the lumen to the second layer was difficult to distinguish from the adjacent tissue. Pathology revealed that the lesion already invaded into the muscularis propria, and it contained a complex composition of inflammatory cells and blood/lymphatic vessels. In addition, using the contrast of submucosal edema or low echoic belt, the layers of the esophagus





**Figure 3** Endoscopic ultrasonography and tissue examination of the normal esophagus in a beagle dog. A: The three layers of a normal esophagus, as visualized by endoscopic ultrasonography (EUS); B: Tissue examination showed that the esophageal wall is composed of the mucosa (including squamous epithelium, lamina propria, and muscularis mucosa), submucosa, and muscularis propria and adventitia.

Table 1 Identification of esophageal lesions with different depths was consistent between the endoscopic ultrasonography findings and pathology results								
	Tissue echogenicity					Echogenicity of submucosal edema		
	Echoic belts of esophagus	Echo strength of lesion	Involved layers	Homogeneity of lesion	Boundary among layers	Submucosal edema belt	Front edge of edema belt	Width of edema belt
EUS findings	H-L-H	/	/	/	Clear	/	/	/
Normal mucosa								
Superficial mucosa	H-L-H	H	1 <sup>st</sup>	Homogeneous	Clear	YES	Smooth	Wide
Deep mucosa	H-L-H	H	1 <sup>st</sup>	Homogeneous	Clear	YES	Less Smooth	Middle
Submucosa	M-L-H	M	1 <sup>st</sup>	Heterogeneous	Clear	YES	Unsmooth	Narrow
Muscularis propria	L-H	L	1 <sup>st</sup> -2 <sup>nd</sup>	Chaotic	Dim	None	None	None
Pathology results								
Normal	Mu/SM-MS-AD	/	/	/	Clear	/	/	/
Superficial mucosa	Mu/SM-MS-AD	Mu	MU	Homogeneous	Clear	Yes	Smooth	Wide
Deep mucosa	Mu/SM-MS-AD	Mu	MU	Homogeneous	Clear	Yes	Less smooth	Middle
Submucosa	SM-MS-AD	SM	Mu-SM	Heterogeneous	Clear	Yes	Unsmooth	Narrow
Muscularis propria	MS-AD	MS	MU-SM-MS	Chaotic	Dim	None	None	None

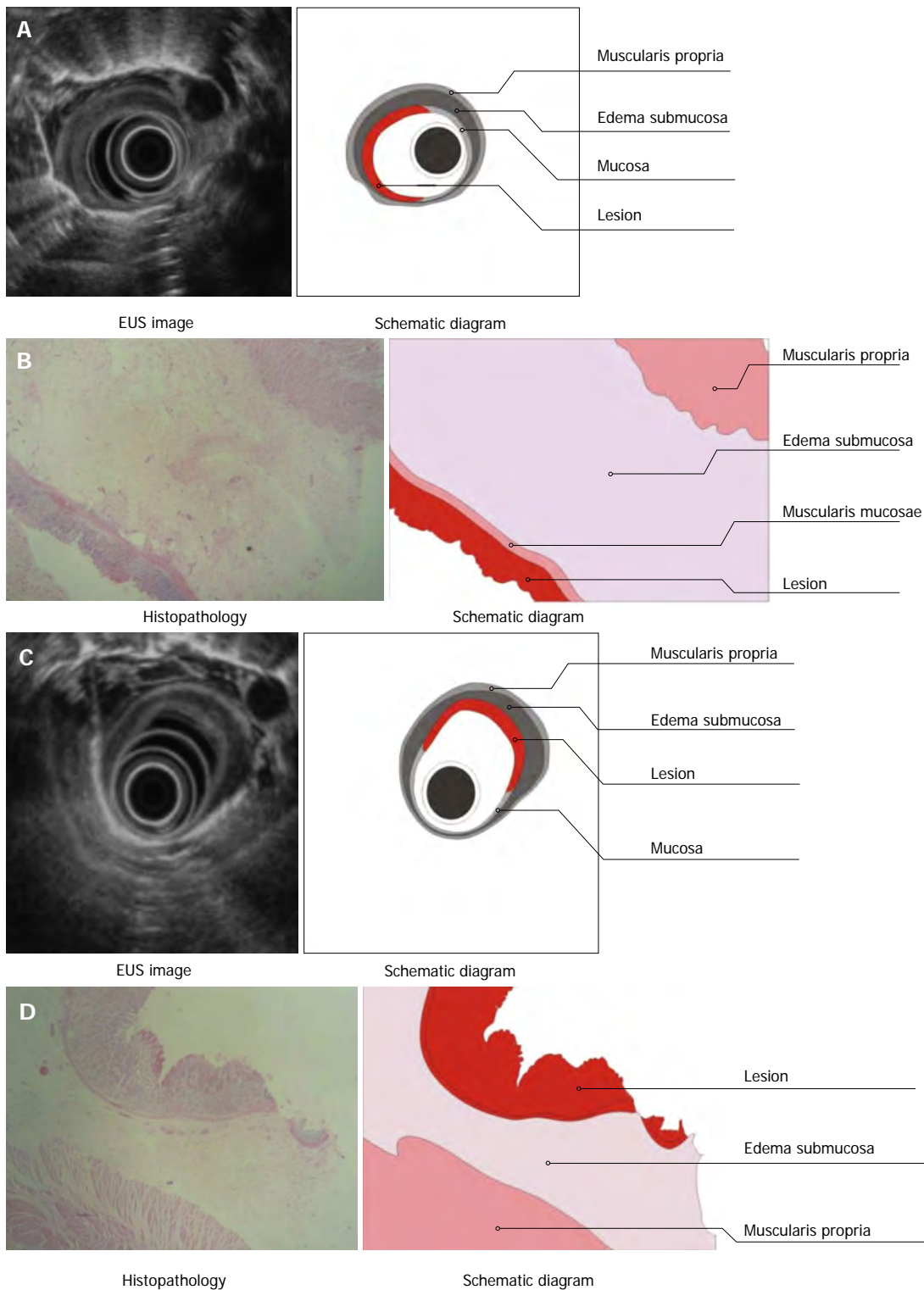
MU: Mucosa; SM: Submucosa; MS: Muscularis propria; AD: Adventitia; H: High echogenicity; L: Low echogenicity; M: Middle echogenicity.

were apparent using both sonography and pathology.

**Submucosal edema serves as an ultrasonic contrast agent**

As stated above, thermal burns in the esophageal mucosa of dogs caused lesions with different depths and different degrees of submucosal edema (except in the case of lesions invading into the muscularis propria). Submucosal edema displayed as a smooth, low echoic

belt between the lesions and the layer of muscularis propria. Because water or liquid is a good medium for ultrasound, with trivial loss in ultrasonic energy, the mucosa was easily distinguished from the submucosa under condition of submucosal edema. The smooth leading edge of the submucosal edema identified lesions that did not invade into the submucosa, whereas an irregular leading edge of the submucosal edema indicated that the lesion invaded into the submucosa.

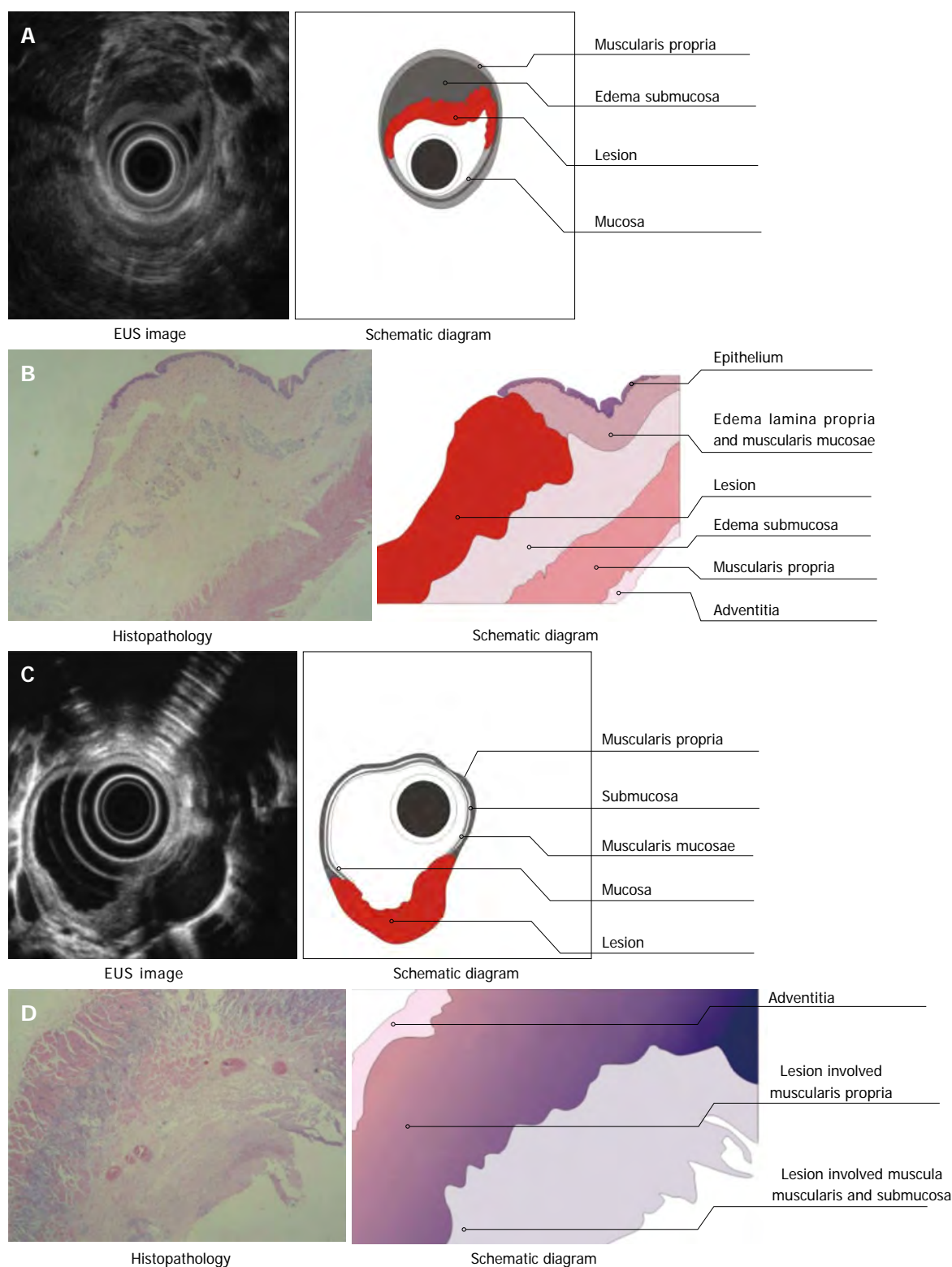


**Figure 4** Endoscopic ultrasonography and tissue examination of esophageal lesions located in the superficial mucosa (A, B) and deep mucosa (C, D). A, C: Endoscopic ultrasonography (EUS) imaging: high echoic lesions located in the mucosa (A) and relatively high echoic lesion (C) located in the mucosa with obvious submucosal edema, as visualized by EUS; B, D: Pathology: tissue examination showed that the lesions were located in the mucosa with complete (B) and incomplete (D) squamous epithelium, intact muscularis mucosa, and obvious submucosal edema.

## DISCUSSION

The strength of echogenicity depends on the energy of the reflection to the ultrasonic probe; when the reflection energy is higher, the gray scale of echogenicity is

stronger<sup>[27,28]</sup>. In addition, the energy of the reflected ultrasound is correlated to the difference in acoustic impedance between the interfaces; when the difference is larger, the reflection is stronger and the echogenicity is higher. The difference of acoustic impedance depends



**Figure 5** Endoscopy, endoscopic ultrasonography, and tissue examination of an esophageal lesion invading into the submucosa (A, B) and muscularis propria (C, D). A, C: Endoscopic ultrasonography (EUS) imaging: Middle echoic lesion (A) and low echoic lesion (C) invading the submucosa with obvious submucosal edema, as visualized by EUS; B, D: Pathology: Lesion invading into the submucosa (B) and muscularis propria (D) was characterized as squamous epithelium with disappearing muscularis mucosa and submucosal edema, as revealed by pathological examination.

on the tissue gradient. Generally, a homogeneous gradient in tissue reflects a small acoustic impedance difference, and the reflection of ultrasonic energy is small and the echoic gray scale is low. Alternatively, complicated

tissue composition means that there are large acoustic impedance differences between interfaces, the ultrasonic energy is high and the echogenicity is strong<sup>[20,22]</sup>. It has been well established that the thickness of the

ultrasonic image is in direct proportion to ultrasonic propagation time. Under conditions of similar ultrasonic transmission speed in soft tissue, we can assume that the thickness of the ultrasonic image is equal to the actual thickness. In fact, the ultrasonic image corresponds to the histological characteristics of the tissue due to the pathway of ultrasound propagation, such as extracellular water, tissue density, histological type, vessels, adipose tissue, keratin pearls, and intercellular bridges in squamous cell epithelium<sup>[23]</sup>.

Therefore, the high echo belt on sonography means that there are complicated gradients and a significant difference in acoustic impedance, thus corresponding to the mucosa (including the squamous cell epithelium with keratin pearl and intercellular bridges and muscularis mucosa, which is thin and has just one-fold muscular tissue) and the submucosal layer (composed of a complex gradient of vessels, lipid, and other soft connective tissue). The second low echoic belt indicates that the homogeneous tissue is mainly composed of muscular cells, with only a trivial difference in acoustic impedance and no significant difference between interfaces; thus, this low echoic echo corresponds to the muscularis propria. The thin, third strong echoic belt is indicative of dense tissue with a significant difference in the acoustic impedance between tissues; thus, it corresponds to the adventitia and other dense connective tissue<sup>[4]</sup>.

The results of this study not only confirmed that thermal burns created at different energy levels caused superficial lesions with different infiltration depths but also that the EUS findings corresponded with pathological results in a canine model. Furthermore, our results indicate that submucosal edema separates the mucosa and submucosa, which caused drastic changes in acoustic impedance between the layers of the esophagus. Moreover, submucosal edema increased the thickness of the esophagus, allowing the layers to be definitively identified by sonography. Therefore, extracellular water or edema served as an ultrasonic contrast agent (negative role). Although the lesions caused by thermal burns in the canine model differ from actual EC, we can detect EC lesions using submucosal extracellular saline or fluid injection to enhance the accuracy of EUS. This is especially useful to distinguish T1a and T1b EC in the clinic. Our team tried to combine SSI with EUS examination to increase the accuracy of EUS for the staging and sub-staging of early esophageal squamous cell carcinoma preoperatively.

The esophagus of dogs has a thicker squamous cell epithelium and muscularis propria, as well as a thinner lamina propria and muscularis mucosa than that of human beings<sup>[23]</sup>. The large number of interfaces in the squamous epithelium, such as keratin pearls, extracellular bridges, vessels and lipid tissue in the submucosa, can cause strong ultrasonic reflection. Therefore, the first layer on sonography displayed as a high echoic belt with a 10 MHz ultrasonic probe; the mucosa and the submucosa were present as a high echoic belt, and they were difficult to distinguish from each other. Hence, the first

low echoic belt includes the mucosa and submucosa in the normal esophagus of dogs. Once the lesion invades the submucosa, sonography cannot easily distinguish the lesion from the submucosa<sup>[15,16]</sup>. In fact, physicians are predominantly concerned with determining whether the lesions have already invaded into the submucosa or into deeper layers because EC patients with submucosa invasion are not eligible for endoscopic resection and must have esophagectomy<sup>[29]</sup>. With the contrast of fluid in the submucosa, lesions invading into the submucosa were easily identified by EUS.

In this study, we planned to perform SSI sequentially with EUS after thermal burning at different energy levels created superficial lesions of different infiltration depths. However, in pre-experiments, we found that lesions caused by thermal burning led to significant submucosal edema, so SSI was not needed to perform in order to enhance EUS. Furthermore, as the mucosa recovered (generally longer than two weeks), the submucosal edema gradually vanished. Therefore, performing SSI after the disappearance of submucosal edema was unnecessary because the mucosa had already recovered, with no remaining lesions. Furthermore, at 72 h post-thermal burning, the superficial lesions were obvious, whereas the mucosal edema had subsided, and the submucosal edema produced an effect similarly to the saline cushion caused by SSI.

The identification of esophageal lesions with different depths using ultrasonic technology is consistent with pathological results, demonstrating that the submucosal edema can serve as an ultrasonic contrast agent.

## COMMENTS

### Background

It is well known that treatment for esophageal cancer (EC) differs according to the depth of the lesion since the T1 stage or sub-stage of EC depends on the invading depth in early EC. Endoscopic ultrasonography (EUS) is the most common modality to stage early EC preoperatively.

### Research frontiers

There are many questions about the effectiveness of EUS for EC diagnosis especially in consistency between sonographic and pathological results.

### Innovations and breakthroughs

This study confirmed that there was consistency between EUS findings and pathological results of esophageal lesions with different depths. Submucosal edema can serve as an ultrasonic contrast agent.

### Applications

Using submucosal saline as an ultrasonic contrast agent, the physicians may employ a novel technique-submucosal saline injection (SSI) to enhance the accuracy of EUS for staging or sub-staging early EC in clinic.

### Terminology

EUS is a medical procedure in which endoscopy (insertion of a probe into a hollow organ) is combined with ultrasound to obtain images of the internal organs in the chest and abdomen. It can be used to visualize the walls of these organs, or to look at adjacent structures. Endoscopic ultrasonography is most commonly used in the upper digestive tract. SSI is a technique prior to endoscopic treatment for early EC to avoid damage to adjacent tissues.

### Peer review

The authors present interesting data on the accuracy of EUS assessment of thermal esophageal burns, facilitated by submucosal edema in a canine model. Further studies will be required to determine the utility of submucosal fluid en-



hanced EUS examination of esophageal carcinoma.

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## Small bowel tumors detected and missed during capsule endoscopy: Single center experience

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### Abstract

**AIM:** To characterize small bowel (SB) tumors detected by capsule endoscopy (CE), and identify missed tumors.

**METHODS:** The study included 145 consecutive patients in whom 150 CEs were performed. Following CE, the medical records of the study population were reviewed. Results of double- or single-balloon enteroscopy performed after CE and the results of surgery in all

patients operated on were retrieved. The patients were contacted through telephone interviews or postal mail. In addition, the national cancer registry and the polish clinical gastrointestinal stromal tumor (GIST) Registry were searched to identify missed neoplasms.

**RESULTS:** Indications for CE included overt and occult obscure gastrointestinal bleeding ( $n = 81$ , 53.7%), anemia ( $n = 19$ , 12.7%), malabsorption ( $n = 18$ , 12%), abnormal CB follow through ( $n = 9$ , 6%), abdominal pain ( $n = 7$ , 5%), celiac disease ( $n = 5$ , 3%), neuroendocrine tumor ( $n = 3$ , 2%), Crohn's disease ( $n = 2$ , < 2%), Peutz-Jeghers syndrome ( $n = 2$ , < 2%), other polyposes ( $n = 2$ , < 2%), and diarrhea ( $n = 2$ , < 2%). The capsule reached the colon in 115 (76.6%) examinations. In 150 investigations, CE identified 15 SB tumors (10%), 14 of which were operated on or treated endoscopically. Malignancies included metastatic melanoma ( $n = 1$ ), adenocarcinoma ( $n = 2$ ), and GIST ( $n = 3$ ). Benign neoplasms included dysplastic Peutz-Jeghers polyps ( $n = 4$ ). Non-neoplastic masses included venous malformation ( $n = 1$ ), inflammatory tumors ( $n = 2$ ), and a mass of unknown histology ( $n = 1$ ). During the follow-up period, three additional SB tumors were found (2 GISTs and one mesenteric tumor of undefined nature). The National Cancer Registry and Polish Clinical GIST Registry revealed no additional SB neoplasms in the post-examination period (follow-up: range 4.2-102.5 mo, median 39 mo). The sensitivity of CE for tumor detection was 83.3%, and the negative predictive value was 97.6%. The specificity and positive predictive value were both 100%.

**CONCLUSION:** Neoplasms may be missed by CE, especially in the proximal SB. In overt obscure gastrointestinal bleeding, complementary endoscopic and/or radiologic diagnostic tests are indicated.

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**Key words:** Capsule endoscopy; Small bowel tumor; Tumor miss rate; Gastrointestinal bleeding; Gastrointestinal stromal tumor

**Core tip:** The aims of this study were to characterize small bowel (SB) tumors detected by capsule endoscopy (CE) and identify SB tumors missed by CE. The study included 150 consecutive CE investigations. Following CE, the medical records of the study population were reviewed and the patients contacted by telephone or postal mail. National cancer registries were searched to identify missed neoplasms. CE detected 15 SB tumors (10%). During the follow-up period, three additional SB tumors were found. The sensitivity of CE for tumor detection was 83.3% and the negative predictive value 97.6%. The specificity and positive predictive value were both 100%.

Zagorowicz ES, Pietrzak AM, Wronska E, Pachlewski J, Rutkowski P, Kraszewska E, Regula J. Small bowel tumors detected and missed during capsule endoscopy: Single center experience. *World J Gastroenterol* 2013; 19(47): 9043-9048 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9043.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9043>

## INTRODUCTION

Capsule endoscopy (CE) has become a first-line diagnostic tool in obscure gastrointestinal bleeding (OGIB) when the small bowel (SB) is a suspected source. Compared with push enteroscopy (PE), which is performed to establish the source of bleeding, CE detects more than twice as many clinically-significant abnormalities (56% *vs* 26%), whereas any abnormalities are detected in 63% with CE *vs* 28% with PE<sup>[1]</sup>. Balloon-assisted enteroscopy (BAE), most often double-balloon enteroscopy (DBE), is performed following both a negative CE or as a complementary procedure guided by the CE findings. Initial studies suggested that CE and DBE have a comparable diagnostic yield in patients with suspected SB disease, including OGIB, when the whole SB is visualized<sup>[2]</sup>. Now evidence is growing that CE misses a significant number of lesions detected on enteroscopy<sup>[3,4]</sup>. In a recent meta-analysis, the yield of DBE after previously-negative CE was 27.5%<sup>[5]</sup>. Nevertheless, CE remains the preferred initial diagnostic test because of its noninvasiveness, better tolerance, and ability to view the entire SB.

SB tumors are source of bleeding in some patients with OGIB, particularly younger patients. In a large series of patients undergoing CE, SB tumors were found in 2.4% (Rondonotti *et al*<sup>[6]</sup>), 8.9% (Cobrin *et al*<sup>[7]</sup>), 6.3% (Bailey *et al*<sup>[8]</sup>), and 4.3% (Cheung *et al*<sup>[9]</sup>) of cases. Malignant tumors were found in 4.2%, 4%, and 2.7% of patients, respectively. In a multicenter Belgian study, the percentage of malignant tumors was 2.5%<sup>[10]</sup>. The percentage of DBE procedures detecting SB tumors is higher than with

CE, increasing up to 12% (27 of 225 patients in Choi *et al*<sup>[11]</sup>) and up to 13.9% in the largest series described, comprising 1035 Japanese patients of whom 42.4% were examined because a SB tumor was suspected<sup>[4]</sup>.

A retrospective review of 183 cases in which DBE was performed at 7 North American centers found that DBE identified SB tumors in 15 patients who had prior CE, whereas lesions were found by CE in only 5 patients, and all 4 cases of primary adenocarcinoma were missed by CE<sup>[3]</sup>.

We performed a retrospective study to characterize SB tumors detected in consecutive patients who underwent CE at our center. The second aim of this study was to identify any SB tumors missed by CE in these patients.

## MATERIALS AND METHODS

The results of all consecutive CE examinations (PillCam SB1, Given Imaging, Israel), which were assessed by two readers between March 2003 and July 2009 at a single center, were reviewed and categorized. In a standard evaluation, CE findings were further classified as negative or positive. Positive findings were also classified as clinically significant or insignificant lesions. Clinically significant lesions included angioectasias, tumors or polyps  $\geq$  10 mm, active bleeding, blood clots, diverticula, mucosal breaks, and features consistent with celiac disease. Clinically insignificant lesions included red spots, white spots, erythema, focal atresia of villi, or small polyps.

As CE allows for only an approximate estimation of polyp size, a cut-off polyp diameter of 10 mm was used; this size is an accepted indication for polyp resection in patients with polyposis syndromes.

The preparation for CE included fasting from lunch-time and ingesting 3 L of glycol polyethylene the day before the examination. The patients ingested the capsule in the morning with 50 mL of water and 0.5 mL of simethicone (Espumisan, Berlin Chemie, Poland). The patients were allowed to drink more water no earlier than 2 h after capsule ingestion and eat no earlier than 4 h after capsule ingestion.

Follow-up data were obtained by reviewing hospital case notes. Results of push, double-balloon, single-balloon, and intraoperative enteroscopy performed following CE, and the results of any surgery performed were retrieved. Following the analysis of records, an attempt to contact the patients by phone or mail was made using a standardized interview. The questions referred to any serious diseases diagnosed following CE, including cancer, and operations performed.

Finally, the National Cancer Registry and the Polish clinical gastrointestinal stromal tumor (GIST) Registry were searched to identify any neoplasms possibly missed in the study population.

The study was approved by the institutional review board in accordance with the guidelines of the Declaration of Helsinki revised in 1989.



**Table 1 Characteristics of the study population *n* (%)**

Gender, males	71/145 (49)
Age	
(min, max)	(8, 85)
mean (SD)	50.1 (19.2)
Main indication for CE <sup>1</sup>	<i>n</i> = 150 <sup>2</sup>
Overt obscure bleeding	58 (38.7)
Occult obscure bleeding	23 (15.3)
Anemia	19 (12.7)
Malabsorption	18 (12.0)
Abnormal SB follow through	9 (6.0)
Abdominal pain	7 (4.7)
Celiac disease	5 (3.3)
Neuroendocrine tumor	3 (2.0)
Peutz-Jeghers syndrome	2 (1.3)
Diarrhea	2 (1.3)
Crohn's disease	2 (1.3)
Polyposis syndrome	2 (1.3)

<sup>1</sup>Primary indication was given; <sup>2</sup>Capsule endoscopy (CE) was performed twice in 5 patients for the following reasons: incomplete examination (2 patients); recurring overt obscure gastrointestinal bleeding (OGIB) in a patient with normal first examination (1 patient) and recurring overt gastrointestinal bleeding in patients with abnormal first CE result and treatment instituted (mucosal breaks, angioectasias, 2 patients).

## RESULTS

Over the study period, 145 patients underwent 150 CEs. The characteristics of the patients, including the indications for CE, are presented in Table 1. The most frequent indication for performing the procedure was OGIB (81 patients; 53.7%), which was occult in 23 patients (15.3%) and overt in 58 (38.4%).

The capsule reached the colon in 115 (76.6%) examinations. CE revealed no abnormalities in 29 (19.3%) procedures, was abnormal and clinically significant in 82 (54.6%), and abnormal but insignificant in 37 (24.7%) procedures. No conclusions were drawn in 2 cases (1.3%). In the initial studies, the cleansing conditions were not routinely assessed by the reader, so this parameter could not be reported for the whole study population. The results of the 150 procedures are shown in Table 2.

Tumors  $\geq 10$  mm were identified in 15 patients (10%). Fourteen tumors were surgically or endoscopically resected. The characteristics of these patients are presented in Table 3. Of the 14 resected tumors, 6 were malignant (4%), 4 were benign (2.6%), and 3 were non-neoplastic (2%) and the precise histology of one non-malignant tumor was not retrieved. The most frequent indication for CE that resulted in tumor detection was overt OGIB (6 patients).

Longer follow-up was available for 139 patients (95.8%). Sixteen patients died (11%). In 6 patients (4.1%), the medical records were unavailable or the patient could not be contacted by phone or mail. However, they were included in the registries search. The median observation time of the living patients in whom the follow-up was performed was 39 mo (*n* = 124, range 4.2-102.5 mo).

It was established that CE missed 2 SB GISTs and

**Table 2 Results of 150 capsule endoscopy examinations *n* (%)**

Findings	<i>n</i> = 150
Significant findings	
Angioectasias	25 (16.7)
Mucosal breaks	20 (13.3)
Tumor or polyp(s) $\geq 10$ mm	15 (10.0)
Diverticula	14 (9.3)
Celiac disease	5 (3.3)
Active bleeding with no visible origin	3 (2.0)
Insignificant findings	
Erythema or red spots	15 (10.0)
White spots	13 (8.7)
Other	7 (4.7)
Modeling of the bowel wall	2 (1.3)
Normal	29 (19.3)
Non-diagnostic	2 (1.3)

one SB mesenteric tumor. All three patients underwent CE due to overt OGIB.

In one patient, PE up to the ligament of Treitz was performed before CE and duodenal lymphangiectasis were seen. CE examination was complete and normal, but cleansing of the distal SB was poor. Following CE, CT angiography was performed and active SB bleeding was observed in the right mid-abdomen and a lesion within the ileocecal artery was suggested. Immediate surgery revealed bleeding in Meckel's diverticulum, and a non-bleeding jejunal 4-cm GIST that was 15 cm behind the ligament of Treitz. The mucosa covering the tumor was normal.

In the second patient CE was complete, but the SB cleansing was poor. On CE a diverticulum in the left mid-abdomen was seen. Subsequent laparoscopy revealed a 4-cm SB tumor that appeared to be a GIST. Unfortunately, the exact tumor location was not assessed. This patient did not undergo enteroscopy.

In the third patient, upper DBE was performed before CE and 150-170 cm of SB inspected. Upon withdrawal, a small clot firmly attached to the mucosa in the proximal jejunum was observed. A possible iatrogenic lesion was suspected and argon plasma coagulation performed. No other abnormalities were detected. Subsequent CE was complete and normal, however, contrast abdominal CT performed 11 mo later revealed a mass located between the pancreatic head and duodenum.

On laparotomy, a diagnosis of non-resectable mesenteric tumor was made, but intraoperative cytology and later histology did not confirm neoplastic disease. After 6 mo of observation without progression of the disease, the patient was lost to follow-up.

In addition, the National Cancer Registry and the Polish Clinical GIST Registry were searched for 144 patients (99.3%) whose national identity number was available, and this search did not identify any other (missed) SB neoplasms during the post-examination period. A plasmocytoma was diagnosed 14 mo after a normal CE in a female who underwent the procedure due to occult OGIB; this patient died 4 mo after the cancer diagnosis.



**Table 3** Characteristics of patients with tumor or polyp(s)  $\geq 10$  mm detected on capsule endoscopy and the results of follow-up

No.	Age (yr)	Sex	Indication for CE	Bleeding on CE	CE reached the colon	DBE result	Surgery result	Neoplasm
1	21	F	PJS	-	√	NA	Dysplastic Peutz-Jeghers polyp	yes
2	25	M	Polyposis	-	-	NA	Dysplastic Peutz-Jeghers polyp	yes
3	30	M	Overt OGIB	-	-	NA	Meckel's diverticulum	no
4	36	F	Occult OGIB	√	-	NA	SB adenocarcinoma	yes
5	37	F	Occult OGIB	-	-	Dysplastic Peutz-Jeghers polyp in jejunum	Not operated on	yes
6	47	M	Malabsorption	√	√	NA	Unknown but benign	unknown
7	50	M	Abnormal SBFT	-	√	NA	Not operated on	unknown
8	53	M	Overt OGIB	√	-	NA	GIST	yes
9	53	M	PJS	-	√	NA	Dysplastic Peutz-Jeghers polyp	yes
10	56	M	Anemia, disseminated melanoma malignum	-	-	NA	Melanoma malignum metastasis	yes
11	59	F	Overt OGIB	√	√	Submucosal tumor	Venous malformation	no
12	60	F	Overt OGIB	-	√	NA	GIST	yes
13	64	F	Overt OGIB	-	√	NA	SB adenocarcinoma	yes
14	68	F	Occult OGIB	-	-	NA	Inflammatory tumor	no
15	68	F	Overt OGIB	-	√	NA	GIST	yes

CE: Capsule endoscopy; NA: Not applicable; DBE: Double-balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding; SBFT: Small bowel follow through; PJS: Peutz-Jeghers syndrome; GIST: Gastro-intestinal stromal tumor; M: Male; F: Female.

Based on these data, in a per patient analysis the sensitivity of CE for tumor detection was 83.3% and the negative predictive value was 97.6%. The specificity and positive predictive value were both 100%.

## DISCUSSION

We performed a retrospective study of consecutive patients who underwent CE at our center for various reasons. We then followed these patients and found three tumors missed by CE. To the best of our knowledge, this is the first study with such a specific, tumor-oriented follow-up. The percentage of tumors found in our study (10%) was higher than in other CE series, which may be explained by the strict selection of patients who undergo CE at our center<sup>[6-10]</sup>. This may be the result of a lack of reimbursement for CE by the national health care system. OGIB, for which CE had the highest diagnostic yield, was the indication for CE in 81 (53.7%) examinations in our series and CE resulted in tumor detection in 9 OGIB patients (11.1%). The diagnostic algorithm included an upper and lower endoscopy and push enteroscopy. The latter was performed in 35 (24.1%) patients before CE and was negative, which eliminated proximal intestinal vascular abnormalities, making a tumor diagnosis more likely. In the studies which analyzed only OGIB patients, a SB mass was found in 10%<sup>[12]</sup> and 7.18%<sup>[13]</sup> of cases. In the CE results in the study by Cobrin *et al*<sup>[7]</sup>, SB tumors were detected in 9% of patients with OGIB and the number of OGIB patients in the other CE series cited was not given.

In our study, the median follow-up was slightly over 3.2 years (39 mo). This seems sufficient for a serious symptomatic diagnosis, which might have been missed by

CE, to be made during complementary investigations.

During follow-up, we found two cases of GIST in the SB not detected by CE. Both lesions were diagnosed intra-operatively. The first lesion was located in the proximity of Treitz's ligament; the mucosa covering the tumor was normal and the source of active bleeding was Meckel's diverticulum. Thus, one might suppose that this tumor would not be recognizable on CE. The exact location of the second GIST could not be given precisely. Notably, the bowel cleansing for CE in these two patients was poor.

The third missed lesion was first found on contrast CT, and was located in the proximal SB. This is in concordance with observations made by others. Postgate *et al*<sup>[14]</sup> described 5 significant lesions missed by CE that were found using other imaging modalities [DBE in 3 patients, CT enterography (CTE), and magnetic resonance enterography (MRE) in the 2 remaining patients]; 4 of which were located in the proximal jejunum. Chong *et al*<sup>[15]</sup> described 4 tumors in the proximal ileum that were missed by CE but found with DBE. This particular location, where many lesions were missed, may be partly explained by a rapid transit of the capsule through the duodenum and the proximal jejunum that enhances the risk of missing a lesion in the proximal SB.

The complementary role of DBE in CE-positive and CE-negative patients is widely accepted. Among our patients, the first with GIST underwent PE that did not reach the segment with the tumor. The second patient with GIST did not undergo enteroscopy. In the third patient, DBE included the involved segment but failed to provide a diagnosis.

Radiological imaging is more readily available than BAE and remains the next diagnostic step at many cen-

ters. With respect to conventional radiological SB imaging, CE is superior in diagnosing mass lesions. A small study comparing CE to barium enterography in children with Peutz-Jeghers syndrome (PJS) showed that polyps with a diameter of 10 mm and more were detected with similar frequency with both modalities, but CE identified significantly more polyps < 10 mm<sup>[16]</sup>. The performance of CE compared with newer radiological SB imaging is still a subject of debate. The first study comparing CE and magnetic resonance imaging (MRI) in patients with PJS (4 patients) or familial adenomatous polyposis syndrome (FAP, 16 patients) showed that smaller polyps were seen much more often with CE, whereas polyps larger than 15 mm were detected at similar rates with both CE and MRI<sup>[17]</sup>. However, a subsequent study performed in 19 PJS patients showed that CE missed large polyps (> 15 mm) detected on MRE in three patients, suggesting that MRE may be less prone to miss large polyps and more reliable in their size assessment<sup>[18]</sup>. With regard to CTE, both CE and CTE were performed in 32 patients with OGIB described in a retrospective study by Khalife *et al*<sup>[19]</sup>. When CTE followed CE, it helped to identify tumors not detected by CE ( $n = 2$ ) and excluded suspected tumors ( $n = 3$ ). In another retrospective study of 17 patients with SB tumors who had both CE and CTE, CE detected SB tumors in 6 patients and CTE in 16, with a significant difference in the sensitivity of the two methods<sup>[20]</sup>. In a prospective comparison of CTE and CE in 58 patients with OGIB, the sensitivity of CTE for detecting SB bleeding sources and SB masses was significantly greater than that of CE<sup>[21]</sup>. In our study, (angio) CT followed CE and helped to establish the source of bleeding in two patients.

The risk of rebleeding in 42 patients with OGIB and negative CE was first evaluated by Macdonald *et al*<sup>[22]</sup> who observed bleeding episodes in only 2 overt OGIB patients during 17.3 mo of follow-up. Subsequently, Park *et al*<sup>[23]</sup> observed 57 OGIB patients, of whom 46 had overt OGIB, for a median time of 31.7 mo. They found a substantial cumulative rebleeding rate of 35.7% in CE-negative patients, recommending further investigation or close observation of such patients<sup>[23]</sup>. The results of these studies suggest that following a negative CE, overt OGIB patients were the most likely to benefit from further investigation.

In summary, in patients with overt OGIB and normal or insignificant CE, the risk of missing a lesion in the SB cannot be underestimated. In our opinion, BAE should be the next diagnostic tool used when symptoms strongly suggest that the source of bleeding is located in the SB. In the remaining cases, or when BAE is not easily available, CT or MRI seem to be a rational choice in further evaluations. According to the most recent studies, CT or MRI enterography may be the best choice. Laparotomy remains a diagnostic option when these tests are normal or not available, with the advantage of therapeutic possibilities.

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## COMMENTS

### Background

Capsule endoscopy (CE) has become a first-line diagnostic tool for obscure gastrointestinal bleeding (OGIB) and small bowel (SB) polyp or tumor detection, but the reliability of negative CE for excluding gross SB pathology is unclear.

### Research frontiers

SB tumors detected by CE were characterized in a retrospective cohort. At follow-up, three additional SB tumors missed by CE were identified. In patients with overt OGIB, negative CE does not exclude significant disease.

### Innovations and breakthroughs

This is the first study of CE with a specific, tumor-oriented, long-term follow-up.

### Applications

In patients with significant clinical symptoms, the risk of missing a lesion in the SB cannot be underestimated. In overt OGIB, supplementary diagnostic methods should be used to visualize SB, including balloon enteroscopy, computed tomography, and magnetic resonance enterography.

### Terminology

CE is a method of visualizing the walls of the gut and used mainly to investigate the SB, which is difficult to access by conventional endoscopy, but is also used to visualize the esophagus and colon. A small, pill-like camera is ingested by the patient and moves naturally throughout the gastrointestinal tract, taking thousands of pictures. These pictures are sent to a detector connected to the patient's skin during the examination and assessed later by a reader on the computer. The camera pill is excreted with the stool.

### Peer review

This study has the strengths of large enrollment and SB tumor-oriented follow-up. It is well written.

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## Serum concentrations of insulin-like growth factor-binding protein 5 in Crohn's disease

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### Abstract

**AIM:** To investigate serum insulin-like growth factor-binding protein 5 (IGFBP-5) levels and intestinal IGFBP-5 expression in patients with Crohn's disease (CD).

**METHODS:** We analyzed the serum concentrations and intestinal expression of IGFBP-5 in 42 patients with CD, of whom 26 had endoscopically or radiologically proven stricture formation. Nine of the 42 patients had active disease, with a Crohn's disease activity index > 150. Serum IGFBP-5 levels were analyzed in 20 healthy controls matched by sex and age to the CD patients.

Serum IGFBP-5 was measured using an enzyme-linked immunosorbent assay. Intestinal tissue was obtained from patients through endoscopic biopsies. IGFBP-5 expression was detected using immunohistochemistry and was scored semiquantitatively.

**RESULTS:** The median serum IGFBP-5 concentrations of CD patients were significantly lower compared with healthy controls [median 7.2 (IQR: 5.5-11.3) ng/mL *vs* 11.3 (8.0-44.6) ng/mL,  $P < 0.001$ ]. There was no significant difference between median serum IGFBP-5 levels in CD patients with or without stricture formation [6.9 (5.5-11.3) ng/mL *vs* 7.8 (5.3-10.1) ng/mL,  $P = 0.815$ ]. The serum IGFBP-5 levels were not significantly different between patients with active disease and inactive disease [7.2 (6.5-7.6) ng/mL *vs* 7.2 (5.5-11.3) ng/mL,  $P = 0.890$ ]. However, a significant correlation was observed between serum IGFBP-5 levels and platelet count (PLT) ( $r = 0.319$ ,  $P = 0.0395$ ). No significant correlation was found between tissue IGFBP-5 immunohistochemical staining intensity scores and serum IGFBP-5 levels. No significant difference was found when comparing the serum IGFBP-5 levels among the patients with different tissue IGFBP-5 staining scores (absent/very weak, weak, moderate or strong). There was a significant correlation between tissue IGFBP-5 staining scores and white blood cell count ( $r = 0.391$ ,  $P = 0.01$ ) and PLT ( $r = 0.356$ ,  $P = 0.021$ ).

**CONCLUSION:** Our results indicate that serum IGFBP-5 concentrations were lower in CD patients compared to healthy controls regardless of disease activity or the presence of stricture formation.

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**Key words:** Crohn's disease; Insulin-like growth factor-binding protein 5; Stricture; Immunohistochemistry;



## Enzyme-linked immunosorbent assay

**Core tip:** Previous studies have suggested that insulin-like growth factors are important for the growth and development of visceral smooth muscle. In particular, increased insulin-like growth factor-binding protein 5 expression has been described in inflamed and fibrotic intestinal tissue. In this study, we aim to investigate the possible role of insulin-like growth factor-binding protein 5 in Crohn's disease with stricture involvement. Crohn's disease patients had lower serum levels of IGFBP-5 compared to healthy controls. The results of the study suggest that additional research is necessary to explain the low circulating levels of IGFBP-5 in Crohn's disease patients.

Adali G, Yorulmaz E, Ozkanli S, Ulasoglu C, Bayraktar B, Orhun A, Colak Y, Tuncer I. Serum concentrations of insulin-like growth factor-binding protein 5 in Crohn's disease. *World J Gastroenterol* 2013; 19(47): 9049-9056 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9049.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9049>

## INTRODUCTION

Crohn's disease (CD), a condition characterized by chronic inflammation of the alimentary tract, arises from a complex interaction between genetic, immunological, and microbial factors<sup>[1]</sup>. More than one-third of CD patients develop a distinct fibrostenosing phenotype with progressive intestinal strictures and potential intestinal obstruction<sup>[2]</sup>. Intestinal obstruction and fistulae are the main indications for surgery in patients with CD<sup>[3]</sup>. Stricture formation is caused by a combination of smooth muscle cell hyperplasia, smooth muscle cell hypertrophy, and excessive net extracellular matrix production by intestinal smooth muscle cells<sup>[4]</sup>. The factors underlying stricture development in CD are not completely understood. It is critical to develop markers that can be used to predict intestinal stricture formation in the early stages of CD.

The insulin-like growth factor (IGF) system has a critical role in regulating the growth and development of visceral and vascular smooth muscle<sup>[5]</sup>. Insulin-like growth factors (IGF- I and IGF- II) are transported in the serum by insulin-like growth factor-binding proteins (IGFBPs; IGFBP-1 to 6), which are produced in the liver. IGFBPs are also produced by non-liver tissues, in which they act in autocrine and paracrine manners to modulate responses to IGFs<sup>[6]</sup>. Despite their structural similarity, each IGFBP has unique characteristics and functions. Insulin-like growth factor-binding protein 5 (IGFBP-5) is the most conserved of the IGFBPs, has several regulatory functions, and is among the IGFBP subtypes that display IGF-independent effects. The most important in vivo regulator of IGFBP-5 expression is IGF- I. In normal adult human serum, IGFBP-5 levels are positively correlated with IGF- I concentrations<sup>[7]</sup>. In patients with CD,

IGF- I expression is specifically upregulated in smooth muscle cells in regions of stricture compared to normal margins; this upregulation is accompanied by upregulated IGFBP-5 expression, which acts synergistically with IGF- I in these cells<sup>[8]</sup>. Increased IGFBP-5 expression has also been described in two human fibrotic disorders: systemic sclerosis and idiopathic pulmonary fibrosis<sup>[9,10]</sup>.

Several studies have investigated the serum concentrations of IGF-1 and IGFBP-3 in active inflammatory bowel disease (IBD) patients and found significantly decreased serum levels<sup>[11-16]</sup>. There are currently no data available regarding serum IGFBP-5 levels in patients with CD. It is unknown whether circulating IGFBP-5 proteins influence local IGFBP-5 tissue expression or whether the protein levels are reflective of stricture formation in patients with CD. Therefore, we aimed to investigate the serum concentration of IGFBP-5 and intestinal IGFBP-5 expression in tissue taken from CD patients with and without stricture formation and to determine the correlation between serum IGFBP-5 levels and intestinal IGFBP-5 expression.

## MATERIALS AND METHODS

*Patients and samples*

Forty-two patients with CD [20 female patients and 22 male patients; mean age ( $\pm$  SD)  $38.79 \pm 13.91$  years; range 19-70 years, mean disease duration  $4.74 \pm 7.46$  years] were enrolled from the inflammatory bowel disease outpatient clinic in Istanbul Medeniyet University, Goztepe Training and Research Hospital, Istanbul, Turkey, between March 2011 and September 2012. The study was conducted in accordance with the Declaration of Helsinki and according to the principles of Good Clinical Practice. The Goztepe Training and Research Hospital ethics committee approved the study (19/S-2012). All subjects gave informed consent. Twenty healthy controls who were matched to study patients by sex and age [10 female and 10 male, mean age ( $\pm$  SD)  $38.4 \pm 8.73$  years; range 26-56 years] were also enrolled, and they provided written consent for the collection of blood samples.

CD was diagnosed based on the established criteria of clinical, endoscopic, and histological findings. All patients ( $n = 42$ ) were evaluated endoscopically or radiologically for the presence of stricture formation. Twenty-six of the 42 patients (61.9%) had endoscopically or radiologically proven stricture formation. Eighteen of these 26 patients (69.2%) had a history of intestinal resection. The Crohn's disease activity index (CDAI) was used in all patients to assess disease activity<sup>[17]</sup>. Nine of the 42 patients (21.4%) had active disease corresponding to a CDAI  $> 150$ . Only four of the 26 patients (15.4%) with stricture formation had active disease corresponding to a CDAI  $> 150$ .

All patients were subdivided into disease phenotypes according to the Montreal Classification<sup>[18]</sup>: ileal disease only (L1) ( $n = 9$ , 21.4%), colonic disease only (L2) ( $n = 2$ , 4.8%), and ileocolonic disease (L3) ( $n = 31$ , 73.8%). Clin-

cal data regarding each patient's duration and localization of the disease, history of bowel resection, and current medications were obtained and recorded. Exclusion criteria included the presence of liver fibrosis or cirrhosis, systemic sclerosis, idiopathic pulmonary fibrosis, or a history of cancer.

The recruited patients were scheduled to undergo an ileocolonoscopy. The reasons for the scheduled endoscopy were as follows: to assess the disease extent and activity ( $n = 24$ , 57.1%), to monitor response to therapy ( $n = 9$ , 21.4%), and to perform stricture dilation ( $n = 9$ , 21.4%). Endoscopy was performed by experienced gastroenterologists, who collected biopsies with standardized flexible endoscopic forceps from areas that were endoscopically strictured and/or ulcerated (ileal and/or colonic) in patients with stricture formation. The biopsies were taken from inflamed or ulcerated areas (ileal and/or colonic) in patients without stricture formation. At least 2 biopsy specimens were collected from each area. One specimen was stained with immunohistochemistry to determine IGFBP-5 expression, and the other was stained with hematoxylin and eosin (HE). Routine histological examination of the biopsy specimens was performed by an experienced pathologist. Blood samples were collected on the same day, and sera were frozen at  $-80^{\circ}\text{C}$  until testing was performed.

#### Determination of IGFBP-5 in serum

Human serum IGFBP-5 levels were determined using an ELISA kit (RayBiotech, Norcross, GA) according to the manufacturer's instructions. The sensitivity of the assay was less than 2 ng/mL. The intra- and inter-assay coefficients of variation (CVs) were  $< 10\%$  and  $< 12\%$ , respectively.

#### Immunohistochemical staining and evaluation

Immunohistochemical staining was performed using polyclonal antibodies against IGFBP-5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Tissue samples from the diagnostic ileocolonoscopy (ileal and/or colonic) were stained with immunohistochemistry to determine IGFBP-5 expression. HE staining was performed on parallel sections of the tissue samples. Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3  $\mu\text{m}$ ) were deparaffinized with xylene for 10 min. After deparaffinization, the sections were incubated in a 3% hydrogen peroxide block for 10 min to reduce nonspecific background staining due to endogenous peroxidase. After a wash in phosphate-buffered saline plus Tween 20 (20  $\times$ ) (PBS; ScyTek Laboratories, Logan, Utah, United States), the sections were incubated in an ultra V block (ScyTek Laboratories, Logan, Utah, United States) for 5 min at room temperature (RT) to block non-specific binding. A primary antibody against IGFBP-5 (dilution 1:50) was added to the tissue sections, and the sections were incubated at RT for 90 min, followed by incubation with a secondary antibody (dilution 1:200, Ultra Tek antipolyvalent biotinylated antibody, ScyTek Labo-

ratories Logan, Utah, United States) at RT for 15 min. After rehydration with PBS, Ultra Tek HRP (ScyTek Laboratories, Logan, Utah, United States) was added to the specimens. The DAB chromogen system (DAB substrate kit, ScyTek Laboratories, Logan, Utah, United States) was added to the specimens after rehydration with PBS. Mayer's hematoxylin stain was used as a counterstain.

IGFBP-5 immunohistochemical staining was scored semiquantitatively by an independent pathologist who was blinded to clinical information. Positive staining for IGFBP-5 was observed as diffuse brown staining. The intensity of staining was scored as follows: 0 = absent or very weak staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining.

#### Statistical analysis

The SPSS statistical software package (SPSS version 19.0, SPSS, Chicago, IL, United States) was used for data management and analyses. CD patients were matched by age and sex with healthy controls to minimize confounding factors; matched controls were included because the numbers of patients in the study was not large enough to carry out the modeling necessary to adjust for possible effects of age and gender. For continuous normally distributed variables, the mean and standard deviation were reported. Median and interquartile range were reported for non-normally distributed continuous variables. Frequencies and percentages were given for categorical variables. The Mann-Whitney test was used to evaluate the median difference between groups, and a  $t$  test was used to compare differences in mean scores. Fisher's exact test was used instead of the typical  $\chi^2$  test to compare the frequencies or categorical variables because there were few subjects in each category ( $n < 10$  subjects). Spearman's correlation coefficient was used to assess the association between continuous variables in the CD group. The Kruskal-Wallis test was used to assess differences in serum concentrations of IGFBP-5 and expression in tissue specimens (*i.e.*, scores of 0, 1, 2, and 3). Statistical significance was set at a 95%CI level using a 2-sided  $P$  value.

## RESULTS

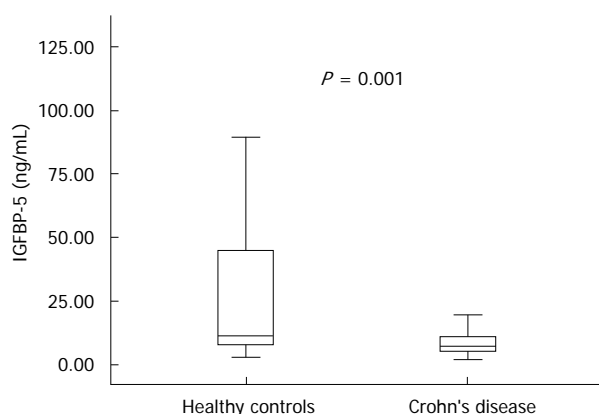
The baseline characteristics of the CD patients and healthy controls are summarized in Table 1. The median duration of CD diagnosis was 1.2 years (IQR: 0.16-7). Of the 42 patients with CD, 31(73%) had ileocolonic disease, and 33 (78.6%) were treated with azathioprine.

The main clinical and biochemical characteristics of CD patients and healthy controls are presented in Table 2. The median values for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cell (WBC) and platelet count (PLT) were significantly higher in CD patients compared to controls (for ESR and CRP,  $P < 0.001$ ; for PLT,  $P = 0.0001$ ; and for WBC count,  $P = 0.036$ ). However, the median levels of albumin (Alb) and hemoglobin (Hb) were significantly lower in the CD

**Table 1** Baseline characteristics of the study population *n* (%)

	Crohn's disease ( <i>n</i> = 42)	Healthy controls ( <i>n</i> = 20)
Age, mean ± SD (yr)	38.7 ± 13.9	38.4 ± 8.7
Gender		
Female	20 (47.6)	10 (50)
Male	22 (52.4)	10 (50)
Disease localization		-
Ileum	9 (21.4)	
Colon	2 (4.8)	
Ileum + colon	31 (73.8)	
Medical treatment		-
None	9 (21.4)	
Corticosteroids	1 (2.4)	
5-aminosalicylate	8 (19)	
Azathioprine	33 (78.6)	
Sulfasalazine	1 (2.4)	
More than one drug	23 (54.8)	
Median disease duration (yr) (IQR)	1.2 (0.17)	-
Prior intestinal resection	18 (42.9)	-

IQR: Interquartile range.

**Figure 1** Serum Insulin-like growth factor-binding protein 5 concentrations in Crohn's disease patients and healthy controls. Serum insulin-like growth factor-binding protein 5 (IGFBP-5) (ELISA) levels were significantly decreased in patients with Crohn's disease compared to healthy controls ( $P < 0.001$ ).  $P$  value from Mann-Whitney test.

group compared to healthy controls, with  $P$  values of  $< 0.001$  and  $0.0063$ , respectively. Serum IGFBP-5 levels were significantly reduced in patients with CD [7.2 (5.5-11.3) ng/mL] compared to healthy controls [11.3 (8.0-44.6) ng/mL,  $P = 0.001$ ] (Figure 1 and Table 2).

Table 3 shows the demographic and biochemical characteristics of CD patients with and without stricture formation. There were no significant differences between the CD patients with stricture formation and those without stricture formation with regards to age, gender, disease activity, disease localization or disease duration. Additionally, there was no significant difference in median values for biochemical parameters in CD patients with or without stricture formation. There was also no significant difference between serum IGFBP-5 levels in CD patients with and without stricture formation [6.9 (5.5-11.3) ng/mL and 7.8 (5.3-10.1) ng/mL ( $P = 0.815$ ), respectively].

The serum median IGFBP-5 levels were not signifi-

**Table 2** Clinical and biochemical parameters of Crohn's disease patients and healthy controls

	Crohn's disease ( <i>n</i> = 42)	Healthy controls ( <i>n</i> = 20)	$P$ value <sup>1</sup>
CDAI	87.0 (52-138)	-	
ESR (mm/h)	35.5 (17.0-48)	12.0 (10.5-14.5)	$< 0.001$
CRP (mg/dL)	0.6 (0.3-1.5)	0.2 (0.1-0.3)	$< 0.001$
Hb (g/dL)	13.0 (11.8-13.9)	14.0 (12.9-15)	0.0063
WBC ( $\times 10^3/\text{mm}^3$ )	7.7 (5.9-9.2)	6.2 (5.3-7.9)	0.0356
PLT ( $\times 10^3/\text{mm}^3$ )	309.5 (245-359)	240.0 (229.0-243.5)	0.0001
Alb (g/L)	3.9 (3.7-4.4)	4.7 (4-5)	$< 0.001$
IGFBP-5 (ng/mL)	7.2 (5.5-11.3)	11.3 (8.0-44.6)	0.0019

<sup>1</sup> $P$  value from Mann-Whitney test. Results are given as median. IQR: Interquartile range; CDAI: Crohn's Disease Activity Index; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; Hb: Hemoglobin; WBC: White blood cell count; PLT: Platelet count; Alb: Albumin; IGFBP-5: Insulin-like growth factor-binding protein 5.

**Table 3** Characteristics of Crohn's disease patients with and without stricture formation *n* (%)

	CD patients with stricture formation ( <i>n</i> = 26)	CD patients without stricture formation ( <i>n</i> = 16)	$P$ value <sup>1</sup>
Age mean ± SD (yr)	41.4 (15.0)	34.6 (11.0)	0.124
Gender (F/M)	11 (42.3)/15 (57.7)	9 (56.3)/7 (43.8)	0.527
Disease localization			0.083
Ileum	3 (11.5)	6 (37.5)	
Colon	1 (3.9)	1 (6.3)	
Ileum + colon	22 (84.6)	9 (56.3)	
Disease duration (yr)	1.25 (0.25-12)	1.2 (0-3.1)	0.254
CDAI	78.5 (50.0-122.0)	112.0 (74.5-165.5)	0.090
ESR (mm/h)	36.5 (17.0-54.0)	35.0 (21.0-39.5)	0.660
CRP (mg/dL)	0.4 (0.3-1.2)	1.0 (0.4-3.0)	0.239
Hb (g/dL)	13.0 (12.4-14.1)	12.4 (11.5-13.7)	0.468
WBC ( $\times 10^3/\text{mm}^3$ )	7.25 (5.5-9.6)	8.8 (6.15-9.15)	0.509
PLT ( $\times 10^3/\text{mm}^3$ )	309.5 (262-383)	288.5 (234.0-334.0)	0.399
Alb (g/L)	3.9 (3.6-4.4)	4.1 (3.8-4.5)	0.233
IGFBP-5 (ng/mL)	6.9 (5.5-11.3)	7.8 (5.3-10.1)	0.815

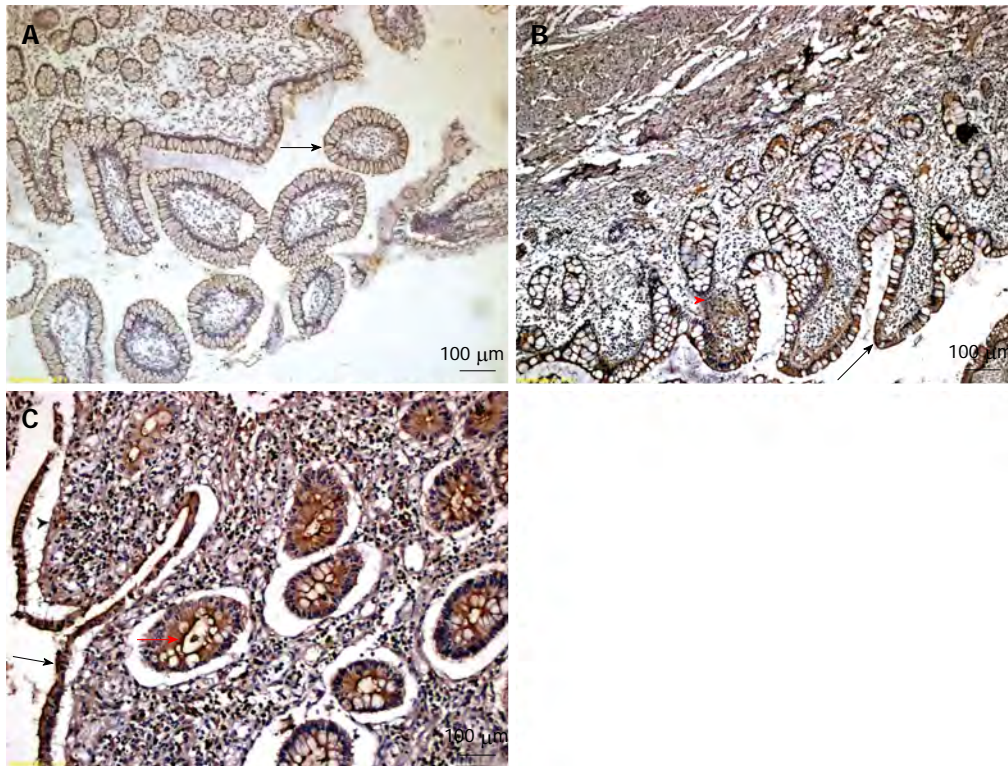
<sup>1</sup> $P$  values are from  $t$  test, Fisher's exact and Mann-Whitney test statistics were appropriate. Results are given as median. IQR: Interquartile range; CDAI: Crohn's Disease Activity Index; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; Hb: Hemoglobin; WBC: White blood cell count; PLT: Platelet count; Alb: Albumin; IGFBP-5: Insulin-like growth factor-binding protein 5.

cantly different in patients with active CD [7.2 (6.3-7.6) ng/mL] compared to patients with inactive CD [7.2 (5.5-11.3) ng/mL;  $P = 0.8901$ ]. However, patients with active disease had lower 75 percentile values compared to patients with inactive disease (7.6 *vs* 11.3) (data not shown).

The serum IGFBP-5 levels were not correlated with clinical baseline parameters reflecting disease activity (CDAI, Alb, and Hb) or the presence of stricture formation. However, there was a significant correlation between serum IGFBP-5 levels and PLT ( $r = 0.319$ ,  $P = 0.0395$ ).

We evaluated IGFBP-5 expression in ileal biopsies using immunohistochemistry in all CD patients. Figure 2 presents representative examples of different immunohistochemical staining intensity scores (scores 1, 2 and





**Figure 2** Representative examples of different immunohistochemical staining intensity scores for Insulin-like growth factor-binding protein 5 expression. Insulin-like growth factor-binding protein 5 (IGFBP-5) was diffusely expressed in the intestinal tissue. A: Weak staining (score = 1) (arrow) in epithelial cells in a Crohn's disease (CD) patient without stricture formation (ileal sample); B: Moderate staining (score = 2) both in epithelial (arrow) and stromal cells (arrowhead) in a CD patient with stricture formation (ileal sample); C: Strong staining (score = 3) in epithelial cells (arrow), stromal cells (arrowhead) and crypt lumen (red arrow) in a CD patient with active disease (colonic sample). Bar = 100 µm. Images were obtained using a light-field microscope, and edited using Adobe Photoshop CS5 (Adobe Systems Incorporated).

**Table 4** Frequency of Insulin-like growth factor-binding protein 5 positive samples and the median intensity score of IGFBP-5 immunostaining (scores 0 to 3) in Crohn's disease patients with and without stricture formation *n* (%)

	CD patients with stricture formation ( <i>n</i> = 26)	CD patients without stricture formation ( <i>n</i> = 16)	<i>P</i> value <sup>1</sup>
Number of IGFBP-5 positive samples	16 (61.5)	8 (50)	0.463
Median intensity score of IGFBP-5 immunostaining	1	0.5	0.405

<sup>1</sup>*P* values are from Fisher's exact and Mann-Whitney test statistics were appropriate. IGFBP-5: Insulin-like growth factor -binding protein 5; CD: Crohn's disease.

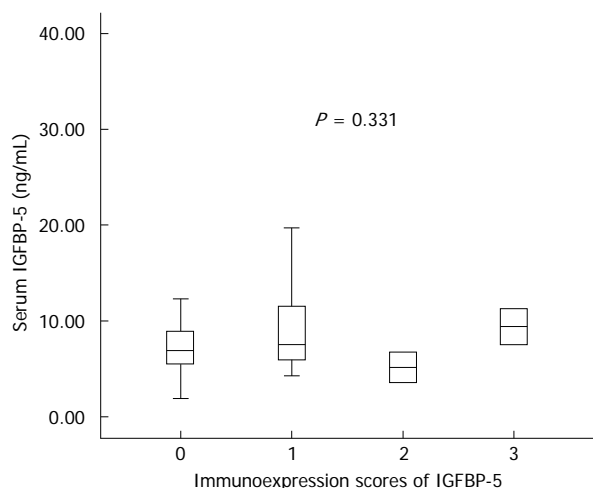
3) for IGFBP-5 expression using tissue samples from 3 different CD patients. Among all 42 CD patients, 24 (57.1%) had tissue samples stain positive for IGFBP-5 expression. The median level of IGFBP-5 intensity score was 1.0. The frequency of IGFBP-5 positive samples and the median intensity score did not differ significantly between CD patients with stricture formation and those without stricture formation (Table 4) or between CD patients with active disease and those with inactive disease (data not shown). The IGFBP-5 intensity scores were positively correlated with WBC count ( $r = 0.391$ ;  $P = 0.01$ ) and PLT ( $r = 0.356$ ;  $P = 0.021$ ).

No significant correlation was found between ileal IGFBP-5 immunohistochemical expression and serum IGFBP-5 levels. The serum IGFBP-5 concentrations were not significantly different for individuals with biopsies with absent/very weak (0), weak (1), moderate (2), or strong (3) IGFBP-5 staining scores (Figure 3).

## DISCUSSION

This study revealed that circulating levels of IGFBP-5 were significantly reduced in patients with CD compared to healthy controls [7.2 (5.5-11.3) ng/mL *vs* 11.3 (8.0-44.6) ng/mL ( $P = 0.002$ ), respectively]. To our knowledge, this is the first study that demonstrates low serum IGFBP-5 levels in patients with CD compared to healthy controls. Despite the low serum IGFBP-5 levels described in CD patients, the authors did not observe any significant differences between the median serum IGFBP-5 levels for patients with and without strictures [6.9 (5.5-11.3) ng/mL *vs* 7.8 (5.3-10.1) ng/mL ( $P = 0.815$ ), respectively]. Previous studies have mainly evaluated serum total and free IGF- I , IGFBP-1, IGFBP-2 and IGFBP-3 levels in the context of disease activity. Our results were similar to these studies, demonstrating low levels of IGF system proteins in IBD patients<sup>[11-16]</sup>. Katsanos *et al*<sup>[11]</sup> reported





**Figure 3** Serum Insulin-like growth factor-binding protein 5 concentrations in patients with different immunohistochemical staining intensity scores for Insulin-like growth factor-binding protein 5 expression. Serum insulin-like growth factor-binding protein 5 (IGFBP-5) concentrations were not significantly different for individuals with absent/very weak, weak, moderate, or strong IGFBP-5 staining intensity scores ( $P = 0.331$ ).  $P$  value from Kruskal-Wallis test.

that circulating levels of IGF-I and IGFBP-3 were reduced in patients with active IBD<sup>[9]</sup>. Grønbek *et al*<sup>[12]</sup> demonstrated reduced serum total and free IGF- I and IGFBP-3 levels in patients with active IBD without complete normalization during high-dose prednisolone treatment and tapering<sup>[10]</sup>. Vespasiani Gentilucci *et al*<sup>[14]</sup> observed low IGF-1 and IGFBP-3 levels in active IBD patients before infliximab therapy, and a repeated drop in levels after normalization and clinical remission. However, we did not observe any significant difference between serum IGFBP-5 levels in patients with active and inactive disease [7.2 (5.5-11.3) ng/mL *vs* 7.2 (6.3-7.6) ng/mL ( $P = 0.8901$ ), respectively]. Moreover, serum IGFBP-5 levels were moderately correlated with PLT, but not with hemoglobin, albumin levels, or CDAI. This finding may be explained by the low number of active CD patients ( $n = 9$ , 21.4%) in our study or by the presence of low-grade subclinical transmural inflammation, which is not detectable with clinical or biochemical markers. The most important in vivo regulator of IGFBP-5 expression is IGF- I<sup>[7]</sup>. In normal adult human serum, IGFBP-5 levels are positively correlated with IGF- I concentrations<sup>[19]</sup>. Although we did not evaluate serum IGF- I levels, the mechanism underlying low IGFBP-5 levels in CD patients may be similar to the mechanism for low IGF- I levels, as IGFBP-5 expression is mainly regulated by IGF- I. Previous studies have suggested that low IGF-1 levels in active IBD patients may be due to the direct adverse effects of circulating inflammatory cytokines<sup>[19,20]</sup>. Katsanos *et al*<sup>[11]</sup> also showed that serum IL-6 levels were increased in IBD patients with active disease compared to healthy controls. IGFBP-5 expression *in vitro* can be regulated by hormones and cytokines<sup>[7]</sup>. However, as inflammatory cytokines were not evaluated in our study, further investigations are needed to examine the effects

of cytokines on circulating IGFBP-5 levels. Previous studies demonstrated partially normalized or unchanged low IGF levels during corticosteroid and infliximab treatments<sup>[12,14-16]</sup>, and the low serum IGFBP-5 levels in our study may be explained by the previously described poor correlation between clinical activity, endoscopic severity and biological parameters in CD patients<sup>[21]</sup>.

To our knowledge, we are the first researchers to investigate the relationship between circulating IGFBP-5 concentrations and intestinal IGFBP-5 expression in CD patients. No correlation was found between circulating IGFBP-5 concentrations and intestinal IGFBP-5 expression. The serum IGFBP-5 concentrations were not significantly different between individuals with biopsies with absent/very weak, weak, moderate, or strong IGFBP-5 staining. Previous studies demonstrated that serum levels of hepatic-derived IGF- I and IGFBP-3 were lower in patients with active CD than in normal subjects, whereas the expression of IGF- I, IGFBP-5, and IGFBP-3 in smooth muscle cells in strictured intestines was increased compared to adjacent nonstrictured intestinal muscle from the margin of resected tissue<sup>[22-24]</sup>; however, the reasons for the discrepancy in the levels of these mediators in the serum compared to the expression in the muscle layer of the intestine remain unclear. It has been suggested that serum levels and intestinal expression of the IGF system are differentially regulated.

Three pathophysiologic events occurring within smooth muscle cells of the muscularis propria contribute to stricture formation in CD: increased smooth muscle cell hyperplasia, increased smooth muscle cell hypertrophy, and excess net extracellular matrix proteins, including collagen. IGF- I up-regulation is accompanied by IGFBP-5 up-regulation and collagen I, III, and V up-regulation<sup>[4,5,25]</sup>. Zimmermann *et al*<sup>[5]</sup> showed that IGF- I and IGFBP-5 mRNA was increased in inflamed/fibrotic intestines compared with normal-appearing intestines. However, we could not demonstrate increased intestinal IGFBP-5 expression in CD patients with stricture formation compared to those without stricture formation. Unfortunately, we were not able to analyze and compare intestinal IGFBP-5 expression in both fibrotic and normal-appearing tissue in CD patients with stricture formation. Moreover, the intestinal tissue was obtained with standard endoscopic biopsies, and the majority of our patients had inactive disease ( $n = 33$ , 78.6%), whereas previous studies analyzed active inflamed intestinal tissues from resection samples. Although we did not observe any significant difference between active and inactive patient groups regarding intestinal IGFBP-5 expression, intestinal IGFBP-5 expression was positively correlated with WBC count and PLT. This finding may be due to a poor correlation between clinical activity indices and actual endoscopic disease activity. One limitation of our study is the small sample size. Additionally, we were unable to obtain biopsies from normal-appearing intestinal mucosa of CD patients to compare with intestinal IGFBP-5 expression in inflamed/strictured mucosa. Moreover, intestinal IGFBP-5 expression could be affected by the area of the

biopsy samples.

In conclusion, our results indicate that serum IGFBP-5 concentrations are lower in CD patients compared to healthy controls regardless of disease activity or the presence of stricture formation. Serum IGFBP-5 concentrations were not associated with intestinal IGFBP-5 tissue expression. Therefore, our results do not answer the question of whether IGFBP-5 is involved in the stricture formation of CD, and thus, more research is necessary. Directions for future research include examination of other serum IGF system components, use of a larger patient population with active and inactive disease, endoscopic determination of disease activity and collection of biopsy tissue from both normal and inflamed/strictured areas.

## COMMENTS

### Background

Crohn's disease (CD) is a multifactorial disorder and its behavior may change throughout the course of the disease. Approximately 30 % of the CD patients will develop strictures and experience complications related to the stricture formation. It is crucial to understand the pathophysiology of bowel-wall stricturing in CD. Members of the insulin-like growth factor (IGF) system have been implicated as central players in stricture formation.

### Research frontiers

It has been shown that both insulin-like growth factor 1 (IGF-1) and insulin-like growth factor-binding protein 5 (IGFBP-5) expressions are increased in inflamed/fibrotic intestine. Many studies showed that circulating levels of IGF-1 and its binding protein proteins (IGFBPs) are decreased in inflammatory bowel disease. In the present study serum IGFBP-5 levels and intestinal IGFBP-5 expression was investigated in CD patients with and without stricture formation.

### Innovations and breakthroughs

The serum levels of IGFBP-5 and intestinal expression of IGFBP-5 with immunohistochemistry in CD patients has not been studied previously. This study, for first time, reports that serum IGFBP-5 levels are decreased in CD patients regardless the presence of stricture formation or disease activity.

### Applications

By understanding the circulating IGFBP-5 profile in CD patients, this study may represent a future strategy for prospective studies to understand the interaction between IGF system and CD pathophysiology, which may in turn aid in finding specific biomolecular targets for treatment of CD.

### Terminology

IGFBP-5 is a member of six IGFBPs. It is binding to IGFs with high affinity and has several regulatory functions. IGFBP-5 stimulates muscle hyperplasia and collagen secretion in human intestinal smooth muscle. It has been suggested that IGFBP-5, may be important in the pathogenesis of intestinal fibrosis in inflammatory bowel disease.

### Peer review

The authors examined the serum levels of IGFBP-5 and its intestinal expression in CD. It revealed that circulating IGFBP-5 levels are lower in CD patients compared to healthy controls. The results are interesting and may address the potential role of circulating IGFBP-5 in the pathophysiology of CD.

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## Alvarado, Eskelinen, Ohlmann and Raja Isteri Pengiran Anak Saleha Appendicitis scores for diagnosis of acute appendicitis

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### Abstract

**AIM:** To assess the reliability and practical applicability of the widely used Alvarado, Eskelinen, Ohlmann and Raja Isteri Pengiran Anak Saleha Appendicitis (RIPASA) scoring systems in patients with suspected acute appendicitis.

**METHODS:** Patients admitted to our tertiary center due to suspected acute appendicitis constituted the study group. Patients were divided into two groups. appendicitis group (Group A) consisted of patients who underwent appendectomy and were histopathologically diagnosed with acute appendicitis, and non-appendicitis group (Group N-A) consisted of patients who underwent negative appendectomy and were diagnosed with pathologies other than appendicitis and patients that were followed non-operatively. The operative findings for the patients, the additional analyses from follow up

of the patients and the results of those analyses were recorded using the follow-up forms.

**RESULTS:** One hundred and thirteen patients with suspected acute appendicitis were included in the study. Of the 113 patients (62 males, 51 females), the mean age was  $30.2 \pm 10.1$  (range 18-67) years. Of the 113 patients, 94 patients underwent surgery, while the rest were followed non-operatively. Of the 94 patients, 77 patients were histopathologically diagnosed with acute appendicitis. Our study showed a sensitivity level of 81% for the Alvarado system when a cut-off value of 6.5 was used, a sensitivity level of 83.1% for the Ohlmann system when a cut-off value of 13.75 was used, a sensitivity level of 80.5% for the Eskelinen system when a cut-off value of 63.72 was used, and a sensitivity level of 83.1% for the RIPASA system when a cut-off value of 10.25 was used.

**CONCLUSION:** The Ohlmann and RIPASA scoring systems had the highest specificity for the diagnosis of acute appendicitis.

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**Key words:** Acute appendicitis; Alvarado; Eskelinen; Ohlmann; Raja Isteri Pengiran Anak Saleha Appendicitis

**Core tip:** Several scoring systems have been devised to aid decision making in doubtful acute appendicitis cases, including the Ohlmann, Alvarado, Eskelinen, Raja Isteri Pengiran Anak Saleha Appendicitis and several others. These scores utilize routine clinical and laboratory assessments and are simple to use in a variety of clinical settings. However, differences in sensitivities and specificities were observed if the scores were applied to various populations and clinical settings, usually with



worse performance when applied outside the population in which they were originally created.

Erdem H, Çetinkünar S, Daş K, Reyhan E, Değer C, Aziret M, Bozkurt H, Uzun S, Sözen S, İrkörücü O. Alvarado, Eskelinen, Ohlmann and Raja Isteri Pengiran Anak Saleha Appendicitis scores for diagnosis of acute appendicitis. *World J Gastroenterol* 2013; 19(47): 9057-9062 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i47/9057.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9057>

## INTRODUCTION

Acute appendicitis is a common surgical condition that requires prompt diagnosis to minimize morbidity and avoid serious complications. Accurate identification of patients who require immediate surgery as opposed to those who will benefit from active observation is not always easy<sup>[1]</sup>.

Several scoring systems have been devised to aid decision making in doubtful cases, including the Ohlmann, Alvarado, Eskelinen, Raja Isteri Pengiran Anak Saleha Appendicitis (RIPASA) and several others<sup>[2-5]</sup>. These scoring systems utilize routine clinical and laboratory assessments and are simple to use in a variety of clinical settings. However, differences in sensitivities and specificities were observed if the scores were applied to various populations and clinical settings, usually with worse performance when applied outside the population in which they were originally created<sup>[2,3,6]</sup>. Additionally, geographic variation of the incidence and clinical pattern of the differential diagnosis of acute abdominal pain may impair their applicability<sup>[7]</sup>. Accurate diagnosis of acute appendicitis is especially difficult in women, where the inaccuracy of available diagnostic methods leads to an unacceptably high negative appendectomy rate due to gynecological disorders that frequently mimic appendicitis<sup>[8]</sup>.

This study aimed to assess the reliability and practical applicability of the widely used Alvarado, Eskelinen, Ohlmann and RIPASA scoring systems in patients with suspected acute appendicitis.

## MATERIALS AND METHODS

### Study design

This prospective study was approved by the local Institutional Review Board (ANEAH 2011/2). Written informed consent was obtained from all subjects. Patients admitted to our tertiary center due to suspected acute appendicitis between October 2011 and March 2012 constituted the study group.

Patients were divided into two groups: appendicitis group (Group A) consisted of patients who underwent appendectomy and were histopathologically diagnosed with acute appendicitis, and non-appendicitis group (Group N-A) consisted of patients who underwent negative appendectomy, patients diagnosed to have patholo-

gies other than appendicitis, and patients that were followed non-operatively.

### Outcome parameters

Patient data including age, gender, height, weight, the duration of hospital stay, accompanying disease history, operation or follow-up findings, and laboratory and imaging findings were recorded. Parameters from the Alvarado, Eskelinen, Ohlmann and RIPASA scoring systems were combined in this form<sup>[2-5]</sup>. Decisions regarding operation and follow up were given according to the preferences of the surgeon, not the scoring results.

The scores were calculated using an automated Microsoft Excel sheet after the patients were discharged. Calculated values were recorded as having a low, medium or high probability for acute appendicitis (Table 1). Operative findings, additional analyses of follow-up patients and the results of those analyses were recorded using the follow-up forms. A diagnosis of appendicitis was given macroscopically during the operation (purulent formations, and edematous- necrotic changes on the appendix wall). The results were confirmed with histopathological findings.

### Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences 19.0 for Windows (SPSS Inc., Chicago, IL, United States) and Medcalc (Mariakerke, Belgium) for Windows. The results for all of the items were expressed as the mean  $\pm$  SD, assessed within a 95% reliance and at a level of  $P < 0.05$  significance. The sample size calculation was based on a significance level of 0.05. We needed a sample of 103 patients to achieve 80% power. A normal distribution of the quantitative data was checked using the Kolmogorov-Smirnov test. Parametric tests were applied to the normally distributed data and non-parametric tests were applied to data with a questionably normal distribution. An independent sample *t* test and Mann-Whitney *U* test were used to compare the independent groups. Receiver operating characteristic curves were used to identify the optimal cut-off points. Cross tables were prepared for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the diagnostic accuracy values of the scoring systems. We used a  $\chi^2$  test to compare categorical measures regarding the diagnosis of acute appendicitis.

## RESULTS

One hundred and thirteen patients with suspected acute appendicitis were included in the study. Of the 113 patients (62 males, 51 females), the mean age was  $30.2 \pm 10.1$  (range 18 to 67) years. Of the 113 patients, 94 patients (83.19%) underwent surgery, while the rest (16.81%) were followed non-operatively. Of the 94 patients, 77 patients (81.91%) were histopathologically diagnosed with acute appendicitis, 6 (6.38%) were diagnosed with pathologies other than appendicitis (ovarian cyst rupture in three patients, inflammatory bowel disease in two patients, and a carcinoid tumor in one patient), and 11 patients (11.71%)

**Table 1 Clinical approaches advised by the authors regarding the scoring systems**

	High probability	Probable/should be followed	Appendicitis with high probability
Alvarado	< 4	5-6	> 7
Eskelinen	< 48	48-57	> 57
Ohlmann	< 6	6-11.5	> 12
RIPASA	< 5	5-7	> 7.5

underwent a negative appendectomy. Among the 19 patients who were followed non-operatively, urinary system disease was diagnosed in eight patients, gastroenteritis was diagnosed in four patients, mesenteric lymphadenitis was diagnosed in one patient, inflammatory bowel disease was diagnosed in one patient and gynecologic problems were diagnosed in one patient. A diagnosis was not established, and clinical improvement was observed in four patients.

Group A included 77 patients (46 males, 31 females) with a mean age of  $29.5 \pm 9$  years, and Group N-A included 36 patients (16 males, 20 females) with a mean age of  $31.8 \pm 12.1$  years. Both groups did not differ significantly in age and gender ( $P = 0.560$  and  $P = 0.157$ , respectively). With respect to the mean height ( $168.9 \pm 8.1$  cm *vs*  $168.1 \pm 8.8$  cm), mean weight ( $71.3 \pm 12.8$  kg *vs*  $71.6 \pm 16.4$  kg), and duration of hospital stay ( $45.3 \pm 20.1$  d *vs*  $57.9 \pm 37.6$  d), the two groups were not significantly different ( $P = 0.634$ ,  $P = 0.894$ , and  $P = 0.065$ , respectively).

Regarding patient symptoms, there was no similar pain history among the 64 patients that were diagnosed with acute appendicitis, while 13 patients had a similar pain history. It was found that not having a similar pain history was statistically significant for acute appendicitis ( $P < 0.001$ ). The studied groups differed significantly from each other with regard to the starting point of pain ( $P = 0.021$ ) and relocation of the pain to the lower right quadrant ( $P = 0.020$ ). As for the examination findings, the defense-rigidity, rebound, and Rowsing findings differed significantly between the groups ( $P < 0.001$ ,  $P < 0.001$ , and  $P = 0.034$ , respectively). Fever was also significantly different between the groups ( $P = 0.015$ ). As for the laboratory results, the neutrophil rate, leukocyte count, and urine analysis results differed significantly between the groups ( $P = 0.001$ ,  $P = 0.009$ , and  $P < 0.001$ , respectively) (Table 2). The operative and follow-up results for the patients were as follows: phlegmonous in 45 patients, catarrhal in 15 patients, gangrenous in 11 patients, vermiformis (negative appendectomy) in 11 patients, and perforated in six patients.

When the sensitivity and specificity levels of the scoring systems were assessed, they were 82% and 75% for the Alvarado, 100% and 28% for the RIPASA, 96% and 42% for the Ohlmann, and 100% and 44% for the Eskelinen scores. When the negative appendectomy rates of the Alvarado, RIPASA Ohlmann and Eskelinen scoring systems were assessed, they were found to be 12%, 25%,

**Table 2 Frequency of symptoms, examination findings and laboratory results *n* (%)**

		Group A	Group N-A	P value
Symptoms				
Loss of appetite	Yes	25 (69)	66 (86)	0.072
	No	11 (31)	11 (14)	
Nausea-Vomiting	Yes	20 (56)	52(68)	0.294
	No	16 (44)	25 (32)	
Time pain started	< 48	21 (58)	59 (77)	0.074
	> 48	15 (42)	18 (23)	
Starting point of pain	Around stomach	8 (22)	37 (48)	0.021
	Lower right quadrant	25 (69)	38 (49)	
	Anywhere	3 (8)	2 (3)	
Relocalization of the pain to the lower right quadrant	Yes	7 (19)	33 (43)	0.020
	No	29 (81)	44 (57)	
Urinary system complaint	Yes	9 (25)	8 (10)	0.052
	No	27 (75)	69 (90)	
Similar pain history	Yes	19 (53)	13 (17)	< 0.001
	No	17 (47)	64 (83)	
Findings				
Sensitivity on lower right quadrant	Yes	36 (100)	76 (99)	0.999
	No	0 (0)	1 (1)	
Defense-rigidity	Yes	23 (64)	77 (100)	< 0.001
	No	13 (36)	0 (0)	
Rebound	Yes	16 (44)	75 (97)	< 0.001
	No	20 (56)	2 (3)	
Rowsing finding	Yes	7 (19)	31 (40)	0.034
	No	29 (81)	46 (60)	
Fever	> 37.3	5 (14)	28 (36)	0.015
	< 37.3	31 (86)	49 (64)	
Laboratory results				
Neutrophil	> %75	10 (28)	49 (64)	0.001
	< %75	26 (72)	28 (36)	
Leukocyte	< 10000	15 (42)	13 (17)	0.009
	≥ 10000	21 (58)	64 (83)	
Urine analysis	Normal	24 (67)	75 (97)	< 0.001
	Abnormal	12 (33)	2 (3)	

Group A: Appendicitis group; Group N-A: Non-appendicitis group.

22% and 21%, respectively (Table 3). When a cut-off value for the Alvarado system was set at 6.5, its sensitivity was calculated as 81%. When a cut-off value for the Ohlmann system was set at 13.75, its sensitivity was calculated as 83.1%. When a cut-off value for the Eskelinen system was set at 63.72, its sensitivity was calculated as 80.5%. When a cut-off value for the RIPASA system was set at 10.25, its sensitivity was calculated as 83.1% (Figure 1 and Table 4).

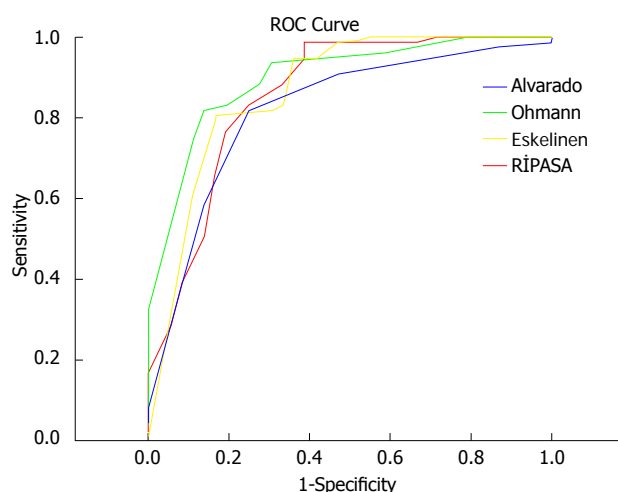
## DISCUSSION

The diagnosis of acute appendicitis still represents one of the most difficult problems in surgery<sup>[7]</sup>. It is generally accepted that the removal of a normal appendix is safer in questionable cases and that delaying surgery leads to an increased rate of perforation<sup>[8]</sup>. There have been many attempts to increase the accuracy of the diagnosis of acute appendicitis. In addition to clinical evaluation, with the

**Table 3** Sensitivity, specificity, positive predictive value, negative predictive value, diagnostic accuracy and negative appendectomy values of the scoring systems

	Alvarado (cut-off = 7)	Ohhmann (cut-off = 12)	Eskelinen (cut-off = 57)	RIPASA (cut-off = 7.5)
Sensitivity	82%	96%	100%	100%
Specificity	75%	42%	44%	28%
PPV	88%	78%	79%	75%
NPV	66%	83%	100%	100%
Diagnostic accuracy	80%	79%	82%	77%
Neg. app. rate	12%	22%	21%	25%

PPV: Positive predictive value; NPV: Negative predictive value; Neg. app. rate: Negative appendectomy rate.



**Figure 1** If we use high cut-off values for diagnostic methods and accept where the majority (at least three methods) are positive as positive and the others as negative, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy values of this new diagnostic method would be 98.7%, 55.6%, 82.6%, 95.2% and 84.9%, respectively.

variety of clinical signs and symptoms, many of the modern diagnostic tools have proved to be effective for the diagnosis of acute appendicitis<sup>[1,7,8]</sup>. Although sonography and CT increase the accuracy of the diagnosis of acute appendicitis, they are unfortunately still often unavailable in some emergency departments<sup>[9,10]</sup>. Several scoring systems that have been devised for the purpose of increasing both the sensitivity and specificity of the diagnosis of acute appendicitis have been repeatedly tested<sup>[2-5]</sup>. Scoring systems represent an inexpensive, non-invasive and easy to use diagnostic aid.

According to previous publications, the criteria for diagnostic quality have been postulated as a 15% rate of negative appendectomies, a 10% rate of negative laparotomies, a 35% rate of potential perforations, a 15% rate of overlooked perforations and a 5% rate of overlooked acute appendicitis<sup>[9,10]</sup>. Although the negative appendectomy rate reported by surgeons advocating early surgical intervention in suspected cases to prevent perforation varies between 20% and 40%, the generally accepted negative appendectomy rate is approximately 15%-20%<sup>[11-13]</sup>. Furthermore, misdiagnosis and late surgical intervention leads to complications with high morbidity and mortal-

**Table 4** Cut-off values for the maximum sensitivity and specificity values of the scoring systems

Measurements	AUC	Cut-off	Sensitivity	Specificity
Alvarado	0.818	6.50	81.8	75.0
Ohhmann	0.899	13.75	83.1	80.6
Eskelinen	0.867	63.72	80.5	83.3
RIPASA	0.857	10.25	83.1	75.0

AUC: Area under the curve.

ity, such as perforation and peritonitis. In the present study, 83.2% of the patients underwent surgery, while 16.8% were followed non-operatively. Of the patients who underwent surgery, 81.9% were histopathologically diagnosed with acute appendicitis, 6.38% were diagnosed to have pathologies other than appendicitis, and 11.71% underwent a negative appendectomy.

Acute appendicitis typically presents itself with pain that starts in the epigastrium or around the stomach and localizes to the lower right quadrant. A study by Ortega-Deballon *et al*<sup>[14]</sup> reported that the acute appendicitis diagnosis rate found in patients presenting with pain in the lower right quadrant was 65%. Similarly, Lane *et al*<sup>[15]</sup> reported this rate as 55%. In the present study, 68% of the patients who presented with pain in the lower right quadrant were histopathologically proven to have acute appendicitis.

Non-surgical pathologies can be found on physical examination and laboratory analyses in 20%-25% of the cases presenting with acute pain in the lower right quadrant, and these cases can be followed using conservative methods<sup>[16,17]</sup>. Furthermore, 5%-15% of cases with suspected acute appendicitis cannot be diagnosed despite an aggressive work-up<sup>[18]</sup>. In the present study, the rate of patients who could be followed non-operatively was 21%. The rate of patients with symptoms that receded clinically was 5%.

The idea of improving the diagnostic accuracy simply by assigning numeric values to defined signs and symptoms has been the goal of some of the scores that were previously described<sup>[1-5]</sup>. The parameters comprising the score usually include general signs of abdominal illness (*e.g.*, type, location and migration of pain, body temperature, signs of peritoneal irritation, nausea, vomiting, *etc.*) and routine laboratory findings (leukocytosis)<sup>[19]</sup>. Ohhmann

*et al*<sup>[3]</sup> performed a multivariate analysis, and of the initial 15 parameters, eight were included in a regression model, resulting in different values being attributed to each parameter. Originally, it was proposed that patients with scores less than six should not be considered to have appendicitis. However, patients with scores of six or more should undergo observation, and those with scores of 12 or more should proceed to immediate appendectomy<sup>[3]</sup>. The Eskelinen score delivered acceptable clinical results after calibration to a cut-off value of 57<sup>[5]</sup>. The Alvarado score is widely used for the diagnosis of acute appendicitis. The score is calculated over 10 points, and a score higher than six is indicative of acute appendicitis. On the other hand, a score of less than four indicates that it is unlikely that the patient has appendicitis. For scores of 4-6, follow-up or imaging with computerized tomography is recommended<sup>[4]</sup>. Chaudhuri *et al*<sup>[20]</sup>, in their series of 175 patients with a mean age of 30 years, reported a negative cut-off point of five. The RIPASA score is a relatively new diagnostic scoring system and has been shown to have a significantly higher sensitivity, specificity and diagnostic accuracy<sup>[2,21]</sup>. The RIPASA score is easy to apply and includes several parameters that are absent in the Alvarado score, such as age, gender and the duration of symptoms prior to presentation<sup>[22,23]</sup>. Our study calculated the sensitivity and specificity of the Alvarado scoring system as 82% and 75%, respectively, and calculated the sensitivity and specificity of the RIPASA scoring system as 100% and 28%, respectively. Although the diagnostic accuracy levels of these two scoring systems were comparable, the RIPASA scoring system is considered less accurate because of the higher negative appendectomy rates. The negative appendectomy rate calculated in our study was 12%. When the accuracy measures of all of the scoring systems included in our study were analyzed, they performed better, especially if the cut-off values were increased. A higher cut-off value leads to 100% sensitivity and a negative predictive value for the RIPASA and Eskelinen methods and leads to 96% sensitivity with an 83% negative predictive value for the Ohlmann method. When these values are assessed, it is found that the Ohlmann and Eskelinen methods are one step ahead in terms of detecting appendicitis, although they fail to meet expectations in terms of specificity. The disadvantage of the Eskelinen scoring system is the practicality of calculations because values in this system are decimals, and in other systems they are integers.

For the scoring systems, sensitivity and specificity values higher than 80% are acceptable<sup>[24,25]</sup>. This is why these scoring systems may prove more advantageous when the cut-off values are customized to clinical populations. Our study showed a sensitivity level of 81% for the Alvarado system when the cut-off value was set at 6.5, a sensitivity level of 83.1% for the Ohlmann system when the cut-off value was set at 13.75, a sensitivity level of 80.5% for the Eskelinen system when the cut-off value was set at 63.72, and a sensitivity level of 83.1% for the RIPASA system when the cut-off value was set at 10.25.

The main limitation of our study is the relatively small number in our series. In addition, some details regarding the history and factors that may influence the outcome may not have been completely documented. Due to these restrictions, associations should be interpreted with caution.

In conclusion, the Ohlmann and RIPASA scoring systems have the highest specificity for the diagnosis of acute appendicitis.

## COMMENTS

### Background

Several scoring systems have been devised to aid decision making in doubtful acute appendicitis cases, including the Ohlmann, Alvarado, Eskelinen, Raja Isteri Pengiran Anak Saleha Appendicitis (RIPASA) and several others.

### Research frontiers

To assess the reliability and practical applicability of the widely used Alvarado, Eskelinen, Ohlmann and RIPASA scoring systems in patients with suspected acute appendicitis.

### Innovations and breakthroughs

The Ohlmann and RIPASA scoring systems have the highest specificity for the diagnosis of acute appendicitis.

### Applications

Accurate identification of patients who require immediate surgery as opposed to those who will benefit from active observation is always useful.

### Terminology

The Alvarado, Eskelinen, Ohlmann and RIPASA are common scoring systems that are used in patients with suspected acute appendicitis.

### Peer review

This study is a prospective one and was conducted over 5-mo period and managed to recruit 113 patients (62 males and 51 females); 94 patients underwent surgery. This could be the first study comparing the four scoring systems in term reliability in diagnosing appendicitis.

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**S- Editor:** Gou SX **L- Editor:** A **E- Editor:** Ma S



## Seasonal variations in the onset of ulcerative colitis in Japan

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### Abstract

**AIM:** To investigate seasonal variations in the onset and relapse of ulcerative colitis (UC) in Japanese patients.

**METHODS:** Between 1994 and 2006, 198 Japanese patients diagnosed with UC according to conventional criteria in an academic hospital were enrolled for onset evaluation. Among 265 Japanese patients with UC who were observed for more than 12 mo, 165 patients relapsed (239 times) and were enrolled for relapse evaluation. The patients' symptoms were recorded each

month for 12 consecutive years.

**RESULTS:** There was monthly seasonality in symptom onset during October and March for UC. The onset of symptoms in UC patients frequently occurred during the winter. Variation in UC onset was observed according to both month ( $P = 0.015$ ) and season ( $P = 0.048$ ). Relapse commonly occurred in October, and variations in relapse were not significant either in month ( $P = 0.52$ ) or season ( $P = 0.12$ ). Upper respiratory inflammation was the main factor responsible for relapse.

**CONCLUSION:** Our results suggest that environmental factors associated with winter and spring seasonality may be responsible for triggering the clinical onset of UC in Japan.

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**Key words:** Japanese population; Onset; Relapse; Seasonal variations; Ulcerative colitis

**Core tip:** Monthly seasonality in the symptomatic onset of ulcerative colitis (UC) during October and March was observed in Japan. The onset of symptoms frequently occurred during the winter, whereas relapse of UC particularly occurred in October. Upper respiratory inflammation was one of the main factors responsible for relapse. Therefore, environmental factors associated with winter and spring seasonality may be responsible for triggering the clinical onset of UC in Japan.

Koido S, Ohkusa T, Saito H, Yokoyama T, Shibuya T, Sakamoto N, Uchiyama K, Arakawa H, Osada T, Nagahara A, Watanabe S, Tajiri H. Seasonal variations in the onset of ulcerative colitis in Japan. *World J Gastroenterol* 2013; 19(47): 9063-9068 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/>

## INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing disease characterized by alternating periods of remission and active disease. UC is the result of a complex interaction between genetic susceptibility<sup>[1]</sup>, stimulation by bacterial antigens<sup>[2]</sup> in the lumen and occasional environmental triggers<sup>[3]</sup> that damage the mucosal barrier. Although the incidence and prevalence of UC is lower in Asia than in the West, recent population-based and referral center cohorts have shown a rising incidence and prevalence of UC in Asia<sup>[3]</sup>. Therefore, it is critical to gain a better understanding of the environmental factors that contribute to the onset and relapse of UC in Asia.

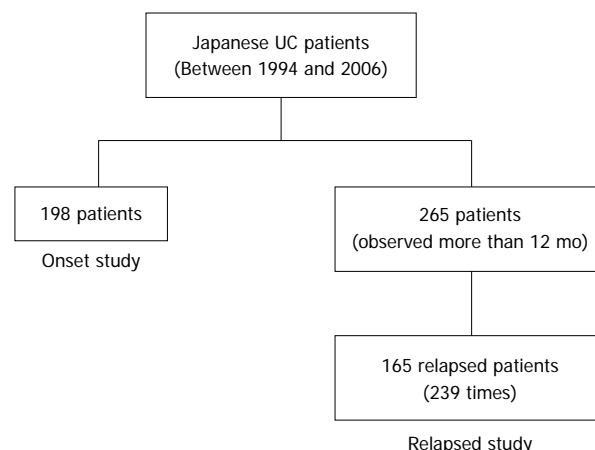
Furthermore, the environmental factors associated with UC are poorly defined. For example, previous studies examining seasonal variations in the onset and relapse of UC in Western populations<sup>[4-10]</sup> have demonstrated conflicting results. These studies used retrospectively collected or hospital admission data, which may have led to a bias in the results. Moreover, due to different cultural backgrounds, racial genetic predisposition and dietary habits, Japanese patients may show different clinical patterns from those of Western populations. Therefore, the present study was designed to investigate whether there were seasonal variations in the onset and relapse of symptoms in Japanese patients with UC.

## MATERIALS AND METHODS

### Patients

We performed an epidemiological cohort study of Japanese patients with UC who were diagnosed according to conventional criteria<sup>[11]</sup> between 1994 and 2006 at an academic hospital in Tokyo. The diagnosis of UC was confirmed by a typical history combined with the appropriate endoscopic, histopathological, and radiologic findings<sup>[11]</sup>. A total of 198 patients were enrolled in the onset evaluation (Figure 1). Data concerning the onset of symptoms were prospectively assessed using a standard interview focusing on symptoms accepted for UC (diarrhea, blood in stool, mucus or pus in stool, abdominal pain, fever, weight loss) and the period of time in which such symptoms occurred for the first time. The date of diagnosis was established according to the first investigation (endoscopy and histology, radiology or surgery) in which a diagnosis of UC could be defined. The onset of symptoms was recorded each month for 12 consecutive years. Relapse was defined as 3 or more increases in the symptom score<sup>[12]</sup>, excluding patients who relapsed due to decreasing doses of steroids, 5-aminosalicylic acid (5-ASA), or salazosulfapyridine (SASP).

Among 265 Japanese patients with UC observed for more than 12 mo between 1994 and 2006, 165 patients relapsed (239 times). The symptom scores of these pa-



**Figure 1** A flow chart of Japanese ulcerative colitis patients. Among 265 Japanese patients with ulcerative colitis observed for more than 12 mo between 1994 and 2006, a total of 198 patients were enrolled in the onset evaluation. Of these, 165 patients relapsed (239 times), and their symptom scores were recorded each month for at least one year in the relapse evaluation.

tients were recorded each month for at least one year (Figure 1). The frequencies of onset and relapse were compared for each month and the following four seasons: winter (December to February), spring (March to May), summer (June to August), and autumn (September to November).

### Statistical analysis

The frequencies of onset and relapse were compared using the  $\chi^2$  test. In addition, the 12-mo seasonality was tested using Rogers' method.  $P < 0.05$  was considered significant.

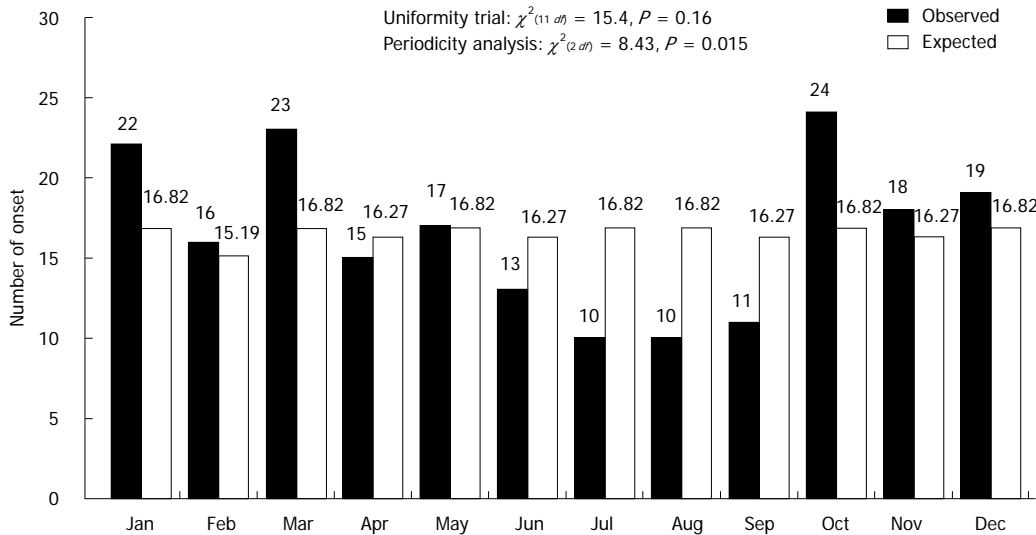
## RESULTS

### Variations in the onset of UC

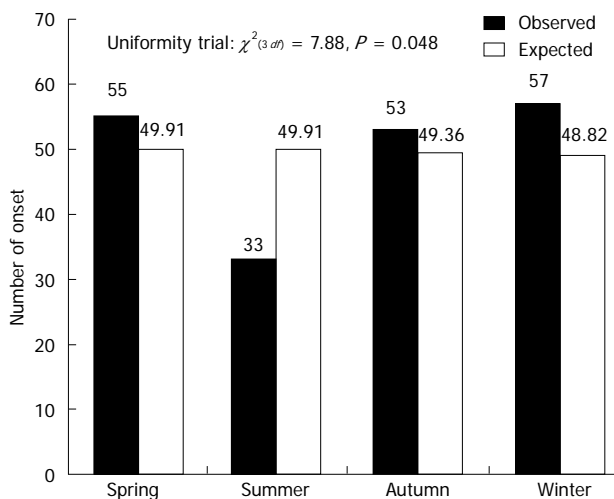
A total of 198 Japanese patients with UC (132 males and 66 females) were investigated in the onset evaluation. The median age at diagnosis was  $35 \pm 14$  years. The distribution of symptom onset according to month is shown in Figure 2. The timing of symptom onset was characterized by a clear monthly variation ( $\chi^2_{(2 df)} = 8.43$ ,  $P = 0.015$ ), with a peak during October and March and a trough during June and September. Moreover, a seasonal pattern was observed ( $\chi^2_{(3 df)} = 7.88$ ,  $P = 0.048$ ), as the onset rate was highest from autumn to spring [observed/expected (O/E): 53/49.36, 57/48.82, 55/49.91, respectively] (Figure 3).

### Variations in the relapse of UC

Among 265 Japanese patients with UC observed for more than 12 mo, 165 patients relapsed (239 times), which was defined as 3 or more increases in the symptom score<sup>[12]</sup>. Patients who relapsed due to decreasing doses of steroids, 5-ASA, or SASP were excluded. The median age at relapse was  $34 \pm 12$  years. Figure 4 shows the 165 observed UC patients according to month, taking into



**Figure 2 Monthly variations in the onset of ulcerative colitis.** The highest onset rate was observed in October (24/198, 12.1%), followed by March (23/198, 11.6%). The lowest onset rate was observed in July and August (10/198, 5.1%). The timing of ulcerative colitis (UC) onset was characterized by a monthly variation ( $\chi^2_{(12 df)} = 8.43, P = 0.015$ ), with a peak during October and March and a trough during June and September.



**Figure 3 Seasonal variations in the onset of ulcerative colitis.** The highest seasonal onset rate was observed in the winter (57/198, 28.8%), followed by the spring (55/198, 27.8%), autumn (53/198, 26.8%), and summer (33/198, 16.7%). A seasonal pattern was also observed ( $\chi^2_{(3 df)} = 7.88, P = 0.048$ ), as the onset rate was highest during autumn to spring (observed/expected: 53/49.36, 57/48.82, 55/49.91, respectively).

consideration the difference in the number of UC relapses each month. Relapse of symptoms in UC patients frequently occurred in October (O/E: 27/20.30). The lowest relapse rate was observed in January (O/E: 11/20.30). Variations in relapse were not found on a monthly basis ( $\chi^2_{(2 df)} = 1.31, P = 0.52$ , Figure 4). Moreover, there was no variation in relapse on a seasonal basis ( $\chi^2_{(3 df)} = 5.75, P = 0.12$ ) (Figure 5).

### Causes of UC relapse

In most cases, the causes of relapse were not identified. However, in cases with an identifiable cause, upper respiratory inflammation was the main factor responsible for

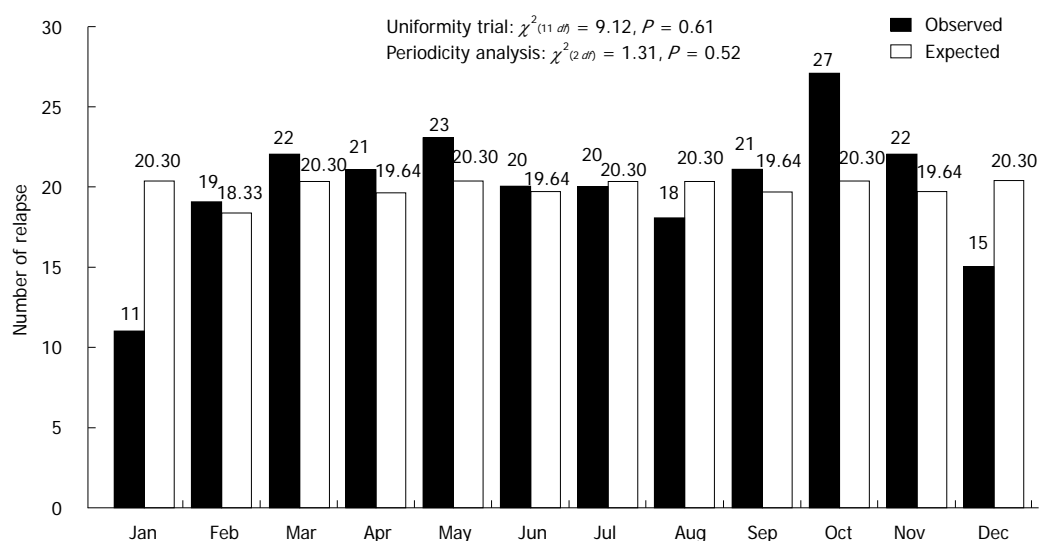
relapse (Figure 6).

## DISCUSSION

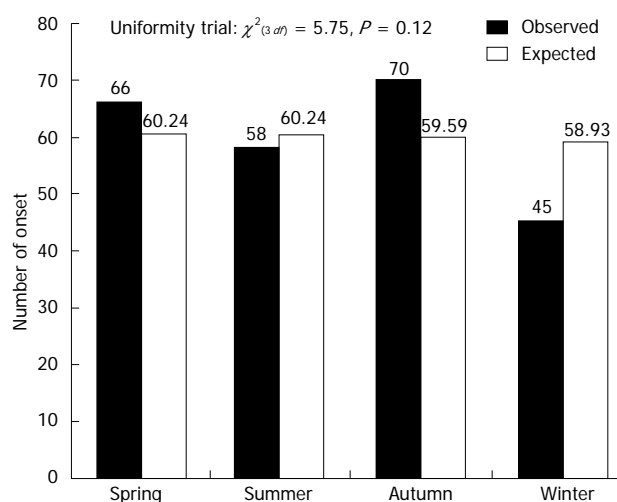
It is well known that environmental factors contribute to the induction of UC, although little is known about the relationship between seasonality and symptom flares in Asian UC patients, especially in the Japanese population. Therefore, we performed an epidemiological cohort study of patients with UC diagnosed between 1994 and 2006 at an academic hospital in Tokyo. The diagnosis of UC was confirmed by a typical history combined with the appropriate endoscopic, histopathological, and radiologic findings<sup>[11]</sup>. A total of 198 patients (132 males and 66 females) were enrolled in the onset evaluation.

A growing number of studies in Asia have reported an equal gender distribution in UC<sup>[12]</sup>, although several studies have also demonstrated a male predominance<sup>[13]</sup>. In our academic hospital-based cross sectional study, there was a preponderance of male UC cases. These conflicting findings may, at least in part, reflect the small population numbers in the present study. Our results revealed seasonal variations in the onset of UC symptoms in Japanese patients, although there was no difference in the timing of relapse. We observed monthly seasonality in the symptomatic onset of UC in October and March, mainly in winter and spring. Previous studies have reported seasonal variations in the symptomatic onset of UC in December in the United Kingdom<sup>[5,14]</sup>, December to January in Norway<sup>[15]</sup>, and June to August in Spain<sup>[16]</sup>. Moreover, increased relapse rates for UC were reported in winter<sup>[4]</sup> and autumn<sup>[5]</sup> in the United Kingdom, spring to autumn in Greece<sup>[8]</sup>, and winter in Sweden<sup>[7]</sup>; however, other studies reported no seasonality in the United Kingdom<sup>[17]</sup>, Spain<sup>[16]</sup>, or the United States<sup>[10,18]</sup>. These conflicting data may, at least in part, reflect the distinct genetic

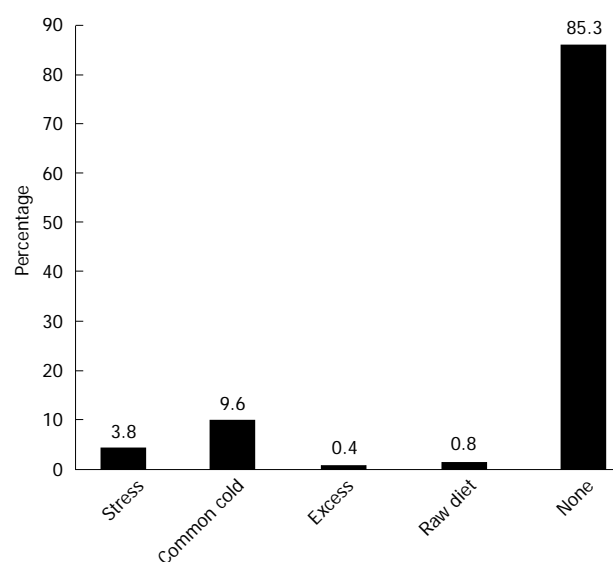




**Figure 4 Monthly variations in the relapse of ulcerative colitis.** The highest monthly relapse rate was observed in October (27/239, 11.3%), followed by May (23/239, 9.6%). The lowest relapse rate was observed in January (11/239, 4.6%). Variations in relapse were not found on a monthly basis ( $\chi^2_{(2 df)} = 1.31, P = 0.52$ ).



**Figure 5 Seasonal variations in the relapse of ulcerative colitis.** The highest seasonal relapse rate was observed in the autumn (70/239, 29.3%), followed by the spring (66/239, 27.6%), summer (58/239, 24.3%), and winter (45/239, 18.8%). There was no variation in relapse on a seasonal basis ( $\chi^2_{(3 df)} = 5.75, P = 0.12$ ).



**Figure 6 Causes of ulcerative colitis relapse.** In most cases (85.3%), the causes of ulcerative colitis (UC) relapse were not identified (none). However, in cases with an apparent cause, upper respiratory inflammation was the main factor responsible for UC relapse in our Japanese sample.

backgrounds of the study populations. In addition, the different seasonal patterns observed in different countries could also reflect variations in the climate and related environmental triggering factors associated with the onset of UC.

The environmental factors that may induce the onset or relapse of UC are not well understood. Patients with UC often experience exacerbations following bacterial and viral infections, and it is interesting that enteric pathogens such as *Salmonella*, *Campylobacter*, *E. coli*, and *Clostridium difficile* may cause relapses in UC. We have also reported that bacteria such as *Fusobacterium varium* (*F. varium*) can modulate the gut immune response and contribute to UC<sup>[19]</sup>; however, *F. varium* infections do not show a seasonal pattern of occurrence. Smoking is a well-known

environmental factor for UC<sup>[20]</sup>, and the use of non-steroidal anti-inflammatory drugs (NSAIDs)<sup>[21]</sup> and antibiotics<sup>[22]</sup> is also a risk factor for UC. In particular, during the winter, due to respiratory tract infections with organisms such as influenza and *Mycoplasma pneumoniae*, these drugs may be associated with the onset of UC. It has also been reported that respiratory and systemic viral infections are associated with the exacerbation of inflammatory bowel disease<sup>[23,24]</sup>. Also during the winter, intake of NSAIDs due to arthritic pain is common in Japan. In this study, cigarette smoking was not found to enhance the onset of UC, which is in contrast to previous reports concerning Crohn's disease<sup>[25]</sup>. Normally, patients with UC experience exacerbation when they attempt to quit smoking<sup>[25]</sup>.

Cigarette smoking may especially enhance the onset of UC in combination with other seasonal environmental factors, such as the intake of NSAIDs and antibiotics in the winter<sup>[20]</sup>, and these variables may explain the high incidence of disease onset observed during the winter. Our data showing that upper respiratory inflammation was the main factor responsible for relapse, with the exception of cases with no apparent cause for relapse, also support the high incidence of UC onset during the winter. Moreover, UC leads to inappropriate immune activation and increased levels of inflammatory cells and mediators, and UC can also be triggered by inappropriate immune activation in genetically predisposed individuals<sup>[26]</sup>. In addition, seasonal variations in immune responses have been reported<sup>[27]</sup>; unlike the summer and spring, immune functions and the levels of pro-inflammatory cytokines are decreased during the winter<sup>[28]</sup>. These differences in immune function across seasons may also explain the seasonal variations in the onset of UC.

In retrospective studies, UC relapse was shown to occur more frequently in spring and autumn in Western populations<sup>[5,8,10]</sup>. In our study of Japanese individuals, there were no significant differences in relapse on a monthly or seasonal basis. For most Japanese patients with UC, there may be no clear seasonal trigger for disease relapse, although long-term follow up studies with detailed microbiological surveillance of UC relapse should be performed, as antibiotic therapy can increase susceptibility to bacteria.

In conclusion, our results support the seasonality of UC onset in Japan. We found that the onset of UC in Japan typically occurred between October and March, although relapse rates did not show consistent seasonal variation. However, the present study was an epidemiological cohort study conducted at an academic hospital in Tokyo, and the study size was too small to draw valid statistical conclusions. Therefore, larger studies are needed in a Japanese population to assess the seasonal variations in UC onset and relapse.

## COMMENTS

### Background

The incidence and prevalence of ulcerative colitis (UC) has increased rapidly in Japan; however, the environmental factors that contribute to the course of UC have not been well defined. Therefore, it is critically important to gain a better understanding of the environmental factors that contribute to the onset and relapse of UC in a Japanese population.

### Research frontiers

Seasonal variations in the onset or relapse of UC have previously been studied in Western populations with conflicting results. Due to different cultural backgrounds, racial genetic predispositions and dietary habits, Japanese patients may show different clinical patterns from those of Western populations. To date, the environmental factors in Japan have not been well defined, although the results of studies in Western populations suggest that there may be seasonal variation in the natural history of UC.

### Innovations and breakthroughs

Environmental factors related to winter and spring seasonality are responsible for triggering the clinical onset of UC in Japan.

### Applications

The symptomatic onset of UC occurred during October and March in Japan,

whereas relapse generally occurred in October. Upper respiratory inflammation was one of the main factors responsible for relapse. Thus, the results of this study shed light on the environmental factors that contribute to the onset and relapse of UC in Japanese patients.

### Peer review

This manuscript reports statistically significant differences in the seasonal variation of UC incidence in a Japanese population. There was monthly seasonality in symptomatic onset during October and March; the onset of symptoms in UC patients generally occurred during the winter. The variation in UC onset was observed for both month ( $P = 0.015$ ) and season ( $P = 0.048$ ). In contrast, the variation in relapse was not significant either in month ( $P = 0.52$ ) or season ( $P = 0.12$ ). These conclusions support similar previous observations in Western populations.

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## Anxiety and depression propensities in patients with acute toxic liver injury

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### Abstract

**AIM:** To investigate anxiety and depression propensities in patients with toxic liver injury.

**METHODS:** The subjects were divided into three groups: a healthy control group (Group 1,  $n = 125$ ), an acute non-toxic liver injury group (Group 2,  $n = 124$ ), and a group with acute toxic liver injury group caused by non-commercial herbal preparations (Group 3,  $n = 126$ ). These three groups were compared and evaluated through questionnaire surveys and using the Hospital Anxiety-Depression Scale (HADS), Beck Anxiety Inventory (BAI), Beck Depression Inventory (BDI), and the hypochondriasis scale.

**RESULTS:** The HADS anxiety subscale was  $4.9 \pm 2.7$ ,  $5.0 \pm 3.0$  and  $5.6 \pm 3.4$ , in Groups 1, 2, and 3, respectively. The HADS depression subscale in Group 3 showed the most significant score ( $5.2 \pm 3.2$ ,  $6.4 \pm 3.4$  and  $7.2 \pm 3.4$  in Groups 1, 2, and 3, respectively) ( $P < 0.01$  vs Group



1,  $P < 0.05$  vs Group 2). The BAI and BDI in Group 3 showed the most significant score ( $7.0 \pm 6.3$  and  $6.9 \pm 6.9$ ,  $9.5 \pm 8.6$  and  $8.8 \pm 7.3$ ,  $10.7 \pm 7.2$  and  $11.6 \pm 8.5$  in Groups 1, 2, and 3, respectively) (BAI:  $P < 0.01$  vs Group 1,  $P < 0.05$  vs Group 2) (BDI:  $P < 0.01$  vs Group 1 and 2). Group 3 showed a significantly higher hypochondriasis score ( $8.2 \pm 6.0$ ,  $11.6 \pm 7.5$  and  $13.1 \pm 6.5$  in Groups 1, 2, and 3, respectively) ( $P < 0.01$  vs Group 1,  $P < 0.05$  vs Group 2).

**CONCLUSION:** Psychological factors that present vulnerability to the temptation to use alternative medicines, such as herbs and plant preparations, are important for understanding toxic liver injury.

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**Key words:** Liver injury; Herb; Toxic; Anxiety; Depression

**Core tip:** In South Korea, the number of toxic liver injuries caused by herbal and folk remedies is increasing. Although positive views on folk remedies are widespread and patients who have been hospitalized with toxic liver injury are often re-hospitalized, no studies have been conducted on the correlation between toxic liver injury and anxiety or depression. This multi-center nation-wide prospective study showed the anxiety and depression propensities in patients with toxic liver injury. Psychological factors that lead to vulnerability to the temptation to use alternative medicines, such as herbs and plant preparations, are important to better understand toxic liver injury.

Suh JI, Sakong JK, Lee K, Lee YK, Park JB, Kim DJ, Seo YS, Lee JD, Ko SY, Lee BS, Kim SH, Kim BS, Kim YS, Lee HJ, Kim IH, Sohn JH, Kim TY, Ahn BM. Anxiety and depression propensities in patients with acute toxic liver injury. *World J Gastroenterol* 2013; 19(47): 9069-9076 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9069.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9069>

## INTRODUCTION

As interest in health is increasing due to the rising average life expectancy, the aging population, and increased income, the use of unconventional medicines processed from various natural substances is increasing<sup>[1]</sup>. The frequency of the use of unconventional medicines is currently far higher than previously reported. The extents of use and costs of such medicines are also more wide and expensive in the United States<sup>[2]</sup>.

In South Korea, liver injuries caused by the abuse of oriental medicines and folk remedies with no clinical study results are increasing because many people still depend on easily accessible oriental medicines and folk remedies<sup>[3]</sup>. In particular, the groundless belief that natural

extracts and plant preparations have less adverse effects is widespread among the general public, and liver injuries caused by the misuse and abuse of such substances are gradually increasing<sup>[4]</sup>. A retrospective multi-center preliminary study, which was performed in 7 university hospitals in South Korea across the country in 2003, reported that the estimated annual incidence of hospitalization of patients with toxic hepatitis at a university hospital in South Korea was 2629.8<sup>[5]</sup>. Four years investigation of patients with acute liver injury in the Gyeongju area, located in southeast South Korea, found that 52% of patients had drug-induced liver injuries, and about 50% of such liver injuries were caused by plant preparations<sup>[6]</sup>. In a recent prospective nationwide study of drug-induced liver injury in South Korea, the most common cause of drug-induced liver injury was found to be "herbal medications"<sup>[7]</sup>. Toxic hepatitis is often seen in clinical settings, but the general public has a lower interest in and less knowledge of toxic hepatitis than viral hepatitis. Even though most people in South Korea are exposed to oriental medicines and supplementary health foods made of plant preparations that may induce hepatotoxicity, basic data about the frequency, the clinical catamnesis and the medical and social costs of hepatotoxicity caused by such substances are insufficient. Therefore, active reports on such clinical experiences are needed; however, it is difficult to definitively diagnose most cases of liver injuries that are presumed to be caused by plant preparations; thus, they are kept idle<sup>[8-10]</sup>. Furthermore, because repetitive exposures to preparations that cause liver injuries have been clinically observed, studies are needed on the psychoneurotic propensities of patients who are exposed to supplementary health foods, oriental medicines, and folk remedies that cause liver injuries.

The symptoms of anxiety and depression are often observed in psychiatry, and they are also often discovered in patients with non-psychiatric physical disease. Considerable research has been conducted regarding anxiety or depression in non-psychiatric general or medical practice<sup>[11-14]</sup>.

As shown by the above-mentioned studies, it is a well-known fact that such medical diseases are accompanied by anxiety and depression symptoms. Even though positive views on folk remedies are widespread and patients who have been hospitalized with toxic liver injury are often re-hospitalized, no studies have been conducted on the correlation between toxic liver injury which is frequently observed in South Korea in patients with anxiety and depression. Thus, this multi-center nationwide study was intended to investigate the propensities associated with anxiety and depression in patients with toxic liver injury.

## MATERIALS AND METHODS

### Study design and population

This is a prospective, multi-center study using questionnaire surveys to determine the anxiety and depression

of patients with toxic liver injury who were selected by their Roussel Uclaf Causality Assessment Method (RUCAM) scores, which were determined from their medical records. The RUCAM system is a means of assigning points for clinical, biochemical, serologic and radiologic features of liver injury, which gives an overall assessment score that reflects the likelihood that the hepatic injury is due to a specific medication<sup>[15]</sup>.

The study groups were enrolled between May 1, 2010 and April 30, 2012. Ten university hospitals in South Korea (Konkuk University, Korea University, Catholic University of Daegu, Dongguk University, Soon Chun Hyang University, Yeongnam University, Chonbuk National University, Chungnam National University, Hallym University, and Hanyang University) participated in this study.

The subjects were divided into three groups: a control group, a non-toxic acute liver injury group, and a toxic acute liver injury group involving toxic hepatitis. The subjects were divided as follows; Control group (Group 1): some patients who visited the medical examination center of each hospital for the purpose of medical examination were selected randomly; Non-toxic acute liver injury group (Group 2): patients with acute liver injury due to non-toxic causes such as virus and metabolic causes; and Toxic acute liver injury group (Group 3): patients with acute liver injury caused by toxic causes.

To identify the cause of acute liver injury, careful history taking, physical examination, liver function test, viral hepatitis serological testing (anti-HAV IgM, HBsAg, anti-HBc IgM, anti-HCV antibody, CMV, EBV, HSV), anti-nuclear antibody, anti-mitochondrial antibody, or imaging studies (abdominal sonography or CT) were performed.

These three groups were compared and evaluated through the questionnaire survey using scales of anxiety and depression, and the causative factors were analyzed. Liver injury was defined as cases in which the serum alanine aminotransferase (ALT) or conjugated bilirubin values were increased more than twice the upper limit of normal, or that aspartate aminotransferase (AST), alkaline phosphatase and total bilirubin were increased together with at least one of them being more than twice the upper limit of normal<sup>[16]</sup>. The acute nature of liver injury was defined as cases in which the liver injury had been recovered within 3 mo.

Toxic liver injury was defined as an acute liver injury, caused by medicinal herbs, plant preparations, health foods and folk remedies, with the exception of commercial drugs, having a score of 4 or higher on the RUCAM scale. After the purpose of this study was explained to patients with liver injury, only the patients, who agreed to participate in the survey, were selected as subjects. The following patients were excluded from this study: patients whose cases have been diagnosed as or who are receiving treatment by a psychiatrist for depression disorder, dysthymic disorder, schizophrenia, schizoaffective disorder, or organic mental disorder, and patients who

did not respond to the survey during outpatient visits.

Questionnaires including the following questions were answered by the selected subjects and were collected, and the characteristics of the clinical study groups were compared and analyzed. The questionnaire included general questions on age, sex, *etc.*; questions from the Hospital Anxiety-Depression Scale (HADS), the Beck Anxiety Inventory (BAI), the Beck Depression Inventory (BDI), and the hypochondriasis scale. The questionnaire surveys were conducted during the hospital visit for Group 1 and at the time of admission for Groups 2 and 3.

After collecting the 448 case questionnaires answered by the subjects, 73 case questionnaires were excluded because the patient failed to answer all of the questions, acute liver injury caused by commercial drugs, chronic liver disease, a RUCAM score of 4 or less, or an AST/ALT value that did not correspond with acute liver injury. The other 375 case questionnaires were analyzed. Accordingly, 125, 124, and 126 subjects were selected for Groups 1, 2, and 3, respectively.

HADS was developed by Zigmond and Snaith in 1983 to identify the caseness (possible and probable) of anxiety disorders and depression among patients in non-psychiatric hospital clinics<sup>[17]</sup>. The tool includes 14 items, seven related to anxiety and seven related to depression, each scored between 0 and 3. The authors recommended that a score above 8 on an individual scale should be regarded as a possible case and a score above 10 a probable case<sup>[18]</sup>. To rule out somatic disorders on the scores, all symptoms of anxiety or depression that were related to a physical disorder, such as dizziness, headaches, insomnia, anergia and fatigue, were excluded.

BAI is a 21 item self-report questionnaire measuring common symptoms of clinical anxiety, such as nervousness and fear of losing control. Respondents indicated the degree to which they are affected by each symptom. Each symptom is scored on a range from 0 to 3, with higher scores corresponding to higher levels of anxiety. Thirteen items assess physiological symptoms, five describe cognitive aspects, and three represent both somatic and cognitive symptoms<sup>[19]</sup>. A score above 21 is considered a breaking point and was distributed among each of the groups.

BDI is a 21 item self-reported questionnaire that measures the status of clinical depression without psychological diagnosis<sup>[20]</sup>. It consists of 8 items of physical depression and 13 items of non-physical depression. The BDI covers cognitive, emotional, and somatic symptoms, and its reliability, sensitivity and specificity have a high affinity for diagnosing depression<sup>[21,22]</sup>. Each item is scored ranging from 0 to 3, with higher scores corresponding to higher levels of depression. A score above 21 is considered a breaking point and was divided evenly among each of the groups.

To measure hypochondriasis, 33 questions corresponding to the hypochondriasis in the Minnesota Multiphasic Personality Inventory, which is widely used in psychia-

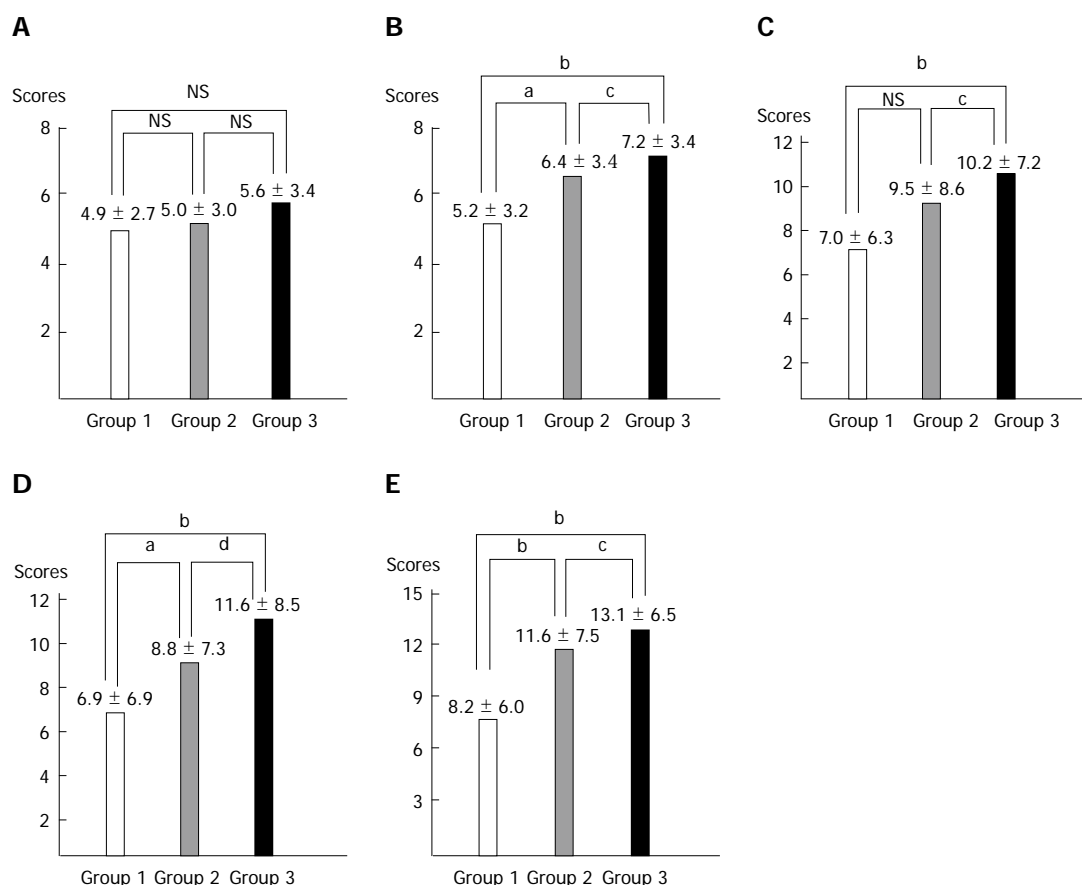


Figure 1 The anxiety and depression subscale of the Hospital Anxiety-Depression Scale, Beck anxiety inventory, Beck depression inventory and the hypochondriasis scores among the study groups. A: Group 3 showed the highest score, but there was no significant difference in the HADS anxiety subscale between groups; B: The HADS depression subscale in Group 3 showed the most significant score; C: The BAI in Group 3 showed the most significant score; D: The BDI in Group 3 showed the most significant score; E: Group 3 showed a significantly higher hypochondriasis score. Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. NS: Non significant; HADS: Hospital Anxiety-Depression Scale; BAI: Beck anxiety inventory; BDI: Beck depression inventory. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs Group 1; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs Group 2.  $P$  value by the Mann-Whitney  $U$  test.

try, were extracted, and the total score was used for data analysis.

### Statistical analysis

The SPSS for Windows 19.0 (IBM, New York, NY, United States) statistics program was used for statistical processing of the data. Main results were presented as mean  $\pm$  SD and the statistical significance was determined by  $P$  values smaller than 0.05. For comparison of continuous variables among the patient groups and the control groups or comparison of various variables depending upon different severity levels in the patient groups, non-parametric analyses (Kruskal-Wallis test and subsequent Mann-Whitney  $U$  test) were performed. The categorical variables were analyzed using the  $\chi^2$  test.

## RESULTS

### Clinical characteristics

Of the 375 total subjects, Groups 1, 2, and 3 consisted of 125, 124, and 126 subjects, respectively. The average age was  $45.2 \pm 13.7$  in Group 1,  $37.7 \pm 14.3$  in Group 2, and  $48.4 \pm 13.1$  in Group 3. The male-female ratio was 1:1.5

in Group 1, 1:1.5 in Group 2, and 1:1.5 in Group 3. In Group 2, the primary cause of acute liver injury was acute viral hepatitis A (Table 1). In Group 3, with toxic liver injury, the mean RUCAM score was  $7.14 \pm 1.6$  (4-11). As a cause of liver cell injuries by types, hepatocellular liver injuries were found in 96 cases (76.1%), cholestatic in 11 cases (8.7%), and a mixed type in 19 cases (15.0%).

### HADS

The anxiety subscale of the HADS was  $4.9 \pm 2.7$  (0-14) in Group 1,  $5.0 \pm 3.0$  (0-15) in Group 2, and  $5.6 \pm 3.4$  (0-16) in Group 3. Group 3 showed the highest score, but there was no significant difference in the HADS between groups (Table 2) (Figure 1). When the breaking point was set at 8, subjects, who had a score of 8 or higher and were thus deemed to have a high anxiety propensity, presented 20 cases (16.0%), 23 cases (18.5%) and 32 cases (25.3%) in Groups 1, 2, and 3, respectively. Group 3 had the largest number (Table 3).

The depression subscale of the HADS was  $5.2 \pm 3.2$  (0-13) in Group 1,  $6.4 \pm 3.4$  (0-16) in Group 2, and  $7.2 \pm 3.4$  (0-15) in Group 3. Group 3 showed the highest score, which was significantly higher than Groups 1 and 2 ( $P <$

**Table 1** Clinical characteristics of the patients

Demographic data	Group 1 ( <i>n</i> = 125)	Group 2 ( <i>n</i> = 124)	Group 3 ( <i>n</i> = 126)
Mean age (yr) (range)	45.2 ± 13.7 (20-78)	37.7 ± 14.3 (22-87)	48.4 ± 13.1 (18-72)
M:F ( <i>n</i> )	1:1.5 (50:75)	1:1.5 (50:74)	1:1.5 (50:76)
Cause ( <i>n</i> )	Healthy control (125)	Acute HAV (75) Unknown (34) Acute HBV (5) Alcoholic hepatitis (3) Gall stone (3) NAFLD (1) Cholecystitis (1) Cholangitis (1) Autoimmune hepatitis (1)	Toxic hepatitis (126)

Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. NAFLD: Non-alcoholic fatty liver disease.

**Table 2** Mean scores of the Hospital Anxiety-Depression Scale, Beck anxiety inventory, Beck depression inventory and Hypochondriasis scores in each groups

Scale	Group 1 ( <i>n</i> = 125)	Group 2 ( <i>n</i> = 124)	Group 3 ( <i>n</i> = 126)	<i>P</i> value
Anxiety mean scores of HADS (range)	4.9 ± 2.7 (0-14)	5.0 ± 3.2 (0-15)	5.5 ± 3.4 (0-16)	0.110
Depression mean scores of HADS (range)	5.2 ± 3.1 (0-13)	6.4 ± 3.4 (0-16)	7.0 ± 3.5 (0-15)	0.000
BAI mean scores (range)	7.0 ± 6.5 (0-34)	9.5 ± 8.5 (0-41)	10.2 ± 7.4 (0-40)	0.000
BDI mean scores (range)	6.8 ± 7.0 (0-38)	8.8 ± 7.3 (0-33)	11.2 ± 8.6 (0-37)	0.000
Hypochondriasis scores (range)	8.2 ± 6.4 (0-27)	11.6 ± 7.5 (0-31)	12.7 ± 6.7 (0-25)	0.000

Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. *P* value by the Kruskal-Wallis *U* test. HADS: Hospital Anxiety-Depression Scale; BAI: Beck anxiety inventory; BDI: Beck depression inventory.

**Table 3** The number of cases with scores of 8 or higher on the Hospital Anxiety-Depression Scale and 21 or higher on the Beck anxiety inventory and Beck depression inventory in each groups *n* (%)

Scale	Group 1 ( <i>n</i> = 125)	Group 2 ( <i>n</i> = 124)	Group 3 ( <i>n</i> = 126)	<i>P</i> value
Anxiety subscale of HADS No. of ≥ 8 scores	20 (16.0)	23 (18.5)	32 (25.3)	0.157
Depression subscale of HADS No. of ≥ 8 scores	33 (26.4)	44 (35.4)	57 (45.2)	0.008
BAI No. of ≥ 21 scores	7 (5.6)	17 (13.7)	13 (10.3)	0.098
BDI No. of ≥ 21 scores	6 (4.8)	10 (8.0)	19 (15.0)	0.017

Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. *P* value by the  $\chi^2$  test. HADS: Hospital Anxiety-Depression Scale; BAI: Beck anxiety inventory; BDI: Beck depression inventory.

0.01 *vs* Group 1, *P* < 0.05 *vs* Group 2) (Table 2) (Figure 1). When the breaking point was set at 8, the number of subjects, who had a score of 8 or higher and were thus deemed to have a high depression propensity, included were 33 cases (26.4%), 44 cases (35.4%) and 57 cases (45.2%) in Groups 1, 2, and 3, respectively. Group 3 had the most significant number (*P* < 0.01) (Table 3).

### BAI

The mean of the BAI score, which was designed to assess

anxiety symptoms, was 7.0 ± 6.3 (0-34) in Group 1, 9.5 ± 8.6 (0-41) in Group 2, and 10.7 ± 7.2 (0-40) in Group 3. Group 3 showed the most statistically significant score (*P* < 0.01 *vs* Group 1, *P* < 0.05 *vs* Group 2) (Table 2) (Figure 1). When the breaking point was set at 21, subjects, who had a score of 21 or higher and were thus deemed to have a high anxiety propensity, were 7 cases (5.6%), 17 cases (13.7%) and 13 cases (10.3%) in Groups 1, 2, and 3, respectively. Group 2 had the largest number, but there was no statistical significance (Table 3).



**BDI**

The mean of the BDI score, which was designed to assess depression symptoms, was  $6.9 \pm 6.9$  (0-38) in Group 1,  $8.8 \pm 7.3$  (0-33) in Group 2, and  $11.6 \pm 8.5$  (0-37) in Group 3. Group 3 showed the most statistically significant score ( $P < 0.01$  *vs* Groups 1 and 2) (Figure 1 and Table 2). When the breaking point was set at 21, subjects, who had a score of 21 or higher and were thus deemed to have a high depression propensity, were 6 cases (4.8%), 10 cases (8.0%) and 19 cases (15.0%) in Groups 1, 2, and 3, respectively. Group 3 had the most statistically significant number ( $P < 0.05$ ) (Table 3).

**Hypochondriasis score**

The mean of the hypochondriasis score was  $8.2 \pm 6.4$  (0-27) in Group 1,  $11.6 \pm 7.5$  (0-31) in Group 2, and  $12.7 \pm 6.7$  (0-25) in Group 3. Group 3 showed the most statistically significant score ( $P < 0.01$  *vs* Group 1,  $P < 0.05$  *vs* Group 2) (Figure 1 and Table 2).

**DISCUSSION**

The present study demonstrates that patients with toxic liver injury had high anxiety and depression propensities. Many studies have recently been conducted on anxiety and depression symptoms accompanying various medical diseases such as diabetes<sup>[23,24]</sup>, cardiovascular diseases<sup>[25,26]</sup>, and chronic obstructive pulmonary disease<sup>[27,28]</sup>. This study has a high value because this was the first study that evaluated patients with toxic liver injury from the psychiatric aspects of anxiety and depression. Furthermore, as a multi-center study, we are confident that the results of this study represent the nationwide pattern in South Korea.

From the above results, although the HADS levels were similar anxiety and depression tend to be observed slightly more in Group 3 compared to that of Group 2 and more in Group 2 compared to that of Group 1. In HADS, the anxiety subscale is not influenced by the 8 cut off point but the depression scale in Group 3 was significantly higher. In BAI and BDI, Group 3 showed a significantly higher rate of anxiety and depression. When subscale 21 was used as a cutoff point in BAI and BDI, the depression scale was particularly very high in the Group 3 patients. These observations show that both anxiety and depression are related to toxic hepatitis but that depression has a particularly high correlation. When the hypochondriasis scale is considered, Group 3 also displays a higher score. This general trend may be the result of the patients' tendency, due to anxiety and depression, to seek herbal supplements or alternative medicine when faced with toxic hepatitis.

Patients with toxic liver injury can be largely divided into two types: those whose liver injury was unavoidably caused by hospital prescriptions and those whose liver injury was caused by voluntary administration of sought out medicines. We can presume that patients of the latter type would have psychiatrically higher anxiety and

depression than the general public, which is the main subject matter in this study.

When this study was designed, we took into consideration that acute liver injury alone could cause anxiety and depression. Therefore, we divided the acute liver injury group into toxic/non-toxic groups. Generally, toxic or drug-induced liver injury displays similar increases in AST and ALT levels. However, causes of the damage are of a different nature: drug-induced liver injury is usually due to the passive intake of prescribed medication as instructed by physicians, whereas toxic liver injury is due to active self medication. Therefore, patients with drug-induced acute liver injury were excluded in this study, and we have a separate study on drug-induced liver injury and toxic liver injury planned.

Generally, there are risk factors such as genetic, non-genetic host susceptibility and environmental factors for idiosyncratic drug-induced liver injury<sup>[29]</sup>. The risk factors for herbal toxic liver injury are not well known. This study is meaningful in that we found that among the risk factors for the herbal or dietary supplement induced toxic liver injury including sex, age, cumulative dosage and herb-drug interaction<sup>[30-33]</sup>, the psycho-behavioral attitude of patients could also be an important risk factor. The reason that toxic liver injury broke out in a large number of females and people aged 50 or older is not known, but the results of this study suggests interpretationally that psychological factors should contribute greatly to such phenomenon.

It is thought that an effective method for preventing toxic liver injury is to treat patients with special care in collaboration with psychiatric treatment rather than simply telling them not to ingest plant preparations or health foods that could cause toxic liver injury. Although it may not be economic to treat all patients by administering drugs in collaboration with psychiatric treatment, it is thought that it could be a good guide to apply these study results, at least generally, to patients who visit the hospital repetitively due to toxic liver injury. This is because the patients, who had visited the hospitals at least 2 or 3 times repetitively due to the toxic liver injury, had higher anxiety and depression scores (data not shown).

This study has one limitation: most subjects in Group 2 were patients with acute hepatitis A. This was unavoidable because acute hepatitis A has recently broken out in a large number in South Korea<sup>[34]</sup>. However, no statistical difference was shown between the group with the acute liver injury caused by acute hepatitis A and the group with the acute liver injury caused by a small number of other causes. The reason for including Group 2 in the comparison was to determine whether anxiety and depression propensities were secondarily accompanied due to the acute liver injury, or whether the acute liver injury broke out because people with previously high anxiety and depression propensities had often searched for invigorants to rejuvenate their body.

This study is a cross-sectional study that was designed to try to understand the psychological states, such as

anxiety and depression, in patients with toxic liver injury who are taking herbal or folk remedies. The primary purpose of our study was achieved by determining the proportion of psychological conditions, such as anxiety and depression, suffered by patients with toxic liver injury taking herbal or folk remedies. However, there are issues that may be pointed out as a limitation of this cross-section study, such as “Does psychological state induce toxic liver injury?” and “Are changes in psychological state caused by toxic liver injury?” The relation can be demonstrated, but this research design may be limit our understanding of causalities. To overcome some of these limitations, we tried to compare the non-toxic acute liver injury group with the normal group. Through this process, we attempted to distinguish between anxiety and depression induced by hospitalization alone. This study demonstrated that the rate of anxiety and depression in patients with toxic liver injury is significantly higher than that of cases without toxic liver injury, even when taking into account the change in the psychological states due to hospitalization. We believe that this finding is a key result of our research. We plan to promote research to clarify the psychological risk factors such as anxiety and depression by comparing healthy individuals who are taking herbal preparations with toxic hepatitis patients taking herbal preparations through a case-control study.

It is thought that it will be necessary to develop scales specifically applicable to anxiety and depression in patients with toxic liver injury. Even though various conventional scales for anxiety and depression were used in this study, we feel that it is necessary to develop specific scales for anxiety and depression in patients with toxic liver injury.

We believe that meaningful results were derived from this study because psychiatric patients who were previously diagnosed with psychiatric disorders were excluded from this study. It is assumed that the results of this study would have a higher significance if a specific scale for patients with toxic liver injury had been used.

In conclusion, patients with toxic liver injury showed high scores on the HADS, BAI, BDI, and hypochondriasis scales. It can be postulated from this result that patients with toxic liver injury have high anxiety and depression propensities. This fact suggests that high anxiety and depression propensities could be the psychological factors that lead patients with toxic liver injury to access factors that may cause toxic liver injury, such as folk remedies and oriental medicines. It is therefore thought that although it is important to treat toxic liver injury itself, it is necessary to take active measures to understand and improve anxiety and depression symptoms of patients in an effort to prevent toxic liver injury from occurring and recurring.

## COMMENTS

### Background

Even though positive views on herbal preparations or folk remedies are widespread and patients who have been hospitalized with toxic liver injury are often re-hospitalized, no studies have been conducted on the correlation between

toxic liver injury which is frequently observed in South Korea and anxiety and depression in patients with toxic liver injury.

### Research frontiers

Patients with toxic liver injury can be largely divided into two types: those whose liver injury was unavoidably caused by hospital prescriptions and those whose liver injury was caused by voluntary administration of sought out medicines. It can be presumed that patients of the latter type would have psychiatrically higher anxiety and depression than the general public, which is the main subject matter in this study.

### Innovations and breakthroughs

This study has a high value because this was the first study that evaluated patients with toxic liver injury from the psychiatric aspects of anxiety and depression. This study indicates that patients with toxic liver injury showed high scores on the Hospital Anxiety-Depression Scale, the Beck Anxiety Inventory, the Beck Depression Inventory, and the hypochondriasis scales. It can be postulated from this result that patients with toxic liver injury have high anxiety and depression propensities. This fact suggests that high anxiety and depression propensities could be the psychological factors that lead patients with toxic liver injury to access factors that may cause toxic liver injury, such as folk remedies and oriental medicines.

### Applications

This study indicates that although it is important to treat toxic liver injury itself, it is necessary to take active measures to understand and improve anxiety and depression symptoms of patients in an effort to prevent toxic liver injury from occurring and recurring.

### Peer review

The authors present patients with herbal preparations-induced acute toxic liver injury had high anxiety and depression propensities. The results are interesting and indicate that psychological factors vulnerable to the temptation to use alternative medicines, such as herbs and plant preparations, are most important for understanding the toxic liver injury.

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## Appropriateness, endoscopic findings and contributive yield of pediatric gastrointestinal endoscopy

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### Abstract

**AIM:** To determine the predictability of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and American Society for Gastrointestinal Endoscopy (ASGE) guideline with regard to appropriate endoscopic practice in children, positive endoscopic findings and contributive yield in clinical practice.

**METHODS:** This was a descriptive, retrospective analysis, conducted at the Department of Paediatrics, University Malaya Medical Centre, Malaysia. All children who had esophagogastroduodenoscopy (EGD) and colonoscopy from January 2008 to June 2011 were included. An endoscopy was considered appropriate when its indication complied with the NASPGHAN and ASGE guideline. All endoscopic findings were classified as either positive (presence of any endoscopic or histo-

logic abnormality) or negative (no or minor abnormality, normal histology); effecting a positive contributive (a change in therapeutic decisions or prognostic consequences) or non-contributive yield (no therapeutic or prognostic consequences).

**RESULTS:** Overall, 76% of the 345 procedures (231 EGD alone, 26 colonoscopy alone, 44 combined EGD and colonoscopy) performed in 301 children (median age 7.0 years, range 3 months to 18 years) had a positive endoscopic finding. Based on the NASPGHAN and ASGE guideline, 99.7% of the procedures performed were considered as appropriate. The only inappropriate procedure (0.3%) was in a child who had EGD for assessment of the healing of gastric ulcer following therapy in the absence of any symptoms. The overall positive contributive yield for a change in diagnosis and/or management was 44%. The presence of a positive endoscopic finding was more likely to effect a change in the therapeutic plan than an alteration of the initial diagnosis. A total of 20 (5.8%) adverse events were noted, most were minor and none was fatal.

**CONCLUSION:** The NASPGHAN and ASGE guideline is more likely to predict a positive endoscopic finding but is less sensitive to effect a change in the initial clinical diagnosis or the subsequent therapeutic plan.

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**Key words:** Pediatric gastrointestinal endoscopy; Contributive yield; Esophagogastroduodenoscopy; North American Society for Pediatric Gastroenterology, Hepatology and Nutrition; American Society for Gastrointestinal Endoscopy

**Core tip:** Since the publication of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and American Society for Gastrointestinal Endoscopy (ASGE) modification of the



guideline on the appropriate use of endoscopy in children, no study has been conducted to ascertain the applicability of this guideline in the pediatric population. The present study addressed the deficiency in the literature by conducting a retrospective review of the gastrointestinal endoscopies conducted in a university setting in an Asian country. The present study showed that the modified NASPGHAN and ASGE guideline is applicable universally, be it in a Western country or an Asian country.

Lee WS, Zainuddin H, Boey CCM, Chai PF. Appropriateness, endoscopic findings and contributive yield of pediatric gastrointestinal endoscopy. *World J Gastroenterol* 2013; 19(47): 9077-9083 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9077.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9077>

## INTRODUCTION

Endoscopy is a useful diagnostic tool in both adult and pediatric populations<sup>[1,2]</sup>. Endoscopy in the pediatric population is usually performed by a pediatric gastroenterologist, and occasionally by a pediatric surgeon. In settings where the expertise of a fully trained pediatric gastroenterologist is not available, an adult gastroenterologist, supported by a pediatrician, can perform simple, diagnostic endoscopy in children safely<sup>[3]</sup>.

Esophagogastroduodenoscopy (EGD) and colonoscopy in children can be either diagnostic or therapeutic<sup>[2]</sup>. Common indications for diagnostic EGD and colonoscopy in children include the presence of symptoms indicative of an underlying organic pathology of the gastrointestinal (GI) tract<sup>[1,2,4,5]</sup>.

Generally, diagnostic pediatric EGD and colonoscopy are safe<sup>[6]</sup>. The risks of therapeutic endoscopy depend on the nature of interventions, but if performed by a pediatric endoscopist with appropriate training, the complication rate is less than 1%<sup>[6,7]</sup>. Potential complications may be encountered in sedation and anesthesia provided during the procedure<sup>[8]</sup>.

In 2000, the American Society for Gastrointestinal Endoscopy (ASGE) published a guideline on the appropriate use of GI endoscopy in the adult population<sup>[9]</sup>. Since then, many studies have found the ASGE guideline to be applicable in the adult population<sup>[10-12]</sup>. ASGE and the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) published a modification of the guideline for the pediatric population, where clear indications for both EGD and colonoscopy in children were recommended<sup>[2]</sup>.

There are several studies on the appropriateness of endoscopy in the adult population<sup>[10-12]</sup>. However, similar studies in the pediatric population are limited<sup>[5,13,14]</sup>. We conducted a retrospective review to assess the appropriateness of GI endoscopy performed in children in our

unit, based on the NASPGHAN and ASGE guideline<sup>[9]</sup>. In addition, the rates of positive and negative endoscopic findings as well as contributive and non-contributive yields to the diagnosis and management of the patients were also studied.

## MATERIALS AND METHODS

This was a retrospective, descriptive study conducted at the Gastroenterology and Nutrition Unit, Department of Paediatrics, University of Malaya Medical Centre (UMMC), Malaysia; from 1<sup>st</sup> January 2008 to 30<sup>th</sup> June, 2011. The present study was approved by the institutional ethical committee of UMMC.

During the study period, all children who required GI endoscopy in the unit, including those who were referred from outside the unit, were screened initially by one of the three practicing pediatric gastroenterologists (Lee WS, Chai PF, Boey CCM). All endoscopic procedures were performed by one of these three pediatric gastroenterologists.

### Case ascertainment

All consecutive patients younger than 18 years of age who had undergone EGD and colonoscopy during the study period were included. Patients were identified from the electronic database of the unit, and were cross-checked with the patient database from the endoscopic unit of the hospital. The case notes were reviewed. Patients who had inadequate data or incomplete procedures were not included.

### Data collection

The following data were collected: basic demographic data, preliminary diagnosis, indication for endoscopy, sedation or anesthesia, endoscopic finding, adverse events encountered during and after the procedure, clinical course and final diagnosis.

### Definitions

“Appropriate” and “inappropriate” indications for EGD and colonoscopy were defined according to the “Modifications in Endoscopic Practice for Pediatric Patients” by ASGE and NASPGHAN, published in 2008<sup>[9]</sup>. The indication for endoscopic procedures performed during the study period, if found to be compliant with the indications listed under “pediatric upper endoscopy” and “pediatric colonoscopy” in the ASGE and NASPGHAN guideline, was considered as “appropriate”. An indication was classified as “inappropriate” if the indication of the procedure was not listed in the guideline.

Anesthetic techniques and drugs used: In the present study, the induction of anesthesia used in children was the inhalational technique with sevoflurane and oxygen. After endotracheal intubation, patient paralysis, if necessary, was achieved by intravenous atracurium. Maintenance anesthesia was achieved by inhalational sevoflurane. Reversal of anesthesia was achieved by neostigmine and atropine.

**Table 1** Characteristics of 310 children undergoing 345 endoscopic procedures *n* (%)

Age	
< 6 mo	3 (1)
6 mo-2 yr	32 (11)
2-10 yr	185 (61)
> 10 yr	81 (27)
Gender	
Male	158 (53)
Female	143 (47)
Weight-for-age	
< 3 <sup>rd</sup> centile	123 (41)
3 <sup>rd</sup> -50 <sup>th</sup> centile	154 (51)
50 <sup>th</sup> -95 <sup>th</sup> centile	22 (6)
> 95 <sup>th</sup> centile	4 (1.4)
Type of procedure	
Esophagogastroduodenoscopy	231 (77)
Colonoscopy	26 (9)
Both	44 (15)

Three hundreds and forty-five endoscopic procedures took place in University Malaya Medical Center, Kuala Lumpur; January 2008 to June 2011.

### Positive and negative findings

By screening the procedures report, the endoscopic findings were divided into positive (presence of any abnormality in the endoscopic findings, or presence of relevant histologic findings), or negative (no abnormality or minor abnormality, normal histology)<sup>[10]</sup>.

### Contributive and non-contributive yields

The endoscopic procedures were divided into two categories: a positive contributive yield (the procedure had a positive effect on therapeutic decisions or prognostic consequences; this included interventional procedures) and non-contributive yield (a procedure which has no therapeutic or prognostic consequences)<sup>[10]</sup>. The patient may have a negative endoscopic finding and yet the procedure may be considered as having a positive contributive yield (example: a negative EGD finding in a child with upper GI bleeding).

### Adverse events

Adverse events which occurred during and after the procedures were noted. These were divided into sedation- or anesthesia-related, or procedure-related.

### Statistical analysis

Data were collected and managed by using statistical software programs (SPSS version 20.0, SPSS Inc., Chicago, IL, United States). Data were analyzed using a two-tailed  $\chi^2$  test; OR and related 95%CI were calculated. A *P* value < 0.05 was considered significant. Multivariate analysis was performed on selected symptoms and signs predicting a positive contributive yield (change) on the initial diagnosis or subsequent therapeutic plan.

## RESULTS

During the study period, a total of 362 procedures were

**Table 2** Indications for esophagogastroduodenoscopy and colonoscopy in 310 children *n* (%)

Indications	Value
Esophagogastroduodenoscopy	
Diagnostic	
Variceal surveillance/eradication	137 (48.9)
Hematemesis	41 (14.9)
Significant recurrent abdominal pain	37 (13.4)
Malenic stool	20 (7.3)
Chronic diarrhea/malabsorption	16 (5.8)
Recurrent vomiting	5 (1.8)
Malignancy surveillance	5 (1.8)
Dysphagia/odynophagia	4 (1.4)
Complicated gastroesophageal reflux disease	3 (1.0)
Unexplained anemia	1 (0.3)
Failure to thrive	1 (0.3)
Therapeutic	
Gastrostomy insertion	2 (0.7)
Foreign body removal	1 (0.3)
Colonoscopy	
Rectal bleeding	19 (27.0)
Monitoring of inflammatory bowel disease	19 (27.0)
Chronic diarrhea/malabsorption	18 (25.7)
Surveillance for polyp syndrome	6 (8.5)
Recurrent abdominal pain	5 (7.1)
Malignancy surveillance	3 (4.2)

performed in 318 children. Of these, 17 procedures involving 17 patients were excluded from analysis: 8 had incomplete data (three for EGD, two each for colonoscopy and percutaneous endoscopic gastrostomy feeding tube, and one for foreign body removal), and 9 had an incomplete procedure (seven had colonoscopy abandoned because of poor bowel preparation, and two patients had EGD abandoned because of esophageal stricture). Thus, a total of 345 procedures involving 301 patients were analyzed. Of these, 231 patients had EGD alone, 26 had colonoscopy alone, while 44 had combined EGD and colonoscopy.

### Patients' characteristics

The median age of these 301 children was 7.0 years old (range 3 mo to 18 years; Table 1). There were 158 (53%) males and 143 (48%) females. Almost half of the patients had a weight-for-age below the 3<sup>rd</sup> centile (*n* = 141, 41%).

### Indications for endoscopy

The two most common indications for EGD were surveillance for esophageal varices (*n* = 137, 50%) and upper GI bleed (*n* = 73, 26%; Table 2), while the two most common indications for colonoscopy were per rectal bleeding (*n* = 19, 27%), and surveillance/diagnosis of inflammatory bowel disease (IBD; *n* = 19, 27%). Of the total 86 therapeutic procedures performed, three-quarters (74%) were rubber banding for esophageal varices (Table 3).

### Appropriateness of endoscopy

Based on the NASPGHAN and ASGE guideline, 99.7% (*n* = 344) of the 345 procedures performed during the

**Table 3 Therapeutic procedures *n* (%)**

Procedures	Value
Esophageal varices eradication	
Rubber banding for esophageal varices	64 (74)
Sclerotherapy	18 (21)
Polypectomy	2 (2.3)
Foreign body removal	1 (1)
Insertion of percutaneous gastrostomy feeding tube	2 (2)
Total	86 (100)

study period were considered as appropriate. The only procedure (0.3%) which was considered as inappropriate was in a child who had an EGD for assessment of the healing of a gastric ulcer following medical therapy in the absence of any signs and symptoms.

### Positive and negative findings

Three-quarters ( $n = 261$ , 76%) of the 345 procedures performed showed a positive (abnormal) endoscopic finding [EGD: 216 (79% of all EGD performed), colonoscopy: 45 (64% of all colonoscopy performed); Table 4] while the remaining 84 (24%) had a negative endoscopic finding. A rapid urease test from a mucosal biopsy taken from the stomach and duodenum for *Helicobacter pylori* (*H. pylori*) infection was performed in 62 patients and was positive in 10 patients (16%).

### Factors predicting a positive endoscopic finding

Six clinical symptoms and four signs were analyzed to predict a positive contributive yield (effecting a change) in the initial diagnosis or subsequent therapeutic plan (Tables 5 and 6). On multivariate analysis, the presence of an enlarged liver or an enlarged spleen were least likely to effect a change in the diagnosis, while vomiting and abdominal pain were most likely to be associated with a change in the initial diagnosis. The presence of hematemesis was most likely to be associated with a change in therapeutic plan.

### Contributive and non-contributive yields

The overall contributive yield was 44.3% (Table 7). All the 79 patients who had a change in the initial diagnosis (positive contributive yield in diagnosis) also had a change in the subsequent therapeutic plan (positive contributive yield in therapeutic plan).

The presence of a positive (abnormal) endoscopic finding confirmed the clinical diagnosis in 57% ( $n = 197$ , negative contributive yield) of patients, while it altered the diagnosis in 19% ( $n = 64$ , positive contributive yield) of patients (Table 7). Conversely, a negative (normal) endoscopic finding confirmed the clinical diagnosis in 20% ( $n = 69$ ) of patients, while it altered the diagnosis in 4.3% ( $n = 15$ ) of patients (Table 7). This was not statistically significant ( $P = 0.234$ ). Of the 15 patients (4.3%) who had an alteration in the final diagnosis despite a negative endoscopic finding, most had an abnormal histology in the presence of normal endoscopic findings.

**Table 4 Probability of positive (abnormal) *vs* negative (normal) endoscopic findings *n* (%)**

Procedures	Endoscopic findings		Total
	Positive	Negative	
Esophagogastroduodenoscopy	216 (79)	59 (21)	275 (100)
Colonoscopy	45 (64)	25 (36)	70 (100)
All	261 (76)	84 (24)	

The presence of a positive endoscopic finding was more likely to effect a change in the management plan of a patient as compared to having a negative endoscopic finding (positive finding:  $n = 145$ , 42% *vs* negative finding:  $n = 8$ , 2.3%,  $P < 0.001$ ; Table 7). Most of those ( $n = 8$ ) who had a negative endoscopic finding but had a change in management plan were found to have a positive urease test for *H. pylori*. All had eradication therapy initiated.

### Adverse events

A total of 20 (5.8%) adverse events were noted; most were minor (Table 8). Secondary bleeding following rubber banding or sclerotherapy for esophageal varices was noted in 12 patients, while the bleeding rate following EGD was 4.3%. All the bleeding episodes were seen in patients with ( $n = 3$ , aged 11 mo to 2 years) biliary atresia and liver cirrhosis who had rubber banding for esophageal varices. None had liver transplantation. All patients needed blood transfusion but none became hemodynamically unstable.

Two patients who had esophageal varices and large ascites complicating liver cirrhosis needed assistance in respiration for a few hours following general anesthesia. Three children developed fever after endoscopy. All recovered uneventfully following a course of oral antibiotics. Another patient developed transient bronchospasm following extubation.

Two iatrogenic perforations following colonoscopy were noted in two children who had Crohn's disease. Both had gross delay in referral, severe malnutrition and extensive colonic disease. Both had fecal diversion surgery and recovered following surgical repair. The perforation rate following colonoscopy was 2.9%. No death occurred as a result of endoscopy in the present study.

## DISCUSSION

Generally, for a procedure to be considered as appropriate, its expected benefit should be greater than its expected negative consequences by a sufficiently wide margin to make the procedure worthwhile<sup>[15]</sup>. Benefit and negative consequences of a procedure are both defined in the broadest terms<sup>[10,15]</sup>.

Guidelines on the appropriateness of endoscopic procedures have been devised to aid clinicians in selecting more appropriate patients for referral, especially to units with limited expertise and financial resources<sup>[2,9]</sup>. Recently, a guideline pertaining to the appropriate use of endoscopy in children was published by NASPGHAN and

**Table 5 Univariate analysis for clinical parameters predicting a positive (abnormal) endoscopic finding**

Clinical parameters	Positive contributive yield (a change in diagnosis)			Positive contributive yield (a change in treatment)		
	P value	OR	95%CI	P value	OR	95%CI
Symptoms						
Vomiting	< 0.001	4.5	2.0-10.3	0.456	1.4	0.5-3.5
Diarrhea	0.010	2.6	1.5-4.2	0.940	0.4	0.4-1.8
Abdominal pain	0.020	2.1	1.1-4.0	0.750	0.9	0.5-1.7
Hematemesis	0.321	1.7	0.5-5.5	0.001	4.3	1.7-10.3
Melena	0.048	0.7	0.05-0.5	0.027	0.6	0.27-1.4
Hematochezia	0.065	2.0	0.9-4.3	0.040	2.8	1.3-5.7
Signs						
Pallor	0.525	1.2	0.5-2.8	0.520	1.2	0.6-2.3
Hepatomegaly	0.551	1.4	0.43-4.7	0.825	0.9	0.5-1.7
Splenomegaly	< 0.001	0.082	0.029-0.2	0.227	0.6	0.3-1.2
Abdominal tenderness	0.396	0.5	0.14-2.1	0.242	0.37	0.07-1.9

**Table 6 Multivariate analysis for clinical parameters predicting a positive (abnormal) endoscopic finding**

Clinical parameters	Positive contributive yield (a change in diagnosis)			Positive contributive yield (a change in treatment)		
	P value	OR	95%CI	P value	OR	95%CI
Symptoms						
Vomiting	< 0.001	4.5	2.0-10.3	0.827	0.9	0.4-2.6
Diarrhea	0.014	2.6	1.5-4.7	0.448	0.8	0.4-1.4
Abdominal pain	< 0.001	3.7	2.1-6.4	0.664	0.6	0.4-1.6
Hematemesis	0.251	1.5	0.7-3.3	< 0.001	4.3	1.8-9.5
Melena	0.080	1.7	0.9-4.3	0.022	2.9	1.0-5.2
Hematochezia	0.010	3.1	1.6-6.3	0.020	1.9	1.1-4.5
Signs						
Pallor	0.768	1.4	0.5-2.1	0.113	2.0	0.8-3.0
Hepatomegaly	0.004	0.2	0.1-0.5	0.629	1.3	0.6-1.9
Splenomegaly	< 0.001	0.08	0.04-0.17	0.198	1.3	0.8-2.0
Abdominal tenderness	0.267	1.9	0.5-2.1	0.097	0.2	0.05-3.0

**Table 7 Endoscopic findings and a subsequent contributive yield *n* (%)**

Endoscopic findings	Positive contributive yield (a change in diagnosis)		Positive contributive yield (a change in management)		Total
	Yes	No	Yes	No	
Positive	64 (18.6) <sup>1</sup>	197 (57.1)	145 (42.0) <sup>2</sup>	116 (33.6)	261 (75.7)
Negative	15 (4.3)	69 (20.0)	8 (2.3)	76 (22.0)	84 (24.3)
Total	79 (22.9)	266 (77.1)	153 (44.3)	192 (55.7)	345 (100)

<sup>1</sup>*P* = 0.234 ( $\chi^2$  test); <sup>2</sup>*P* < 0.001 ( $\chi^2$  test).

ASGE<sup>[2]</sup>. We believe that, although there are unavoidable socio-cultural and geographical differences as well as pattern of diseases, the NASPGHAN and ASGE guideline can be applied universally. Thus, for the present study the NASPGHAN and ASGE guideline was chosen.

In addition, little is known about pediatric endoscopic practice and its appropriateness in Asian countries, where human and financial resources, funding model, pattern of GI and liver diseases are different from the more advanced Western countries.

There have been several studies on the appropriateness of EGD in various clinical situations in children<sup>[13,14]</sup>. However, none are based on the NASPGHAN and ASGE guideline. For example, Jantchou *et al*<sup>[13]</sup>, based on the recommendations by the French-language Pediatric Hepatology, Gastroenterology and Nutrition Group (GF-HGNP), noted that 18% of the 251 EGD procedures

performed were considered as inappropriate, a figure which was higher among outpatient referrals. Guariso *et al*<sup>[5]</sup>, using a model of expert consensus from theoretical scenarios, noted that except in cases with a positive family history of peptic ulcer and/or *H. pylori* infection, children aged 10 years of older, or with persistent symptoms, not all EGD in children with dyspeptic symptoms could be considered as appropriate. Miele *et al*<sup>[14]</sup> found that the publication of Rome II criteria for functional GI disorders has a positive impact on the appropriateness of GI endoscopy, with inappropriate procedures reduced significantly after its publication. Nevertheless, 26% of all procedures were still considered as inappropriate<sup>[14]</sup>.

In contrast, although using different standards, the overall inappropriateness for pediatric endoscopy in the present study was 0.3%, with an overwhelming 99.7% of the cases being considered as appropriate. The only case



**Table 8 Adverse events encountered in 345 endoscopic procedures**

Complications	n
Procedure-related	
Secondary bleeding following rubber banding or sclerotherapy	12
Bowel perforation during colonoscopy	2
Anesthesia/sedation-related	
Delayed extubation due to ascites	2
Post-extubation bronchospasm	1
Secondary fever	3
Total	20

in the present study which was deemed to be inappropriate as an EGD reassessment of a healing gastric ulcer, in the absence of any symptoms and signs. This figure compares favorably with 18% of inappropriateness noted by Jantchou *et al.*<sup>[13]</sup> and 26% found by Miele *et al.*<sup>[14]</sup>.

There is, at present, limited availability of human resources in pediatric gastroenterology practice in Malaysia. The pediatric gastroenterology and nutrition unit of UMMC is only one of two pediatric gastroenterology units in Malaysia providing regular pediatric endoscopic services. The model of practice is not an open-access system. Thus, in the present study, all referrals for GI endoscopy from office-based pediatricians were screened initially by one of the practicing gastroenterologists before being subjected to endoscopy, hence reducing potentially inappropriate cases.

Nevertheless, some authors argued that the probability of detecting a clinically relevant lesion is considered as important as the appropriateness of the procedure<sup>[16,17]</sup>. Gonvers *et al.*<sup>[18]</sup> found that when applying the ASGE criteria to 450 outpatients who underwent EGD, there were no significant differences in clinically relevant findings in those patients who had an appropriate *vs* an inappropriate EGD.

Thus, we also studied the probability of finding a positive endoscopic finding in addition to studying the appropriateness of endoscopy. In the present study, the overall probability of detecting a positive endoscopic finding was 76%, higher in EGD (79%) than in colonoscopy (64%).

In the present study, the positive contributive yield for a change in the initial diagnosis was only 23% (Table 5). This is mainly because in over half of the cases (57%), a positive endoscopic finding confirmed the initial diagnosis, thus the contributive yield was considered as negative. However, what is equally important was a negative finding which has a positive contributive yield. Examples included the reassuring negative EGD finding in a child with upper GI bleeding. In the present study, a positive endoscopic finding was more likely to effect a change in the management plan than to effect a change in the initial diagnosis.

Although endoscopic procedures in the pediatric population are generally safe, adverse events and complications related to anesthesia and the endoscopic procedure itself are well documented<sup>[7,8,19]</sup>. Most of the adverse events encountered in the present study were minor and transient in nature. The perforation rate of colonoscopy

in the present study was 2.9%, higher than similar figures in the literature<sup>[7,19]</sup>. Both cases were children with Crohn's disease who had severe delay in referral, advanced malnutrition and total colonic involvement. Nevertheless, efforts should be initiated to reduce the complications rate further by improving the training of endoscopy in the unit<sup>[20]</sup>.

The main shortcoming in the present study was its retrospective nature. Thus, it may not be entirely accurate in ascertaining whether an endoscopic finding effected any alteration in the initial diagnosis and subsequent therapeutic plan. In addition, the age range of the patients in the present study was wide, and the indications for endoscopy in young children may not be similar to adolescents. Thirdly, the present study was conducted in a university hospital setting and the procedures were performed by experienced pediatric gastroenterologists. Thus, the findings of the present study may not be entirely applicable in other settings.

In conclusion, the present study showed that the modified NASPGHAN and ASGE guideline is applicable universally, be it in a Western country or an Asian country. Although the NASPGHAN and ASGE guideline on the appropriateness of pediatric endoscopy is useful in helping clinicians selecting the most appropriate patient for GI endoscopic procedures, nevertheless its predictability of a positive endoscopic finding is moderate, and it is not very sensitive in predicting whether a procedure has any positive contributive yield in the diagnosis and management of the patients.

## COMMENTS

### Background

Esophagogastroduodenoscopy (EGD) and colonoscopy in children can either be diagnostic or therapeutic. Generally, diagnostic pediatric EGD and colonoscopy are safe, but the risks of therapeutic endoscopy depend on the nature of interventions. If performed by an experienced pediatric endoscopist with appropriate training, the complication rate is less than 1%. Generally, for a procedure such as gastrointestinal endoscopy to be considered as appropriate, its expected benefit should be greater than its expected negative consequences by a sufficiently wide margin to make the procedure worthwhile. There have been several publications on the appropriateness of gastrointestinal endoscopy in the adult population. But studies of a similar nature in the pediatric population are limited.

### Research frontiers

The present study planned to address the deficiency in the literature on the appropriateness of pediatric gastrointestinal endoscopy by conducting a retrospective review on the gastrointestinal endoscopies conducted in a university setting in an Asian country.

### Innovations and breakthroughs

The present study was the first major study to ascertain the applicability of the Modified Guidelines on the Appropriate use of Gastrointestinal Endoscopy in children by North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and American Society for Gastrointestinal Endoscopy (ASGE). It is also the first study from an Asian country to determine the indications of pediatric gastrointestinal endoscopy in children. There have been several publications on the indications of childhood gastrointestinal endoscopy from the Western countries, but none from an Asian country.

### Applications

The results of the present study showed that the vast majority of the pediatric gastrointestinal endoscopies performed in a university hospital setting were appropriate according to the modified guidelines. Thus, other pediatric endoscopists performing pediatric gastrointestinal endoscopy should consider referring

to the "Modified Guidelines" for the purpose of benchmarking.

### Terminology

"Appropriate" and "inappropriate" indications for pediatric gastrointestinal endoscopies were defined according to the "Modifications in Endoscopic Practice for Pediatric Patients" by the American Society for Gastrointestinal Endoscopy and North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. A contributive yield was defined as a procedure that had a positive effect on therapeutic decisions or prognostic consequences in a patient. A non-contributive yield was defined as a procedure that had no therapeutic or prognostic consequences.

### Peer review

This article from an Asian country aimed to determine the predictability of the NASPGHAN and ASGE guideline in endoscopic practice for children on positive endoscopic finding and contributive yield in clinical practice in children. Although there are some unavoidable socio-cultural and geographical differences as well as pattern of diseases, the NASPGHAN and ASGE guidelines, as the present study shows, can be applied universally. The overall study is interesting, and no similar study was detected in the literature.

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## Routine lymph node dissection may be not suitable for all intrahepatic cholangiocarcinoma patients: Results of a monocentric series

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### Abstract

**AIM:** To investigate the indications for lymph node dissection (LND) in intrahepatic cholangiocarcinoma patients.

**METHODS:** A retrospective analysis was conducted on 124 intrahepatic cholangiocarcinoma (ICC) patients who had undergone surgical resection of ICC from January 2006 to December 2007. Curative resection was attempted for all patients unless there were metastases to lymph nodes (LNs) beyond the hepatoduodenal ligament. Prophylactic LND was performed in patients in whom any enlarged LNs had been suspicious for metastases. The patients were classified according to the LND and LN metastases. Clinicopathologic, operative, and long-term survival data were collected retrospectively. The impact on survival of LND during primary resection was analyzed.

**RESULTS:** Of 53 patients who had undergone hepatic resection with curative intent combined with regional

LND, 11 had lymph nodes metastases. Whether or not patients without lymph node involvement had undergone LND made no significant difference to their survival ( $P = 0.822$ ). Five patients with multiple tumors and involvement of lymph nodes underwent hepatic resection with LND; their survival curve did not differ significantly from that of the palliative resection group ( $P = 0.744$ ). However, there were significant differences in survival between patients with lymph node involvement and a solitary tumor who underwent hepatic resection with LND and the palliative resection group (median survival time 12 mo vs 6.0 mo,  $P = 0.013$ ).

**CONCLUSION:** ICC patients without lymph node involvement and patients with multiple tumors and lymph node metastases may not benefit from aggressive lymphadenectomy. Routine LND should be considered with discretion.

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**Key words:** Intrahepatic cholangiocarcinoma; Lymph node dissection; Lymph node metastases; Postoperative survival

**Core tip:** The indications for lymph node dissection (LND) in patients with intrahepatic cholangiocarcinoma (ICC) are still controversial. Our findings may provide a reference to the criterion for LND in ICC patients. Routine LND should be considered with discretion for ICC patients without lymph node involvement and patients with multiple tumors and lymph node metastases.

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## INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC), arising from second order or more peripheral branches of the intrahepatic bile duct, is the second most common primary liver cancer after hepatocellular carcinoma (HCC), accounting for 5%-10% of primary malignancies of the liver<sup>[1,2]</sup>. It is considered a highly malignant neoplasm because it is frequently associated with lymph node (LN) involvement, intrahepatic metastasis, and peritoneal dissemination<sup>[3,4]</sup>.

Hepatic resection remains the most effective therapy for patients with ICC. LN status, a definite prognostic factor in oncologic surgery, significantly affects long-term survival, as reported by the tumor staging system of the International Union Against Cancer<sup>[5]</sup>. Regional lymph node dissection (LND) is already a standard procedure, in combination with hepatic resection, for carcinoma arising from the extrahepatic bile duct<sup>[6,7]</sup>. Although LN metastasis is considered to be the most important prognostic factor for survival of ICC patients<sup>[8,9]</sup>, the indications for, and roles of, LND in patients with ICC are still subject to discussion. It is important to define the role of LND because it is a modifiable factor by a surgeon during hepatic resection, but no clear guidelines yet exist. Although some consider the standard surgical procedure for ICC is hepatectomy combined with extensive nodal dissection, not all centers support routine LND<sup>[10]</sup>. Some institutions have reported selective LND and limited application of this procedure<sup>[11]</sup>. Concerns remain about routine performance of LND in patients with liver tumors because it is reportedly associated with an increased operative risk compared with hepatic resection alone<sup>[3,12]</sup>.

We performed a retrospective analysis of consecutive patients at our hospital to examine the outcomes of ICC patients undergoing hepatic resection. We assessed the influence of LND on patient survival to clarify the indications for this procedure in surgical treatment of ICC, especially when LN metastases are absent.

## MATERIALS AND METHODS

### Patients

Altogether, 152 patients were diagnosed with ICC and underwent surgical dissection at Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China) from January 2006 to December 2007. Twelve patients only underwent laparotomy and biopsy because they had peritoneal dissemination. The remaining 140 patients were included in the present study. Among them, only 124 (88.6%) were followed sufficiently to allow subsequent data analysis, and the remaining 16 patients were lost to follow-up. The reasons for their loss to follow-up are unknown but include inability to contact them and possibly death. ICC was defined as adenocarci-

noma arising from second order or more distal branches of the intrahepatic bile ducts<sup>[10,11]</sup>. Patients with combined HCC and cholangiocarcinoma or bile duct cystadenocarcinoma were excluded from this study. The study protocol was approved by the Clinical Research Ethics Committee of our hospital. Written informed consent was obtained from all patients in the study according to the requirements of this committee.

### Preoperative investigations

Resectability of the ICCs was assessed by ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) before a decision to perform surgery was made. Liver function was evaluated according to the Child-Pugh classification. Patients aged over 60 years were routinely subjected to formal cardiopulmonary evaluation and evaluation of their general condition preoperatively. Resection criteria were constant over the study period and included the number of resectable tumors, presence or absence of tumor thrombi and gross metastatic foci, and adequate hepatic functional reserve, as described in our previous study<sup>[13]</sup>. Patients were deemed to have resectable disease only if the tumor could be completely removed while preserving a sufficient functional liver remnant with adequate vascular inflow and hepatic venous outflow. If the estimated liver resection volume exceeded 60% of the whole liver as calculated by CT, preoperative percutaneous transhepatic portal embolization was performed on the liver segment to be resected, in order to induce compensatory hypertrophy of the future remnant liver.

### Surgical procedures and definitions of parameters

Patients with peripheral ICC underwent hepatectomy while patients with hilar ICC underwent hemihepatectomy or trisectionectomy. Bisectionectomy or more was defined as a major hepatectomy. Sectionectomy or less was defined as a minor hepatectomy. Extended hepatectomy was defined as removal of 5 or more segments. Liver resection was performed using finger fracture and clamp crushing with intermittent Pringle's maneuver at room temperature. Initial intraoperative assessment consisted of careful examination and palpation of the hepatic hilum and hepatoduodenal ligament by the chief surgeons to detect any enlarged LNs. Any enlarged LN was considered suspicious for metastases. Because of the patient's old age, poor general condition, peripheral tumor location in the liver and the known increased risk of adding this procedure to hepatic resection, prophylactic LND was not performed in patients in whom LN involvement had not been identified by preoperative imaging (CT and MRI) and intraoperative assessment. These patients were clinically defined as not having LN metastases. If LN metastases were clinically recognized, regional LND was performed. However, curative resection was not attempted when there were metastases to LNs beyond the hepatoduodenal ligament. Regional LND included complete excision of soft tissue and LNs at the hepatic hilum, hepatoduodenal liga-



**Table 1** Operative procedures for intrahepatic cholangiocarcinoma

Operative modality	<i>n</i>
Hepatic resection ( <i>n</i> = 124)	
Major resection ( <i>n</i> = 65)	
Partial hepatectomy	38
Right trisectionectomy	1
Left trisectionectomy	2
Extended left hemihepatectomy	2
Right hemihepatectomy	4
Left hemihepatectomy	15
Central bisectionectomy	3
Minor resection ( <i>n</i> = 59)	
Partial hepatectomy	37
Right anterior sectionectomy	4
Right posterior sectionectomy	3
Left lateral sectionectomy	8
Bisegmentectomy	7
Additional procedures ( <i>n</i> = 67)	
Spleen resection	2
Gallbladder resection	12
Lymph node dissection	53

ment, posterior to the upper portion of the pancreatic head, and common hepatic artery stations. The extent of LND was similar for right- and left-sided tumors, except that dissection of LNs along the lesser curvature of the stomach was added for tumors located in the left lobe of the liver.

Intrahepatic cholangiocarcinoma was classified by gross appearance, as proposed by the Liver Cancer Study Group of Japan<sup>[14]</sup>. These types include mass-forming (MF), periductal infiltrating (PI), and intraductal growth (IG), with mixed types being expressed as MF + PI or MF + IG. Tumor-node-metastasis (TNM) staging of tumors followed the guidelines of the seventh edition of the American Joint Committee on Cancer/International Union against Cancer. In this study, multiple tumors were defined as more than one involved node (including micrometastases that were discovered only on pathological examination); tumor size referred to the maximum tumor diameter; resection of three or more hepatic segments was classified as major liver resection; and resection of one or two hepatic segments as minor liver resection. Curative resection was defined as negative surgical margins on microscopic examination of the resected specimen, surgical findings of macroscopic absence of intrahepatic metastases in the residual liver, and absence of visible abdominal dissemination.

### Follow-up

Clinical data for all patients were collected retrospectively. After resection, follow-up included routine blood tests, physical examination, and abdominal ultrasonography every 3 mo postoperatively for the first 2 years and twice a year thereafter at our hospital. Suspected recurrences were confirmed by CT or MRI. If the patients were unable to attend for these assessments, they were followed by telephone or letter yearly.

### Statistical analysis

Overall survival (OS) was measured from the date of surgery. Recurrence-free survival (RFS) was calculated from the date of surgery to the date of the first clinically documented disease recurrence. Comparison between groups was examined by the  $\chi^2$  test or Fisher's exact test. The OS and RFS were calculated using the Kaplan-Meier method. The log-rank test was used to assess differences. All statistical analyses were performed with software package SPSS 18.0 (SPSS, Chicago, IL, United States). Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### Clinicopathological characteristics

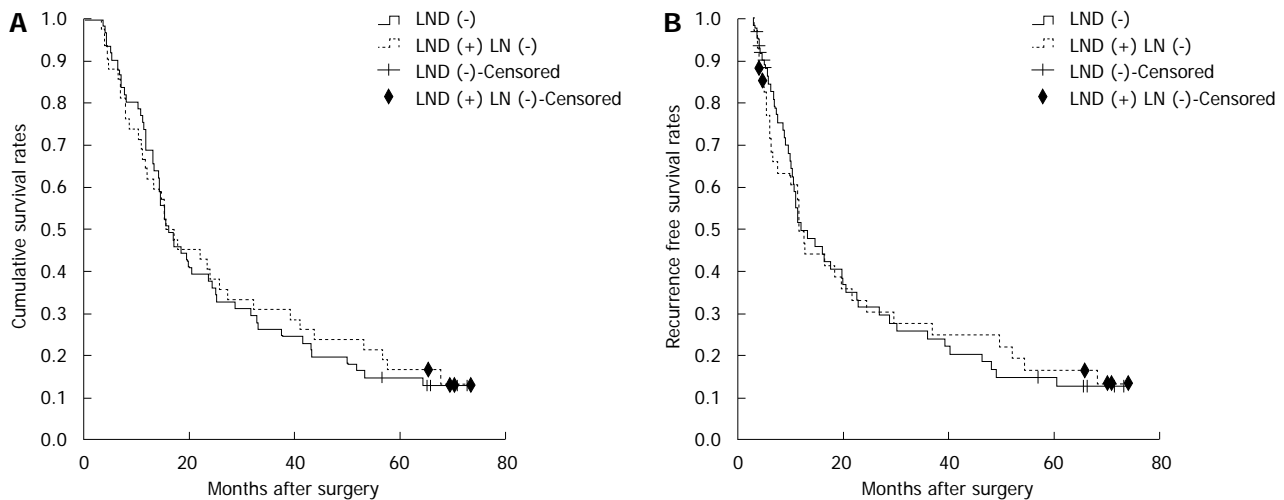
We continued follow-up of patients until death or the final date of the study, June 30, 2012. Data for analysis were available for 124 patients, including 96 men and 28 women with a median age of 56 years (range, 28-79). According to the Union for International Cancer Control TNM classification, 65 patients had stage I disease, 8 had stage II, 51 had stage III, and none had stage IV. As for liver function as defined by the Child-Pugh classification, 113 patients had class A, 11 had class B, and none had class C.

Of the 124 patients, 65 underwent major liver resection and 59 underwent minor resection. We performed additional procedures in 67 patients (Table 1). Surgical complications occurred in eight patients, including biliary leakage in three, subphrenic infection in two, liver abscess in one, bowel obstruction in one, and bleeding in one. LND did not increase the rates of postoperative complications or death.

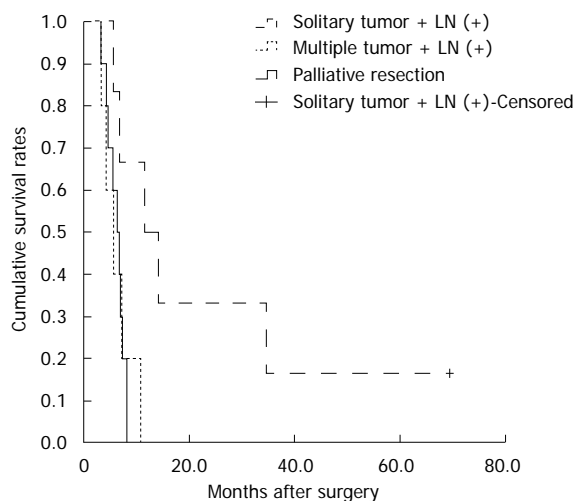
Of the 124 patients, 10 had microscopically positive resection margins (palliative resection group). Of the 114 patients who underwent resection with curative intent, 61 did not undergo LND [LND (-) group]. Of the 53 patients who underwent LND, 42 did not have LN metastases [LND (+) LN (-) group] and 11 did [LND (+) LN (+) group]. In all, 318 LNs were analyzed histologically. The median number of retrieved LNs was 6 (1-16). We found LN metastases in the hepatoduodenal ligament in 10 patients and along the common hepatic artery in three patients. We found a single LN metastasis in nine patients.

### OS and RFS of patients who did not undergo LND and those who did and had no LN metastases detected

The clinical and pathological characteristics of the patients in the LND (-) and LND (+) LN (-) groups are summarized in Table 2. There were no significant differences between these groups. Figure 1 presents the Kaplan-Meier survival analysis comparing patients in the LND (-) group with those in the LND (+) LN (-) group. There were no differences in their survival curves ( $P = 0.822$ ). The 1-, 3-, and 5-year OS rates were 69%, 26% and 15%, respectively, in the LND (-) group and 64%, 31%, and 17%, respectively, in the LND (+) LN (-) group. Recurrence occurred in 69 of these patients (67.0%).



**Figure 1** Overall survival and recurrence-free survival curves of intrahepatic cholangiocarcinoma patients without lymph node involvement. A: Survival curves of patients in the lymph node dissection (LND) (-) and LND (+) LN (-) groups. There is no significant survival difference between the two groups ( $P = 0.822$ ). The censored represented the cases who were still alive at the endpoint; B: Recurrence-free survival curve of patients in LND (-) and LND (+) LN (-) groups. There is no significant survival difference between the two groups ( $P = 0.970$ ). The censored represented the cases who were still alive at the endpoint or died for other reasons instead of tumor recurrence.



**Figure 2** Survival curves of patients in the palliative resection and lymph node dissection (+) lymph node (+) groups. There are significant differences between the palliative resection group and patients with lymph node (LN) involvement and a solitary tumor ( $P = 0.013$ ). There are no significant differences between the palliative resection group and patients with LN involvement and multiple tumors ( $P = 0.744$ ).

RFS rates at 1-, 3-, and 5-year were 53%, 25%, and 15%, respectively, in the LND (-) group and 52%, 29%, and 17%, respectively, in the LND (+) LN (-) group. There was no significant difference in RFS between the LND (-) and LND (+) LN (-) groups ( $P = 0.970$ ) (Figure 1). The sites of recurrence are shown in Table 3. The most common recurrence site was the remnant liver. Among the 61 patients who did not undergo LND, the initial recurrence site was LNs in nine. Recurrence in LNs occurred in four patients who had undergone LN dissection.

### OS of patients in the palliative resection group and patients who underwent LND and had positive LNs

Five patients with LN involvement and multiple tumors underwent hepatic resection with LND. As for the subgroup analysis, the median survival times of the palliative resection group and patients with LN involvement and multiple tumors were 6.0 mo and 5.5 mo, respectively (Figure 2). There were no significant survival differences between the two groups ( $P = 0.744$ ). However, there was a significant difference between patients with a solitary tumor and LN involvement who underwent hepatic resection with LND and the palliative resection group ( $P = 0.013$ ), and their median survival times were 12 mo and 6.0 mo, respectively (Figure 2).

## DISCUSSION

Although curative resection provides the only chance of long-term survival for patients with ICC, the prognosis after surgical resection remains poor because this tumor exhibits aggressive invasion locally and frequently metastasizes, tending especially to spread via the lymphatic system<sup>[3,15-25]</sup>. The rate of perihepatic LN positivity detected at surgery reportedly ranges from 36% to 62%<sup>[3,15-25]</sup>. In our current study of 53 patients who underwent regional lymphadenectomy, the incidence of LN metastasis was 20.8% (11/53), which is slightly lower than those reported in previous studies. The most common site of LN metastases was the hepatoduodenal ligament (10/11).

Many investigators have used multivariate analysis to determine useful prognostic factors for ICC after surgical resection in recent 5 years (Table 4)<sup>[8,9,26-37]</sup>. According to these reports, potentially significant prognostic factors

**Table 2 Clinicopathologic characteristics of patients in the lymph node dissection (-) and lymph node dissection (+) lymph node (-) groups**

Factor	LND (-) n = 61	LND (+) LN (-) n = 42	P value
Gender			1.000
Female	13	9	
Male	48	33	
Age			0.516
≤ 60	44	27	
> 60	17	15	
Viral hepatitis			1.000
Yes	36	24	
No	25	18	
Cirrhosis			0.694
Yes	28	21	
No	33	21	
Child-Pugh class			0.735
A	55	39	
B	6	3	
CA19-9 (U/mL)			0.553
≤ 37	30	18	
> 37	31	24	
Histologic differentiation			0.498
Well or Moderate	44	33	
Poor	17	9	
Gross type			0.433
MF	43	28	
PI	4	7	
IG	5	2	
MF + PI	5	4	
MF + IG	4	1	
Tumor number			0.510
Single	45	28	
Multiple	16	14	
Tumor size (cm)			0.318
< 5	33	18	
≥ 5	28	24	
TNM classification			0.080
Early (stage I, II)	47	25	
Advanced (stage III, IV)	14	17	
Width of resection margin (cm)			0.229
< 1	33	17	
≥ 1	28	25	
Surgical procedure			0.153
Major hepatectomy	41	22	
Minor hepatectomy	20	20	

CA19-9: Carbohydrate antigen 19-9; IG: Intraductal growth; LN: Lymph node; LND: Lymph node dissection; MF: Mass-forming; PI: Periductal infiltrating; TNM: Tumor-node-metastasis.

include multiple tumors, LN metastasis, serum CA 19-9 level, vascular invasion, tumor size, histological grade, intrahepatic metastases, histological grade, and resection margin. LN metastasis was confirmed to be one of the most significant independent prognostic factors for patients with ICC. Although LN metastasis was considered a significant prognostic factor, whether routine LND should be adopted is still controversial.

It is unclear whether prophylactic clearance of the route of LN metastasis improves survival. Ribero *et al*<sup>[8]</sup> reported that LN metastases and multiple tumors are associated with decreased survival rates. Lymphadenectomy should be considered for all patients according to its

**Table 3 Sites of initial recurrence in intrahepatic cholangiocarcinoma patients after resection with curative intent**

Site of initial recurrence	LND (-) n = 61	LND(+) LN (-) n = 42	LND(+) LN (+) n = 11
Liver, lymph nodes	6	3	2
Liver, lung	2	0	1
Liver	25	18	6
Lymph nodes	3	1	0
Peritoneum	5	2	2
Wound site	0	1	0
Bone	0	1	0
Lung	1	1	0
Total No. of recurrence	42	27	11

LN: Lymph node; LND: Lymph node dissection.

theoretical potential to improve long-term survival. LND for nodal metastases has reportedly resulted in a few long-term survivors<sup>[38,39]</sup>. But some authors have reported that extended LN dissection in patients with ICC does not seem to offer any advantage without control of liver metastases, because most recurrences are in the liver<sup>[3,40]</sup>.

In the current study, we showed that patients who did not undergo LND and those who did, but had negative LNs, had similar survival (1-year: 69% *vs* 64%; 3-year: 26% *vs* 31%; 5-year: 15% *vs* 17%, *P* = 0.822). These findings suggest that LND does not improve the survival significantly in LN negative patients. The commonest recurrence pattern was intrahepatic, which is similar to other reported findings<sup>[5,25,41,42]</sup>. We also found no statistically significant difference in RFS between patients who did and did not undergo LND (*P* = 0.970). It seems that LND does not improve the prognosis because it has no effect on liver metastases.

Prophylactic LND has been advocated to prevent LN recurrence, not only because there can be microscopic LN metastases around the perihepatic LNs, but also because it allows removing a frequent site of recurrence. Among the 61 patients who did not undergo LND, three developed LN recurrence as the primary recurrence site. The chance of benefiting from LND seems to be only about 3/61 (4.9%) of all patients with ICC. Choi *et al*<sup>[37]</sup> found that the patients who underwent LND but had negative LDs appear to show slightly worse survival than LND (-) group in the earlier time of the follow-up period, although it was not statistically significant because of the small sample size. Thus, prophylactic LND may be not beneficial to the clinically LD negative patients.

One might question the reliability of our intra-operative LN examination and indications for lymphadenectomy. For patients who had not undergone LN dissection, the N status cannot be ascertained. Clearly, some patients have microscopic nodal involvement that is beyond detection by conventional radiographic imaging or even direct palpation. Some authors have advocated routine lymphadenectomy for all patients undergoing hepatic resection as a staging procedure<sup>[8,9,40,43]</sup>. However, clinical assessment of LN negativity without histopathologic

**Table 4** Selected published series of intrahepatic cholangiocarcinoma patients after resection

Author	Year	Cases	Prognostic factors	Median survival time	5-yr survival rate	Routine LND
Ribero <i>et al</i> <sup>[8]</sup>	2012	434	LN metastases	33	32.90%	No
de Jong <i>et al</i> <sup>[9]</sup>	2011	449	Multiple tumors CA19.9 level	27.3	30.7	No
Saxena <i>et al</i> <sup>[26]</sup>	2010	88	Tumor number Vascular invasion LN metastasis CA 19.9 level Clinical stage Histological grade	33	28	No
Ercolani <i>et al</i> <sup>[27]</sup>	2010	72	LN metastases LN metastasis Blood transfusion	57.1	48	Yes
Cho <i>et al</i> <sup>[28]</sup>	2010	63	Old age CA19-9 level LN metastasis	Not available	31.8	Yes
Shirabe <i>et al</i> <sup>[29]</sup>	2010	60	Narrow resection margin Lymphatic invasion index Histological grade	Not available	30.6	No
Guglielmi <i>et al</i> <sup>[30]</sup>	2009	81	LN metastasis Vascular invasion	40	20	Not available
Tamandl <i>et al</i> <sup>[31]</sup>	2009	93	Lymph node ratio	25.5	Not available	No
Choi <i>et al</i> <sup>[37]</sup>	2009	64	LN metastasis	39	39.5	No
Shimada <i>et al</i> <sup>[32]</sup>	2009	104	Intrahepatic metastases LN metastasis	25	37	No
Yedibela <i>et al</i> <sup>[33]</sup>	2009	67	Resection margin LN metastasis Blood transfusion	26	27	No
Nakagohri <i>et al</i> <sup>[34]</sup>	2008	56	Intrahepatic metastasis	22	32	Not available
Uenishi <i>et al</i> <sup>[35]</sup>	2008	133	Intrahepatic metastasis LN metastases	18.4	29	Yes
Shimada <i>et al</i> <sup>[36]</sup>	2007	57	Tumor at the margin LN metastasis	62	56.8	No

LN: Lymph node; LND: Lymph node dissection; ICC: Intrahepatic cholangiocarcinoma.

confirmation appears to be associated with a small risk of subsequent LN metastases. Grobmyer *et al*<sup>[44]</sup> stated that the incidence of truly occult metastatic disease to perihepatic LNs is low in patients with primary and metastatic liver cancer. Of patients with negative preoperative imaging and intraoperative assessment, none had involved perihepatic nodes. This conclusion is consistent with another report of a low incidence of missed diagnosis of LN metastases<sup>[45]</sup>. In addition to the increased operative time associated with lymphadenectomy, surgeons should factor potential complications into decisions about performing this procedure in these patients without LN involvement.

In addition, hepatectomy with LND might not contribute to long-term survival in patients with multiple tumors and LN metastases. These patients had similar survival to patients who underwent palliative resection ( $P = 0.744$ ), possibly because both LN involvement and multiple tumors are poor prognostic factors<sup>[8]</sup>. However, patients with a solitary tumor and LN involvement might benefit from LND. Suzuki *et al*<sup>[21]</sup> reported that hepatic resection with LND may be curative for patients with a solitary tumor and a single LN metastasis. Nakagawa *et al*<sup>[24]</sup> also reported that curative resection with LND could improve the prognosis of patients with a solitary tumor

and no more than two LN metastases. In our series of patients with ICC, those with a solitary tumor and LN involvement had better survival than did palliative resection patients. The median survival time was 12 mo *vs* 6.0 mo ( $P = 0.013$ ). Although LN metastasis is an independent prognostic factor, it seems that LND can prolong the survival time of patients with a solitary tumor and LN metastases. However, more studies are needed.

Because our study was based on retrospectively available medical records, and more than five surgeons were involved in treating the study patients, it is difficult to draw definite conclusions about the indications for LND.

In conclusion, ICC patients without LN involvement and patients with multiple tumors and LD metastases may not benefit from aggressive lymphadenectomy. Without sufficient evidence, routine LND for all the ICC patients would be dogmatic. Routine LND should be considered with discretion.

## COMMENTS

### Background

Surgical resection is considered to improve the survival of patients with intrahepatic cholangiocarcinoma (ICC). Lymph node (LN) involvement significantly affects survival adversely. However, the benefit of lymph node dissection (LND) is still controversial.



## Research frontiers

Although LN metastasis was considered one of the most significant prognostic factors for patients with ICC, whether routine LND should be adopted is still controversial. Some consider that LN metastasis should not be considered a selection criterion that prevents patients from undergoing a potentially curative resection. Lymphadenectomy should be considered for all patients. On the other hand, some consider that routine use of LND in patients with ICC is not recommended, because no difference in survival was observed in patients with negative LN metastases, irrespective of the use of LND.

## Innovations and breakthroughs

The indications for, and roles of, LND in patients with ICC are still subject to discussion. It is important to define the role of LND because it is a modifiable factor by a surgeon during hepatic resection, but no clear guidelines yet exist. In this study, they found that ICC patients without LN involvement and patients with multiple tumors and LN metastases may not benefit from aggressive lymphadenectomy.

## Applications

It is unclear whether prophylactic clearance of the route of LN metastasis improves survival. Thire findings may provide a reference to the criterion for LND in ICC patients. Routine LND should be considered with discretion for ICC patients without LN involvement and patients with multiple tumors and LN metastases. Routine LND can be performed for the patients with a solitary tumor and LN metastases for a better survival.

## Peer review

The authors investigated the benefit of LND in the patients with ICC. They concluded that ICC patients without LN involvement and patients with multiple tumors and LN metastases may not benefit from aggressive lymphadenectomy, so routine LND should be considered with discretion. It is a well written manuscript that addresses an interesting topic. It also provides useful data on recurrences. The design is appropriate and the conclusion is reasonable.

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## Separate calculation of DW-MRI in assessing therapeutic effect in liver tumors in rats

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### Abstract

**AIM:** To explore whether the antitumor effect of a vascular disrupting agent (VDA) would be enhanced by combining with an antiangiogenic agent, and whether such synergistic effects can be effectively evaluated with separate calculation of diffusion weighted magnetic resonance imaging (DW-MRI).

**METHODS:** Thirty-seven rats with implanted liver tumors were randomized into the following three groups: (1) ZD6126, a kind of VDA; (2) ZDTHA, ZD6126 in combination with an antiangiogenic, thalidomide; and (3) control. Morphological DW-MRI were performed

and quantified before, 4 h and 2 d after treatment. The apparent diffusion coefficient (ADC) values were calculated separately for low  $b$  values ( $ADC_{low}$ ), high  $b$  values ( $ADC_{high}$ ) and all  $b$  values ( $ADC_{all}$ ). The tissue perfusion contribution,  $ADC_{perf}$ , was calculated as  $ADC_{low} - ADC_{high}$ . Imaging findings were finally verified by histopathology.

**RESULTS:** The combination therapy with ZDTHA significantly delayed tumor growth due to synergistic effects by inducing cumulative tumor necrosis. In addition to delaying tumor growth, ZDTHA caused tumor necrosis in an additive manner, which was verified by HE staining. Although both  $ADC_{high}$  and  $ADC_{all}$  in the ZD6126 and ZDTHA groups were significantly higher compared to those in the control group on day 2, the entire tumor  $ADC_{high}$  of ZDTHA was even higher than that of ZD6126, but the significant difference was not observed for  $ADC_{all}$  between ZDTHA and ZD6126. This indicated that the perfusion insensitive  $ADC_{high}$  values calculated from high  $b$  value images performed significantly better than  $ADC_{all}$  for the monitoring of tumor necrosis on day 2. The perfusion sensitive  $ADC_{perf}$  derived from  $ADC_{low}$  by excluding high  $b$  value effects could better reflect the reduction of blood flow due to the vessel shutdown induced by ZD6126, compared to the  $ADC_{low}$  at 4 h. The  $ADC_{perf}$  could provide valuable perfusion information from DW-MRI data.

**CONCLUSION:** The separate calculation of ADC is more useful than conventional averaged ADC in evaluating the efficacy of combination therapy with ZD6126 and thalidomide for solid tumors.

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**Key words:** Diffusion weighted imaging; Magnetic resonance imaging; Therapeutic assessment; Liver tumor; Rats; Vascular disrupting agent; Antiangiogenic agent;



## Animal model; Rodents

**Core tip:** The combination therapy with ZD6126 and thalidomide significantly delayed liver tumor growth due to synergistic effects by inducing cumulative tumor necrosis in rodents. The apparent diffusion coefficient (ADC)<sub>high</sub> performed significantly better than ADC<sub>all</sub> for the monitoring of tumor necrosis on day 2. The ADC<sub>perf</sub> could better reflect the reduction of blood flow due to the vessel shutdown induced by ZD6126, compared to the ADC<sub>low</sub>. The ADC<sub>perf</sub> could provide valuable perfusion information from diffusion weighted magnetic resonance imaging data.

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## INTRODUCTION

Tumor vasculature has become an attractive target for therapy. One of such therapies is to use vascular disrupting agents (VDAs), which can selectively destroy existing tumor blood vessels by disrupting the microtubules of the cytoskeleton in endothelial cells; this leads to ischemic central necrosis of the tumor<sup>[1]</sup>. However, tumors can rapidly rebound from the residual viable rim when VDAs are used alone; this compromises the therapeutic utility of these agents<sup>[2]</sup>. Another therapy is to prevent new tumor blood vessel formation with antiangiogenic agents. Therefore, current efforts have gradually shifted from the single use of VDA to the combination of a VDA with an antiangiogenic agent<sup>[3,4]</sup>. As the latter may inhibit the growth of new tumor vessels, the combination of two approaches thus is likely to have synergistic therapeutic efficacy.

As an established non-invasive technique, *in vivo* magnetic resonance imaging (MRI) has played an important role in the evaluation of tumor response to treatment. Diffusion-weighted MRI (DW-MRI), due to its ability to detect molecular water motion at the cellular level, *i.e.*, the measurement of tissue apparent diffusion coefficient (ADC), has become a favorite choice of measures in a variety of oncological studies and tissue viability assessments<sup>[5]</sup>. Technological innovations in recent years have enabled the increasing use of high-quality and quantitative DW-MRI in monitoring tumor treatment. However, it has been realized that the information acquired from conventional calculation of DW-MRI data actually represents the combined effects of tissue microcirculation perfusion and pure tissue diffusivity in each imaging voxel at DW-MRI. This may hinder the appropriate interpretation of the DW-MRI data<sup>[6]</sup>. Therefore, there is growing interest in applying more sophisticated approaches, such as separate ADC (calculating different

ADC values based on various combinations of *b* values with a monoexponential fitting algorithm)<sup>[7-9]</sup> and intra-voxel incoherent motion (IVIM)<sup>[10,11]</sup>, to differentiate the fraction of microcirculation perfusion from pure diffusivity within the DW-MRI data.

The purpose of the present study was to test our hypotheses that the antitumor effect of a VDA, ZD6126, would be enhanced by combining with an antiangiogenic agent, thalidomide, and that the effect can be monitored and better elucidated with separate calculation of ADC values compared to conventional ADC value in a rat liver tumor model. To our knowledge, the application of separate calculation of ADC maps has not been reported in such a combined antitumor therapy.

## MATERIALS AND METHODS

## Experimental design

A total of 37 rats were randomly assigned into the following 3 groups: (1) ZD6126 group (*n* = 14): ZD6126 (AstraZeneca, Cheshire, United Kingdom) was dissolved with 4 portions of 8.4% sodium carbonate and 1 portion of phosphate-buffered saline (PBS), pH 7.4. On day 0, one dose of 50 mg/kg ZD6126 was injected *iv* into each animal; (2) ZDTHA group (*n* = 13): Stock solutions of thalidomide (Pharmaceutical Factory, Changzhou, China) were prepared in DMSO (Sigma-Aldrich NV/SA, Bornem, Belgium) and injected *ip* at a dose of 200 mg/kg three times at a interval of one day during the entire experiment<sup>[12]</sup>; the first dose of thalidomide was injected 24 h prior to ZD6126 administration, and the second and third doses of thalidomide were given immediately and 2 d after ZD6126 administration, respectively; and (3) Control group (*n* = 10): Animals were *iv* and *ip* injected with the vehicles (solvents) of both agents at the same time points that the other groups were injected. For all groups, MRI was performed before, and 4 h and 2 d after the initial ZD6126 treatment. At the end of the experiment, all animals were sacrificed for histopathological examinations.

## Animal model

This study was approved by the institutional ethical committee for the use and care of laboratory animals. Adult WAG/Rij rats (Iffa Credo, Brussels, Belgium) with existing subcutaneous rhabdomyosarcomas were used as donors. The tumor tissues were excised and implanted into 37 normal adult WAG/Rij rats that weighed 225-275 g, as described previously<sup>[13]</sup>.

## MRI

All rats were initially anesthetized by inhalation of 2% isoflurane and maintained with 0.8% isoflurane for MRI. A clinical 1.5T MRI system (Sonata, Siemens, Erlangen, Germany) was used with a maximum gradient capability of 40 mT/m. The following sequences were acquired in the transverse plane for all rats, with a slice thickness of 2 mm and an inter-slice gap of 0.2 mm: (1) Fat saturated



T2-weighted fast spin echo MRI (T2W-MRI) with a repetition/echo time (TR/TE) of 3860/106 ms, a turbo factor of 19, a field of view (FOV) of 140 mm × 70 mm, and an acquisition matrix of 256 × 256 (in-plane resolution: 0.5 mm × 0.3 mm). Three signals were acquired, in a scan time of 1 min 25 s; (2) Contrast-enhanced fat saturated T1-weighted fast spin echo MRI (CE-T1W-MRI) immediately after an *iv* bolus of 0.3 mmol/kg gadoterate meglumine (Dotarem®, Guerbet, France), with the following parameters: a TR/TE of 535/9.2 ms, a turbo factor of seven, a FOV of 140 mm × 70 mm, and an acquisition matrix of 256 × 256 (in-plane resolution: 0.5 mm × 0.3 mm). Four signals were acquired, in a scan time of 1 min 24 s; (3) DW-MRI with a 2-dimensional (2D), spin echo, echo-planar imaging sequence. We used a TR/TE of 1700/83 ms, a FOV of 140 mm × 82 mm, and an acquisition matrix of 192 × 91 (in-plane resolution: 0.7 mm × 0.9 mm). For the DW-MRI, six signals were acquired, including repeated measurements for 10 different *b* values (0, 50, 100, 150, 200, 250, 300, 500, 750, and 1000 s/mm<sup>2</sup>) in 3 directions (*x*, *y*, and *z*) and averaged for the calculation of the isotropic ADC value. A parallel imaging technique was applied to reduce susceptibility artifacts and examination times. The total examination time was 4 min 51 s.

### Tissue processing and histology

All rats were euthanized for tissue processing and histology at the end of the experiment. First, animals were over-anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) (Nembutoal, Sanofi Sante Animale, Brussels, Belgium). Then, the livers were collected, fixed with formalin, embedded in paraffin, and sliced into transverse sections. The sections were 2 mm thick, and were positioned on the same planes used for the MRI scans, based on a grid (Agar Scientific, England). The tumor slices (5 μm thick) were stained with hematoxylin and eosin (HE).

### MRI analysis

An off-line LINUX workstation with dedicated software (Biomap, Novartis, Basel, Switzerland) was used for image analyses. Two experienced radiologists delineated the entire tumor with operator-defined regions of interest (ROI) in consensus to obtain robust measurements and to facilitate comparisons between different treatment approaches. All ROIs were larger than 10 pixels in size. For each imaging parameter, tumor and normal liver were measured with ROIs on all tumor-containing image slices, and mean values were obtained for each tumor and the liver, respectively. After that, the mean value and standard deviation for each parameter were calculated for each group at each time point for statistical analysis.

**T2 weighted and contrast enhanced-T1 weighted MRI:** The residual viable tumor or rim after treatment was visualized as contrast-enhanced, high signal region on the CE-T1W-MRI. The tumor necrotic areas were

contoured on CE-T1W-MRI based on the unenhanced, low-signal areas within the tumors that were observed after injection of a contrast agent. Relative volumes (%) of tumor necrosis were calculated by normalizing them to the entire tumor volume. For each lesion, the tumor areas were delineated at T2W-MRI on all slices and automatically combined into the total tumor volume. The tumor volume change (%) was calculated using the following formula:  $[(\text{volume}_{\text{post}} - \text{volume}_{\text{pre}}) / \text{volume}_{\text{pre}}] \times 100$ .

**Separate calculation of tumor ADC:** For calculating different ADC values, the first step was to measure the entire tumor signal intensity (SI) from original DW-MRI images of 10 *b* values, respectively. Briefly, for each tumor, freehand delineations were performed on all slices of the original DW-MRI at a *b* value of 1000 s/mm<sup>2</sup>. These delineations were merged to form one 3D volume of interest per lesion. The volume of interest was then automatically copied to all images with different *b* values and the average SI of each lesion per *b* value was determined. The second step was to obtain separate ADC values according to a monoexponential model using all 10 *b* values<sup>[14]</sup>. To differentiate the individual contributions of tissue microcapillary perfusion and pure tissue diffusivity, the ADC values of each tumor were obtained separately for low *b* values (*b* = 0, 50, and 100 s/mm<sup>2</sup>; ADC<sub>low</sub>) and high *b* values (*b* = 500, 750, and 1000 s/mm<sup>2</sup>; ADC<sub>high</sub>) from the average SI per tumor and per *b* value. Each ADC value was calculated by using a least squares solution of the following system of equations: ADC<sub>all</sub>:  $S_k = S_0 \times \exp(-b_k \times \text{ADC}_{\text{all}})$ , for *k* = 0, 50, 100; 150, 200, 250, 300, 500, 750, 1000; ADC<sub>low</sub>:  $S_i = S_0 \times \exp(-b_i \times \text{ADC}_{\text{low}})$ , for *i* = 0, 50, 100; ADC<sub>high</sub>:  $S_j = S_0 \times \exp(-b_j \times \text{ADC}_{\text{high}})$ , for *j* = 500, 750, 1000; where *S<sub>k</sub>*, *S<sub>i</sub>*, and *S<sub>j</sub>* are the SI measured on the DW-MRI acquired with the corresponding *b* values *b<sub>k</sub>*, *b<sub>i</sub>* and *b<sub>j</sub>*, *S<sub>0</sub>* represents the exact SI (without the influence of noise induced by the MR measurement) with *b* value equal to 0 s/mm<sup>2</sup>. ADC<sub>low</sub> is perfusion sensitive, while ADC<sub>high</sub> is perfusion insensitive. Although ADC<sub>low</sub> is perfusion sensitive, it is also affected by diffusion effects in tissue<sup>[15]</sup>. Therefore, an approximate indicator, ADC<sub>perf</sub>, for the tissue perfusion contribution can be calculated as ADC<sub>low</sub>-ADC<sub>high</sub><sup>[7]</sup>. Imaging software (MeVisLab 2.2.1, MeVis Medical Solutions AG, Bremen, Germany) was used to generate the maps of ADC<sub>all</sub>, ADC<sub>high</sub>, ADC<sub>low</sub> and ADC<sub>perf</sub>.

### Microscopic analysis

Microscopic image analyses were performed by a pathologist blinded to the experimental detail with magnifications ranging from × 50 to × 400. On HE stained macroscopic sections, image analysis software (ImageJ 1.34s, NIH, United States) was used to quantify the percentages of amorphous eosinophilic necrosis in the total tumor area.

### Statistical analysis

Statistical analysis was carried out with the SPSS for win-

dows software package (release 18.0, SPSS Inc., Chicago, United States). A general linear model, with repeated-measures, was used to compare changes in various parameters over time among groups. The nonparametric Kruskal-Wallis analysis of variance was performed for comparing parameters between groups at certain time points, followed by post-hoc group-wise comparisons using a Bonferroni correction for multiple tests. A  $P$  value  $< 0.05$  was considered statistically significant.

## RESULTS

A total of 37 rats (72 tumors) were included in the study. Four rats in the ZDTHA group were found to have minor hemorrhage around the eye socket and perianal area on day 1 after the first thalidomide treatment. This was probably due to a venous thromboembolism induced by thalidomide<sup>[16]</sup>.

### Tumor volume growth

As shown on T2W-MRI images, ZD6126 and ZDTHA both induced a significant tumor volume growth delay from pretreatment to 2 d after administration, compared to the control group ( $P < 0.0001$  for both). Furthermore, ZDTHA performed significantly better than ZD6126 in delaying tumor growth on day 2 after treatment ( $P < 0.0001$ ) (Figure 1).

**Perfusion insensitive  $ADC_{high}$ :** Before treatment: there were no significant differences in  $ADC_{high}$  among the three groups ( $P > 0.05$  for all). At 4 h, there was no significant change in the  $ADC_{high}$  ( $P > 0.05$  for both) in both the ZDTHA and ZD6126 groups, compared to the control group. On day 2, the therapy-induced tumor necrosis caused a significant rise in the  $ADC_{high}$  in both ZDTHA and ZD6126 groups compared to the control group ( $P < 0.0001$  and  $= 0.0004$ , respectively). Furthermore, the  $ADC_{high}$  was much higher in the ZDTHA group than in the ZD6126 group ( $P = 0.03$ ) (Table 1 and Figure 2A-C).

**$ADC_{all}$ :** At 4 h, the  $ADC_{all}$  in both the ZDTHA and ZD6126 groups was significantly reduced compared to the control group ( $P = 0.01$  and  $0.02$ , respectively). In contrast, the  $ADC_{all}$  in both the ZDTHA and ZD6126 groups showed a sharp increase on day 2 compared to the control group ( $P < 0.0001$  for both). However, no difference in  $ADC_{all}$  was found between the ZDTHA and ZD6126 groups ( $P = 0.08$ ) (Table 1 and Figure 2A-C).

**Comparison of  $ADC_{high}$  with  $ADC_{all}$ :** The performance of  $ADC_{all}$  was different with that of  $ADC_{high}$  at the following time points. At 4 h, the  $ADC_{all}$  in both the ZDTHA and ZD6126 groups showed a significant decrease compared to the control group; however, this was not observed for  $ADC_{high}$  in the same two groups. On day 2, the  $ADC_{high}$  of ZDTHA was significantly greater than that of ZD6126 ( $P = 0.03$ ), but the significant difference was not observed for  $ADC_{all}$  between the ZDTHA and

ZD6126 groups ( $P = 0.08$ ) (Table 1 and Figure 2A-C).

### $ADC_{low}$ and perfusion sensitive $ADC_{perf}$

**$ADC_{low}$ :** The  $ADC_{low}$  of ZDTHA was significantly lower than that of ZD6126 before treatment ( $P < 0.05$ ), but it was not for the control group ( $P = 0.12$ ). No significant differences in  $ADC_{low}$  were observed among the three groups at 4 h ( $P > 0.05$  for all). On day 2, the  $ADC_{low}$  of ZDTHA was much higher compared to the control group ( $P = 0.02$ ) (Table 1 and Figure 2D-F).

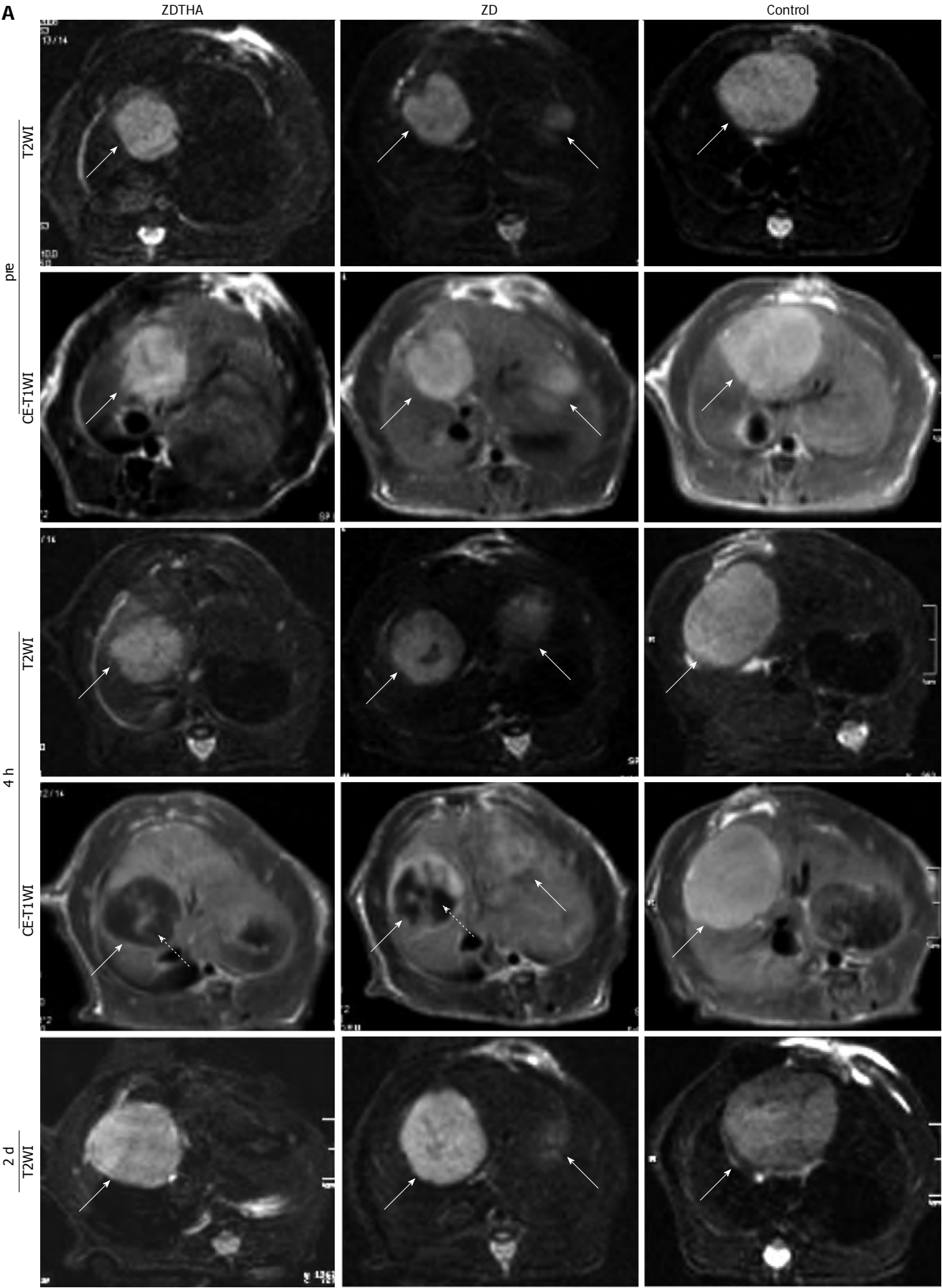
**$ADC_{perf}$ :** Compared to the control group, tumor  $ADC_{perf}$  in both the ZD6126 and ZDTHA groups decreased dramatically at 4 h, most likely due to a rapid vascular shutdown induced by ZD6126 ( $P = 0.016$  and  $0.047$ , respectively). This was followed by a rapid rebound on day 2 in both the ZD6126 and ZDTHA groups (no longer significantly different compared to the control group,  $P = 0.979$  and  $0.525$ , respectively) (Figure 2D-F). A significant reduction in the tumor  $ADC_{perf}$  of ZDTHA was noted at 4 h compared to the ZD6126 group ( $P = 0.025$ ). The  $ADC_{perf}$  of ZDTHA still showed a lower level compared to the ZD6126 at 2 d, although there was no significant difference ( $P = 0.44$ ) (Table 1 and Figure 2D-F).

### Histology

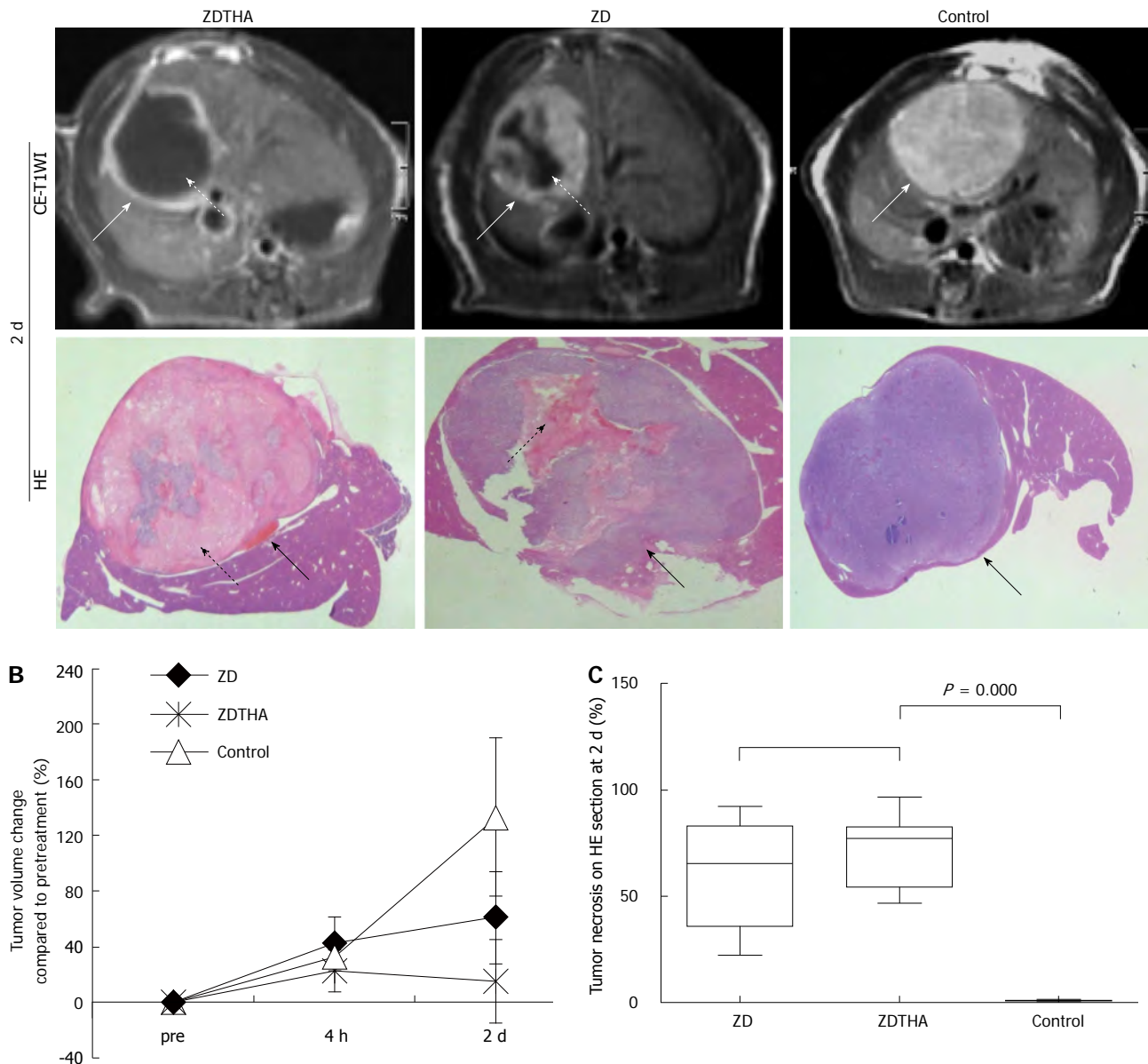
Two days after treatment, the percentages of necrosis compared to the total tumor areas on HE stained tumor sections were significantly higher in both the ZDTHA and ZD6126 groups compared to the control group ( $P = 0.000$  for both). No significant difference was found in the necrotic areas of the ZDTHA and ZD6126 groups ( $P = 0.09$ ) (Figure 1).

## DISCUSSION

We have demonstrated three main findings in the present study. First, tumor growth was significantly delayed by both ZD6126 and ZDTHA treatments compared to the control group, and a significant delay could be observed only two days after application of a single dose of ZD6126. In addition, ZDTHA significantly delayed tumor growth than ZD6126, indicating a synergistic anticancer effect of ZD6126 and thalidomide. It has been known that tumors can rapidly regrow due to the residual viable rim when ZD6126 was used alone<sup>[2]</sup>. It has also been reported that thalidomide, which was reintroduced into clinical practice with its antiangiogenic properties, had little or no effect on full-grown tumors like those in our patients, when used alone<sup>[4]</sup>. Therefore, the synergistic effects of ZDTHA may have contributed to the enhanced antitumor effect in this study. In the combination therapy, ZD6126-induced tumor necrosis, which may then promote tumor angiogenesis, could provide the appropriate conditions for THA to indirectly inhibit angiogenesis<sup>[17]</sup>, because thalidomide is effective only in the early stages of tumor formation<sup>[4]</sup>. Thalidomide indirectly inhibits angiogenesis *via* tumor necrosis factor and







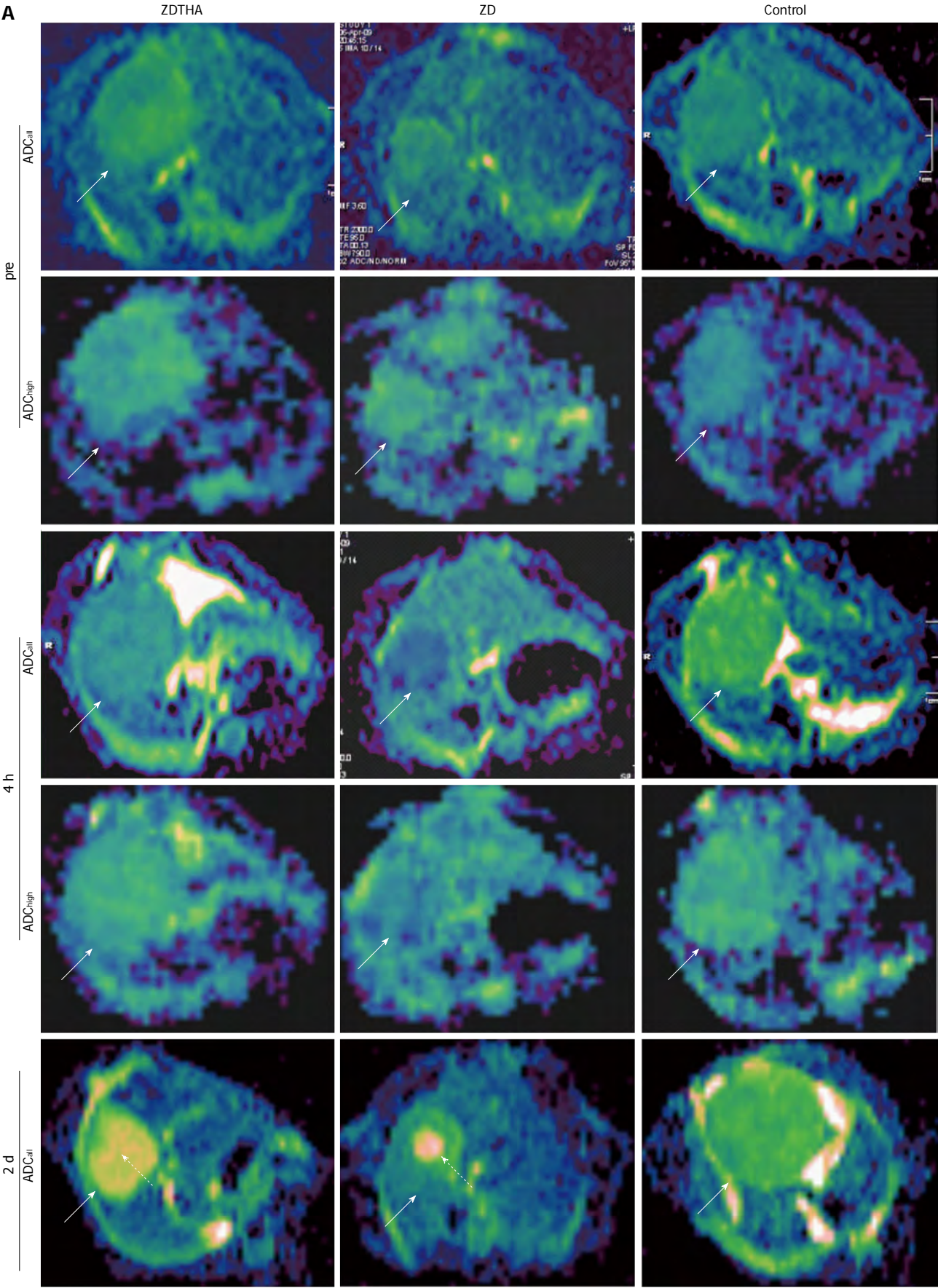
**Figure 1 Tumor growth delay.** A: Representative axial magnetic resonance images of liver tumors acquired with T2-weighted images (T2WI) [repetition/echo time (TR/TE) = 3860/106 ms], contrast enhanced T1-weighted images (CE-T1WI) (TR/TE = 535/9.2 ms) and HE stained sections. Top row magnetic resonance images: After the combination therapy with ZDTHA, the tumor (solid arrow) in the right liver lobe showed delayed growth with massive central necrotic area (dotted arrow) compared to the control tumor on day 2; Middle row magnetic resonance images: After ZD6126 treatment, the right tumor also showed delayed growth compared to the control tumor on day 2, however, the tumor necrotic area was reduced (dotted arrow) because the tumor regrew from viable rim; Bottom row magnetic resonance images: In a control animal, the tumor grew remarkably at 2 d; HE sections: The tumor (solid arrow) and central necrotic areas (dotted arrow) in different groups were verified by HE staining; B: The graph indicated that ZDTHA induced a significant tumor volume growth delay during the experiment, compared to both the ZD6126 and control groups ( $P < 0.01$  for both); C: The box plots showed significantly higher percentages of necrotic area (necrosis/tumor) on HE stained sections in both the ZDTHA and ZD6126 groups compared to the control group ( $P = 0.000$  for both). No significant difference in necrosis was found between the ZDTHA and ZD6126 groups ( $P = 0.09$ ).

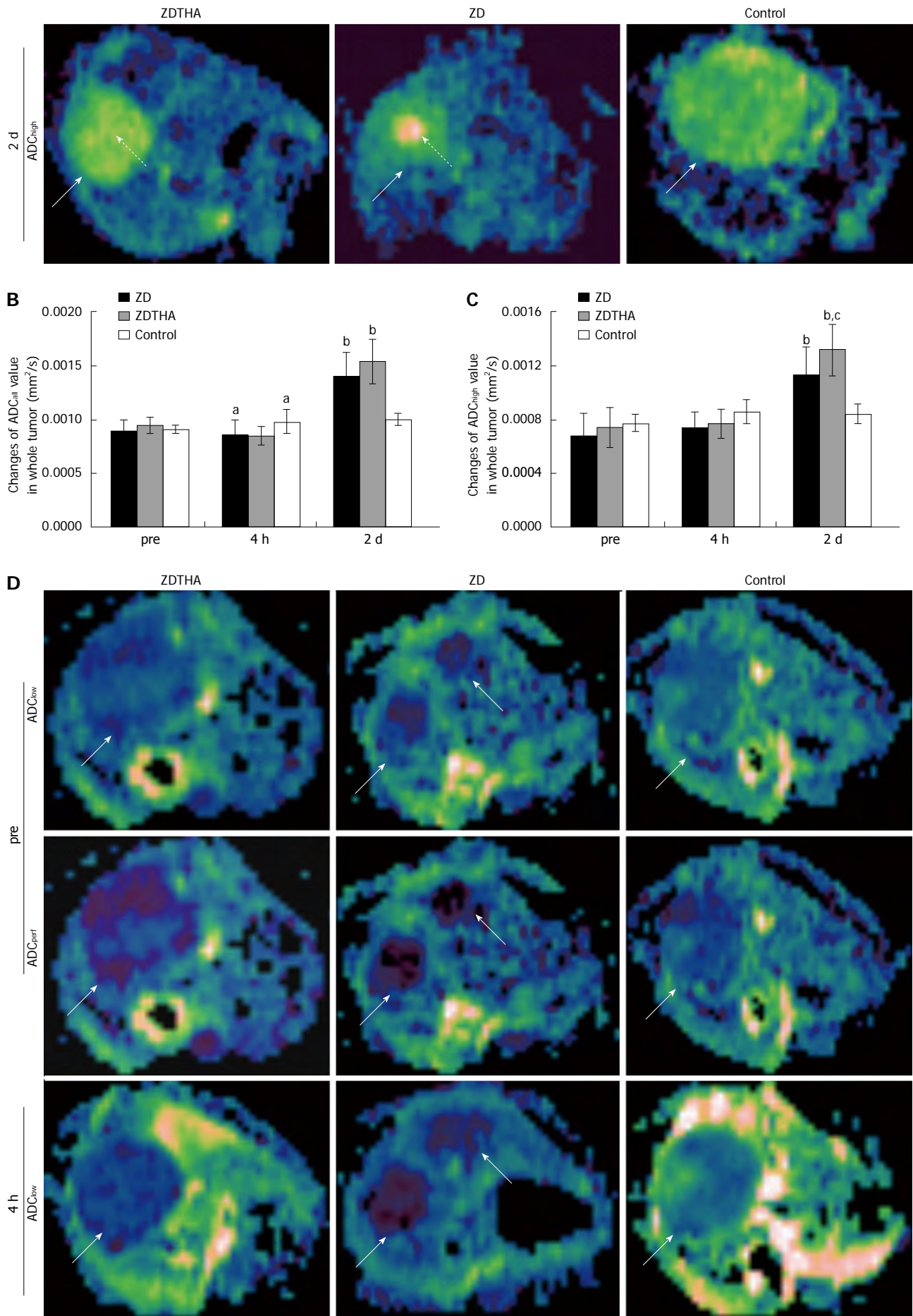
the prostaglandin E pathway<sup>[17]</sup>. Therefore, the proposed combination therapy with ZD6126 and thalidomide may have some potential applications for solid tumor treatment in clinic.

Second,  $ADC_{high}$ , a separate ADC value calculated from high  $b$  value images, performed significantly better than  $ADC_{all}$  for the monitoring of tumor necrosis. In addition to delaying tumor growth, ZDTHA caused tumor necrosis in an additive manner, which was verified by HE staining. Our results showed that although both the  $ADC_{high}$  and  $ADC_{all}$  of ZDTHA and ZD612 were significant-

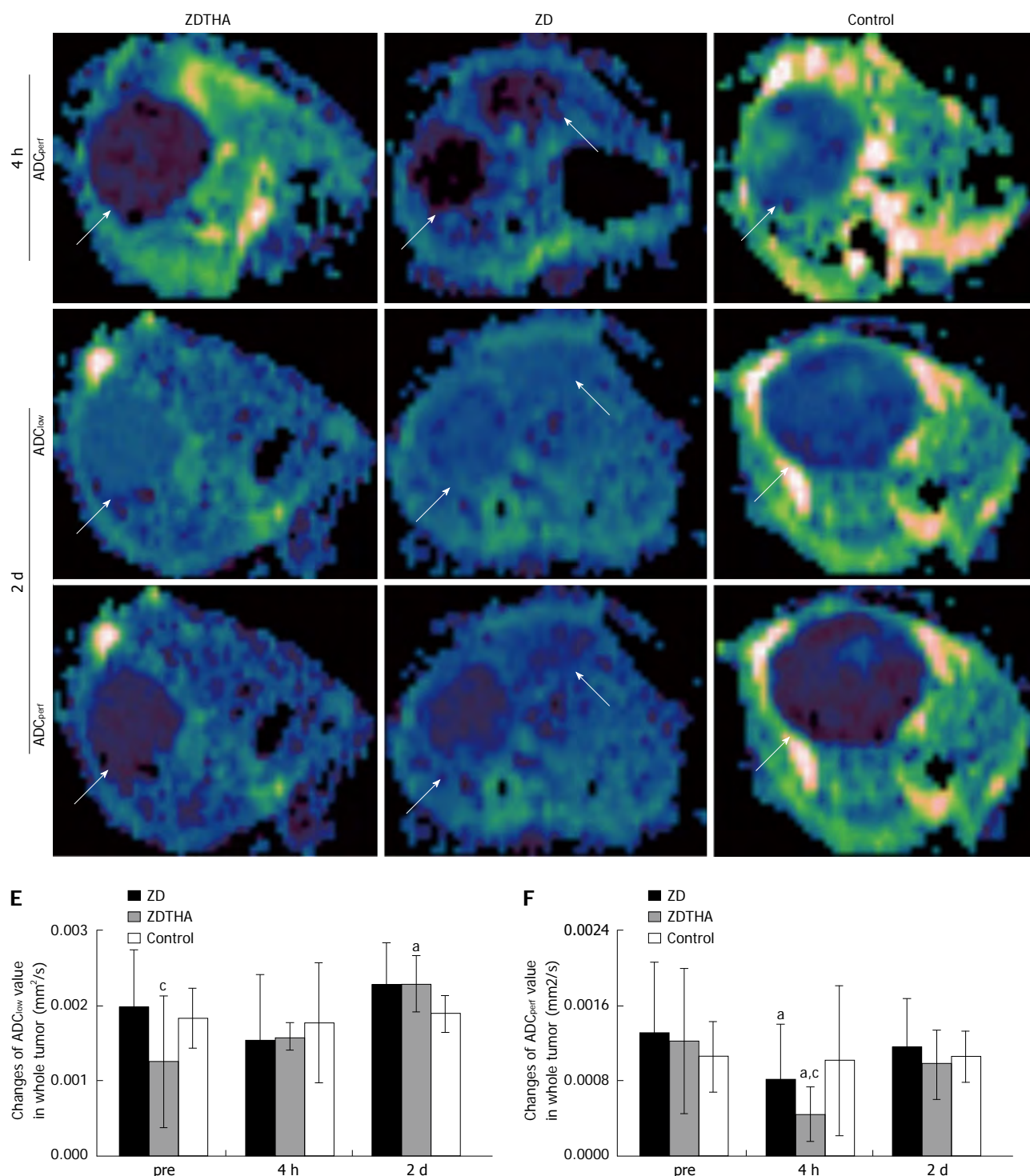
ly higher compared to those of the control group on day 2, the entire tumor  $ADC_{high}$  of ZDTHA was even higher than that of ZD6126, but the significant difference was not observed for  $ADC_{all}$  between ZDTHA and ZD6126. This was due to that  $ADC_{high}$  was more sensitive to the diffusion change resulting from the therapeutic necrosis of the tumor on day 2. It has been reported that VDA can cause massive central necrosis 2 d after treatment<sup>[18]</sup>. Thalidomide can directly induce apoptosis or G1 phase arrest<sup>[19]</sup>. Consequently, tumor cells treated with ZD6126 and thalidomide underwent increased necrosis compared











**Figure 2** Apparent diffusion coefficient maps for tumors. A: Representative maps of apparent diffusion coefficient (ADC)<sub>all</sub> and ADC<sub>high</sub> for liver tumors (solid arrow) in three groups. The area of therapy-induced necrosis (dotted arrow) was significantly larger in the ZDTHA group than those observed in both the ZD6126 and control groups; B: The dynamic change of ADC<sub>all</sub> during the experiment (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control; respectively); C: The dynamic change of ADC<sub>high</sub> during the experiment. Compared to the ADC<sub>all</sub>, the increased diffusion due to therapeutic necrosis was better reflected with ADC<sub>high</sub> in both the ZDTHA and ZD6126 groups on day 2 (<sup>a</sup> $P < 0.01$  vs control; <sup>c</sup> $P < 0.05$  vs ZD6126); D: Representative maps of ADC<sub>low</sub> and ADC<sub>perf</sub> for liver tumors (arrow) in the three groups. The signal intensities observed on ADC<sub>low</sub> maps were always higher than those observed on ADC<sub>perf</sub> maps at each time point in each group, because ADC<sub>low</sub> combines both the perfusion and diffusion effects; E: The dynamic change of ADC<sub>low</sub> during the experiment (<sup>a</sup> $P < 0.05$  vs control; <sup>c</sup> $P < 0.05$  vs ZD6126); F: The dynamic change of ADC<sub>perf</sub> during the experiment. Compared to the ADC<sub>low</sub>, the perfusion reduction due to the shutdown of tumor vessels was better reflected with ADC<sub>perf</sub> in both the ZDTHA and ZD6126 groups at 4 h (<sup>a</sup> $P < 0.05$  vs control). Furthermore, the ADC<sub>perf</sub> in the ZDTHA group was even lower compared to the ZD6126 group at 4 h (<sup>c</sup> $P < 0.05$  vs ZD6126).

to the single use of ZD6126 at the end of the study; this synergistic effect on necrosis could be better reflected with ADC<sub>high</sub> as shown in this study.

Third, ADC<sub>perf</sub>, a separate ADC value calculated as ADC<sub>low</sub> minus ADC<sub>high</sub>, can provide valuable perfusion information from DWI data. Although the ADC<sub>low</sub> was

**Table 1** Average apparent diffusion coefficient of entire tumor before and after treatment (mean  $\pm$  SD)

Group and time	ADC <sub>all</sub> ( $\times 10^{-3}$ mm <sup>2</sup> /s)	ADC <sub>high</sub> ( $\times 10^{-3}$ mm <sup>2</sup> /s)	ADC <sub>low</sub> ( $\times 10^{-3}$ mm <sup>2</sup> /s)	ADC <sub>perf</sub> ( $\times 10^{-3}$ mm <sup>2</sup> /s)
ZD6126				
Pre	0.90 $\pm$ 0.10	0.68 $\pm$ 0.16	1.98 $\pm$ 0.75	1.30 $\pm$ 0.74
4 h	0.86 $\pm$ 0.13	0.73 $\pm$ 0.12	1.54 $\pm$ 0.87	0.81 $\pm$ 0.70
2 d	1.40 $\pm$ 0.22	1.13 $\pm$ 0.21	2.28 $\pm$ 0.54	1.15 $\pm$ 0.52
ZDTHA				
Pre	0.95 $\pm$ 0.08	0.70 $\pm$ 0.15	1.25 $\pm$ 0.87	1.22 $\pm$ 0.77
4 h	0.85 $\pm$ 0.08	0.77 $\pm$ 0.11	1.21 $\pm$ 0.59	0.44 $\pm$ 0.50
2 d	1.54 $\pm$ 0.21	1.31 $\pm$ 0.19	2.28 $\pm$ 0.37	0.97 $\pm$ 0.36
Control				
Pre	0.91 $\pm$ 0.04	0.77 $\pm$ 0.07	1.82 $\pm$ 0.39	1.06 $\pm$ 0.37
4 h	0.98 $\pm$ 0.11	0.85 $\pm$ 0.09	1.76 $\pm$ 0.79	1.01 $\pm$ 0.80
2 d	1.00 $\pm$ 0.06	0.95 $\pm$ 0.08	1.90 $\pm$ 0.24	1.06 $\pm$ 0.27

Pre: Pretreatment; ZD: ZD6126; ZDTHA: ZD6126 + thalidomide; ADC: Apparent diffusion coefficient; ADC<sub>all</sub>: Calculated from the entire  $b$  value setting ( $b = 0, 50, 100, 150, 200, 250, 300, 500, 750, 1000$  s/mm<sup>2</sup>); ADC<sub>high</sub>: calculated from the high  $b$  values ( $b = 500, 750, 1000$  s/mm<sup>2</sup>); ADC<sub>low</sub>: Calculated from the low  $b$  values ( $b = 0, 50, 100$  s/mm<sup>2</sup>); ADC<sub>perf</sub>: Calculated from ADC<sub>low</sub>-ADC<sub>high</sub>.

calculated from low  $b$  value images and perfusion sensitive, it was still contaminated with diffusion effects in tissues<sup>[15]</sup>. Therefore, ADC<sub>low</sub> was not satisfactory in evaluating the tumor response to treatment as indicated in this study. However, ADC<sub>perf</sub> was calculated from ADC<sub>low</sub> by excluding high  $b$  value effects; it would be more perfusion sensitive. In this study, for instance, strikingly reduced perfusion in response to treatment was detected with ADC<sub>perf</sub> at 4 h, but not with ADC<sub>low</sub> for both the ZDTHA and ZD6126 groups compared to the control group. Furthermore, the reduction of ADC<sub>perf</sub> in ZDTHA was even lower; this indicated a more pronounced decrease in blood perfusion induced by ZDTHA. The ADC<sub>perf</sub> of ZDTHA still showed a lower level compared to ZD6126 on day 2, although there was no significant difference. This could be explained by the fact that besides the vascular shutdown effect of ZD6126, thalidomide may also induce a transient normalization of tumor vasculature *via* aggressive vascular pruning and improve pericyte coverage on vessels. As a result, tumor perfusion was reduced<sup>[20,21]</sup>. Our results indicated that ADC<sub>perf</sub> allowed the early monitoring of therapeutic effects, because it was more sensitive to the microcapillary perfusion and could detect perfusion in response to therapy before the appearance of tumor necrosis without the administration of contrast media.

Similarly, such a significant perfusion reduction at 4 h was also detected with ADC<sub>all</sub> in both the ZDTHA and ZD6126 groups, however, this was not observed for ADC<sub>high</sub>, compared to the control group. The reason is that ADC<sub>all</sub> was derived from 10  $b$  values including low and high  $b$  values; consequently it was affected by both diffusion and perfusion in the tumor. Even though, the perfusion change measured with ADC<sub>all</sub> was not as striking as that noted with ADC<sub>perf</sub> due to the influence of diffusion contribution. Despite the delayed growth and the massive central necrotic areas in both the ZDTHA and ZD6126 groups, tumors began to relapse evidenced by the recovery of tumor ADC<sub>perf</sub> and ADC<sub>low</sub>, as well as the enhanced rim visualized on CE-T1WI, due to residue

viable tumor cells on day 2 after therapy. These results are consistent with previous findings<sup>[18,22]</sup>. However, ZDTHA demonstrated significantly less tumor relapse than ZD6126, suggesting the benefit of applying the combination therapy.

It remains controversial regarding the option of mono- or biexponential model in extracting diffusion and perfusion information from DWI data. Because each model has its own advantages and drawbacks<sup>[15,23]</sup>. As a pioneering work in the mid-1980s, Le Bihan *et al.*<sup>[24,25]</sup> proposed the concept of IVIM to address the microscopic movements in image voxel in MRI. In biologic tissue, the motions include the molecular diffusion of water and the microcirculation of blood or capillary perfusion. With the biexponential model of IVIM, the fraction of capillary perfusion can be separated from diffusion. Therefore, there is growing studies using IVIM from DWI data<sup>[26-29]</sup>. However, the clinical benefit of the biexponential model as compared to the monoexponential model has not been comprehensively established<sup>[10,15]</sup>. Our study supports that the ADC<sub>high</sub> values are similar to the diffusion coefficient derived from IVIM model. The separate calculations of ADC<sub>all</sub>, ADC<sub>high</sub>, ADC<sub>low</sub> and ADC<sub>perf</sub> using a monoexponential fitting algorithm are relatively simple to estimate and are readily available for most users of clinical MR scanners. However, a lack of direct comparison of diffusion parameters derived from mono- and biexponential model may be a limitation of the present study.

In conclusion, we have demonstrated that ZDTHA combination treatment significantly delayed tumor growth due to synergistic effects by inducing cumulative tumor necrosis. The perfusion insensitive ADC<sub>high</sub> values calculated from high  $b$  value images performed significantly better than ADC<sub>all</sub> values for the monitoring of tumor necrosis. The perfusion sensitive ADC<sub>perf</sub> values derived from ADC<sub>low</sub> by excluding high  $b$  value effects could provide valuable perfusion information from DWI data. Therefore, the *in vivo* separate calculations of ADC values derived from monoexponential model are more useful than conventional averaged ADC values in the



successful evaluation of tumor therapeutic effects.

## COMMENTS

### Background

Diffusion weighted magnetic resonance imaging (DW-MRI), due to its ability to detect molecular water motion at the cellular level, *i.e.*, the measurement of apparent diffusion coefficient (ADC), has become a favorite choice of measures in a variety of oncological studies and tissue viability assessments.

### Research frontiers

Technological innovations in recent years have enabled the increasing use of high-quality and quantitative DW-MRI in monitoring tumor treatment. However, it has been realized that the information acquired from conventional calculation of DW-MRI data actually represents the combined effects of tissue microcirculation perfusion and pure tissue diffusivity in each imaging voxel at DW-MRI. This may hinder the appropriate interpretation of the DW-MRI data. Therefore, there is growing interest in applying more sophisticated approaches, such as separate ADC (calculating different ADC values based on various combinations of *b* values with a monoexponential fitting algorithm) and intravoxel incoherent motion, to differentiate the fraction of microcirculation perfusion from pure diffusivity within the DW-MRI data.

### Innovations and breakthroughs

The combination therapy with ZD6126 and thalidomide significantly delayed liver tumor growth due to synergistic effects by inducing cumulative tumor necrosis in rodents. The ADC<sub>high</sub> performed significantly better than ADC<sub>all</sub> for the monitoring of tumor necrosis on day 2. The ADC<sub>perf</sub> could better reflect the reduction of blood flow due to the vessel shutdown induced by ZD6126, compared to the ADC<sub>low</sub>. The ADC<sub>perf</sub> could provide valuable perfusion information from diffusion weighted MRI data.

### Applications

The separate calculation of ADC is more useful than conventional averaged ADC in evaluating the efficacy of combination therapy with ZD6126 and thalidomide for solid tumors.

### Terminology

DW-MRI is an *in vivo* imaging technique to detect molecular water motion at the cellular level by using the ADC parameter. Separate ADC measurement is to calculate the different ADC values based on various combinations of *b* values with a monoexponential fitting algorithm.

### Peer review

The authors present ADC is more useful than conventional averaged ADC in evaluating the efficacy of combination therapy with ZD6126 and thalidomide for solid tumors. It would be of interest to perform the same study in a vascular tumor such as infantile hemangioma or a malignant vascular tumor such as angiosarcoma or hemangiopericytoma.

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## <sup>131</sup>I-labeled metuximab combined with chemoembolization for unresectable hepatocellular carcinoma

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### Abstract

**AIM:** To investigate the safety and effectiveness of combined <sup>131</sup>I-metuximab and transcatheter arterial chemoembolization (TACE) for hepatocellular carcinoma (HCC).

**METHODS:** One hundred and eighty-five patients (159 men and 26 women) with advanced HCC were enrolled in this study from February 2009 to July 2011. There were 95 patients in the combined metuximab and TACE group, and 90 patients in the TACE only group. The patients were followed for 12 mo. Clinical symptoms, blood cell counts, Karnofsky Performance Score (KPS) evaluation and therapeutic effects according to the Response Evaluation Criteria in Solid Tumors were recorded and evaluated.

**RESULTS:** The 1-mo effective rates (complete re-

sponse + partial response + stable disease) of the test group and control group were 71.23% and 38.89%, respectively ( $P < 0.001$ ). The 6-, 9- and 12-mo survival rates were 86.42%, 74.07% and 60.49% for the test group and 60.0%, 42.22% and 34.44% for the control group ( $P < 0.001$ ). The incidence of adverse events (gastrointestinal symptoms, fever and pain) and blood cell toxicity were significantly higher for the test group than for the control group ( $P < 0.001$ ). No severe <sup>131</sup>I-metuximab-related complications were identified. With respect to efficacy, patients in the test group had greater improvement in tumor-related pain ( $P = 0.014$ ) and increase in KPS ( $P < 0.001$ ) than those in the control group.

**CONCLUSION:** Combination of <sup>131</sup>I-metuximab and TACE prolonged the survival time in patients with HCC compared with TACE alone. The combination treatment was safe and effective.

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**Key words:** Hepatocellular carcinoma; <sup>131</sup>I-metuximab; Transcatheter arterial chemoembolization; Radioimmunotherapy

**Core tip:** <sup>131</sup>I-metuximab has high affinity with a target antigen highly expressed on hepatocellular carcinoma (HCC) cells and a limited area of action. The combination of metuximab and transcatheter arterial chemoembolization had a synergistic effect in the treatment of HCC. It may represent a promising treatment modality for patients with advanced HCC, especially for those patients with multiple nodules who have a heavy tumor burden.

He Q, Lu WS, Liu Y, Guan YS, Kuang AR. <sup>131</sup>I-labeled metuximab combined with chemoembolization for unresectable hepatocellular carcinoma. *World J Gastroenterol* 2013;

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## INTRODUCTION

Hepatocellular carcinoma (HCC) has traditionally been regarded as a radioresistant tumor because external beam radiation does great harm to the surrounding normal tissue. On the contrary, since the 1980s, radioimmunotherapy has become a promising treatment modality for HCC, due to the specificity of the antibodies and the killing power of the radionuclides, resulting in improvement of clinical efficacy with fewer side effects.

A therapeutic anti-HCC radioimmunological agent,  $^{131}\text{I}$ -metuximab, generated by  $^{131}\text{I}$  labeling of the murine monoclonal antibody (mAb) fragment HAb18 F(ab')<sub>2</sub> derived from HAb18G/CD147, has been approved for the treatment of primary HCC by the China State Food and Drug Administration (Registration No. S20050039).

Transcatheter arterial chemoembolization (TACE) is currently one of the widely used treatment modalities for unresectable advanced HCC. However, the long-term survival rate of such patients remains low, with a reported 5-year survival rate of 17%<sup>[1]</sup>. Although  $^{131}\text{I}$ -metuximab monotherapy has been shown to be effective, both in the treatment of HCC and in the prevention of HCC recurrence after orthotopic liver transplantation<sup>[2]</sup>, its efficacy in combination with other established treatment modalities such as TACE has seldom been tested. Theoretically, the TACE can enhance the antitumor effects of  $^{131}\text{I}$ -metuximab, because of substantially reduced blood flow to the tumor that prolongs retention of  $^{131}\text{I}$ -metuximab in the tumor tissues. Radioimmunotherapy combined with TACE may provide a new concept in radiotherapy for patients with HCC.

In this study, the safety and efficacy of  $^{131}\text{I}$ -metuximab in combination with TACE were evaluated in patients with advanced HCC to demonstrate that the combination of  $^{131}\text{I}$ -metuximab with TACE could produce better results than TACE alone.

## MATERIALS AND METHODS

### $^{131}\text{I}$ -metuximab injection

$^{131}\text{I}$ -metuximab injection (Licartin; Chengdu Hoist Hi-tech Co. Ltd., Chengdu, China) is an  $^{131}\text{I}$ -labeled HAb18 F(ab')<sub>2</sub> fragment of murine mAb against the HCC-associated antigen HAb18G/CD147. Before metuximab therapy, 0.5 mL of iodine solution should be taken orally tid for 3 d and continued for 7 d after treatment for thyroid protection. A vial of  $^{131}\text{I}$ -metuximab injection solution that had been prepared at the standard dose of 0.75 mCi/kg was removed from a lead box containing ice at a temperature of 0 °C. The thawed solution was diluted with 1 mL of saline and sucked into a 5-mL syringe for arterial injection.

### Patient cohort

One hundred and eighty-five patients (159 men and 26 women, aged 12-87 years) with advanced unresectable HCC were enrolled in this study from February 2009 to July 2011. Patients with a Karnofsky Performance Score (KPS) < 60 or severe heart, kidney or hematological disease were excluded to ensure at least a 3-mo lifespan in the enrolled patients, so as to have enough time for follow-up. Patients with a history of allergy to biological products, pregnant or breast-feeding women, or patients receiving other therapies within 4 wk of the clinical trial were also excluded. All patients in our study gave written informed consent. Patients in the test group underwent  $^{131}\text{I}$ -metuximab therapy and TACE while those in the control group received TACE only. The patients received local ethanol injection, microwave coagulation, resection or liver transplantation before and after TACE or  $^{131}\text{I}$ -metuximab therapy if needed. All tumors were diagnosed according to pathological examination or distinctive findings on computed tomography (CT), conventional angiography, magnetic resonance imaging (MRI), or serum tumor markers [ $\alpha$ -fetoprotein (AFP)].

### Procedure of TACE and $^{131}\text{I}$ -metuximab intra-arterial injection

TACE and  $^{131}\text{I}$ -metuximab injection were performed through the femoral artery using the Seldinger technique with local anesthesia. Arteriography of the celiac trunk and superior mesenteric artery was performed to visualize the arterial vascularization of the liver and evaluate portal vein patency, and anticancer drugs were injected. The angiographic catheter was superselected into the hepatic artery where the target tumor was located. An embolic agent (mainly Lipiodol) was continuously injecting through the artery until the rate of blood flow to the tumor mass fell below 25%, or minimal hepatic vein appeared to protect the liver tissues around the tumor so that Licartin is more liable to stay in tumor tissues by minimizing the effect of quick or slow blood flow. Patients in the test group underwent  $^{131}\text{I}$ -metuximab therapy immediately after TACE. At each injection,  $^{131}\text{I}$ -metuximab was administered at a dose of 0.75 mCi/kg according to the patient's weight and the intra-arterial injection usually lasted 1-2 min. Patients in the control group received TACE only. In both groups, the dose of Lipiodol, ranging from 3-20 mL, was determined according to the size and number of tumors and functional hepatic reserve. Anticancer drugs for each patient enrolled in this trial were 5-fluorouracil (800-1000 mg) and epirubicin-adriamycin (30-40 mg) according to the body surface area. Therapy for patients in both groups was repeated according to the patient's clinical condition and the iconography exams at a 1-6-mo interval.

### Follow-up protocol and efficiency evaluation

Clinical symptoms, blood cell counts and KPS evaluation were recorded before and after treatment. After treatment, ultrasound, CT scan or MRI was performed every



**Table 1** Baseline characteristics

Characteristics	Test group ( <i>n</i> = 95)	Control group ( <i>n</i> = 90)	Statistical analysis
Age (yr)	50.2 (22-80)	51.4 (12-87)	NS
Sex (M/F)	83/12	76/14	NS
Child-Pugh classification			NS
Child class A	91	88	
Child class B	4	2	
BCLC stage			NS
C	95	90	
Size of main tumors			
≥ 5 cm	80	81	NS
< 5 cm	15	9	NS
Tumor/liver volume ratio			
0%-50%	10	13	NS
≥ 50%	85	77	NS
Hepatitis B/C	70	76	NS
KPS	75.16 ± 7.42	73.89 ± 11.39	NS

BCLC: Barcelona clinic liver cancer; KPS: Karnofsky performance status; NS: Not significant.

1-3 mo, with or without contrast enhancement, to evaluate the features of Lipiodol deposit and the therapeutic effect according to the Response Evaluation Criteria in Solid Tumors (RECIST). If elevated tumor marker (AFP), diminished Lipiodol, enlarged lesions or new nodules were observed, the patients were readmitted for angiography and treatment. The starting point of survival analysis was regulated as the day of initial treatment. The Kaplan-Meier method was used to analyze the survival rates in the two groups.

### Statistical analysis

The primary endpoint of this study was overall survival and the secondary endpoint was short-term (1 mo) treatment response. Survival analysis was estimated by the Kaplan-Meier method. Survival probabilities were estimated using the life-table method, and between-group differences in survival rates were compared using the log-rank test. All statistical analyses were carried out with SPSS version 17.0 (SPSS, Chicago, IL, United States). All reported *P* values were two-sided, with *P* < 0.05 considered statistically significant.

## RESULTS

### Patient population

The patients were divided into the test group (*n* = 95) with a mean age of 50.2 years (range: 22-80 years) and the control group (*n* = 90) with a mean age of 51.4 years (range: 12-87 years). All the patients in this trial were classified as Barcelona Clinic Liver Cancer Stage C. Both the test group and control group had a high percentage of patients (89.47% and 85.56%, respectively) with a tumor/liver volume ratio > 50%. Thus, the patients enrolled in this clinical trial had advanced HCC. Although this was a nonrandomized prospective cohort study, no significant difference was observed in baseline characteristics between the two groups (Table 1).

**Table 2** Clinical symptoms immediately after treatment *n* (%)

Group	Fever <sup>1</sup>	Gastrointestinal <sup>1</sup> symptoms	Pain <sup>1</sup>	Sudden death
Test group	90 (94.74)	74 (77.89)	93 (97.89)	0
Control group	28 (31.11)	16 (17.78)	19 (21.11)	1 (1.11)

<sup>1</sup>*P* < 0.001.

The patients were followed for 12 mo. At the time of analysis, 46 and 80 patients had died while 14 and zero were lost to follow-up in the test and control groups, respectively. Causes of death in the test and control groups included tumor progression in 46 and 72 patients, digestive tract hemorrhage in zero and four, tumor rupture in zero and one, acute renal failure in zero and one, and other causes in zero and two, respectively; none was possibly related to treatment.

Two hundred and forty-one (mean: 2.56 procedures) and 261 (mean: 2.90 procedures) procedures of interventional therapy were performed in the test and control groups, respectively. Arterial portal vein shunt (AVS), arterial hepatic vein shunt (APS) and/or portal vein involvement, which indicate high invasion and poor prognosis were found in 35.79% (34/95) of patients in the test group and 33.3% (30/90) of patients in the control group. No difference was observed in the time of therapy and the incidence of malignancy signs such as AVS, APS or portal vein involvement between the two groups.

### Safety

The clinical symptoms were carefully recorded after treatment (Table 2). Overall, although <sup>131</sup>I-metuximab in combination with TACE was well tolerated, the patients in the test group obviously suffered more frequent adverse events than those in the control group. The most frequent adverse event in the test group was abdominal pain. Of the 95 patients in the test group, 93 (97.89%) suffered from abdominal pain, 90 (94.74%) had fever of 37.2 °C-40 °C, which usually occurred 0.5-10 h after <sup>131</sup>I-metuximab injection and lasted for 1-14 d, and 74 (77.89%) had anorexia and/or vomiting, which often faded away in several days. The corresponding numbers of patients in the control group were 19 (21.11%), 28 (31.11%) and 16 (17.78%) (*P* < 0.001). The changes in blood cell count and liver function before and 1 mo after treatment were evaluated. Statistical analysis showed that the changes in leukocytes and platelets were significant. Changes in total bilirubin, albumin, aspartate aminotransferase, alanine transaminase and hemoglobin were not significant (Table 3).

One patient in the test group had hypothyroidism and was prescribed oral thyroxine, and one sudden death occurred in the control group, possibly because of liver rupture. No human anti-murine antibody immune responses, anaphylactic reaction and changes in myocardial zymograms were observed.

**Table 3** Changes in blood cells and liver function before and 1 mo after treatment

Group	Test group	Control group	P value
Leukocytes	-1.25 ± 1.79	2.04 ± 11.51	0.0270
Platelets	-36.69 ± 49.62	12.74 ± 52.59	< 0.001
TB	1.7	0.95	0.860
Alb	-0.86 ± 6.89	-1.30 ± 5.36	0.6708
AST	5	7.5	0.631
ALT	-1	0	0.5137
HGB	-5.86 ± 16.42	-7.98 ± 20.26	0.515
KPS			< 0.001
Mass-associated pain			0.014

Total bilirubin (TB), Aspartate aminotransferase (AST), Glutamic-pyruvic transaminase (ALT), Karnofsky and pain (Wilcoxon rank sum test); others (*t* test). Alb: Albumin; HGB: Hemoglobin; KPS: Karnofsky performance score.

**Table 4** Therapeutic effect evaluated according to Response Evaluation Criteria in Solid Tumors at 1 mo after treatment *n* (%)

Group	CR	PR	SD	PD	Effective rate (CR + PR + SD)
Test group	4 (4.11)	38 (39.73)	26 (27.40)	27 (28.77)	68 (71.23)
Control group	1 (1.11)	14 (15.56)	20 (22.22)	55 (61.11)	35 (38.89)

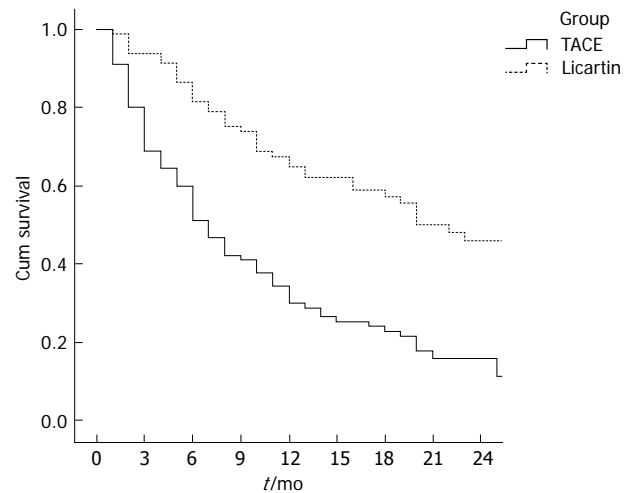
$P < 0.001$ . CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

### Efficacy

The palliative rate of mass-associated pain 1 mo after treatment was 71.1% (27/38) for patients in the test group, which was higher than that in the control group (31.6%, 24/76) ( $P = 0.014$ ). For changes in KPS, the patients in the test group had a greater increase than those in the control group ( $P < 0.001$ , Table 3). The therapeutic effect was evaluated following the RECIST after treatment. Rates of complete response (CR), partial response (PR), stable disease (SD) and progressive disease in the two groups are listed in Table 4. The total effective rates (CR + PR + SD) were 71.23% and 38.89% for the test group and control group, respectively. Wilcoxon rank sum test showed that the therapeutic effects in the two groups were significantly different ( $P < 0.001$ ). The survival rates at 6, 9 and 12 mo after treatment were 86.42%, 74.07% and 60.49% in the test group, and 60.0%, 42.22% and 34.44% in the control group, suggesting that the survival rates for the test group were significantly higher than for the control group ( $P < 0.001$ , Figure 1).

## DISCUSSION

HCC is a highly malignant tumor with high morbidity and mortality rates. Although TACE, as a palliative treatment for unresectable HCC, has become one of the most common interventional therapies<sup>[3-6]</sup>, its effect is limited due to the lack of appropriate and reliable embolic agents, and the infiltrative or hypovascular nature, too large or



**Figure 1** Kaplan-Meier curves of survival of patients in the test group receiving combination therapy (Licartin) and those in the control group receiving transcatheter arterial chemoembolization only ( $P < 0.001$ ). TACE: Transcatheter arterial chemoembolization.

small in size<sup>[7-9]</sup>. Another limitation of TACE is the need for repeated treatment, thus resulting in deterioration of liver function<sup>[10]</sup>. Therefore, many efforts have been made to explore other new therapies in order to achieve better efficacy. Percutaneous ethanol injection, radiofrequency ablation, targeted molecular therapies, and gene therapy in combination with TACE have been found to improve survival in patients with advanced HCC<sup>[11-13]</sup> and decrease the risk of liver failure<sup>[14-16]</sup>. However, in spite of all the above, advanced HCC with wide metastasis, especially in patients with multiple lesions who always have a high tumor/liver volume ratio, still has a poor prognosis and lack of efficient therapeutic modalities.

The target antigen for <sup>131</sup>I-metuximab, HAB18G/CD147, a member of the CD147 family, is highly expressed on HCC cells. The binding rate of HAB18 to human 7721 hepatoma cells, determined by flow cytometry, is up to 99.55%<sup>[17,18]</sup>. Immunohistochemistry performed with HAB18 showed that the positive rate of HCC staining was 75% and had no cross-reaction to normal tissues<sup>[17,19]</sup>. Moreover, the results of drug safety studies showed that <sup>131</sup>I-metuximab injection caused no impairment to cardiovascular, respiratory, or nervous systems<sup>[2]</sup>.

The mechanism by which <sup>131</sup>I-metuximab may benefit patients with HCC has been investigated both *in vitro* and *in vivo*, as well as in clinical trials<sup>[2,17,20]</sup>. <sup>131</sup>I-metuximab is specific to and has high affinity for a target antigen highly expressed on HCC cells. This allows for concentration of conjugated <sup>131</sup>I in HCC tissues, both in the liver and metastatic nodules, which kills tumor cells directly. In addition, the target antigen, HAB18G/CD147, is a cell adhesion molecule with multiple functions and is closely related to tumor metastasis. This antigen is involved in the adhesion and motion of tumor cells, angiogenesis, and signal transduction and can induce fibroblasts to produce matrix metalloproteinases (MMPs), including MMP-1, MMP-2, and MMP-9. These MMPs can degrade the ex-

tracellular matrix and promote the metastasis of HCC cells. Injection of  $^{131}\text{I}$ -metuximab into HCC cells inhibits oncogenesis and metastasis within and outside the liver, blocking and destroying cells carrying HAB18G/CD147 and inhibiting HCC metastasis<sup>[1]</sup>.

The combination of  $^{131}\text{I}$ -metuximab and TACE ought to have a synergistic effect in the treatment of HCC. First, TACE may enhance the efficacy of  $^{131}\text{I}$ -metuximab due to its arterial embolization effect, substantially reducing blood flow to the HCC and resulting in prolonged retention of  $^{131}\text{I}$ -metuximab in the tumor. Second, retention of the anticancer drug in the tumor may have a radiosensitizing effect on  $^{131}\text{I}$ -metuximab. Third,  $^{131}\text{I}$ -metuximab can eliminate residual cancer cells after TACE for its continuous radiation. Taken together, these mechanisms may explain, at least in part, the ability of combination therapy to enhance survival, compared with conventional TACE alone, in patients with advanced HCC. Of course, they are the reasons why the test group suffered more blood cell toxicity and adverse events.

In the present study, the results for the control group were similar to those of earlier trials of TACE in patients with HCC. In those studies, the tumor response rate according to WHO criteria ranged from 12%-57.9%<sup>[1,21-23]</sup>, median survival ranged from 7-19 mo (mean:  $13.63 \pm 6.10$  mo), and 1- and 2-year survival rates ranged from 42%-72% (mean:  $58.30 \pm 10.14\%$ ) and from 0%-55% (mean:  $28.74\% \pm 15.88\%$ ), respectively<sup>[24-28]</sup>. Moreover, treatment of patients with HCC with  $^{131}\text{I}$ -metuximab alone resulted in 6-mo and 12-mo survival rates of 82.63% and 58.68%, respectively, with a median survival time of 19 mo and an objective response rate (CR + PR) of 15.53% according to World Health Organization (WHO) criteria<sup>[1]</sup>. In comparison, patients receiving combination therapy in our study showed 6- and 12-mo survival rates of 86.42% and 60.49%, respectively; a median survival time of 20.0 mo; and an objective response rates of 43.84% according to WHO criteria. All of these studies were performed in patients staged as Child-Pugh class A/B, and their baseline characteristics were similar to those of our patients, therefore, it can be suggested that the combination of  $^{131}\text{I}$ -metuximab and TACE tested here exhibited better clinical efficacy than either treatment alone.

Given the high level of expression of the high-affinity target antigen on HCC cells and the limited area of action of  $^{131}\text{I}$ -metuximab, we thought we would observe more advantages in our clinical trial because most of the enrolled patients had multiple nodules and a high tumor/liver volume ratio. And we did find that the median survival of patients in the test group was significantly longer than that in the control group (20.0 mo *vs* 7.0 mo). This improvement was more marked than that in two similar earlier trials (21.15 mo *vs* 17.73 mo and 26.7 mo *vs* 20.6 mo). The most notable difference between the present and previous studies is that our patients had more advanced HCC and a greater tumor burden (tumor/liver volume ratio  $\geq 50\%$ ; 87.56% *vs*  $< 29\%$ )<sup>[29,30]</sup>.

It is commonly accepted that HCC patients with countable nodules often have more treatment choices, better treatment efficacy, and longer lifespan than those with countless nodules. Our clinical trial proved that  $^{131}\text{I}$ -metuximab combined with TACE had an extensive range of therapeutic function, especially for advanced liver cancer with wide metastasis and multiple lesions. The combination of  $^{131}\text{I}$ -metuximab and TACE may greatly improve the treatment efficacy in these patients and extend their poor life expectancy.

The present study had several limitations. First, the relatively short follow-up period may have resulted in underestimation of survival. Second, most patients were treated with combination therapy only once, a few twice and followed by TACE again. Repeated combination therapy may have a more significant effect on survival. Third, the effects of Chinese traditional medicine, which the patients used when discharged from the hospital, were uncertain. These were hard to control and might have affected the final results.

In conclusion, our findings indicate that combination of  $^{131}\text{I}$ -metuximab and TACE was safe and more effective than TACE alone. It may represent a promising treatment modality for patients with advanced HCC. Nevertheless, caution should be exercised, and a few questions remain. What is the best method for delivering  $^{131}\text{I}$ -metuximab with the TACE procedure? Does the 25% be the best point to administer Licartin? Whether and how much could the Licartin injected into the hepatic artery concentrate in the metastasis outside the liver? All these questions require well-designed, prospective randomized controlled trials.

## COMMENTS

### Background

Transcatheter arterial chemoembolization (TACE) is currently one of the widely used treatment modalities for unresectable advanced hepatocellular carcinoma (HCC). However, the long-term survival rate of such patients remains poor. At the same time,  $^{131}\text{I}$ -metuximab monotherapy has been shown to be effective, both in the treatment of HCC and in the prevention of HCC recurrence after orthotopic liver transplantation. However, its efficacy in combination with TACE has seldom been tested. Theoretically, TACE can enhance the antitumor effects of  $^{131}\text{I}$ -metuximab, because of substantially reducing blood flow to the tumor, which prolongs retention of  $^{131}\text{I}$ -metuximab in the tumor tissues.

### Research frontiers

$^{131}\text{I}$ -metuximab (Licartin) has high affinity with a target antigen highly expressed on HCC cells and has a limited area of action. The combination of  $^{131}\text{I}$ -metuximab and chemoembolization has a synergistic effect in the treatment of HCC. It may especially benefit patients with multiple nodules who always have a high tumor/liver volume ratio.

### Innovations and breakthroughs

It is commonly accepted that HCC patients with countable nodules often have better outcome than patients with countless lesions. At present, patients with multiple lesions still have a poor prognosis and lack of efficient therapeutic modalities. However, in our trial, they managed to treat the same types of patients with  $^{131}\text{I}$ -metuximab combined with TACE and prolonged their lifespan significantly. This improvement was more notable than in earlier similar trials. The combination of  $^{131}\text{I}$ -metuximab and TACE had an extensive range of therapeutic function, especially for advanced liver cancer with wide metastasis and multiple lesions. Combination therapy may greatly improve the treatment efficacy in these patients and extend their poor life expectancy.



## Applications

Radioimmunotherapy combined with TACE may provide a new concept in radiotherapy for patients with HCC.

## Peer review

The authors performed a study on 185 patients (159 men and 26 women) with advanced HCC. The data indicate that combination of <sup>131</sup>I-metuximab with TACE was safe and more effective than TACE alone. The title of the paper accurately reflects the content of the article. The abstract contains a short description of the study. The abstract and article are written in accordance with the journal requirements. The introduction gives sufficient information about the research objectives. It is important that authors state their motivation to conduct this investigation. The design of the study is simple and understandable.

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## Efficacy of mosapride plus proton pump inhibitors for treatment of gastroesophageal reflux disease: A systematic review

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### Abstract

**AIM:** To assess the potential benefits of mosapride plus proton pump inhibitors (PPIs) in the treatment of gastroesophageal reflux disease.

**METHODS:** A literature search was performed through MEDLINE, EMBASE, and the ISI Web of Knowledge. The clinical trials that compared the benefit of mosapride plus PPI treatment with that of PPI monotherapy were analyzed. The rate of responders was evaluated by the pooled relative risk (PRR) and improvement in symptom scores was assessed by single effect size of a standardized mean, while Hedges'g was used as the effect size. Pooled effect sizes with 95% CIs were calculated using a fixed-effects model. Between-study heterogeneity was assessed using *Q* test and *I*<sup>2</sup> analyses. In addition, studies that assessed the additional efficacy of mosapride in PPI-resistant patients were also

reviewed.

**RESULTS:** This systematic review included information on a total of 587 patients based on 7 trials. Four trials compared the efficacy of combination therapy of mosapride plus a PPI with that of PPI monotherapy. The statistical analysis for the effect of additional mosapride showed equivocal results (PRR = 1.132; 95%CI: 0.934-1.372; *P* = 0.205; Hedges'g = 0.24; 95%CI: 0.03-0.46; *P* = 0.023). No heterogeneity and publication bias were found among the studies. Three open-labeled trials assessed the additional efficacy of mosapride in PPI-resistant patients. However, since these trials did not set the control group, the results may be considerably biased.

**CONCLUSION:** Mosapride combined therapy is not more effective than PPI alone as first-line therapy. Whether it is effective in PPI-resistant patients needs to be determined.

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**Key words:** Mosapride; Proton pump inhibitor; Gastroesophageal reflux disease; Systematic review; Combined therapy

**Core tip:** Prokinetic agents have been widely used to relieve the gastroesophageal reflux disease (GERD) symptoms, and mosapride is a selective 5-HT<sub>4</sub> receptor agonist that can be safely used. We conducted a systematic review and meta-analysis to assess the potential benefits of the addition of mosapride to proton pump inhibitors (PPIs) in the treatment of GERD. Based on this research, mosapride combined therapy seems to be not more effective than PPI alone as first-line therapy. Whether it is effective in PPI-resistant patients needs to be determined.

Liu Q, Feng CC, Wang EM, Yan XJ, Chen SL. Efficacy of mosapride plus proton pump inhibitors for treatment of gastroesophageal reflux disease: A systematic review. *World J Gastroenterol* 2013; 19(47): 9111-9118 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9111.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9111>

## INTRODUCTION

Gastroesophageal reflux disease (GERD) encompasses a spectrum of clinical presentations in which gastric content refluxes into the esophagus leading to symptoms with or without visible damage to the esophageal mucosa. It is the most common gastrointestinal diagnosis recorded during visits to outpatient clinics<sup>[1]</sup>. Population-based studies suggest that GERD is a common condition with a prevalence of 10%-20% in Western Europe, while in Asia it is lower, less than 5%<sup>[2,3]</sup>. Traditionally, the treatment for GERD should be focused on symptom control, and abundant data from randomized trials show benefits of inhibiting gastric acid secretion in patients with GERD. Treatment with proton pump inhibitors (PPIs) heals reflux esophagitis in 83% of patients with comparable symptom relief, an outcome that is superior to treatment with histamine 2-receptor antagonists<sup>[4]</sup>.

However, GERD patients present with a wide range of symptom severity and frequency, sometimes do not respond to PPI therapy. Several mechanisms have been proposed for the pathogenesis of refractory GERD, including weakly acidic reflux, visceral hypersensitivity and delayed gastric emptying<sup>[5]</sup>.

An Asia-Pacific consensus on the management of GERD showed that the use of prokinetic agents either as monotherapy or adjunctive therapy to PPIs may have a role in the treatment of GERD in Asia<sup>[6]</sup>. Prokinetic agents like cisapride, which act on the 5-hydroxytryptamine (5-HT)<sub>1</sub>-receptor, have been found to be associated with potentially fatal heart rhythm abnormalities. However, mosapride, a selective 5-HT<sub>4</sub> receptor agonist, is an alternative prokinetic agent that can be safely used in patients with upper gastrointestinal disorders<sup>[7-9]</sup>, while stimulating gastrointestinal motility and gastric emptying<sup>[10-12]</sup>. Many studies have shown that mosapride can reduce acid reflux episodes and esophageal clearance of refluxate, theoretically, suggesting potential efficacy in the treatment of GERD<sup>[13,14]</sup>. In a randomized trial, mosapride combined with PPIs achieved a better therapeutic effect than use of a PPI alone<sup>[15]</sup>. However, another clinical trial showed the additional effect of mosapride was limited<sup>[16]</sup>.

In this study, our aim was to clarify the data on the treatment of GERD by systematically reviewing the literatures on the efficacy of mosapride plus PPIs with regard to initial symptom relief. The additional treatment effect of mosapride in PPI-resistant GERD patients was also assessed.

## MATERIALS AND METHODS

### Study retrieval and selection

The present meta-analysis follows the guidelines for Preferred Reporting Items for Systematic Reviews and Meta-Analyses PRISMA<sup>[17]</sup>. We performed a literature search using the following databases: MEDLINE, EMBASE and the ISI Web of Knowledge. The search pool was enlarged by references found in these initial articles. Three authors (Liu Q, Feng CC and Wang EM) independently searched from the beginning of indexing for each database to May 10<sup>th</sup>, 2013, using the key terms ("gastroesophageal reflux disease" or "reflux esophagitis" or "non-erosive reflux disease") and ("mosapride" or "mosapride citrate" or "prokinetic" or "prokinetics"). Only the articles written in English were included.

Three authors (Liu Q, Feng CC and Wang EM) independently evaluated all of the retrieved studies according to pre-specified selection criteria. Discrepancies between the three investigators were resolved by discussion. Studies were included based on the following criteria: (1) published as original articles; (2) investigations of adults; (3) clinical trials that evaluated the efficacy of mosapride. Studies were excluded if they had the following features: (1) without specific description for the diagnosis of GERD; (2) reported duplicated results that have been published in other articles as repeated data; (3) other primarily identifiable causes of GERD symptoms such as esophageal neoplasm and esophageal stricture; (4) use of mosapride was not designed as an additional drug in combination with a PPI; and (5) included participants who were taking medications that could have complicated interpretation of results.

### Data extraction and analysis

The following data were abstracted from each article: the author(s), publication year, country, study design, numbers of enrolled patients, age, gender distribution and body mass index of the subjects, definition of GERD, treatment dose and duration, effects of treatment. Data extraction was performed independently by two reviewers (Liu Q and Feng CC). We validated a priority of data from intention-to-treat (ITT) analysis other than per-protocol (PP) analysis when the data obtained from two approaches were both available in certain studies.

Subsequently, we arranged the clinical trials using thematic analysis. The overarching categories included the controlled trials showing parallel comparisons between efficacy of mosapride and PPI group with that of PPI alone group, and open trials to assess the additional efficacy of mosapride to PPI-resistant patients.

Using the data from the controlled trials in which treatment efficacy was evaluated by comparing the rate of responders and improvement in symptom scores in a group receiving mosapride plus a PPI with those in patients receiving PPI alone, we assessed the drug effect based on the pooled relative risk (PRR) and single effect

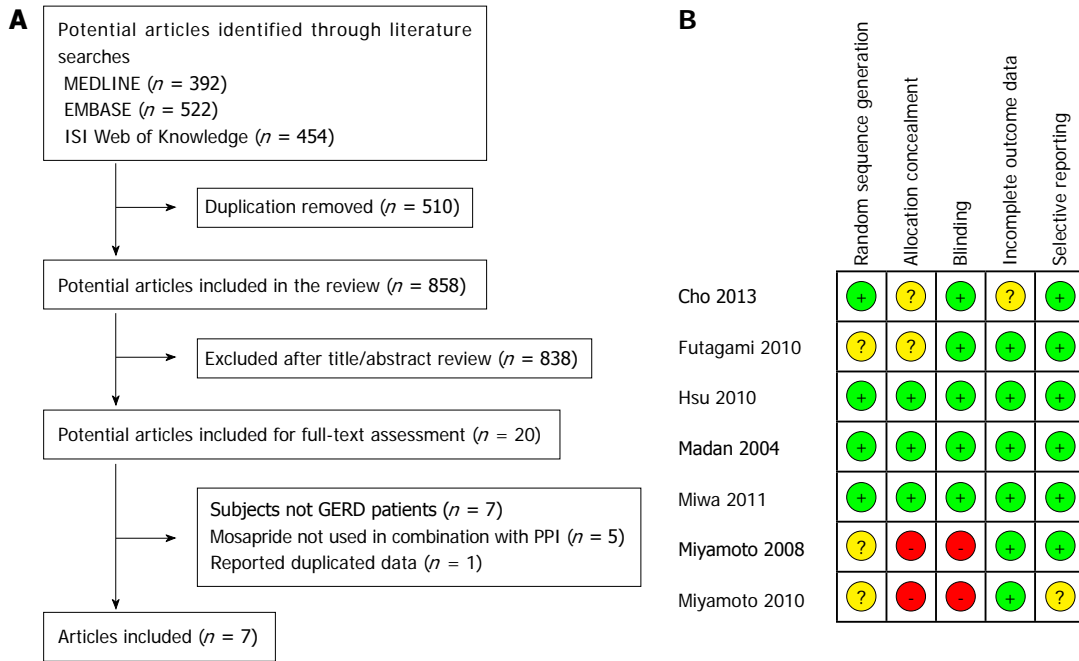


Figure 1 Flow chart of study selection and risk of bias summary. A: Flow chart of study selection; B: Risk of bias summary.

size of a standardized mean. The PRR was calculated using the Mantel-Haenszel method<sup>[18]</sup>, and continuous variables were transformed from the means and standard deviation to determine a standardized effect size. We used the Hedges'g effect size, which is a variation of Cohen's *d*, to correct for bias associated with small sample sizes<sup>[19]</sup>. Statistical heterogeneity across the various studies was then tested with the use of Q-statistic<sup>[20]</sup>. A *P*-value < 0.10 indicated a significant statistical heterogeneity across studies, allowing for the use of a random effects model. Additionally, we calculated *I*<sup>2</sup> statistics, which quantifies the percentage of variation across studies caused by heterogeneity, rather than chance, and, therefore are less biased by the number of studies included in a meta-analysis<sup>[21]</sup>. Finally, publication bias was quantified using Egger's test<sup>[22]</sup>. A two-tailed *P* < 0.05 was considered to be statistically significant. The above analyses were performed using Stata 11.0 (Stata Corp, College Station, TX, United States). Risk of bias was assessed using Cochrane Review guidelines<sup>[23]</sup>.

## RESULTS

### Search results and study characteristics

The search strategy generated 858 references, 20 of which were selected for further assessment by full-text reading (Figure 1A). In this step, 7 articles were excluded because the subjects in the study were not GERD patients<sup>[24-30]</sup>, 5 articles were excluded because mosapride was not used in combination with PPI<sup>[13,14,31-33]</sup>, and one trial reported duplicated data<sup>[34]</sup>. Ultimately, 7 studies were included in this systematic review which contained information on a total of 587 patients, with the characteristics shown in Table 1. The diagnostic criteria of

GERD in the 7 articles we included were basically based on typical reflux-associated symptoms (heartburn and/or regurgitation) which occurred at least twice a week, although the duration was obscure in three studies<sup>[16,37,39]</sup>. The subjects in 3 articles were non-erosive reflux disease (NERD) patients<sup>[16,38,39]</sup>, but one study focused on reflux esophagitis (RE) patients<sup>[35]</sup>. With respect to the dose of mosapride, only one trial used this agent at a dose of 10 mg thrice daily<sup>[36]</sup>. All others employed 5 mg three times per day. Various PPIs were used in these studies including rabeprazole, omeprazole, pantoprazole, lansoprazole and esomeprazole.

### Quality and methodology of trials

Risk of bias was assessed using criteria specified by the Cochrane group. Overall, the risk of bias was high in some studies<sup>[37,39]</sup> and low in others<sup>[15,16,36,38]</sup> (Figure 2). A summary of individual quality assessment can be found in Figure 1B.

There was significant heterogeneity between trials with regard to methodology. In 3 studies<sup>[35,38,39]</sup>, symptom evaluation was based on a frequency scale for the symptoms of GERD (FSSG), a GERD-specific questionnaire developed in Japan has been used for screening GERD patients<sup>[40]</sup>. The gastrointestinal symptom rating scale (GSRS) questionnaire<sup>[41]</sup> was adopted from another trial<sup>[37]</sup>. Two articles presented an explicit symptom assessment approach<sup>[15,36]</sup>, and one used a visual analogue scale to evaluate the symptom<sup>[16]</sup>.

### Trials comparing mosapride plus PPI combination therapy with PPI monotherapy

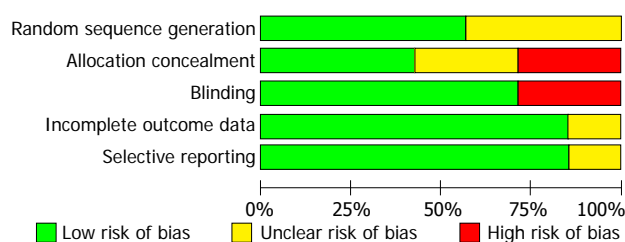
Four trials compared the efficacy of combination therapy of mosapride plus a PPI with that of PPI monothera-



**Table 1** Characteristics of the included studies

Ref.	Year	n	Male (%)	Mean age	BMI	Study design	Treatment agent (daily), dose	Treatment duration	Outcome measures
Trials comparing mosapride plus PPI combined therapy with PPI monotherapy									
Madan <i>et al</i> <sup>[15]</sup> / India	2004	Cases 33 Controls 28	57.6 75.0	34.7 36.5	Unclear Unclear	Double-blind Randomized controlled trial	Pantoprazole 80 mg + mosapride 15 mg	8 wk	Symptom Questionnaire
Hsu <i>et al</i> <sup>[35]</sup> / Taiwan	2010	Cases 50 Controls 46	46.0 54.3	47.0 47.0	23.7 ± 3.6 23.9 ± 4.6	Double-blind Randomized Crossover trial	Pantoprazole 80 mg + placebo Lansoprazole 30 mg + mosapride 15 mg	4 wk	FSSG Questionnaire
Miwa <i>et al</i> <sup>[16]</sup> / Japan	2011	Cases 97 Controls 95	38.1 36.8	52.1 52.2	22.3 ± 3.3 22.0 ± 3.6	Double-blind Randomized Controlled trial	Lansoprazole 30 mg + placebo Omeprazole 10 mg + mosapride 15 mg	4 wk	VAS
Cho <i>et al</i> <sup>[36]</sup> / South Korea	2012	Cases 24 Controls 19	62.5 47.4	49.0 43.0	21.3 ± 2.3 21.5 ± 2.3	Double-blind Randomized Controlled trial	Omeprazole 10 mg + placebo Esomeprazole 40 mg + mosapride 30 mg Esomeprazole 40 mg + placebo	4 wk	Reflux- symptoms Questionnaire
Trials on addition of mosapride to PPIs for the treatment of PPI-resistant GERD patients									
Miyamoto <i>et al</i> <sup>[37]</sup> / Japan	2008	34	Unclear	53.1 <sup>1</sup>	23.0 ± 0.3 <sup>1</sup>	Open trial	Rabeprazole 10 mg + mosapride 15 mg	12 wk	FSSG Questionnaire
Futagami <i>et al</i> <sup>[38]</sup> / Japan	2010	44	50%	42.8	23.0 ± 1.9	Open trial	Omeprazole 20 mg + mosapride 15 mg	12 wk	GSRS Questionnaire
Miyamoto <i>et al</i> <sup>[39]</sup> / Japan	2010	117	Unclear	47.4 <sup>1</sup>	23.0 ± 3.6 <sup>1</sup>	Open trial	PPI therapy <sup>2</sup> + mosapride 15 mg	4 wk	FSSG Questionnaire

<sup>1</sup>Data calculated based on the included participants at the beginning of study; <sup>2</sup>Patients were randomly administered rabeprazole 10 mg or lansoprazole 30 mg or omeprazole 20 mg or lansoprazole 15 mg or omeprazole 10 mg. PPI: Proton pump inhibitor; GERD: Gastroesophageal reflux disease; FSSG: Frequency scale for the symptoms of GERD; GSRS: Gastrointestinal symptom rating scale; VAS: Visual analogue scale; BMI: Body mass index.

**Figure 2** Risk of bias in trials.

py<sup>[15,16,35,36]</sup>, all of which were designed as double-blind, randomized, placebo-controlled trials.

Madan *et al*<sup>[15]</sup> demonstrated that the combination therapy with pantoprazole and mosapride was more effective than pantoprazole alone in providing symptomatic relief to patients with erosive GERD. However, the number of patients who responded to therapy was not statistically different between combination therapy and monotherapy with pantoprazole (89.2% *vs* 69.7%). However, at the end of the treatment duration, the mean symptom score was significantly lower in patients receiving combination therapy (1.67 *vs* 3.78,  $P = 0.009$ ).

Hsu *et al*<sup>[35]</sup> conducted a double-blind randomized trial studying the effects of adding mosapride to lansoprazole for the management of reflux esophagitis. The reduction in symptom score after 4 wk of treatment with lansoprazole and mosapride was not significantly higher compared with lansoprazole plus placebo (13.42 *vs* 10.85,  $P = 0.103$ ), indicating little benefit from the addition of mosapride to a PPI in RE patients. However, in the subgroup of severely symptomatic patients, the difference

was marginally significant ( $P = 0.039$ ), indicating that mosapride as an adjunct to PPI may be beneficial in patients with severe symptoms.

Miwa *et al*<sup>[16]</sup> targeted on patients with NERD in a double-blind placebo-controlled study and found that there was no significant difference between the rates of responders from omeprazole plus mosapride, and omeprazole plus placebo groups in ITT (46% *vs* 44%) and PP (50% *vs* 43%) analyses. The change in symptom score in the treatment group was not significantly different from the placebo group in ITT analysis (-3.8 *vs* -3.4,  $P = 0.128$ ). Therefore, the addition of mosapride to omeprazole was not found to be more effective than omeprazole alone in NERD patients.

Theoretically, prokinetic drugs can improve GERD by increasing lower esophageal sphincter basal pressure, improving esophageal peristalsis, accelerating esophageal acid clearance and facilitating gastric emptying. Cho *et al*<sup>[36]</sup> focused on the change of high-resolution manometry parameters to evaluate the efficacy of mosapride on esophageal motility and reflux symptoms in patients with GERD when used in combination with a PPI. The authors found that a combination of mosapride with esomeprazole affected esophageal peristalsis by improving esophageal contractibility and lowering intrabolar pressure that could lead to facilitation of esophageal bolus transit in patients with GERD. However, with regard to symptom assessment, treatment responsiveness in the combined therapy group was not different from that of the monotherapy group (79% *vs* 68%).

Of note, for the statistical analysis, one study was

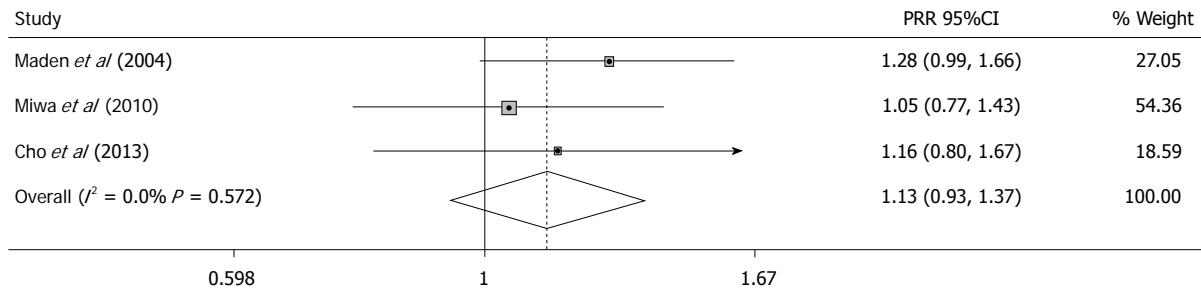


Figure 3 Meta-analysis of three trials that used mosapride as combined therapy with proton pump inhibitor compared with placebo in gastroesophageal reflux disease, a fixed-effects model was used and pooled relative rate was the measure of effect size.  $I^2$ , total variation across studies that is attributable to heterogeneity rather than to chance; PRR: Pooled relative rate.

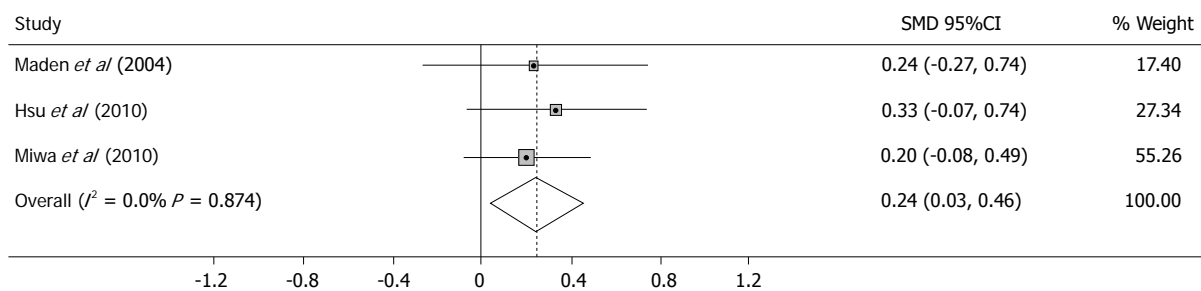


Figure 4 Meta-analysis of three trials that used mosapride as combined therapy with proton pump inhibitor compared with placebo in gastroesophageal reflux disease, a fixed-effects model was used and Hedges'g was the measure of effect size.  $I^2$ , total variation across studies that is attributable to heterogeneity rather than to chance; SMD: Standardized mean difference.

excluded from the above 4 trials for the calculation of responder rate<sup>[35]</sup> and change in symptom scores<sup>[36]</sup> respectively because of insufficient information. Concerning the comparison between mosapride combined therapy and PPI monotherapy, use of mosapride did not significantly elevate the rate of responders (PRR = 1.132; 95%CI: 0.934-1.372;  $P = 0.572$ ;  $I^2 = 0.0\%$ ) (Figure 3). However, the treatment arm achieved a greater symptom relief than that in placebo arm (Hedges'g = 0.24; 95%CI: 0.03-0.46;  $P = 0.874$ ;  $I^2 = 0.0\%$ ) (Figure 4). No heterogeneity was found among the studies, both Egger's tests ( $P = 0.587$ ;  $P = 0.636$ ) failed to show significance for these studies, indicating no statistically significant publication bias. Only one trial<sup>[16]</sup> reported a safety analysis, which showed a similar incidence of adverse effects in the two groups.

#### ***Trials on addition of mosapride to PPIs for the treatment of PPI-resistant GERD patients***

Three open-labeled trials evaluated the additional efficacy of mosapride in PPI-resistant patients. Miyamoto *et al*<sup>[37]</sup> used an FSSG questionnaire which comprised 12 questions concerning not only acid-related symptoms, but also dysmotility symptoms. They treated 163 GERD patients with rabeprazole 10 mg daily for 3 mo. Thirty-four patients were dissatisfied with the PPI monotherapy and, therefore, were considered to be PPI-resistant. Three months of combined therapy with mosapride resulted in high efficacy. Futagami *et al*<sup>[38]</sup> explored the function of gastric emptying in PPI-resistant NERD patients, and found that PPI-resistant NERD patients showed

significant disturbances of gastric emptying compared to healthy volunteers. Moreover, administration of mosapride in addition to omeprazole alleviated reflux symptoms and improved gastric emptying in PPI-resistant NERD patients. Another study by Miyamoto *et al*<sup>[39]</sup> analyzed FSSG-reflux score (RS) and -dyspeptic score (DS) of PPI-resistant NERD patients. Significant improvement in FSSG-total score and FSSG-DS was observed after the addition of mosapride in PPI non-response NERD patients. These results indicate that patients with significant dysmotility and functional dyspepsia were more likely to be PPI-resistant and suggest the need for the addition of a prokinetic agent to PPI therapy.

## **DISCUSSION**

With respect to the comparison between mosapride combined treatment with PPI and PPI monotherapy, similar efficacy was found between these two groups in most<sup>[16,35,36]</sup> of the four randomized controlled trials, the meta-analysis showed similar treatment responsiveness but a significant difference in symptom score improvement between the treatment arm and the placebo arm. However, all the four trials used different symptom scores, the one point improvement should not mean the same symptom relief in different scoring systems. They cannot be standardized, compared or combined easily. Therefore, the rate of responders is more appropriate as the measure of effect size, since the criteria of improvement in each paper was decided to be feasible at least by

the author of each paper. The results indicated that the addition of mosapride to PPI therapy might be useful for patients with GERD, but could not achieve satisfactory effects. Of note, type II error should also be considered as a reason for the failure to show a significant difference in the rate of responders. The number of patients may not have been enough. In addition, the analysis of open-label trials showed that mosapride plus PPIs might be of benefit to PPI-resistant GERD patients. However, since these trials did not set the control group, the results may be considerably biased.

Mosapride is a selective 5-HT<sub>4</sub> receptor agonist with no affinity for 5-HT<sub>1</sub>, 5-HT<sub>2</sub> or dopamine D<sub>2</sub> receptors<sup>[42]</sup>. It is devoid of anti-dopaminergic and direct cholinomimetic effects. It is well tolerated. Diarrhea, dry mouth, malaise and headache are the most frequent side effects and they were reported in < 5% of patients<sup>[43]</sup>. Currently, mosapride is commercially available in many Asian countries, but not in United States and Europe. An interesting feature of mosapride is that its action seems to differ along the gastrointestinal tract. Mosapride decreases acid reflux to the esophagus by modulating esophageal motor function in patients with GERD<sup>[14]</sup>, or improving gastric emptying for both solids and liquids in healthy volunteers and diabetic patients<sup>[44,45]</sup>. It is known that gastric motility is impaired in some NERD patients, and mosapride improves the symptoms in such patients<sup>[32]</sup>. Mosapride has a distinctly lower affinity to receptors located in the colon<sup>[13]</sup>. These findings indicate that mosapride selectively stimulates upper gastrointestinal motility, and interacts heterogeneously with 5-HT<sub>4</sub> receptors. Mosapride has also been shown to modulate visceral sensation *via* raising the threshold of visceral pain caused by balloon expansion in rat stomach<sup>[46]</sup>. Moreover, it has been reported that mosapride increased the pharmacokinetics of PPIs such as rabeprazole<sup>[47]</sup>, indicating that it is able to facilitate the acid inhibitory effect of PPIs. However, the current results showed that mosapride as an add-on therapy was not more effective than PPI alone in the treatment of GERD. This may be partially due to the effect of the PPI, which might be beneficial to the relief of dyspeptic symptoms<sup>[48,49]</sup> and obscure the effect of prokinetic treatment.

The strengths of our systematic review could be summarized as follows. We sought to find as many publications as possible using various search approaches. The explicit, detailed eligibility criteria were set up to minimize the selection bias. And we used Cochrane Review Guidelines to assess the quality of the evidence. We also placed emphasis on calculating the possibility of publication bias by Egger's test and evaluating bias across studies, while no heterogeneity was found in our statistical analysis.

There are several limitations of this review. First, the number of patients in the individual studies was relatively small. Second, with respect to PPI-resistant subjects, all of the three studies were open trials, without a placebo control group who did not receive the additional prokinetic agents, therefore, the results could be considerably biased by placebo effect of mosapride administration in

this setting. Further randomized, placebocontrolled trials should be performed. Moreover, in two<sup>[38,39]</sup> out of these three studies, the investigators focused mainly on dyspeptic rather than reflux symptoms. Third, the subtypes of GERD (RE and NERD) patients were not discussed in this review due to little available information. Moreover, there was significant heterogeneity between trials with regard to methodology. Standardized methodology for GERD symptom questionnaire are needed to facilitate the comparison of outcomes and minimize the operating bias.

In conclusion, this review shows that mosapride combined therapy is not more effective than PPI alone as the first-line therapy in GERD patients. Whether it is effective in the treatment of PPI-resistant reflux disease needs to be determined. However, the results of this review are still at the level of descriptive analysis. Further clinical studies with better design and larger number of participants are needed to verify the efficacy of this combined therapy.

## COMMENTS

### Background

Gastroesophageal reflux disease (GERD) is the most common gastrointestinal diagnosis recorded during visits to outpatient clinics, and the patients sometimes do not respond to proton pump inhibitor (PPI) therapy. Prokinetic agents have been widely used to relieve the GERD symptoms, and mosapride is a selective 5-HT<sub>4</sub> receptor agonist that can be safely used. However, the potential benefit of the addition of mosapride to PPIs in the treatment of GERD is unclear.

### Research frontiers

Mosapride is a selective 5-HT<sub>4</sub> receptor agonist devoid of anti-dopaminergic and direct cholinomimetic effects. Many studies have shown that mosapride can reduce acid reflux episodes and esophageal clearance of refluxate, suggesting potential efficacy in the treatment of GERD.

### Innovations and breakthroughs

Based on this systematic review and meta-analysis, mosapride combined therapy was not more effective than PPI alone as the first-line therapy. This has not been identified clearly in previous studies.

### Applications

The addition of mosapride to PPI therapy might be useful for patients with GERD, but could not achieve satisfactory effects. Moreover, the fact that mosapride is not yet available in Europe and the United States makes this study more relevant giving physicians some basis for the use in these countries once it is licensed.

### Peer review

This is a well-performed systematic review of currently available studies on the potential benefits of the addition of mosapride to PPIs in the treatment of GERD. The authors found that mosapride combined therapy was not more effective than PPI alone as first-line therapy. The conclusions are unbiased and give informative clues to the readers.

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## Fast-track rehabilitation vs conventional care in laparoscopic colorectal resection for colorectal malignancy: A meta-analysis

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### Abstract

**AIM:** To evaluate the fast-track rehabilitation protocol and laparoscopic surgery (LFT) vs conventional care strategies and laparoscopic surgery (LCC).

**METHODS:** Studies and relevant literature comparing the effects of LFT and LCC for colorectal malignancy were identified in MEDLINE, the Cochrane Central Register of Controlled Trials and EMBASE. The complications and re-admission after approximately 1 mo were

assessed.

**RESULTS:** Six recent randomized controlled trials (RCTs) were included in this meta-analysis, which related to 655 enrolled patients. These studies demonstrated that compared with LCC, LFT has fewer complications and a similar incidence of re-admission after approximately 1 mo. LFT had a pooled RR of 0.60 (95%CI: 0.46-0.79,  $P < 0.001$ ) compared with a pooled RR of 0.69 (95%CI: 0.34-1.40,  $P > 0.5$ ) for LCC.

**CONCLUSION:** LFT for colorectal malignancy is safe and efficacious. Larger prospective RCTs should be conducted to further compare the efficacy and safety of this approach.

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**Key words:** Laparoscopic surgery; Fast-track rehabilitation; Enhanced recovery; Colorectal surgery; Complications; Readmission

**Core tip:** Fast-track rehabilitation in laparoscopic colorectal resection has become the most fashionable way to treat colorectal malignancy. Complications after fast-track rehabilitation protocol and laparoscopic surgery (LFT) and conventional care strategies and laparoscopic surgery (LCC) of colorectal resection have generally been discussed in China, as well as in other countries. This study clarified that compared with LCC, LFT has fewer complications and has a similar incidence of re-admission after approximately 1 mo.

Li P, Fang F, Cai JX, Tang D, Li QG, Wang DR. Fast-track rehabilitation vs conventional care in laparoscopic colorectal resection for colorectal malignancy: A meta-analysis. *World J Gastroenterol* 2013; 19(47): 9119-9126 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9119.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9119>

## INTRODUCTION

Fast-track rehabilitation in laparoscopic colorectal resection has become the most fashionable way of treating colorectal malignancy. During the mid-1990s, fast-track rehabilitation, involving dieticians, nurses, surgeons and anesthesiologists, was developed by Kehlet *et al*<sup>[1,2]</sup>, Wilmore *et al*<sup>[3]</sup> and Basse *et al*<sup>[4]</sup>. Common to the other enhanced recovery rehabilitations, it is an attempt to reduce the stress response, decrease complications, speed up recovery, shorten the hospital stay and reduce health costs, all without compromising patient safety. The laparoscopic approach to colorectal surgery has been shown to accelerate dietary intake and return of bowel function<sup>[5]</sup>, to facilitate postoperative mobilization<sup>[6]</sup>, to reduce the length of stay in hospital<sup>[5,7]</sup> and to have a positive effect on postoperative mortality<sup>[5,7-9]</sup>.

Recently, laparoscopic surgery has been generally applied in the treatment of gastrointestinal cancer, which can significantly attenuate trauma and accelerate the rehabilitation of patients after surgery. It was reported that the hospital stay time is shorter and the complication and readmission rate are lower after laparoscopic surgery<sup>[10,11]</sup>.

Despite all the major benefits of laparoscopy, elective colorectal resection is still associated with a morbidity rate between 20% and 30% and a postoperative hospital stay of 7-10 d<sup>[12]</sup>. Both laparoscopic surgery and FT perioperative care have been reported to be safe and effective, and to result in a shorter hospital stay with earlier recovery of gastrointestinal function<sup>[13-16]</sup> and lower morbidity than open colorectal surgery and standard care<sup>[17-19]</sup>. Many recently published randomized controlled trials are available that have compared fast-track rehabilitation to conventional care in laparoscopic colorectal resection for colorectal malignancy. The safety after fast-track rehabilitation protocol and laparoscopic surgery (LFT) of colorectal resection has generally been discussed; therefore, this study analyzed and compared the complications and re-admission between LFT and conventional care strategies and laparoscopic surgery (LCC). The primary aim of this meta-analysis was to evaluate LFT *vs* LCC; the secondary aim was to assess LFT.

## MATERIALS AND METHODS

### Publication search

PubMed, the Cochrane Central Register of Controlled Trials and EMBASE were searched for all relevant literature, including articles referenced in the publications. The medical subject headings (MeSH) and keywords collected for individually and in combination were as follows: "laparoscopic surgery" "open surgery" and "fast track" or "enhanced recovery" and "colorectal". The last search was done on May 10<sup>th</sup>, 2013. References, lists of retrieved articles, reviews and meta-analyses were then scanned for

additional articles. Internet search engines were also used to perform a manual search for abstracts from international meetings, which were then downloaded and studied.

### Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) randomized controlled trials; (2) studies that provided information on at least one of the outcome measures; (3) studies published in English. When a study reporting the same patient cohort was included in several publications, only the most recent or complete study was selected; and (4) detailed patient information provided. The exclusion criteria were as follows: (1) case reports; (2) articles that were not full text, or non-comparative studies; and (3) open operations, not by laparoscopic surgery.

### Study selection

The inclusion criteria were met in studies if they involved LFT for colorectal malignancy in adult patients (*i.e.*, those 18 years and older) and used LCC as a control. All studies that used chemotherapy, or a rehabilitation protocol had to include less than seven of the seventeen FT items among the interventions in the FT group (programs using epidural or local anesthesia, minimally invasive techniques, optimal pain control and aggressive postoperative care) to achieve early recovery after colorectal surgery; and more than two of the conventional care strategies were included, were excluded. Studies that could not provide actual frequencies of complications or re-admission after approximately 1 mo were also excluded. Both full-length publications and abstract publications were selected. Letters, reviews without original data, non-English papers and animal studies were excluded. If any doubt regarding the suitability remained after the abstract was examined, the full manuscript was obtained.

### Data extraction

All included studies were assessed for the quality of their methodology and relevance to the objective of our meta-analysis. Conduct and reporting were in accordance with the QUOROM statement. Data on complications or re-admission approximately 1 mo from each trial were extracted and compared independently by the two investigators.

### Statistical analysis

In statistical analysis, Review Manager (RevMan) software version 5.0.0 was used (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). A pooled RR and a pooled Mean Difference with 95%CI were used to assess the outcomes of the studies. Statistical heterogeneity was tested by the  $\chi^2$  test. According to the forest plot, heterogeneity was limited, so the Mantel-Haenszel fixed effect model was adopted. The significance of the pooled RR was determined by the Z test and statistical significance was considered at  $P < 0.05$ . Publication bias was estimated by the use of a funnel plot with an Egger's linear regression test, and funnel plot

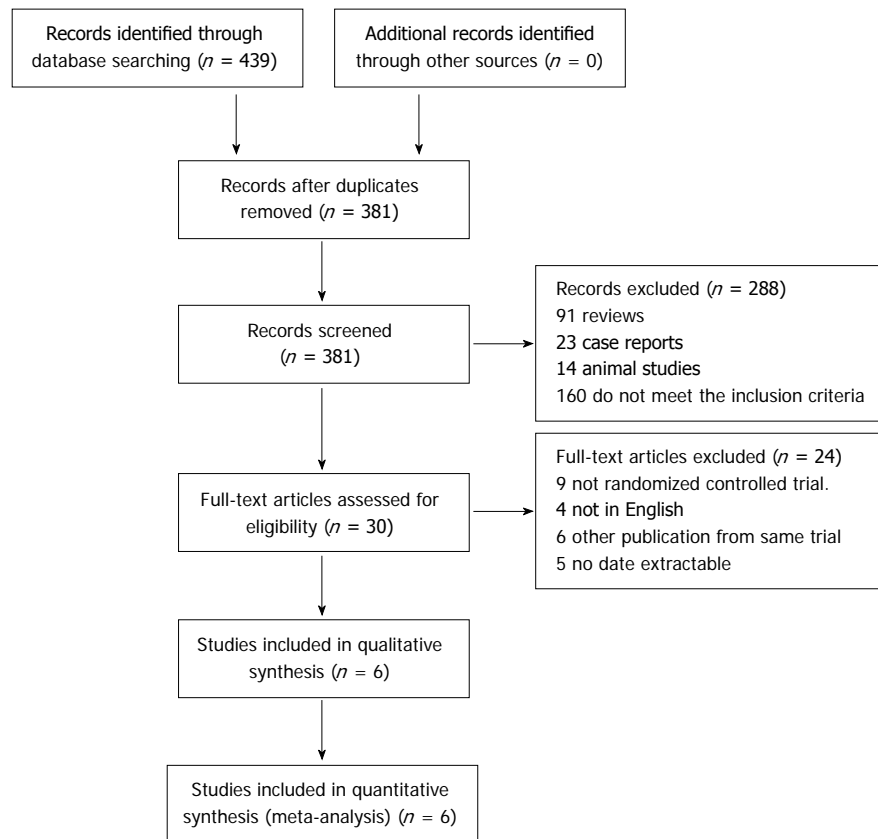


Figure 1 Selection of studies.

Table 1 Main characteristics of the six included studies

	<i>n</i>		Age (yr)		Sex (male/female)	
	LFT	LCC	LFT	LCC	LFT	LCC
Wang <i>et al</i> <sup>[20]</sup>	41	42	57.2 ± 18.1	55.4 ± 16.8	24/17	25/17
Vlug <i>et al</i> <sup>[21]</sup>	93	98	66 ± 10.3	66 ± 7.1	54/39	59/39
van Bree <i>et al</i> <sup>[22]</sup>	18	18	64 ± 10.1	66 ± 6.9	11/7	11/7
Veenhof <i>et al</i> <sup>[23]</sup>	17	20	65	68	9/8	14/6
Wang <i>et al</i> <sup>[24]</sup>	40	38	71	72	22/18	20/18
Wang <i>et al</i> <sup>[25]</sup>	106	104	57	55	65/41	60/44

LFT: Fast-track rehabilitation protocol and laparoscopic surgery; LCC: Conventional care strategies and laparoscopic surgery.

asymmetry on the natural logarithm scale of the RR was measured by a linear regression approach.

## RESULTS

### Search results

A total of 439 references were identified from medical journal databases. Upon examination of the abstracts, 409 articles were rejected based on the rejection criteria outlined in Figure 1. A study of the complete manuscripts for the 30 remaining articles led to elimination of 14 papers that contained no data pertaining to the outcome of LFT for colorectal resection, four papers not in English and six papers explaining the effect of analgesia. The remaining six non-duplicated randomized controlled trials (RCTs) that compared LFT with LCC were included

in the meta-analysis.

### Characteristics of the selected RCTs

Characteristics of the six RCTs<sup>[20-25]</sup> included in the meta-analysis are summarized in Table 1. These studies were published between 1985 and 2013 and investigated a total of 665 patients: 323 received LFT and 332 received LCC.

### Meta-analysis results

**Complication:** Data were collected from six studies (655 patients) on complications for LFT *vs* LCC. In the LFT group, 19.81% patients (64/323) had complications, while in the LCC group, 33.13% patients (110/332) had complications. Pooling the results indicated that LFT could significantly reduce complications compared with LCC. The weighted mean difference (WMD) was 0.60 (95%CI: 0.46-0.79,  $P < 0.05$ ),  $\chi^2 = 12.33$  ( $P = 0.03$ ) and  $I^2 = 59\%$ , indicating heterogeneity among the studies.

**Anastomotic leak:** Data were collected from four studies (497 patients) on anastomotic leak for LFT *vs* LCC. 4.94% (12/243 patients) had an anastomotic leak in the LFT group and 4.72% (12/254) in the LCC group. Pooling the results indicated that LFT and LCC had similar risks of anastomotic leak. The WMD was 1.07 (95%CI: 0.50-2.32,  $P > 0.05$ , Figure 2),  $\chi^2 = 2.13$  ( $P = 0.55$ ) and  $I^2 = 0\%$ , which excludes heterogeneity among the studies.

**Wound infection:** Data were collected from four stud-



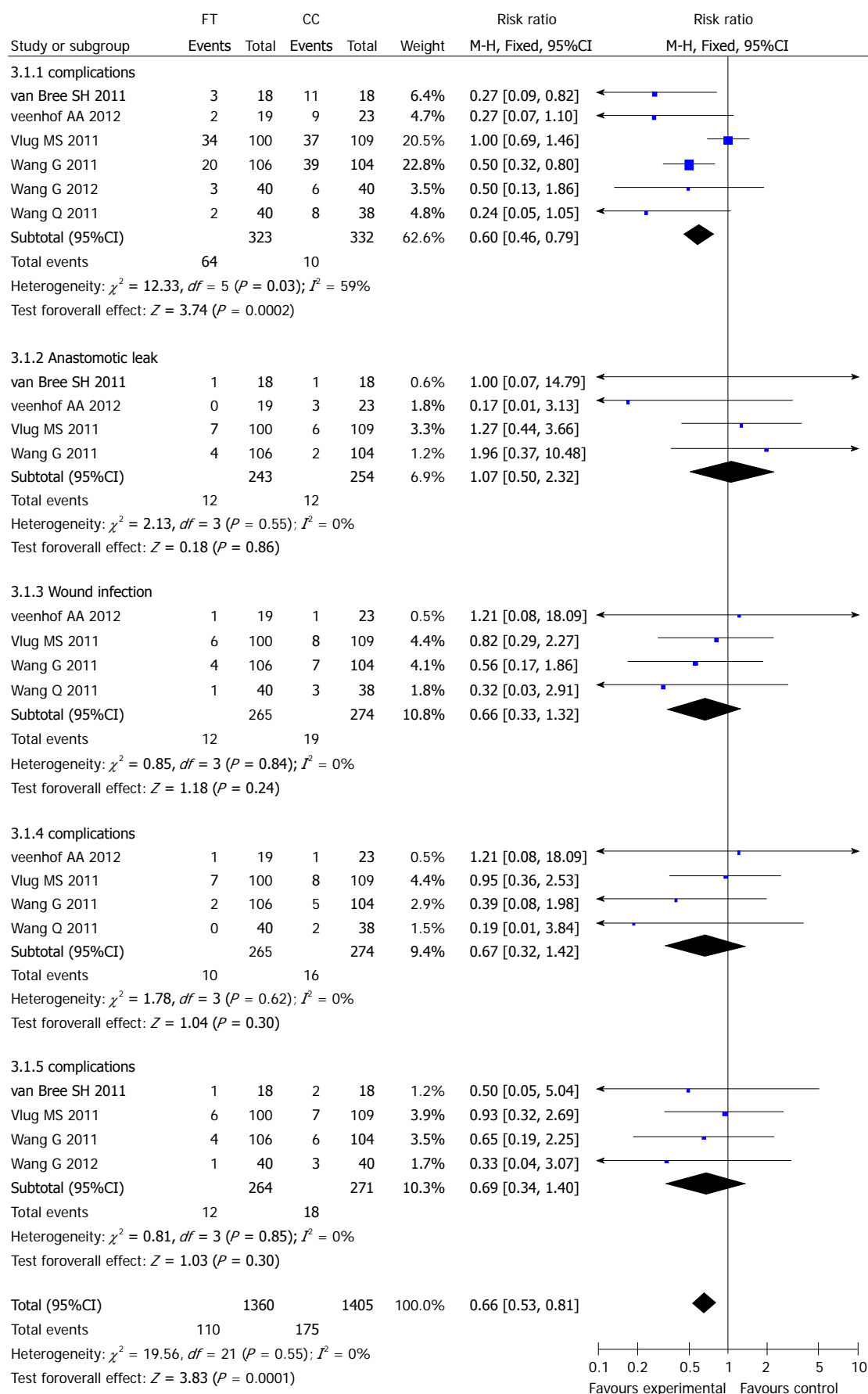


Figure 2 Forest plot comparing fast-track rehabilitation protocol and laparoscopic surgery vs conventional care strategies and laparoscopic surgery in colorectal resection, outcome: complications. LFT: Fast-track rehabilitation protocol and laparoscopic surgery; LCC: Conventional care strategies and laparoscopic surgery.

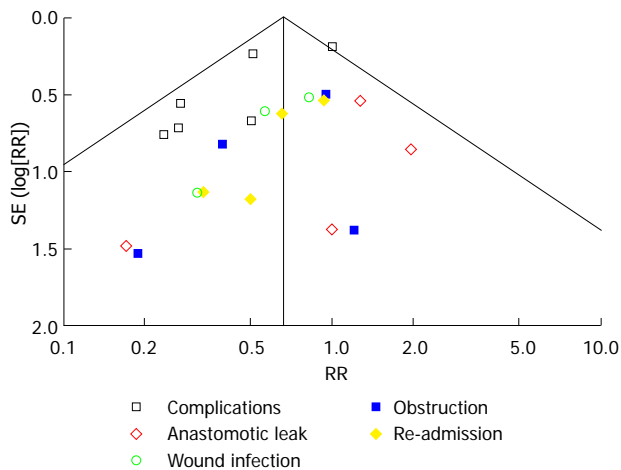


Figure 3 Comparison between the fast-track rehabilitation protocol and laparoscopic surgery, and conventional care strategies and laparoscopic surgery in laparoscopic colorectal resection for colorectal malignancy, outcome: complications.

ies (539 patients) on wound infection for LFT *vs* LCC; 4.53% (12/265 patients) had wound infection in the LFT group and 6.93% (19/274) in the LCC group. Pooling the results indicated that LFT did not significantly reduce wound infections compared with LCC. The WMD was 0.66 (95%CI: 0.33-1.32,  $P > 0.05$ ),  $\chi^2 = 0.85$  ( $P = 0.84$ ) and  $I^2 = 0\%$ , which excludes statistical heterogeneity among the studies.

**Obstruction:** Data were collected from four studies (539 patients) on obstruction for LFT *vs* LCC. 3.77% (10/265 patients) had obstructions in the LFT group and 5.84% (16/274) in the LCC group. Pooling the results indicated no significant difference in the risk of obstruction. The WMD was 0.67 (95%CI: 0.32-1.42,  $P > 0.05$ ),  $\chi^2 = 1.78$  ( $P = 0.62$ ) and  $I^2 = 0\%$ , which excludes heterogeneity among the studies.

**Re-admission:** Data were collected from four studies (535 patients) on re-admission for LFT *vs* LCC. 4.55% (12/264 patients) were readmitted in the LFT group and 6.64% (18/271) in the LCC group. Pooling the results indicated no apparent difference in re-admission. The WMD was 0.69 (95%CI: 0.34-1.4,  $P > 0.05$ ),  $\chi^2 = 0.81$  ( $P = 0.85$ ) and  $I^2 = 0\%$ , which excludes heterogeneity among the studies.

### Publication bias

Funnel plots were created to access the publication bias of the literature. The shapes of the funnel plots did not show any obvious asymmetry (Figure 3).

## DISCUSSION

The straightforward conclusion from the six included studies is that LFT is a more reliable treatment for colorectal malignancy, compared with LCC. LFT reduced complications, but carried similar risks of anastomotic

leak, wound infection, obstruction and re-admission.

Complications after LFT and LCC of colorectal resection have generally been discussed in China, as well as in other countries. A recently published multivariate analysis identified male gender<sup>[26]</sup>, preoperative education, anesthesia<sup>[27]</sup> and early postoperative oral nutrition<sup>[28]</sup> as potential risk factors for complications after colorectal surgery. In addition, some studies have found an increased risk of anastomotic leaks in males, which is consistent with the results of this study (10.1% of the men required re-operation for anastomotic leak *vs* 3.3% of the women)<sup>[29-32]</sup>.

Preoperative education of patients has a crucial role in LFT. It is necessary to demonstrate the detailed treatment program, the different steps of fast-track rehabilitation program and relevant measures for the patients to make them better understand and accept the fast-track rehabilitation program.

Better cooperation of patients can bring better outcomes of LFT. Generally, since the gastric emptying time of solid meal and fluid are 6 and 2 h, respectively<sup>[33]</sup>, the patients should be encouraged to have liquid meal 2 h before the operation instead of fasting. It has been shown that preoperative oral carbohydrate is safe and can efficiently reduce complications<sup>[34-36]</sup>.

The role of epidural anesthesia or regional anesthesia in LFT should be stressed. Postoperative epidural analgesia can avoid stress-induced neurological, endocrinological and homeostatic changes or the blocking of sympathetic nerve-related surgical stress response, reduce complications such as nausea, vomiting and enteroparesis after operation, promote early ambulation, improve the intestinal function and shorten the hospital stay time of patients after resection of colorectal cancer<sup>[26,37-42]</sup>.

Early postoperative oral nutrition is regarded as an essential part of LFT. Food intake can stimulate gastrointestinal peristalsis, and early feeding during the first 24 h after surgery promotes the recovery of an obstruction. It has been illustrated that early postoperative oral nutrition attenuates catabolism and potentially decreases infectious complications<sup>[27,43]</sup>.

Several studies have shown that American Society of Anesthesiologists grade III or higher is associated with increased postoperative morbidity<sup>[44-46]</sup>.

LFT can improve the rehabilitation of patients after resection of colorectal cancer better than LCC, thus benefiting their surgery, anesthesia, pain management, physical therapy and social work. The primary work of LFT is the preoperative education of patients to make them understand the whole plan and the aim of each stage. Therefore, it is vital to obtain cooperation from the nursing staff.

However, we should still regard these outcomes with caution and evaluate them critically for the following reasons. Firstly, although there was no detectable publication bias, as tested by the funnel plots, the overall methodological quality and reporting of the included studies were poor. Secondly, the number of studies found was relatively low, and the aforementioned quality issues may have biased the results significantly. Therefore, more

large trials with better separation between LFT and LCC for colorectal malignancy seem necessary. Furthermore, in light of current evidence, LFT should not yet be considered the new standard for colorectal malignancy. Long-term data on outcome, as well as important other factors in making a decision for an intervention, are also lacking. Quality of life data and data on physiological performance after 5 years have never been described, nor have data on cost-effectiveness or economic evaluations of LFT. These parameters may play an important part in recommending LFT treatment in colorectal resection. However, we believe that, with greater awareness and the increasing popularity of LFT, more long-term follow-up reports will eventually be published.

There have been eight previously published systematic reviews, including meta-analyses on this topic<sup>[16,17,47-52]</sup>. These included three reviews of controlled clinical trials and randomized controlled trials<sup>[17,47,49]</sup>, and five reviews of randomized controlled trials only<sup>[47,50-52]</sup>. The present study is the first meta-analysis to compare fast-track rehabilitation with conventional care in laparoscopic colorectal resection for colorectal malignancy. This also the first meta-analysis of patients undergoing elective colorectal surgery to demonstrate that LFT is associated with a significant reduction in postoperative complications, but no significant reduction in readmission rates. The increased number of included studies supported the quality of the evidence from the present study.

In conclusion, this meta-analysis demonstrated that LFT is safe and feasible for colorectal surgery. As LFT comes into even wider use, additional large, prospective RCTs should be conducted to further compare the efficacy and safety of this approach.

## COMMENTS

### Background

Fast-track rehabilitation in laparoscopic colorectal resection has become the most fashionable way of treating colorectal malignancy. Complications after fast-track rehabilitation protocol and laparoscopic surgery (LFT) and conventional care strategies and laparoscopic surgery (LCC) of colorectal resection have generally been discussed in China, as well as in other countries.

### Research frontiers

Over the past three decades, many studies have assessed the performance of LFT. However, comparisons of LFT and LCC have not been published.

### Innovations and breakthroughs

Based on this meta-analysis, LFT for colorectal malignancy is safe and efficacious. Similar associations were indicated in subgroup analyses of East Asian, Western, cohort, and high-quality studies. These findings were not presented clearly in previous systematic reviews.

### Applications

LFT appears to be neither directly nor indirectly associated with risk. Further studies should seek to clarify this conclusion.

### Peer review

LFT is rapidly becoming the focal point of attraction for specialists worldwide. This article shows the advantages of the procedure. This analysis has great practical value for clinicians.

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## Localized type 1 autoimmune pancreatitis superimposed upon preexisting intraductal papillary mucinous neoplasms

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### Abstract

A 70-year-old woman was found to have 2 cystic lesions in the head of the pancreas on abdominal ultrasonography during a routine medical examination. Endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography showed multilocular cysts in the head of the pancreas without dilation of the main pancreatic duct. The patient was followed-up semiannually with imaging studies for suspected branch duct-type intraductal papillary mucinous neoplasm (IPMN). At 3 years after initial presentation, hypoechoic lesions were observed around each pancreatic cyst by EUS. Diffusion-weighted imaging

showed high-intensity regions corresponding to these lesions. Therefore, a diagnosis of invasive carcinoma derived from IPMN could not be excluded, and subtotal stomach-preserving pancreaticoduodenectomy was performed. The macroscopic examination of the surgical specimen showed whitish solid masses in the head of the pancreas, with multilocular cysts within each mass. Microscopically, each solid mass consisted of inflammatory cells such as lymphocytes and plasma cells. Furthermore, immunochemical staining revealed immunoglobulin G4-positive cells, and many obliterating phlebitides were observed. The cysts consisted of mucus-producing epithelial cells and showed a papillary growth pattern. Based on these findings, we diagnosed multiple localized type 1 autoimmune pancreatitis occurring only in the vicinity of the branch duct-type IPMN.

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**Key words:** Autoimmune pancreatitis; Intraductal papillary mucinous neoplasm; Immunoglobulin G4; Endoscopic ultrasonography; Diffusion-weighted imaging

**Core tip:** We herein report a case of localized type 1 autoimmune pancreatitis (AIP) superimposed upon preexisting multifocal intraductal papillary mucinous neoplasms (IPMNs) of the branch duct. Although few reports have shown AIP associated with IPMN, in our case, AIP had developed only around the IPMN, which was under progressive observation. Therefore, the IPMN may have influenced the pathogenesis of AIP.

Urata T, Naito Y, Izumi Y, Takekuma Y, Yokomizo H, Nagamine M, Fukuda S, Notohara K, Hifumi M. Localized type 1 autoimmune pancreatitis superimposed upon preexisting intraductal papillary mucinous neoplasms. *World J Gastroenterol* 2013; 19(47): 9127-9132 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

Autoimmune pancreatitis (AIP) is classified into 2 groups according to the International Consensus Diagnostic Criteria reported in 2011<sup>[1]</sup>. Type 1 AIP is characterized by the pathological condition termed lymphoplasmacytic sclerosing pancreatitis, whereas type 2 AIP is characterized by idiopathic duct centric pancreatitis. Although the pathogenesis of AIP remains unknown, it is considered to involve certain immune mechanisms. Typical parenchymal imaging features of AIP include diffuse enlargement with delayed enhancement and occasional segmental or focal enlargement. Given these imaging characteristics, distinguishing AIP with focal enlargement (f-AIP) from carcinoma of the pancreas is particularly difficult.

Although intraductal papillary mucinous neoplasm (IPMN) is recognized as a cystic mucus-producing tumor, its association with AIP has not been reported thus far. Herein, we report a rare case of AIP occurring only in the vicinity of IPMN, which was diagnosed during the follow-up examinations for IPMN.

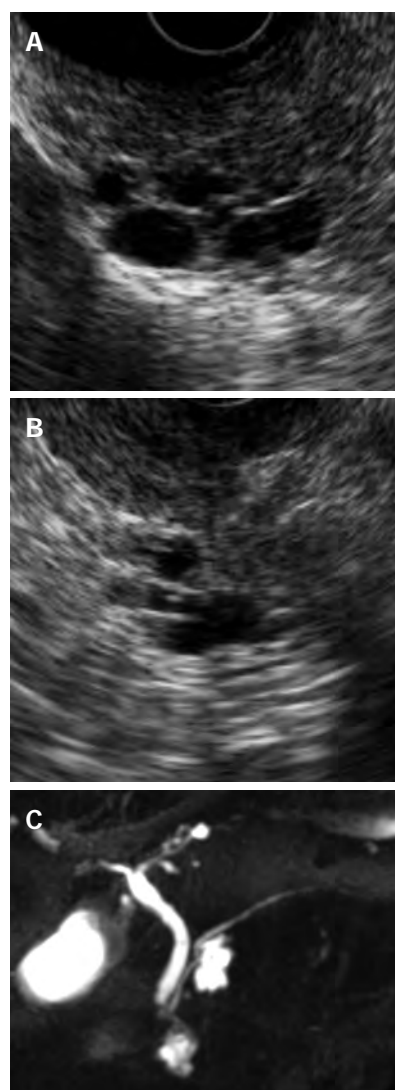
## CASE REPORT

A 70-year-old woman was found to have cystic lesions in the head of the pancreas on abdominal ultrasonography (US) during a routine medical checkup in December 2008. She had no symptoms, and the blood test results were essentially normal. Endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography (MRCP) showed multilocular cysts in the head of the pancreas without dilation of the main pancreatic duct (Figure 1). Diffusion-weighted imaging (DWI) showed no uptake in the pancreas (Figure 2A and B). The patient was followed-up by semiannual imaging studies for suspected branch duct-type IPMN.

In December 2011, solid lesions were observed in or around both the IPMNs on EUS (Figure 3A and B). DWI showed high-intensity signals corresponding to these lesions (Figure 2C and D), and MRCP showed a reduction in the diameter of the IPMNs (Figure 3C). Contrast-enhanced computed tomography (CT) revealed that these lesions had a lower density than the surrounding pancreatic parenchyma during the pancreatic parenchymal phase and showed iso-density in the equilibrium phases (Figure 4). ERP revealed a normal main pancreatic duct and no communication between the main pancreatic duct and the cystic lesions. The pancreatic juice cytology was negative for malignancy.

Based on these results, our principal differential diagnoses included inflammatory pseudo-tumors such as AIP; however, the possibility of invasive carcinoma derived from IPMN could not be excluded.

We performed a subtotal stomach-preserving pan-

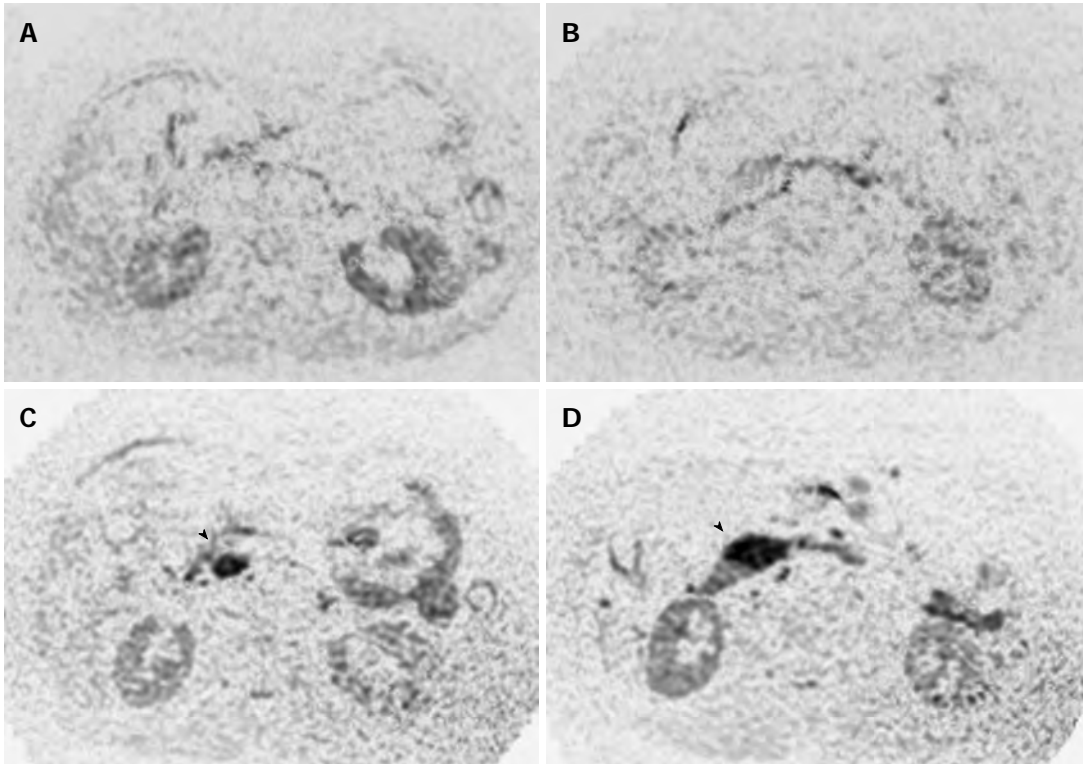


**Figure 1** Imaging studies from the initial examination. A, B: Endoscopic ultrasonography showed 2 multilocular cysts in the pancreas head; C: Magnetic resonance cholangiopancreatography showed 2 cystic lesions without dilation of the main pancreatic duct in the pancreas head.

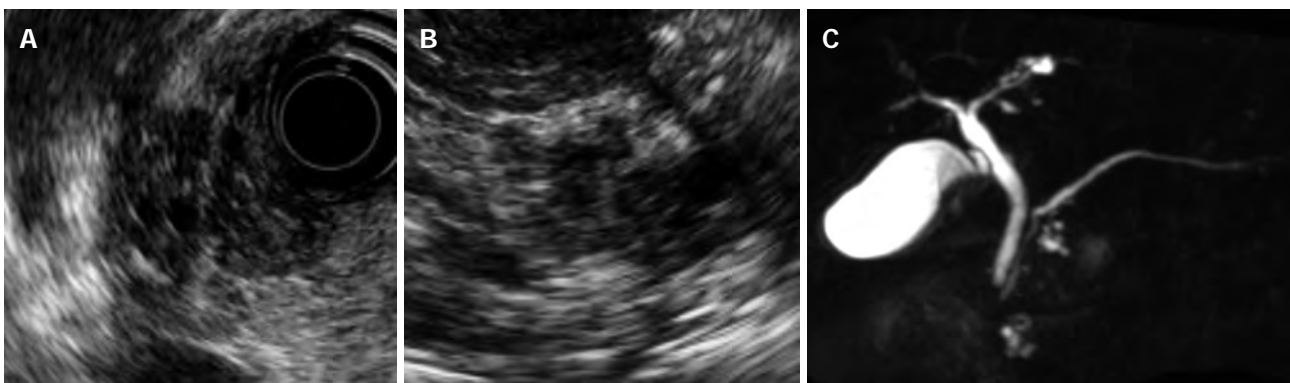
atoduodenectomy after fully explaining the possibility of malignancy and the risks of the surgery to the patient.

### Pathological findings

The macroscopic examination of the surgical specimen showed multilocular cysts with 2 whitish solid lesions (Figure 5). The 2 lesions were solitary and indicated no gross continuity with each other. Microscopically, each solid lesion presented a striform pattern consisting of fibroblasts/myofibroblasts mixed with lymphoid follicles and inflammatory cells (Figure 6A), particularly lymphocytes and plasma cells that tested positive for immunoglobulin IgG4 on immunochemical staining (Figure 6B). In addition, many obliterating phlebitides (Figure 6C) were observed. The multilocular cysts demonstrating periductal inflammation consisted of mucus-producing epithelial cells and showed a papillary growth pattern (Figure 6D). The epithelial cells were MUC2-negative



**Figure 2** Diffusion-weighted magnetic resonance imaging. A, B: In 2008, diffusion-weighted imaging showed no signal in the pancreas; C, D: In 2011, diffusion-weighted imaging showed high-intensity signals (arrow-head) corresponding to both cystic lesions in the pancreas.



**Figure 3** Imaging studies at follow-up examination. A, B: Endoscopic ultrasonography revealed solid lesions in or around both cystic lesions in the pancreas head. Hyperechoic foci and strands were observed in these lesions; C: Magnetic resonance cholangiopancreatography showed a reduction in the diameter of both cystic lesions.

and MUC5AC-positive on immunochemical staining. We therefore diagnosed multiple localized type 1 AIP involving only the vicinity of branch duct-type IPMN.

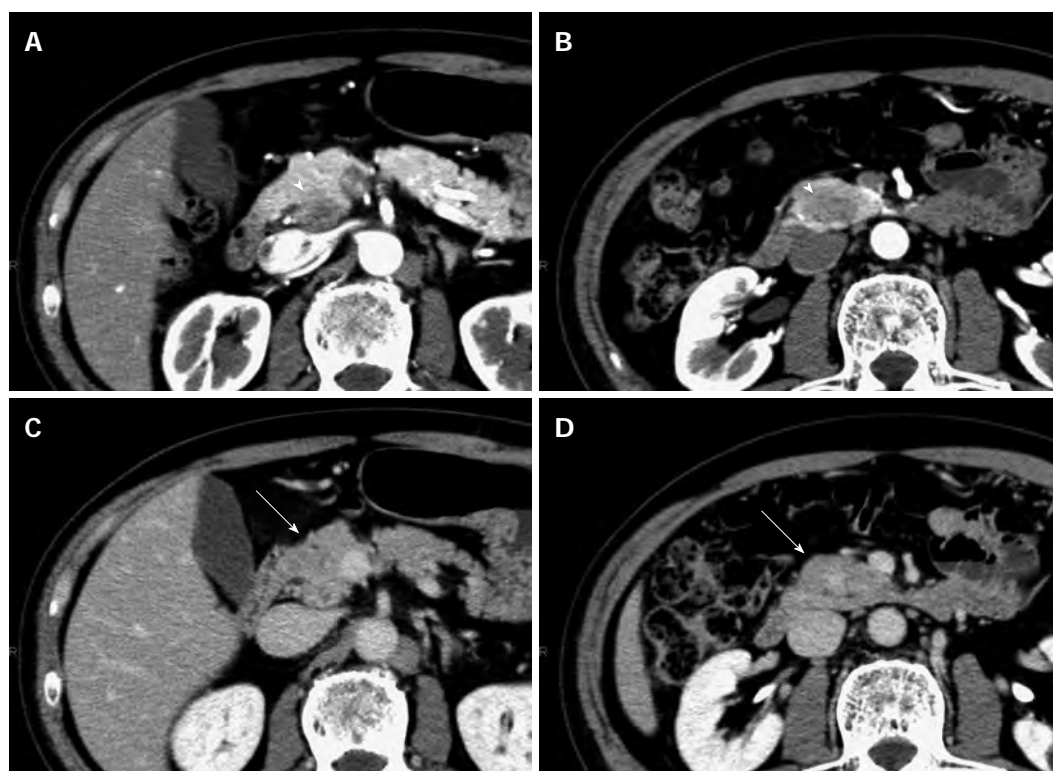
## DISCUSSION

Type 1 AIP is histologically characterized by the following 4 characteristic features: (1) Dense infiltration of plasma cells and lymphocytes, particularly in the periductal regions; (2) Peculiar storiform fibrosis; (3) Venulitis with lymphocytes and plasma cells often leading to obliteration of the affected veins; and (4) Abundant [ $> 10$  cells per high-power field (HPF)] IgG4-positive plasma cells<sup>[1]</sup>. Our case fulfilled all of these histological features,

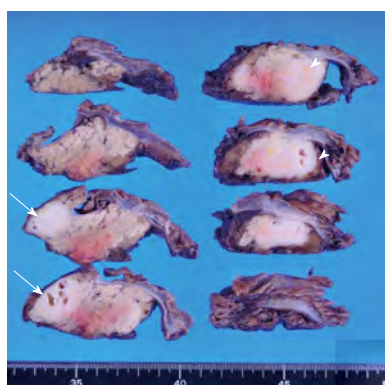
indicating a diagnosis of AIP.

In diagnostic imaging, typical AIP is characterized by the diffuse enlargement of the pancreas with stenosis of the main pancreatic duct. However, at times, AIP can present as segmental or focal enlargement in the pancreas, and differentiating such cases of f-AIP from pancreatic carcinoma is rather difficult<sup>[2]</sup>. Although US and EUS show hypoechoic masses in both f-AIP and pancreatic carcinoma, the characteristic findings of f-AIP include hyperechoic foci and lobularity, reflecting an inflammatory tumor environment. In the Rosemont criteria<sup>[3]</sup>, the EUS-based major criteria for the diagnosis of chronic pancreatitis (CP), including AIP, comprise hyperechoic foci with shadowing, calculi in the main





**Figure 4** Computer tomography scan. A, B: Both cystic lesions (arrow-head) showed a lower density than the surrounding pancreatic parenchyma during the pancreatic parenchymal phase; C, D: Both cystic lesions (arrow) appeared as iso-dense in the equilibrium phases.



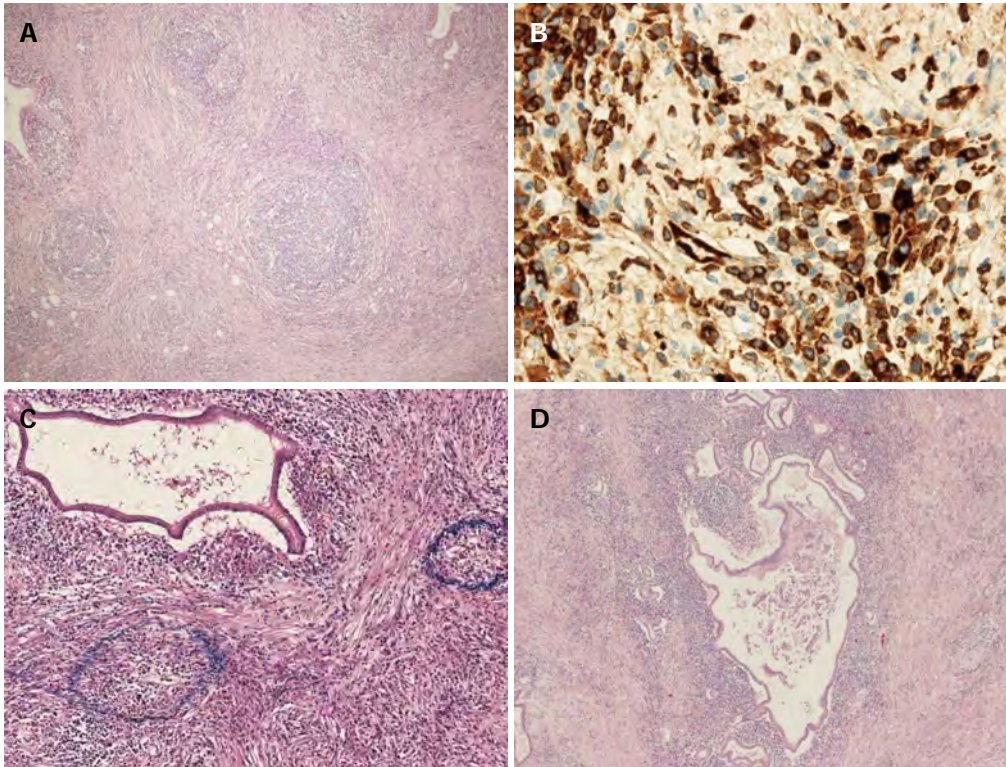
**Figure 5** Macroscopic findings. The macroscopic examination revealed 2 whitish solid lesions with multilocular cysts (arrow, and arrow-head). Each lesion was solitary and indicated no gross continuity.

pancreatic duct, and lobularity with a honeycomb appearance. The minor criteria for CP include the presence of cysts, dilated ducts  $\geq 3.5$  mm, irregular pancreatic duct contours, dilated side branches  $\geq 1$  mm, a hyperechoic duct wall, the presence of strands, non-shadowing hyperechoic foci, and lobularity with noncontiguous lobules. Our case revealed features similar to CP, as the hypoechoic tumor observed within the vicinity of the cystic lesions demonstrated certain characteristic findings of CP, including hyperechoic foci and the presence of strands. However, distinguishing between f-AIP and PC on other imaging modalities may be difficult<sup>[2,4-7]</sup>. In the present case, the tumor showed high-intensity signals

on DWI and delayed enhancement on dynamic CT, although both f-AIP and PC may present similar findings in these types of imaging studies<sup>[7,8]</sup>. However, simultaneous carcinomatous changes in 2 IPMN lesions are very rare; in such a case, the possibility that such lesions are inflammatory rather than carcinomatous is rather high.

Among other diagnostic methods, Kanno *et al*<sup>[9]</sup> reported that EUS-guided fine-needle aspiration (EUS-FNA) with a 22-G needle may provide adequate tissue for histological examination. However, many reports have suggested that the material required to perform a confirmed diagnosis of AIP cannot be obtained with this approach, although EUS-FNA in cases of AIP can eliminate the possibility of carcinoma<sup>[10-12]</sup>. In our case, we could not perform EUS-FNA because the cyst was located near the puncture site.

The pathogenesis of AIP has not yet been clarified. The involvement of certain immunological mechanisms is suspected due to the presence of autoantibodies, the predominant infiltration of CD4 and CD8 T cells, and the expression of HLA-DR antigens in the pancreas in such cases<sup>[13]</sup>. In our patient, AIP was restricted to the vicinity of the IPMNs and did not develop in the remaining pancreatic parenchyma. Although this finding suggests that IPMN is related to the pathogenesis of AIP, proving a causal relationship is difficult because few studies have reported an association between these 2 clinical conditions<sup>[14]</sup>. According to previous reports, Naitoh *et al*<sup>[14]</sup> supported the hypothesis in which IPMN appears in the background of AIP. However, in our



**Figure 6 Microscopic findings.** A: Each solid lesion presented a striiform pattern with lymphoid follicles and inflammatory cells (HE, original magnification  $\times 12.5$ ); B: The plasma cells showed positivity for immunoglobulin G4 (HE, original magnification  $\times 200$ ); C: Many obliterating phlebitides were observed (HE, original magnification  $\times 100$ ); D: The multilocular cysts produced mucus and demonstrated a papillary pattern (HE, original magnification  $\times 12.5$ ).

case, because AIP developed during the progressive observation of IPMN, IPMN may have influenced the pathogenesis of AIP. However, the further examination of such cases of AIP and IPMN for an improved understanding of the relationship between these clinical entities is necessary.

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**S- Editor:** Zhai HH **L- Editor:** A **E- Editor:** Liu XM





## DOG1 is useful for diagnosis of KIT-negative gastrointestinal stromal tumor of stomach

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### Abstract

Approximately 80%-95% of gastrointestinal stromal tumors (GISTs) show positive staining for KIT, while the other 5%-20% show negative staining. If the tumor is negative for KIT, but is positive for CD34, a histological diagnosis is possible. However, if the tumor is negative for KIT, CD34, S-100, and SMA, a definitive diagnosis is often challenging. Recently, Discovered on GIST-1 (DOG1) has received considerable attention as a useful molecule for the diagnosis of GIST. DOG1, a membrane channel protein, is known to be overexpressed in GIST. Because the sensitivity and specificity of DOG1 are higher than those of KIT, positive staining for DOG1 has been reported, even in KIT-negative GISTs. KIT-negative GISTs most commonly arise in the stomach and are mainly characterized by epithelioid features histologically. We describe our experience with a rare case of a KIT-negative GIST of the stomach that was diagnosed by positive immunohistochemical staining for

DOG1 in a patient who presented with severe anemia. Our findings suggest that immunohistochemical staining for DOG1, in addition to gene analysis, is useful for the diagnosis of KIT-negative tumors that are suspected to be GISTs.

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**Key words:** KIT negative; Gastrointestinal stromal tumors; Discovered on gastrointestinal stromal tumor-1; Platelet-derived growth factor receptor alpha

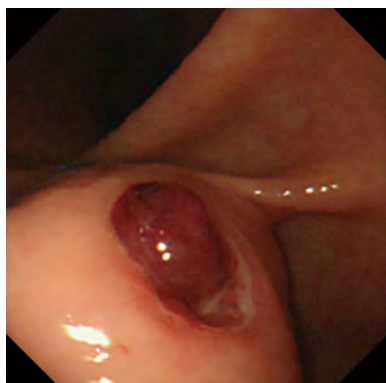
**Core tip:** We describe our experience with a rare case of a KIT-negative gastrointestinal stromal tumor (GIST) of the stomach that was diagnosed by positive immunohistochemical staining for Discovered on GIST-1 (DOG1) in a patient who presented with severe anemia. Our findings suggest that immunohistochemical staining for DOG1, in addition to gene analysis, is useful for the diagnosis of KIT-negative tumors that are suspected to be GISTs.

Wada T, Tanabe S, Ishido K, Higuchi K, Sasaki T, Katada C, Azuma M, Naruke A, Kim M, Koizumi W, Mikami T. DOG1 is useful for diagnosis of KIT-negative gastrointestinal stromal tumor of stomach. *World J Gastroenterol* 2013; 19(47): 9133-9136 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i47/9133.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i47.9133>

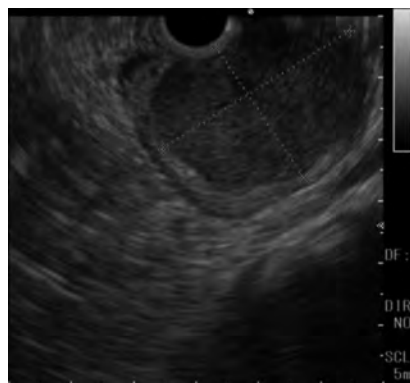
### INTRODUCTION

A gastrointestinal stromal tumor (GIST) is a mesenchymal tumor derived from the mesoderm that arises in the gastrointestinal tract. The estimated incidence is 2 cases per 100000 people per year. The most common age at





**Figure 1** Findings on upper gastrointestinal endoscopy. A submucosal tumor accompanied by an ulcer with an adherent clot was found in the superior portion of the anterior wall of the gastric antrum.



**Figure 2** Findings on upper endoscopic ultrasonography. A homogeneous, hypoechoic, well-demarcated mass, approximately 4 cm in diameter with a flat border, arising from the fourth layer of the gastric wall.

diagnosis is 50-60 years. KIT protein is characteristically expressed by immunohistochemical staining. Gain-of-function mutations of the *c-kit* gene (approximately 90%) or the platelet-derived growth factor receptor alpha (*PDGFR4*) gene (approximately 5%) are the major causes of GISTs<sup>[1]</sup>. Immunohistochemical staining and gene analysis are considered useful for diagnosis, but if the tumor is negative for KIT, CD34, S-100, and smooth muscle actin (SMA), a definitive diagnosis is often challenging. We describe our experience with a patient in whom immunohistochemical staining for Discovered on GIST-1 (DOG1) enabled the diagnosis of a KIT-negative GIST<sup>[2]</sup>.

## CASE REPORT

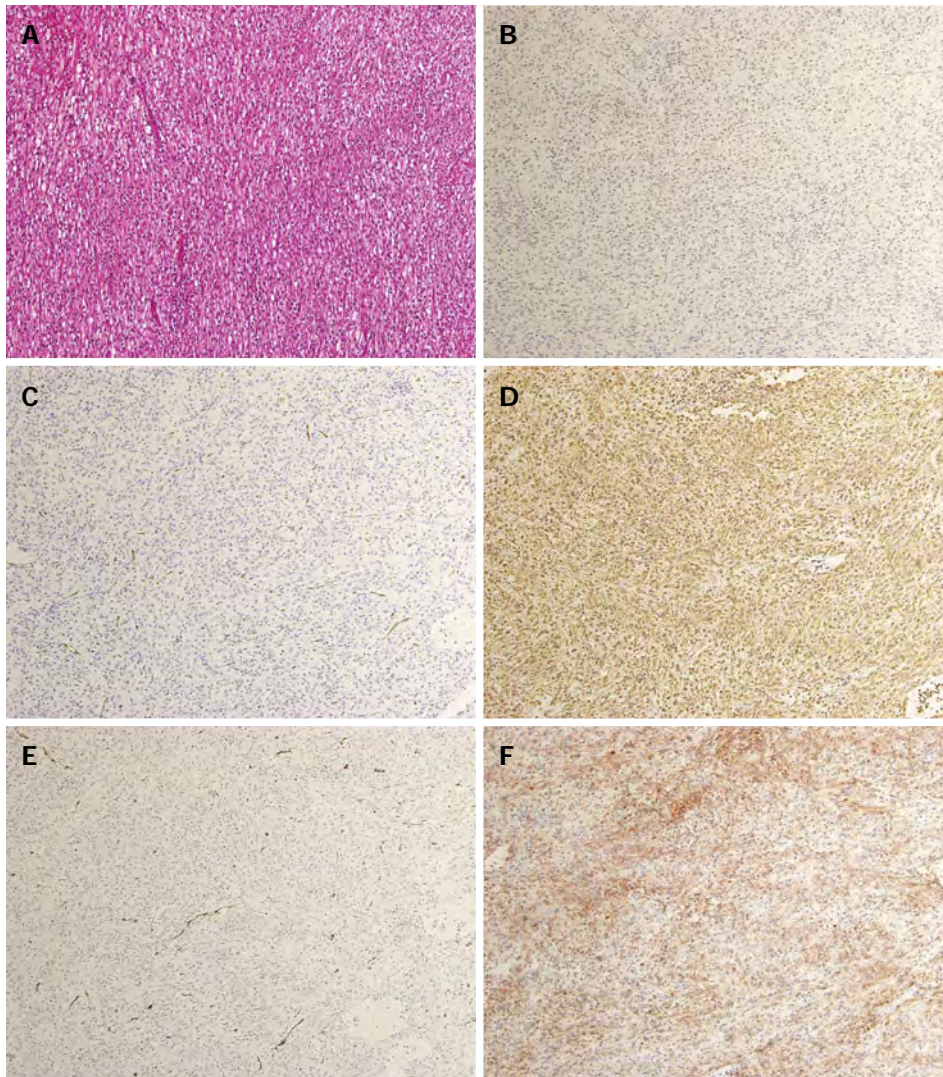
A 60-year-old man was referred to the Department of Gastroenterology of our hospital because of wooziness, shortness of breath on effort, and tarry stools. A blood test showed that the hemoglobin level was 3.6 g/dL, indicating severe anemia. Upper gastrointestinal endoscopy disclosed a submucosal tumor accompanied by an ulcer with an adherent clot, arising in the superior portion of the anterior wall of the gastric antrum (Figure 1). Endoscopic ultrasonography (EUS) revealed a well-demarcated, homogeneous, hypoechoic mass with a flat border. The mass was approximately 4 cm in diameter and arose from the fourth layer of the gastric wall (Figure 2). Endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNAB)<sup>[3,4]</sup> was performed to obtain a definitive diagnosis and showed aggregations of cells with spindle-like or polygonal nuclei. However, immunohistochemical staining was negative for KIT, CD34, S-100, and SMA. A GIST was strongly suspected, but a definite diagnosis was not reached. Gene analysis could not be performed because the tissue sample was too small. However, the patient had a symptomatic, submucosal tumor with no distinct evidence of distant metastasis or direct invasion on enhanced computed tomography of the chest and abdomen. Surgery was, therefore, indicated according to the clinical practice guidelines for GIST in Japan<sup>[5]</sup>, and a distal gas-

trectomy was performed. On macroscopic examination, the surgically resected specimen showed no evidence of bleeding or necrosis. The tumor measured 45 mm in diameter, and the resection margins were tumor negative. Histopathological examination showed that the tumor consisted of mixed components, including diffuse proliferations of spindle cells with eosinophilic cytoplasm, as well as epithelioid cells in some regions. One mitosis was found per 50 high-power fields, and the MIB-1 index was 3%. Immunohistochemical staining was negative for KIT, CD34, S-100, and SMA, but it was positive for vimentin and DOG1, a membrane channel protein (Figure 3). The tissue specimen obtained by EUS-FNA also stained positively for DOG1 (Figure 4). Genetic analysis showed a mutation in exon 18 (D842V) of the *PDGFR4* gene, with no mutation in the *c-kit* gene. On the basis of these results, a KIT-negative GIST with low risk according to Fletcher's classification<sup>[6]</sup>, and very low risk according to Miettinen's classification<sup>[7]</sup>, was diagnosed<sup>[7]</sup>. The patient recovered uneventfully after surgery. As of 3 years after surgery, the patient has been followed up on an outpatient basis and remains free of metastasis and recurrence.

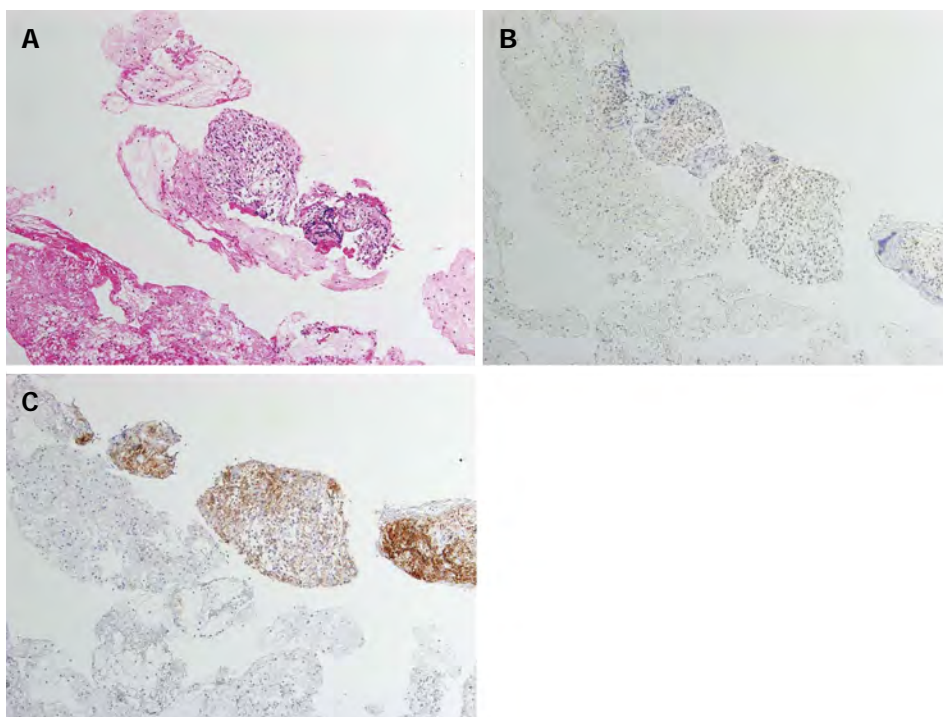
## DISCUSSION

GIST is a mesenchymal tumor of the mesoderm arising from the interstitial cells of Cajal in the gastrointestinal tract. The most common site is the stomach (60%), followed by the small intestine (30%), duodenum (5%), and large intestine (4%)<sup>[8]</sup>. GIST can be associated with diverse clinical symptoms, such as gastrointestinal bleeding, abdominal pain, and tumor obstruction. Histopathologically, GIST can be classified into 3 categories: spindle-cell type, epithelioid-cell type, and mixed type. Epithelioid-cell type accounts for approximately 70% of all GISTs, epithelioid-cell type accounts for approximately 20%, and mixed type, as was found in our patient, accounts for approximately 10%<sup>[8]</sup>.

At present, specific tumor markers for the diagnosis of GIST are unavailable. A definite diagnosis is established by immunostaining tissue specimens obtained by



**Figure 3** Histopathological findings of the surgically resected specimen. A: Hematoxylin and eosin staining ( $\times 100$ ). The tumor consisted of mixed components, including spindle cells with eosinophilic cytoplasm, as well as epithelioid cells in some regions; B: KIT staining ( $\times 100$ ), KIT staining was negative; C: CD34 staining ( $\times 100$ ), CD34 staining was negative; D: Vimentin staining ( $\times 100$ ); vimentin staining was positive. E: Smooth muscle actin (SMA) staining ( $\times 100$ ), SMA staining was negative; F: DOG1 staining ( $\times 100$ ); Immunostaining for DOG1 was positive mainly in the cell membrane and cytoplasm. DOG1: Discovered on gastrointestinal stromal tumor-1.



**Figure 4** Histopathological findings (specimen obtained by endoscopic ultrasound-guided fine needle aspiration biopsy). A: Hematoxylin and eosin staining ( $\times 100$ ). The tumor consisted of mixed components, consisting of spindle cells with eosinophilic cytoplasm, as well as cells with epithelioid features in some regions; B: KIT staining ( $\times 100$ ), KIT staining was negative; C: DOG1 staining ( $\times 100$ ). Immunostaining for DOG1 was positive mainly in the cell membrane and cytoplasm. DOG1: Discovered on gastrointestinal stromal tumor-1.



EUS-FNAB or at surgery for KIT, CD34, SMA, desmin, S-100, and Ki-67<sup>[4,6]</sup>. Approximately 80%-95% of GISTs show positive staining for KIT, while the other 5%-20% show negative staining. If the tumor is negative for KIT but positive for CD34, a histological diagnosis is possible; however, if the tumor is negative for KIT, CD34, S-100, and SMA, similar to our patient, a definitive diagnosis is often challenging.

Recently, DOG1 has received considerable attention as a useful molecule for the diagnosis of GIST<sup>[2]</sup>. DOG1, a membrane channel protein, is known to be overexpressed in GIST. Because the sensitivity and specificity of DOG1 are higher than those of KIT, positive staining for DOG1 has been reported even in KIT-negative GIST<sup>[9-11]</sup>. KIT-negative GISTs most commonly arise in the stomach and are mainly characterized by epithelioid features histologically. KIT-negative GISTs are often associated with *PDGFR*A gene mutations<sup>[8]</sup>. Rizzardi *et al.*<sup>[12]</sup> genetically analyzed a DOG1-positive, KIT-negative GIST of the stomach and reported the presence of a deletion in exon 14 of the *PDGFR*A gene, with no mutation in the *c-kit* gene.

In our patient, pathological examination of the surgically resected specimen showed a mixed-type GIST, including epithelioid cells. Immunostaining was negative for both KIT and CD34 but was positive for DOG1. Consistent with these findings, a mutation was found in exon 18 (D842V) of the *PDGFR*A gene, with no mutation in the *c-kit* gene. Because the D842V mutation of the *PDGFR*A gene is resistant to imatinib, sunitinib is prescribed when recurrence is found<sup>[1,13]</sup>.

In histological specimens obtained by EUS-FNAB before surgery, immunostaining was negative for KIT. A definite diagnosis could not be made. Immunohistochemical staining for DOG1 was additionally performed and showed that the cytoplasm of the tumor cells was positively stained. Hwang *et al.*<sup>[14]</sup> reported that DOG1 was a useful marker for the cytologic diagnosis of GIST in tissue specimens obtained by EUS-FNAB. However, one study reported that approximately 30% of KIT-negative GISTs are negative for DOG1, suggesting that tumors suspected to be GIST should be comprehensively evaluated, including analysis of other genes<sup>[10]</sup>.

We have described our experience with a rare case of KIT-negative GIST of the stomach that was diagnosed by positive immunostaining for DOG1 in a patient who presented with severe anemia. Our findings suggest that immunostaining for DOG1, in addition to gene analysis, is useful for the diagnosis of KIT-negative tumors suspected to be GISTs.

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## Assessment of proximal gastric accommodation in patients with functional dyspepsia

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### Abstract

Impaired gastric accommodation is one of the most important etiologic factors in the pathophysiology of functional dyspepsia. Ultrasound is a potential alternative method to study changes in gastric volume as a reflection of gastric accommodation. Ultrasound is suitable for patients because it is a non-invasive, easily repeated and non-radioactive procedure, and a previous study has demonstrated the feasibility of 3-dimensional ultrasound in examining functional dyspepsia. The brief article by Fan *et al* demonstrated that both the proximal gastric area and volume, measured by 2- and 3-dimensional ultrasound respectively, were significantly smaller in patients with functional dyspepsia than in healthy controls. These results are very interesting, but we raise the relevant point that it should have been mandatory to study both changes in gastric volume and their relationship with upper gastrointestinal symptoms in functional dyspepsia. In fact, the relationship between cardinal symptoms and several pathophysiologic mechanisms in functional dyspepsia remains a matter of debate. Moreover, further evaluation of distal gastric volume that has been previously implicated in the origin of functional dyspeptic symptoms is advisable.

Therefore, impaired gastric accommodation does not serve as a clear marker of the cardinal symptoms experienced by patients with functional dyspepsia in daily life.

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**Key words:** Proximal gastric function; Gastric accommodation; 2-Dimensional ultrasound; 3-Dimensional ultrasound; Functional dyspepsia; Rome III criteria

**Core tip:** Proximal gastric area and volume measured respectively by 2- and 3-dimensional ultrasound were significantly smaller in patients with functional dyspepsia compared to those of healthy controls. Hence, they could be used to assess accommodation impairment, but further prospective studies are needed to establish their clinical role in diagnosis of functional dyspepsia.

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### TO THE EDITOR

We read with great interest the article by Fan *et al*<sup>[1]</sup> showing that post-prandial measurement of both proximal gastric area, measured by 2-dimensional ultrasound (US), and proximal gastric volume, measured by 3-dimensional US, could be useful for assessment of the proximal gastric accommodation in healthy controls and in patients with functional dyspepsia (FD). The authors, therefore, concluded that US measurement of gastric area and volume could help to predict FD.

This article is welcomed because US is a potential al-



ternative method for studying changes in gastric volume as a measure of gastric accommodation that is impaired in a subgroup of about 40% of patients with FD<sup>[2]</sup>. US is suitable for patients because it is a non-invasive, easily repeated and non-radioactive procedure, and a previous study has demonstrated the feasibility of 3-dimensional US in FD<sup>[3]</sup>.

Nevertheless, the clinical significance of the conclusions of the study by Fan *et al*<sup>[1]</sup> should be regarded with a degree of caution, as the isolated determination of the lower gastric area and volume in FD compared with healthy controls is not sufficient to prove a clinical impact of this methodology in predicting FD as the authors suggest in the core tip of the study.

Dyspeptic symptoms are very common in the general population, with prevalence estimates ranging between 10% and 45%<sup>[4]</sup>. The results of prevalence studies are strongly influenced by the criteria used to define dyspepsia. FD, according to Rome III criteria, is a common disorder seen in daily clinical practice, and is characterized by the presence of pain or discomfort in the upper abdomen in the absence of organic, systemic, or metabolic disease<sup>[5]</sup>. FD patients complain about a variety of symptoms, which are frequently intermittent, and mostly related to food intake<sup>[5,6]</sup>. Therefore, it should have been mandatory to study both changes in gastric volume and their relationship with upper gastrointestinal sensations. Previous studies on this topic demonstrated that the relationship between specific upper abdominal sensations and several pathophysiologic mechanisms such as delayed gastric emptying, impaired proximal gastric accommodation, and visceral hypersensitivity, remain a matter of debate. Moreover, a further evaluation of the distal gastric volume is advisable on the basis of the previous results that showed that the distal gastric volume was larger in patients with functional dyspepsia<sup>[7]</sup> - an indirect finding in line with the observation by Caldarella *et al*<sup>[8]</sup> of antro-fundic dysfunctions in FD.

Consequently, impaired gastric accommodation does not serve as a clear marker for the symptoms experienced by FD patients in daily life. These new findings warrant further research on this interesting topic that could also expand our knowledge in other subgroups of patients suffering dyspeptic symptoms.

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## Posterior tibial nerve stimulation for fecal incontinence: Where are we?

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**Key words:** Posterior tibial nerve stimulation; Percutaneous; Transcutaneous; Faecal incontinence; Efficacy of treatment; Neurostimulation

**Core tip:** Posterior tibial nerve stimulation though in its infancy, holds promise to be an effective, patient friendly and cheap treatment for faecal incontinence refractory to available conservative options. However, several questions remain unanswered and pose dilemmas regarding the delivery of this treatment. Solving these dilemmas could hold the key for unlocking the pathway for this treatment to be brought into the limelight.

### Abstract

Neurostimulation remains the mainstay of treatment for patients with faecal incontinence who fails to respond to available conservative measures. Sacral nerve stimulation (SNS) is the main form of neurostimulation that is in use today. Posterior tibial nerve stimulation (PTNS) - both the percutaneous and the transcutaneous routes - remains a relatively new entry in neurostimulation. Though in its infancy, PTNS holds promise to be an effective, patient friendly, safe and cheap treatment. However, presently PTNS only appears to have a minor role with SNS having the limelight in treating patients with faecal incontinence. This seems to have arisen as the strong, uniform and evidence based data on SNS remains to have been unchallenged yet by the weak, disjointed and unsupported evidence for both percutaneous and transcutaneous PTNS. The use of PTNS is slowly gaining acceptance. However, several questions remain unanswered in the delivery of PTNS. These have raised dilemmas which as long as they remain unsolved can considerably weaken the argument that PTNS could offer a viable alternative to SNS. This paper reviews available information on PTNS and focuses on these dilemmas in the light of existing evidence.

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### INTRODUCTION

Neuromodulation is here to stay. Neurostimulation remains at present the first choice treatment for fecally incontinent patients who have failed to improve with biofeedback, except for the small minority in whom where there is an underlying surgically repairable sphincter defect<sup>[1-3]</sup>. The first reported use of the sacral nerve stimulation (SNS) for faecal incontinence (FI) was just under two decades ago<sup>[4]</sup>. However, over the past decade not only has the use of neurostimulation increased exponentially but the remit of neurostimulation has widened to include the stimulation of other nerves- primarily the posterior tibial nerve<sup>[5]</sup>. SNS for faecal incontinence remains a time tested treatment with more than 50 series reporting on its use. A large meta-analysis has confirmed



on its use in improving the symptoms of FI as well as improving the quality of life of the patients<sup>[6]</sup>. Posterior tibial nerve stimulation (PTNS) for faecal incontinence is relatively new with just under 20 studies being reported<sup>[7]</sup>. PTNS has been used mainly in the management of urinary incontinence<sup>[8,9]</sup>. Shafik *et al*<sup>[5]</sup> has been credited with attempting PTNS for faecal incontinence. PTNS can be performed either by using a more invasive percutaneous approach<sup>[5]</sup> where an inserted 34 gauge needle forms the route of stimulation or by the less invasive transcutaneous “Qualtero” approach<sup>[10]</sup> where cutaneous pads replace the needle. Studies that have been done looking at the efficacy of the percutaneous PTNS approach are far more than those which have looked at the less invasive transcutaneous approach. Though there have been no studies so far which have directly compared these two routes of stimulation, indirect evidence points to a better efficacy for the percutaneous approach<sup>[11]</sup>.

PTNS is usually delivered unilaterally, at the nerve’s most superficial position which lies just above and behind the medial malleolus. The area of the nerve stimulated is quite small as the grounding electrode is usually placed in the instep. No evidence exists as to any dominance of the left or right tibial nerve unlike the pudendal nerve<sup>[12]</sup>.

## DILEMMAS IN TREATMENT

### *Treatment protocols dilemmas*

There remains a lack of an effective and standardised treatment protocol for both percutaneous and transcutaneous PTNS (Table 1).

Shafik *et al*<sup>[5]</sup> in 2003 reported giving 30 min of percutaneous PTNS stimulation on alternate days for a period of four weeks. Though there is now a general consensus that patients require 12 wk of continuous treatment and that each treatment episode should last 30 min, there is no uniformity on how this should be given. Studies have given a single 30 min session of PTNS once a week for 12 wk while others have given two 30 min sessions a week for 6 wk<sup>[13-15]</sup>. Three prospective studies of percutaneous PTNS from the same institution have used either once a week or twice a week patterns of treatment with no apparent differences in efficacy<sup>[16-18]</sup>. The superiority of one approach over the other remains yet remains to be demonstrated. The National Institute of Clinical Excellence (NICE) suggests both patterns could be adapted depending on patient response<sup>[19]</sup>. It is logical that the onset of symptom improvement for the patient will only occur later on into the treatment using the once a week regime compared to the twice a week regime. The once a week treatment can help alleviate hospital workloads and may be more acceptable to the patient. However, the onset of symptom improvement for the patient on a once a week regime could be delayed which may have a potential for more patient dropouts. All percutaneous PTNS studies so far have utilised unilateral stimulation. There remains the unexplored question as to whether bilateral percutaneous PTNS could be more effective-

given that a recent pilot study on bilateral transcutaneous PTNS has shown better efficacy compared to unilateral stimulation<sup>[14,20]</sup>.

The same treatment protocol dilemma exists for transcutaneous PTNS as well. Queraltó provided patients with unilateral daily stimulation for 20 min for 4 wk and showed an 80% improvement in incontinence severity scores<sup>[10]</sup>. Eléouet *et al*<sup>[21]</sup> reported 63% improvement following a 20 min of unilateral twice daily stimulation for 1 mo. Vitton *et al*<sup>[22,23]</sup> attempted transcutaneous PTNS once daily for 3 mo on two groups of patients and reported a 41% and 54% improvement in symptoms. George *et al* attempted unilateral transcutaneous PTNS twice a week for 6 wk and reported a 45% improvement in symptoms<sup>[11]</sup>. Leroi *et al*<sup>[24]</sup> reported no improvements in the transcutaneous arm compared to the sham group following 20 min twice daily sessions for 3 mo. Thomas *et al*<sup>[25]</sup> suggested in a pilot study that daily stimulation may offer a better response compared to a twice weekly regime. A more recent variation has been the application of transcutaneous PTNS as a daily bilateral stimulation for 6 wk which has been reported to be more effective than the unilateral approach<sup>[14,20]</sup>. Only in one study was the transcutaneous PTNS stimulation provided in a hospital setting<sup>[11]</sup> while all the other studies required patients to apply the stimulation themselves at home after being trained.

### *Stimulation endpoint dilemmas*

The stimulation end point for the transcutaneous PTNS was to look for a motor response which was visualization of rhythmic flexion of toes during stimulation<sup>[10]</sup>. Intensity of stimulation was then turned down to just below the threshold required for motor contraction. This seems to be a common end point for stimulation in most of the transcutaneous PTNS studies except the published RCT<sup>[11]</sup> where a sensory and a motor response was sought and a study by Vitton *et al*<sup>[23]</sup> where a sensory response was looked for.

However, the end point for stimulation for percutaneous PTNS remains uncharted with no specific end points described to confirm effective stimulation. Percutaneous PTNS can cause both a sensory and a motor response. The motor response is flexion of the big toe or fanning of all toes; the sensory response is a tingling sensation felt on the foot radiating to all of the toes<sup>[26]</sup>. The original paper by Shafik *et al*<sup>[5]</sup> looked for a motor response following stimulation. However, subsequent studies introduced a sensory response as an endpoint for stimulation<sup>[16-18]</sup>. The voltage used and the intensity of stimulation to achieve a sensory response remains lower than the intensity required to achieve a motor response<sup>[26]</sup>. This could imply that the voltage used for eliciting a sensory response alone could be sub-optimal without the full potential of the treatment being realised. This could in turn be reflected in lower treatment response rates.

Using the presence of either a motor or a sensory response could imply different treatment levels for differ-

**Table 1** Posterior tibial nerve stimulation evidence summary *n* (%)

Ref.	Patient ( <i>n</i> )	Type of PTNS	Time, frequency and duration of therapy	Follow-up	Stimulation endpoints	Efficacy	Study classification
Shafik <i>et al</i> <sup>[5]</sup>	32	Pct	30 min, alternate days 4 wk	22 mo	Motor	27 (84)	Nonrandomised controlled
Queralto <i>et al</i> <sup>[10]</sup>	10	Tct	20 min, daily 4 wk	4 mo	Motor	8 (80)	Prospective uncontrolled
Mentes <i>et al</i> <sup>[43]</sup>	2 <sup>1</sup> (spinal)	Pct	30 min, alternate days 4 wk	3 mo	Motor	2 (100)	Prospective uncontrolled
Vitton <i>et al</i> <sup>[22]</sup>	12 <sup>2</sup> (IBD)	Tct	20 min, daily 12 wk	3 mo	Sub sensory	5 (42)	Prospective uncontrolled
Babber <i>et al</i> <sup>[44]</sup>	8	Pct	30 min, weekly 12 wk	3 mo	Not specified	7 (87)	Prospective uncontrolled
De La Portilla <i>et al</i> <sup>[41]</sup>	16	Pct	30 min, weekly 12 wk	6 mo	Motor and sensory	10 (62)	Prospective uncontrolled
Vitton <i>et al</i> <sup>[23]</sup>	24	Tct	20 min, daily 12 wk	15 mo	Sub sensory	13 (54)	Prospective uncontrolled
Govaert <i>et al</i> <sup>[42]</sup>	22	Pct	30 min, twice weekly 6 wk	12 mo	Motor and/or sensory	18 (82)	Prospective uncontrolled
<sup>3</sup> Boyle <i>et al</i> <sup>[18]</sup>	31	Pct	30 min, weekly 12 wk	14 mo	Motor or sensory	21 (68)	Prospective uncontrolled
Findlay <i>et al</i> <sup>[45]</sup>	13	Pct	30 min, weekly 12 wk	4 mo	Sub motor	12 (92)	Retrospective uncontrolled
Eléouet <i>et al</i> <sup>[21]</sup>	32	Tct	20 min, twice daily 4 wk	6 mo	Motor	20 (63)	Prospective uncontrolled
<sup>3</sup> Allison <sup>[17]</sup>	90	Pct	30 min, twice weekly or weekly; 6 or 12 wk	21 mo	Motor or sensory	69 (77)	Prospective uncontrolled
<sup>3</sup> Hotouras <i>et al</i> <sup>[16]</sup>	100	Pct	30 min, twice weekly or weekly; 6 or 12 wk	6 mo	Motor or sensory	85 (85)	Prospective uncontrolled
Leroi <i>et al</i> <sup>[24]</sup>	144	Tct	20 min, twice daily 3 mo	3 mo	Sub motor	34 (47)	Randomised controlled trial
George <i>et al</i> <sup>[11]</sup>	11	Pct	30 min, twice weekly 6 wk	6 mo	Motor and sensory	9 (82)	Randomised controlled trial
	11	Tct	30 min, twice weekly 6 wk	6 mo	Motor and sensory	5 (45)	
Thomas <i>et al</i> <sup>[25]</sup>	15	Tct	30 min, daily 6 wk	6 wk	Sensory	3 (20)	Prospective randomised
	15	Tct	30 min, twice weekly 6 wk	6 wk	Sensory	0 (0)	
Moreira <i>et al</i> <sup>[46]</sup>	10	Pct	30 min, weekly 12 wk	3 mo	Not specified	6 (60)	Prospective uncontrolled
<sup>3</sup> Hotouras <i>et al</i> <sup>[30]</sup>	150	Pct	30 min, twice weekly or weekly; 3 mo	26 mo	Motor or sensory	60 (52)	Prospective uncontrolled

<sup>1</sup>Study included spinal injury patients; <sup>2</sup>Study included patients with inflammatory bowel disease (IBD); <sup>3</sup>Studies from the same institution - possibility of duplication of results. PTNS: Posterior tibial nerve stimulation; Pct: Percutaneous posterior tibial nerve stimulation; Tct: Transcutaneous posterior tibial nerve stimulation.

ent patients. In addition, patients with diabetes mellitus or with peripheral neuropathy could have an impaired sensory response or none at all. The published RCT used the presence of both a motor and sensory response as the end point for effective stimulation<sup>[11]</sup>. The presence of a combined motor and sensory response on PTNS has been reported to be better associated with a successful outcome than the presence of either a motor or a sensory response alone<sup>[27]</sup>. However, this could cause patient discomfort as higher voltages required for achieving a motor response may have the potential to cause discomforting sensory stimulations in some patients. The CONFIDENT multicentre randomised controlled trial (ISRCTN 88559475) presently underway in the United Kingdom utilises either a sensory or a motor response as

an endpoint for stimulation.

### Efficacy dilemmas

Percutaneous PTNS for FI remains a relatively new and untested treatment with only 12 studies, one randomised controlled trial<sup>[11]</sup> and one review<sup>[28]</sup> having been published to date on its use. The only published RCT on PTNS only reports on a 6 mo follow-up<sup>[11]</sup>. There remains no doubt regarding the short term efficacy of PTNS which are comparable to that of SNS. However, the true test of the effectiveness of PTNS would be its efficacy in the medium and long term. This is crucial as this could validate its effectiveness as a treatment option for faecal incontinence rather than a stepping stone towards SNS. There is a dearth of information on such results though

early reports from Hotouras *et al.*<sup>[15,16]</sup> who has published on the largest group of PTNS patients so far ( $n = 100$ ) reports a possible sustained efficacy for PTNS after 42 mo of follow-up<sup>[29]</sup>. However, this group<sup>[16-18]</sup> provided percutaneous PTNS as the first line therapy for fecally incontinent patients without assessing whether they were refractory to other non-interventional treatments<sup>[19]</sup>. This could perhaps imply that some of their patients would have had improvement in symptoms with other less invasive treatments had this been attempted. The CONFIDENT multicentre randomised controlled trial (ISRCTN 8855947) which is presently underway across 14 centres in the United Kingdom may shed more light on the true short term efficacy of PTNS though only the percutaneous approach is compared to a sham route of stimulation. Though this study recruits patients who have been refractory to other less invasive therapies, the lack of any form of standardisation nationally for such therapies nationally remains notable.

The efficacy of transcutaneous PTNS remains even more untested with only a handful of studies which have looked at this approach to PTNS. Though several studies have reported symptoms improvements in patients a recent multicentre trial reported no improvements following stimulation and concluded that unilateral transcutaneous PTNS was no more effective than sham stimulation<sup>[24]</sup>. Patients were exposed to stimulation for 20 min twice daily for 3 mo<sup>[24]</sup>. However, a new pilot study has looked at bilateral transcutaneous PTNS and found it to be effective compared to unilateral stimulation<sup>[20]</sup>.

## FOLLOW-UP DILEMMAS

There remain no standardised follow-up and top-up regimes that can be used for percutaneous and transcutaneous PTNS. Most studies report efficacy only at the end of the 6 or 12 wk treatment period. The first percutaneous PTNS study reported a relapse of symptoms in 29% of patients with the majority of patients improving with further treatment though the exact regime for such follow up treatment was not reported<sup>[5]</sup>. Almost all studies on PTNS mention the need for “top-up” treatments. However there remains no clarity as to whether such top-up sessions should be offered only when patients report back due to recurrence of symptoms or whether such sessions should be offered at lengthening intermittent intervals after the intense initial treatment period. One study on percutaneous PTNS reported good efficacy with a median of one 12 monthly top-up session<sup>[15]</sup>. Regular percutaneous PTNS top-ups at lengthening intermittent intervals resulted in a sustained therapeutic effect for urological dysfunction<sup>[13]</sup>. New studies on PTNS make inroads into this aspect though this has to be verified through more independent trials<sup>[30]</sup>.

The same dilemmas exist for transcutaneous PTNS as well. The efficacy following transcutaneous PTNS lasts for about 3 wk post treatment<sup>[20]</sup>. Though there is no definite top-up regimes recommended there remains the

advantage that such treatments can be undertaken by the patient in the comfort of their own homes as well as the fact that the costs for such top-ups will be very low<sup>[20]</sup>.

In comparison to SNS where the treatment effects are short-lived following the withdrawal of treatment, PTNS appears to confer a slightly longer lasting effect (albeit with a declining efficacy). However, a recent study on SNS has shown persisting efficacy even after the device was switched off which may bring it to par with the longer effects of PTNS<sup>[31]</sup>.

The heterogeneity of follow-up regimes for PTNS makes it difficult to assess exactly the long-term effects of its treatment. Furthermore, only a few studies have performed rigorous assessment of “top-up” regimes to maintain efficacy. Further work needs to be done on the follow-up of patients who benefit from PTNS to accurately assess the duration of efficacy.

## COST IMPLICATIONS

The present worldwide financial crisis has thrown into stark view the cost implications of neurostimulation. The direct medical costs for PTNS remain nearly ten times cheaper compared than those for SNS<sup>[17,32,33]</sup>. In PTNS itself the costs between percutaneous and transcutaneous PTNS also varies significantly. Percutaneous PTNS requires a re-usable stimulator 9V stimulator (Urgent PC®, Uroplasty Inc., United States) along with 12 disposable single-use leads. The disposable kits with 12 individually packed sterile stimulation units and a disposable battery for the Urgent PC stimulator unit costs £480 and are sufficient for the full treatment of 12 sessions<sup>[26,34]</sup>. The cost for the Urgent PC stimulator unit (Uroplasty, Berkshire, United Kingdom) is £1000. However, the reusable nature of the stimulator unit can reduce the costs of multiple treatments.

The costs for transcutaneous PTNS remain even smaller with the 50 mm × 50 mm self-re-usable adhesive surface electrode stimulation pads (Model VS.5050; Premier Medical Products, Bedford, United Kingdom) costing £1 per pair. The stimulator unit used is the NeuroTrac Continence Neurostimulator (Verity Medical Ltd, United Kingdom) costs \$80 and can be re-used as the percutaneous stimulator<sup>[20]</sup>.

SNS involves the *in-vivo* implantation of highly advanced technological devices and both the temporary and permanent wires were implanted under general anaesthesia. The higher costs for SNS arise due to the two-stage procedure along with associated pre- and post-operative care. The equipment only costs of SNS (2008 tariffs) were \$526 for the temporary implant and \$13500 for the permanent implant<sup>[35]</sup>. However, the actual charges levied for these procedures vary. Reports of costs for the initial temporary procedure for SNS vary from \$1300<sup>[35]</sup> to about \$5300<sup>[33]</sup>. Costs for the permanent implant procedure also varies from \$14500<sup>[35]</sup> to about \$21200<sup>[33]</sup>. Performing the initial stage of SNS under local anaesthesia appears to be more patient friendly and cheaper<sup>[36-38]</sup>.

One of the underlying concerns regarding PTNS remains on the follow up post treatment and the hidden costs for these which may outweigh the initial costs savings. Running an SNS service is expensive<sup>[39]</sup>. However, there remains the possibility that the costs for maintaining the efficacy of PTNS in patients may be higher as they remain yet unknown. Conflicting reports on the cost effectiveness of both procedures are available<sup>[7]</sup>. A two-year follow up of percutaneous PTNS in patients with faecal incontinence from one center reported that PTNS became cost effective after the first year of treatment<sup>[17]</sup>. However, another study which compared SNS to PTNS at 5 years post treatment for urological dysfunction reported that SNS therapy became much more cost efficient compared to PTNS<sup>[40]</sup>. Unlike SNS, running costs and long term follow up expenses for PTNS lacks clarity given the absence of a uniform and universally accepted follow up protocol along with the dearth of independent medium and long term follow up data on “successfully” treated PTNS patients.

#### Future for PTNS?

There remains no question that SNS is less patient friendly and more expensive than PTNS in the short term<sup>[33]</sup>. Early attempts to make SNS more patient friendly have experimented at less invasive forms of SNS administration using a transcutaneous Percutaneous PTNS though minimally invasive does not require any operative procedures or a hospital inpatient stay. Patients also do not require a 3 wk trial phase which presently exists for SNS with insertion of a temporary SNS wire and a permanent implant subsequently if successful. Percutaneous PTNS has the potential to be delivered through a primary care setting using perhaps the abilities of specialist nurses who could provide these services on an outpatient basis. This could drive the costs of PTNS down even further.

Transcutaneous PTNS has the unique potential of being a treatment which is truly “by the patient, for the patient”. FI can be socially crippling with patients sometimes being unwilling to leave the safety of their own homes for fear of incontinent episodes<sup>[41]</sup>. Transcutaneous PTNS may hold promise as a treatment which patients can self-administer safely, cheaply and effectively in the comfort of their own homes<sup>[20]</sup>.

Presently PTNS appears to have the role as a stepping stone towards SNS in patients with faecal incontinence. Efficacy of transcutaneous PTNS has been used as a predictor for suggesting efficacy of SNS<sup>[42]</sup>. However, the question remains as to why patients should choose a potentially less patient friendly and clinicians should offer a more expensive and invasive treatment in the form of SNS when PTNS is available-albeit, in its infancy. This seems to have arisen as the strong, coherent, uniform and evidence based data on SNS remains to have been unchallenged yet by the weak, incoherent, disjointed and unsupported evidence for PTNS. A pilot study comparing SNS and percutaneous PTNS (UKCRN ID 10479/MREC ID 10/H 0808/38) may help shed more light on

direct comparison between the two treatments.

The true role for PTNS remains yet to be validated and time tested - as SNS has been. However, the question as to whether SNS and PTNS become “brothers in arms” in treating FI or whether this may yet turn out to be the “David *vs* Goliath” battle will be answered only once PTNS has come into its prime.

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## WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

# Non-alcoholic fatty liver disease and liver transplantation: Outcomes and advances

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## Abstract

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent causes of chronic liver disease worldwide. In the last decade it has become the third most common indication for liver transplantation in the United States. Increasing prevalence of NAFLD in the general population also poses a risk to organ donation, as allograft steatosis can be associated with non-function of the graft. Post-transplant survival is comparable between NAFLD and non-NAFLD causes of liver disease, although long term outcomes beyond 10 year are lacking. NAFLD can recur in the allograft frequently although thus far post transplant survival has not been impacted. *De novo* NAFLD can also occur in the allograft of patients transplanted for non-NAFLD liver disease. Predictors for NAFLD post-transplant recurrence include obesity, hyperlipidemia and diabetes as well as steroid dose after liver transplantation. A polymorphism in PNPLA3 that mediates triglyceride hydrolysis and is linked to pre-transplant risk of obesity and NAFLD has also been linked to post transplant NAFLD risk. Although immunosuppression side effects potentiate obesity and the metabolic syndrome, studies of immunosuppression

modulation and trials of specific immunosuppression regimens post-transplant are lacking in this patient population. Based on pre-transplant data, sustained weight loss through diet and exercise is the most effective therapy for NAFLD. Other agents occasionally utilized in NAFLD prior to transplantation include vitamin E and insulin-sensitizing agents. Studies of these therapies are lacking in the post-transplant population. A multimodality and multidisciplinary approach to treatment should be utilized in management of post-transplant NAFLD.

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**Key words:** Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver transplantation; Metabolic syndrome; Outcomes; Management

**Core tip:** Non-alcoholic fatty liver disease (NAFLD) is a prevalent indication for liver transplantation. It also poses a risk to organ donation, with decreasing rates of suitable allografts. NAFLD frequently recurs in the allograft or develops *de novo*. Post-transplant recurrence is related to obesity and immunosuppression associated metabolic derangements. A polymorphism in PNPLA3 also increases recurrence risk. Pre-transplant data favors sustained weight loss through diet and exercise as the most effective therapy for NAFLD. Vitamin E and insulin-sensitizing agents are occasionally used. Trials on immune-suppression regimens in this population are sorely needed. A multimodality approach to treatment should be utilized in management of post-transplant NAFLD.

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## EPIDEMIOLOGY OF NON-ALCOHOLIC FATTY LIVER DISEASE AND ASSOCIATED ADVANCED LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the developed world with a prevalence averaging 20% in the ulcerative colitis<sup>[1,2]</sup>. Its incidence in the developing world is also increasing sharply<sup>[3]</sup>. Prevalent in adults, it has also become the most common chronic liver disease in children<sup>[4]</sup>. Mirroring the epidemic of obesity, it is closely related to the metabolic syndrome particularly diabetes and dyslipidemia in association with truncal obesity<sup>[5]</sup>. Prior to the widespread recognition of NAFLD which was first described as a separate clinic-pathologic entity in 1980<sup>[6]</sup>, many cases of NAFLD were likely classified as cryptogenic liver disease and cryptogenic cirrhosis (CRC). In a study where 39 liver transplant candidates diagnosed with CRC were carefully re-evaluated, 44% had prior biopsy consistent with NAFLD or clinical features of the metabolic syndrome<sup>[7]</sup>.

Although NAFLD has been associated with excess mortality compared to the general population (Hazard ratio 1.34)<sup>[8]</sup>, the natural history of NAFLD is often one of slow progression. In patients with isolated steatosis (fatty liver) the course of liver disease can be frequently benign<sup>[9,10]</sup>. The progressive form of NAFLD known as Non-alcoholic steatohepatitis (NASH) is associated with hepatocyte damage and consequently can lead to fibrosis as well as cirrhosis and end-stage liver disease<sup>[11]</sup>. Recently data about the natural history of NAFLD related cirrhosis was reported from four international referral centers. In this study, patients with NAFLD or hepatitis C virus (HCV) associated compensated (Childs A) cirrhosis were enrolled. Over the long term (mean follow up 86 mo for NAFLD and 75 mo for HCV), the incidence of liver related complications and hepatocellular carcinoma (HCC) was lower for NAFLD than for HCV. The probability of remaining free from liver related decompensation was 81.5% in the NAFLD cohort and 76.5% in the HCV cohort at 120 mo of follow up with a higher incidence of complications in HCV when adjusted for age, sex, body mass index and diabetes ( $P = 0.03$ ). The incidence of HCC over follow up was 2.4% in the NAFLD cohort and 6.8% in HCV. Despite these differences, the incidence of cardio-vascular disease and overall mortality were similar between NAFLD and HCV patients (82% survival at 120 mo in both cohorts)<sup>[12]</sup>.

## LIVER TRANSPLANTATION INCIDENCE FOR NAFLD

The incidence of liver transplantation related to NAFLD has exploded in the last decade<sup>[13]</sup>. Although some of the reported increase in incidence of NAFLD related liver transplantation is due to increased recognition of patients previously classified as CRC, the increased incidence of NAFLD related liver transplantation is real. Even if the

majority of CRC related liver transplants in prior eras were due to unrecognized NAFLD, the magnitude of increase in transplants for NAFLD far outweighs any classification bias<sup>[13]</sup>. In an audit of United States national transplant data (SRTR), liver transplants attributed to NAFLD related liver disease increased from 1.2% in 2001 to 9.7% by 2009 and this is now the third most common indication for liver transplantation in the United States<sup>[13]</sup>. In this study patients with NAFLD receiving a liver transplant were older, more likely to be females, had higher body mass index (BMI) and were less likely to have HCC at transplant compared to all other recipients.

There have been concerns about bias in transplant evaluation and listing of patients with NAFLD related cirrhosis. NAFLD patients are on average older at presentation and have higher rates of obesity and metabolic syndrome raising concerns about worse outcomes of transplant in these patients including increased risks of cardiovascular disease and chronic kidney disease. In a study from a single liver transplant center, the cohort of NAFLD patients with MELD less than 15 at listing were found to progress more slowly compared to patients with HCV and were more likely to die on the waiting list or be taken off the transplant list due to becoming "too sick"<sup>[14]</sup>. However for patients who were listed with MELD scores over 15 there were no differences in rate of progression of end-stage liver disease, listing rate and receipt of liver transplantation. In another study, patients with NAFLD were equally likely than non-NAFLD patients to undergo liver transplant evaluation, listing and transplantation. In this single center study, NASH patients were older, had similar rates of HCC but increased rates of other prior cancers by history. In addition diabetes and complications of metabolic syndrome were more prevalent in NASH patients. NAFLD patients also had higher creatinine levels at transplant listing than non-NAFLD patients<sup>[15]</sup>. Routine audits of multicenter and national data will have to be done to see if NAFLD patients are indeed at a disadvantage for evaluation and listing due to these concerns.

## OUTCOMES AFTER LIVER TRANSPLANTATION FOR NAFLD

### *Survival after liver transplantation for NAFLD*

Outcomes after liver transplantation in patients with NAFLD have been reported in both large national database audits as well as from single center studies. These studies have been restricted to adult recipients (> 18 years) of liver transplants. In the pediatric population although NAFLD is common, it is a rare indication for liver transplantation<sup>[16]</sup> (Table 1).

The national databases (UNOS and SRTR) studies have looked at outcomes at 1 year and beyond after liver transplantation (Table 1). Overall 1-year, 3-year and 5-year survival has been comparable between NAFLD and non-NAFLD recipients<sup>[13]</sup>. In more specific sub-analyses of the same databases post-transplant survival for NAFLD



Table 1 Liver transplantation for non-alcoholic fatty liver disease

Ref.	Patient	Population	Follow up	Graft survival	Patient survival	NAFLD recurrence in graft	Predictors of NAFLD recurrence	Predictors of survival
National registry data Charlton <i>et al</i> <sup>[13]</sup>	35781 adults	SRTR (US national data)	3 yr post-transplant survival reported	NASH 3-yr survival 76% (similar to other indications)	NASH 1-yr survival 84% and 3-yr 78% CRC 1 -yr survival 86% and 3 -yr 79%Other Diagnoses 1-yr survival 87% and 3-yr 78% ( <i>P</i> = 0.67)	Not reported	Not reported	Not reported
	adult liver transplant recipient NASH primary or secondary indication for 1959 recipient	of liver transplant recipients from 2001 to 2009 Included NASH plus 50% of CRC and NASH plus CRC with BMI > 30 kg/m <sup>2</sup>						
Singal <i>et al</i> <sup>[18]</sup>	54687 adult liver transplant recipient NASH 1368 recipients	UNOS adult liver transplant recipients from 1994 to 2009	10-yr survival reported	1-yr, 3-yr, 50-yr and 10-yr survival NASH: 86%, 82%, 80% and 80%NAFLD post-transplant survival similar to cholestatic liver disease, HBV and better than ALD, CRC, HCV and HCC	1-yr, 3-yr, 5-yr and 10-yr survival NASH: 89%, 85%, 84% and 84% NAFLD post-transplant survival similar to cholestatic liver disease, HBV and better than ALD, CRC, HCV and HCC	Not reported	Not reported	For all recipients, age of recipient, male recipient black race, ventilator support pre transplant and MELD score as well as donor risk index associated with worse patient survival
Afzali <i>et al</i> <sup>[17]</sup>	53738 adult liver transplant recipients NASH 1810 recipients	UNOS adult liver transplant recipients from 1997 to 2010	5-yr survival reported	Not reported HCV and HCC	1-yr, 3-yr and 5-yr survival NASH: 88%, 82% and 77% Overall adjusted HR for NASH post-transplant mortality compared to other etiologies was 0.75 (95%CI: 0.66-0.85) Adjusted survival was better for NASH than for ALD, HCV, and HCC. NASH survival was worse than cholestatic liver disease, AIH, HBV	Not reported	Not reported	Not specified- although state survival adjusted for several donor, recipient characteristics (individual Hazards ratios not reported)
Single center studies Tanaka <i>et al</i> <sup>[27]</sup>	7 patient with NAFLD (425 total LDLT recipients)	Patients with NAFLD that underwent Live donor liver transplant at a single center in Japan between 1996 and 2013	Median follow up 5.3 yr	100% at last follow up	100% at last follow up	1/7 (14%) had recurrent NASH	Not reported	Not reported
El Atrache <i>et al</i> <sup>[27]</sup>	83 recipient, NALFD <sup>[66]</sup> and CRC <sup>[67]</sup>	Liver transplant recipients at a single US center between 1996 and 2008	Mean follow up 46 mo	12/ 83 underwent re-transplantation	12 recipients died. Overall survival not reported	NAFLD recurrence in 20/83 recipients (15 with NASH pre-transplant and 5 with CRC pre-transplant	Predictors of recurrence were metabolic syndrome, drome, hypertension and insulin use as well as hyperlipidemia after transplant	Five year survival worse for those with metabolic syndrome, hypertension and insulin use No difference in survival between those with NASH recurrence and those without

Dureja <i>et al</i> <sup>[20]</sup>	88 recipients with NAFLD	Liver transplant recipients at a single US center between 1993 and 2007	Mean follow up 82 mo	Not reported	5-yr patient survival similar between those with NAFLD recurrence and those without NAFLD recurrence ( $P = 0.78$ )	NAFLD Disease Recurrence in 34/88 (39%)	Pre and post-transplant triglyceride levels and prednisone dose was higher in those with NAFLD recurrence	Post-transplant survival was worse in NAFLD patients with post-transplant cardiac disease (HR 3.2, 95%CI: 1.3-7.7)
Agopian <i>et al</i> <sup>[20]</sup>	144 recipients with NAFLD (total 1294 transplants)	Liver transplant recipients at a single US center between 1993 and 2011	Mean follow up 2.3 yr	Graft survival similar between NAFLD and non NAFLD (90 d survival 86% for NASH) and lower only than PBC/P SC (90 d graft survival of 94%)	Patient survival similar between NASH and non-NASH. 90 d survival 90% for NASH 5-yr patient survival for NASH (70%) similar to ALD, HBV, CC and PBC/PSC but better than HCV	NASH recurrence in 23 (16%)	Not reported	Post-transplant BMI > 35 kg/m <sup>2</sup> independent factor for mortality in NAFLD recipients only. Pretransplant dialysis also had worse survival in NASH patients
Kennedy <i>et al</i> <sup>[21]</sup>	129 recipients with NAFLD and 775 with other liver disease	Liver transplant recipients at a single US center between 1999 and 2009	5-yr survival reported	Graft survival not reported	1-yr, 3-yr, and 5-yr survival NASH: 90%, 88% and 85% Non-NASH: 92%, 86% and 80% ( $P = NS$ ) Mortality within 4 mo higher in NASH -8.5% vs non-NASH 4.2%, $P = 0.04$	Not reported	Not reported	No predictors of survival found
Barritt <i>et al</i> <sup>[24]</sup>	21 recipients with NAFLD and 97 with other liver disease	Liver transplant recipients at a single US center between 2004 and 2007	3-yr survival reported	30-d graft survival worse in NAFLD (81%) vs non-NAFLD (95%), $P = 0.02$ 1-yr survival for NAFLD and non-NAFLD patients was 76% vs 90%, $P = 0.06$ 3-yr survival was 76% for NAFLD vs 84% for non-NAFLD, $P = 0.23$	30-d patient survival worse in NAFLD (81%) vs non-NAFLD (97%), (81% vs 97%, $P = 0.001$ ) 1-yr survival for NAFLD and non-NAFLD patients was 76% vs 90%, $P = 0.06$ 3-yr survival was 76% for NAFLD vs 84% for non-NAFLD, $P = 0.23$	Not reported	Not reported	3-yr survival was significantly worse for diabetic patients compared to non-diabetics (63% vs 89%, $P = 0.006$ )
Yalamanchili <i>et al</i> <sup>[22]</sup>	40 recipients with NAFLD	Liver transplant recipients at a single German center between 2007 and 2011	1-yr survival reported	Not reported	30 d mortality for NAFLD patients was 25% and 1-yr mortality was 35%	Not reported	Not reported	Patients with BMI > 35 kg/m <sup>2</sup> had worse graft survival (1-yr graft failure 55%) than those with lower BMI

Contos <i>et al</i> <sup>[23]</sup>	30 recipients with CRC and NASH compared to patients with ALD <sup>[6]</sup> and PBC/PSC <sup>[2]</sup>	Liver transplant recipients at a single US center between 2004 and 2007	Median follow up 3.5 yr	Graft survival similar between NAFLD and non-NAFLD patients ( <i>P</i> = 0.32)	Patient survival similar between NAFLD and non-NAFLD patients ( <i>P</i> = 0.32)	100% of the 30 NAFLD patients had steatosis in the graft by 5-yr post-transplant compared to 25% in the ALD and PBC/PSC groups	Steroid dose post-transplant associated with NAFLD recurrence	Not reported
Ong <i>et al</i> <sup>[31]</sup>	51 recipients with CRC	Liver transplant recipients at a single US center between 2004 and 2007	> 6 mo post-transplant, not reported	Not reported	Not reported	Post-transplant NAFLD developed in 25% and NASH in 16%	Predictors of post-transplant NAFLD was pre or post-transplant diabetes and triglyceride levels	Not reported
Bhagat <i>et al</i> <sup>[30]</sup>	71 NAFLD patients compared to 83 ALD patients	Liver transplant recipients at a single US center between 1997 and 2007	Median follow up 1517 d in NAFLD group and 1686 d in ALD group	Graft survival similar between NAFLD (76%) and ALD (82%)	1-yr, 3-yr, 5-yr, and 9-yr survival NASH: 82%, 79%, 75%, and 62% ALD: 92%, 86%, 86%, and 76% ( <i>P</i> = 0.17)	NAFLD recurrence was 33% (21/64 NASH patients)	None reported	No predictive factors in NAFLD
Malik <i>et al</i> <sup>[23]</sup>	98 NAFLD recipients compared to 196 with PBC/PSC 196 with ALD 196 with HCV 98, with CR	Liver transplant recipients at a single US center between 2004 and 2007	Mean follow-up was 994 d	Not reported	Survival similar between NAFLD and non-NAFLD recipients 30-d mortality in NAFLD 6.1% 1-yr mortality 21.4% in NAFLD (similar to controls) 3-yr mortality in NAFLD 25% similar to controls, less in PBC (15%) 5-yr mortality (28%) similar in NAFLD patients and controls	Not reported	Not reported	Sepsis accounted for more deaths in NAFLD transplant recipients Liver recipients transplanted for NASH cirrhosis who died within the first post-transplant year were more likely to be older ( $\geq$ 60 yr), more obese (BMI $\geq$ 30 kg/m <sup>2</sup> ) and have both pretransplant diabetes and hypertension

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; ALD: Alcoholic liver disease; PBC: Primary biliary cirrhosis; CRC: Cryptogenic cirrhosis; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis; CR: Cryptogenic cirrhosis; LDLT: Living donor liver transplantation.

was better as compared to HCV, alcohol, CRC, and HCC related liver disease<sup>[17,18]</sup>. When compared to primary biliary cirrhosis (PBC) one study showed similar survival<sup>[18]</sup> and another study showed worse survival for NAFLD<sup>[17]</sup>.

In single center studies, post transplantation outcomes were also similar between NAFLD and non-NAFLD patients<sup>[19-22]</sup>. Survival at 1, 3, 5 and 10-year was reported as similar in the studies, although some have demonstrated higher early mortality (30 d) after transplantation in NAFLD than in non-NAFLD patients. Kennedy *et al*<sup>[21]</sup> reported a twofold higher mortality for NAFLD patients at 4 mo after transplantation than for non-NAFLD patients (8.5% mortality *vs* 4.2% for others). The commonest causes of mortality in NAFLD patients were infectious and cardiac disease. Another study confirmed higher 30-d mortality and 1-year mortality in NAFLD patients, although by 3 years survival were comparable in NAFLD and non-NAFLD patients<sup>[23]</sup>. In this study infections accounted for the majority of deaths. Factors associated with decreased survival in the cohort of NAFLD patients have included age of recipient post-transplant, diabetes<sup>[24]</sup>, obesity and post-transplant metabolic syndrome<sup>[25]</sup>; and post-transplant cardiovascular disease<sup>[19,23,26]</sup>. Close monitoring and critical analysis of early and late outcomes after liver transplantation for NAFLD is thus necessary to further refine criteria and improve outcomes for liver transplantation in NAFLD.

### NAFLD recurrence after liver transplantation

Recurrent NAFLD is common<sup>[22,26,27]</sup>. The recurrence rate depends to some extent on the methodology chosen for detection, (*i.e.*, evaluation of abnormal liver enzymes, liver biopsy, imaging techniques). Use of liver enzymes alone is fairly insensitive as a significant proportion of patients with NAFLD recurrence have normal liver enzymes.

Metabolic syndrome including obesity, diabetes, hyperlipidemia and hypertension are all increased in prevalence after transplantation linked largely to immune-suppression use, particularly steroid use and calcineurin inhibitors. Other factors include post-transplant weight gain due to reduced mobility, at least in the early period and these factors all contribute to recurrence of NAFLD in the allograft<sup>[28]</sup>.

In some studies the risk of allograft steatosis was increased by the presence of the rs738409 single nucleotide polymorphism (SNP) in the *PNPLA3* gene in the recipient<sup>[29]</sup> as well as post-transplant obesity and diabetes<sup>[28]</sup>. This polymorphism (rs738409:1148M) in *PNPLA3* has been associated with reduced triglyceride hydrolysis in the adipocyte and increases the risk of developing NAFLD and NASH in the general population<sup>[30]</sup>. The presence of this SNP in *PNPLA3* in the donor has not been associated with development of allograft steatosis, obesity and diabetes. Thus the role of peripherally mediated triglyceride hydrolysis (in extrahepatic adipose tissue) seems to account for risk of NAFLD recurrence rather than liver related triglyceride hydrolysis, at least in post-transplant NAFLD<sup>[28,29]</sup>.

In a study that systematically re-examined post-transplant biopsies and imaging, recurrent NAFLD was seen in 39% (34/88), with NASH in 25 and isolated steatosis in 9 of these 34 patients within 5 years post-transplant. Severe recurrence (NAS score  $\geq 5$ ) or advanced fibrosis was seen in 6 of the 34 with recurrent NAFLD<sup>[26]</sup>. NAFLD recurrence was correlated with pre and post-transplant BMI and post-transplant triglyceride levels and prednisone dose at 6 mo post-transplant. In this study post-transplant survival was similar between those with NAFLD recurrence *vs* those without.

Other studies have showed similar rates of NAFLD recurrence with one study showing recurrent NAFLD in 20 of 83 (24%). The metabolic syndrome and insulin use were linked to recurrent NAFLD in this study<sup>[27]</sup>.

Yalamanchili *et al*<sup>[22]</sup> reported long term outcomes with post-transplant NAFLD recurrence. In this study, recurrent steatosis was reported in 45% of NAFLD transplant recipients and NASH was less common occurring in 4%. Advanced allograft fibrosis or cirrhosis was reported in 5% by 5 years and 10% by 10 years post transplantation and was more common in those with recurrent NASH (31%) *vs* those with steatosis alone (6%) or no steatosis (3%). In this study survival was similar at 1, 5 and 10 years in those with NAFLD and those with other liver diseases at transplant. Death from cardiovascular disease was more common than due to recurrent liver disease attesting to the strong link between the factors that predict development

of NAFLD (Metabolic syndrome) and cardiac disease<sup>[22]</sup>.

Other studies have also not shown reduced survival with NAFLD recurrence so far<sup>[26]</sup>, although studies have been limited by a dearth of long term follow up (10 years or more) for large number of patients.

In patients transplanted for CRC, NAFLD has been reported to occur post transplantation and may be due to recurrent disease in a significant number of these patients who likely had undiagnosed NAFLD prior to transplantation. In one study steatosis alone developed in 25% and NASH in 16% of patients transplanted for CRC<sup>[31]</sup>. Predictors for post-transplant NAFLD in this population included pre or post-transplant diabetes, hypertriglyceridemia and higher BMI. In another study of thirty CRC patients who had the NAFLD phenotype (metabolic syndrome) prior to liver transplantation, recurrent steatosis was seen in 100% by 5 years post-transplant. Steroid dose was correlated with development of post-transplant NAFLD<sup>[32]</sup>.

Very few if any data exist on risk of HCC in NAFLD and outcomes for these patients after transplantation. In a single center study, 17% of NASH cirrhosis patients referred for liver transplantation had HCC (6 noted incidentally on explant) which was higher than the number of patients with PBC/PSC with HCC and similar to ALD and HCV with HCC. Survival in NASH and HCC patients was good after liver transplant with 88% survival at a mean follow-up of 2.5 years<sup>[33]</sup>.

### DEVELOPMENT OF *DE NOVO* NAFLD AFTER LIVER TRANSPLANTATION

*De novo* NAFLD has been reported after liver transplantation in recipients who did not carry the diagnosis of NAFLD prior to liver transplantation. The incidence of *de novo* NAFLD after liver transplantation has ranged from 18% to 33%<sup>[34-36]</sup> with the progressive form NASH reported in 9% in one report<sup>[34]</sup>. In a study with liver biopsies done as protocol at 1, 5 and 10 years post-transplantation, as well as for clinical indications, the incidence of *de novo* NAFLD (defined as steatosis greater than 5% after more than 6 mo post liver transplantation) was 31% in 599 recipients with an average follow up of 40 mo. Histological NASH was present in only 3.8%, but perisinusoidal fibrosis was present in 29% and advanced fibrosis/cirrhosis in 2.25%<sup>[37]</sup>. The increased incidence of perisinusoidal fibrosis without steatohepatitis has not been well described in non-transplant populations and may represent a modified presentation in immunosuppressed individuals who may not present with brisk inflammatory response. In addition 51% of the recipients with *de novo* NAFLD had normal liver enzymes in this study attesting to the importance of liver biopsies and possibly imaging in accurately diagnosing NAFLD.

Factors associated with *de novo* NAFLD include post-transplant obesity, post-transplant diabetes, hyperlipidemia and hypertension<sup>[37]</sup>. In addition tacrolimus was also associated with recurrent NAFLD and this drug has



been well described as having an increased risk for developing diabetes<sup>[38]</sup>.

In addition in this study a pretransplant diagnosis of alcoholic cirrhosis was associated with an increased risk of *de novo* NAFLD. In this study patients with recurrent alcoholism and recurrent hepatitis C or hepatitis B were excluded from the analysis as these conditions can lead to steatosis. The increased risk of *de novo* NAFLD in patients with prior ALD may reflect an underlying predisposition to NAFLD that could not be diagnosed prior to transplantation due to the concomitant alcoholic steatohepatitis. Donor allograft steatosis was also more prevalent in the group that developed *de novo* NAFLD (30%) as compared to the group that did not develop NAFLD (12.65%). This study did not quantify the degree of hepatic steatosis and nor were any genetic polymorphisms tested for in the donor. Other studies have suggested that donor polymorphisms that regulate cytokine release, inflammation and microsomal triglyceride transfer may be important in risk of developing NAFLD<sup>[39]</sup>. Protective factors against *de novo* NAFLD may include use of Angiotensin converting enzyme inhibitors<sup>[40]</sup>, although this approach has not been tested in a trial.

The consequences of *de novo* NAFLD are not well known. In the study mentioned above complete regression occurred in 13 % (all with grade 1 steatosis initially), reduction of steatosis was seen in 35%, stability in 22%, and exacerbation in 30%. Higher prevalence of obesity was present in those with progression of histological liver disease<sup>[34]</sup>.

In patients with hepatitis C the risk of developing *de novo* NAFLD is higher and can be linked to recurrence of hepatitis C<sup>[35]</sup>. Development of *de novo* NAFLD in the allograft can reduce the response rate to current antiviral therapy for hepatitis C and thus impact graft and patient outcomes<sup>[35]</sup>.

## MANAGEMENT OF NAFLD AFTER LIVER TRANSPLANTATION

There have been no published trials of pharmacotherapy specifically for post-transplant NAFLD. Analysis of the predictors of post-transplant NAFLD recurrence and data from non-transplant therapeutic studies on NAFLD suggest that sustained weight loss through a combination of dietary changes and exercise are most successful in reversing the histological findings of NAFLD<sup>[40]</sup>, and improving biochemical and metabolic parameters including liver enzymes, insulin resistance, lipid levels and blood pressure in this condition<sup>[41]</sup>.

Studies on pharmacotherapeutic agents in non-transplant patients suggest a role for vitamin E in selected individuals. In non-diabetics a large randomized controlled trial over 48 wk improved the histological features and liver enzymes in NAFLD<sup>[42]</sup>. Recent concerns about risk of prostate cancer<sup>[43]</sup> and risk of cardiac disease in susceptible individuals<sup>[44]</sup>, as well as lack of long term data on sustained efficacy and safety may limit its usefulness in the post-transplant population.

The use of PPAR-gamma agonists (*e.g.*, Pioglitazone) improves insulin resistance and has shown some promise in reversing NAFLD in non-transplant patients<sup>[45,46]</sup>. In a large randomized controlled trial however it was not superior to placebo and inferior to vitamin E in reversing NAFLD<sup>[42]</sup>. This class of agents is also associated with weight gain and this also limits its utility in treatment of NAFLD<sup>[45,46]</sup>.

Pharmacologic treatment of clinically overt diabetes, dyslipidemia and hypertension should be carried out as per best practice guidelines for managing these conditions<sup>[46]</sup> and in multidisciplinary teams involving the transplant team, primary care providers<sup>[47]</sup>, diabetes specialists and preventive cardiologists.

Given that to a large extent immune-suppression exacerbates or promotes the development of the metabolic syndrome, immunosuppression modulation should be considered in patients with recurrent NAFLD or at risk of developing recurrent or *de novo* NAFLD. In particular minimization or avoidance of steroids, minimization of calcineurin inhibitor dose and levels and avoiding sirolimus in patients with hyperlipidemia is important in the management of NAFLD, obesity and metabolic syndrome post liver transplantation.

Bariatric surgery for obesity and morbid obesity has shown promising results in non-transplant patients and can reverse some of the metabolic consequences related to obesity such as diabetes<sup>[48]</sup>. Limited series have reported successful bariatric surgery specifically in patients with NAFLD<sup>[49]</sup>, and in case reports in patients with NAFLD with compensated cirrhosis<sup>[50]</sup>.

For NAFLD patients undergoing liver transplantation there are limited case reports of the utility of bariatric surgery after recurrence of NAFLD post transplantation<sup>[51]</sup>. There are also risks of exacerbation of NASH after bariatric surgery due to excessive weight loss as well as risks of impaired drug absorption and bacterial overgrowth that can impact post-transplant outcomes. At this point more evidence is needed before advocating bariatric surgery in transplant recipients.

## DONORS WITH NAFLD

An adverse consequence of the epidemic of obesity and fatty liver in the population is the impact on suitable donors for liver transplantation. There is an increased risk of primary non-function of the allograft with fatty donors<sup>[52]</sup>. This data suggest that greater than 30% steatosis in the donor organ increases the risk of primary non-function. As NAFLD in the populations increases, the pool of potentially suitable organs for liver transplantation may diminish as a consequence.

In a Korean paper that evaluated steatosis in potential donors over a year, NAFLD (> 5% steatosis) was present in 51% and greater than 30% steatosis was present in 10.4% with NASH in 2.2%. The prevalence of steatosis was higher in donor over the age of 30, and those donor

with obesity and elevated triglyceride levels. In this study ultrasonography and CT both had limitations in diagnosis of NAFLD (> 30% steatosis in donors) with sensitivity of 92% for ultrasound but positive predictive value of only 34.5% and for CT a sensitivity of 64% and PPV of 45%. More recently the use of MRI Quantification methods for steatosis have been developed and validated independently against liver biopsy showing excellent correlation with histological steatosis grading<sup>[53,54]</sup>. Although donor biopsies should still be considered before excluding donors as unsuitable due to steatosis, utilization of MRI, particularly for liver donors may in the near future supplant the need for liver biopsies<sup>[55]</sup>.

Although patient and graft survival can be diminished due to use of steatotic grafts, this is possibly not a risk factor for diminished graft survival if it exists in isolation<sup>[56]</sup>. Selection bias also confounds the picture as grafts that are not utilized due to steatosis may have different outcomes than steatotic grafts that are transplanted<sup>[57]</sup>.

## FUTURE DIRECTIONS

With increasing numbers of transplants in patients with NAFLD, current data support a careful audit of both short and long term post-transplant outcomes. Rigorous studies on immune-suppression regimens designed to decrease the incidence of metabolic complications for this population are needed. In addition post-transplant therapy for NAFLD including diet and exercise regimens, pharmacologic agents and bariatric surgery all warrant prospective study. With increasing numbers of donors with fatty livers, outcomes with these grafts should be tracked in prospective databases that include both donor and recipient variables.

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## WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

# Pharmacogenetic considerations for optimizing tacrolimus dosing in liver and kidney transplant patients

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studies the variability of drug response in relation to the genetic factors involved in the processes responsible for the pharmacokinetics and/or the action mechanism of a drug in the body. This variability seems to be correlated with the presence of genetic polymorphisms. Genotyping is an attractive option especially for the initiation of the dosing of tacrolimus; also, unlike phenotypic tests, the genotype is a stable characteristic that needs to be determined only once for any given gene. However, prospective clinical studies must show that genotype determination before transplantation allows for better use of a given drug and improves the safety and clinical efficacy of that medication. At present, research has been able to reliably show that the *CYP3A5* genotype, but not the *CYP3A4* or *ABCB1* ones, can modify the pharmacokinetics of tacrolimus. However, it has not been possible to incontrovertibly show that the corresponding changes in the pharmacokinetic profile are linked with different patient outcomes regarding tacrolimus efficacy and toxicity. For these reasons, pharmacogenetics and individualized medicine remain a fascinating area for further study and may ultimately become the face of future medical practice and drug dosing.

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**Key words:** Pharmacogenetics; Calcineurin inhibitors; Tacrolimus; Liver transplant; Kidney transplant; Single nucleotide polymorphisms; *CYP3A4*; *CYP3A5*; *ABCB1*

## Abstract

The introduction of tacrolimus in clinical practice has improved patient survival after organ transplant. However, despite the long use of tacrolimus in clinical practice, the best way to use this agent is still a matter of intense debate. The start of the genomic era has generated new research areas, such as pharmacogenetics, which

**Core tip:** As researchers continue to evaluate the influence of single nucleotide polymorphisms on tacrolimus dosing and on the response to the drug, the challenge now becomes to assess the potential clinical implications of this research for medical practice. Sufficient data have been accumulated to be certain that the liver donor and kidney recipient *CYP3A5* genotype has an important influence on tacrolimus dosing and on the

observed blood trough levels of the drug. However, the question remains, should genotyping become a standard of practice in transplantation?

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## INTRODUCTION

Transplantation is typically the standard of therapy for all patients with end-stage liver or kidney disease. Almost sixty years have passed since the first kidney transplant between identical twins was successfully performed, in 1954<sup>[1]</sup>. The first human liver transplant was performed almost 10 years later, in 1963. Another decade would pass before it was performed again. Since then, a significant effort has been made to improve the graft survival, as well as the patient outcome.

Despite the significant advances in terms of surgical techniques, tissue typing and patient care, most of the progress in organ transplantation is largely attributable to the recognized importance of immunosuppressive therapy<sup>[2,3]</sup>.

Since the success of the transplant depends on a delicate balance between immunosuppression and rejection, reaching and maintaining an adequate therapeutic level by giving appropriate doses of immunosuppressive drugs is extremely important, especially in the first phases after the transplant.

The introduction of tacrolimus into clinical practice has undoubtedly improved patient survival after organ transplant. However, this drug is characterized by a restricted therapeutic index, a high inter- and intra-individual pharmacokinetic variability, including irregular oral bioavailability, and a series of severe adverse effects<sup>[4,5]</sup>.

Given the high variability in blood levels and clinical response after administering fixed doses of tacrolimus, several studies have recently been conducted to find the optimal dosage of tacrolimus and thus to minimize its toxicity and to improve its risk/benefit ratio<sup>[6]</sup>.

A number of studies have found a close correlation between the pharmacokinetic parameters of tacrolimus and the clinical outcome<sup>[7,8]</sup>. However, despite the long use of the drug in clinical practice, the best way to use tacrolimus is still a matter of intense debate<sup>[9,10]</sup>.

The start of the genomic era has generated new research areas, such as pharmacogenetics, which studies the variability of drug response in relation to the genetic factors involved in the processes responsible for the pharmacokinetics and/or action mechanisms of a drug in the body<sup>[11,12]</sup>.

This variability seems to be correlated with the presence

of genetic polymorphisms, where, for example, some of the genes of the enzymes of phase I and II drug metabolic processes present, in at least 1%-2% of the population, allelic variants<sup>[13,14]</sup>.

These variants can encode for different molecular isoforms of the same protein and, in most cases, consist of single nucleotide polymorphisms (SNPs), which may determine the production of isoforms differing by a single amino acid<sup>[14]</sup>.

The variations in the DNA sequence of genes encoding for drug metabolizing enzymes can cause significant phenotypic differences in their expressivity and activity<sup>[15-17]</sup>.

The clinical implications of genetic polymorphisms can include other aspects of drug bioavailability and elimination, as well as the pharmacodynamics of the drug and/or its metabolites and the therapeutic index. Despite the many genetic polymorphisms, only a small number of them have clinically significant consequences in terms of drug metabolism. This occurs mainly when two conditions coexist: the concerned metabolic pathway is the only pathway for the biotransformation of the drug and the drug has a low therapeutic index.

For all these reasons, pharmacogenetics research has begun to delve into genotyping approaches which may help to optimize the initiation and maintenance dosing of tacrolimus, to attain faster its target concentrations and to limit its dose-related adverse reactions<sup>[18]</sup>. This paper will review various studies that highlight the current genetic considerations in the dosing of tacrolimus and the future implications that this data may have for best individualizing the treatment with this drug.

## TACROLIMUS PHARMACODYNAMICS AND PHARMACOKINETIC CHARACTERISTICS AND CONSIDERATIONS

The two calcineurin inhibitors utilized in transplantation are cyclosporine and tacrolimus.

Tacrolimus is a macrolide containing a 23-membered lactone ring produced by the *Streptomyces tsukubaensis* fungus (Figure 1). Its molecular weight is 822.05 Da<sup>[19]</sup>. Tacrolimus is now preferred to cyclosporine for its potency (10-100 times higher in *in vitro* and *in vivo* immunosuppression models) and for the reduction in episodes of rejection; it allows the use of lower doses of combination corticosteroids, thus reducing the possibility of adverse effects associated with such drugs<sup>[20,21]</sup>.

Tacrolimus becomes biologically active only when it forms a complex with the immunophilin FK binding protein 12 (FKBP-12), that is different from the immunophilin (cyclophilin) to which cyclosporine binds. The complex FKBP-12-tacrolimus interferes with the transduction pathway of the intracellular calcium-dependent signal, which is a fundamental processes for the activation of T lymphocytes. The biological target of the complex

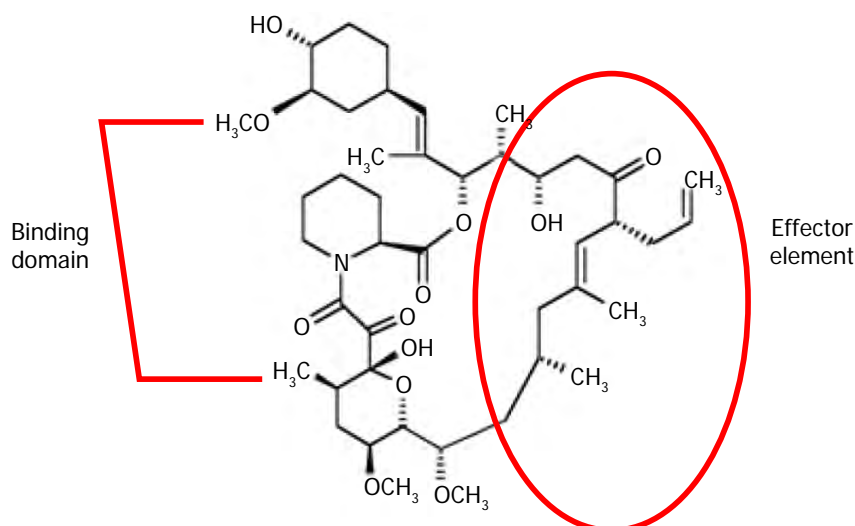


Figure 1 Functional active groups of tacrolimus.

is the calcium/calmodulin-dependent protein phosphatase calcineurin, a fundamental molecule for the reactions necessary to the synthesis of various cytokines, including IL-2.

Tacrolimus acts as a molecular linker between the calcineurin/calmodulin complex and immunophilin, which are molecules that in normal conditions would not interact. The tacrolimus-FKBP-12 complex has a strong inhibitory dose-related effect on calcineurin phosphatase activity and consequently on IL-2 expression. The passage of the signal from the cytoplasm to the nucleus to activate the transcription of the *IL-2* gene involves in fact a protein named nuclear factor of activated T-cell (NF-ATc). This protein is a T lymphocyte-specific transcription factor, the activity of which is correlated with the level of transcription of IL-2 after the T-cell receptor is activated<sup>[20,22]</sup>. The NF-ATc has two subunits, one of which is confined to the cytoplasm, while the other is mostly nuclear. An increase in intracellular calcium allows the cytoplasmic unit to move into the nucleus, where it combines with the nuclear component and allows the formation of the IL-2 transcription factor. The immunophilin/drug complexes would inhibit NF-ATc transcription activities, hindering the formation of the functional transcription factor. The signal transduction cascade starts at the presentation of the antigen to the T-cell receptor, which induces an increase in intracellular calcium, the activation of the calcium/calmodulin complex and the formation of the competent T-cell transcription factor (NF-ATc). The specific role of calcineurin is not entirely clear, but it is widely accepted that dephosphorylation induces the translocation into the nucleus of cytoplasmic NF-ATc, a process that can be blocked by the immunophilin/drug complexes. The liaison of DNA and the genetic transcription of IL-2 require both nucleic and cytoplasmic subunits of NF-ATc. Tacrolimus stops transduction pathways of the signal and therefore hinders the IL-2 production by means of

the intracellular action of the drug-FKBP-12 complex. The tacrolimus molecule can therefore be divided into two separate functional groups<sup>[22]</sup>: a binding group for the drug-FKBP-12 complex, and an effector group to bind to calcineurin (Figure 1).

Tacrolimus, a lipophilic drug, exhibits variable absorption and first pass metabolism when administered orally and this can influence its efficacy and toxicity. P-glycoprotein (P-gp, also known as ABCB1), an ATP-dependent membranous transporter which helps to protect the body against toxic xenobiotics by extruding these compounds out of cells and into the intestinal lumen and bile<sup>[23]</sup>, can limit the oral bioavailability and influence the disposition of the calcineurin inhibitors<sup>[24-28]</sup>. In particular, the presence of P-gp in the intestine can limit tacrolimus absorption. Also, its presence in liver and kidney promotes tacrolimus efflux into bile and urine, respectively.

However, the conclusions drawn so far on the actual influence of P-gp SNPs on tacrolimus pharmacokinetics are highly controversial<sup>[29-31]</sup>.

Additionally, CYP3A4 and CYP3A5, which exhibit variable levels of activity among transplant patients, are the primary enzymes responsible for the metabolism of the calcineurin inhibitors. The same drugs are also known inhibitors of P-gp and of the CYP enzyme system, so that they inhibit their own metabolism and excretion<sup>[32]</sup>. Other factors that can influence the pharmacokinetics of the calcineurin inhibitors include, but are not limited to, transplant type, baseline renal and hepatic function, concomitant use of corticosteroids, which induce both CYP3A and P-gp activity, patient age and race, time after transplantation, albumin and hematocrit concentration, trauma and food administration<sup>[33-40]</sup>.

As a result, therapeutic drug monitoring is typically initiated after transplantation to facilitate the choice of the dosage of the calcineurin inhibitors and ensure appropriate levels of exposure to these medications. To date the most widely used parameter for the therapeutic monitor-

ing of tacrolimus is its trough whole blood concentration ( $C_0$ ), which is measured 12 h after the dose administration and correlates well with the area under the concentration-time curve ( $AUC_{0-12}$ )<sup>[41,42]</sup>.

In practice, target trough levels for tacrolimus are typically set at around 10 ng/mL, but this can vary depending on individual patient characteristics, type of transplant and time after transplantation. Pharmacogenetics is estimated to account for between 20%-95% of drug variability in patients and this has prompted research to assess the feasibility of genotyping as a mean to more rapidly and accurately determine the appropriate starting and maintenance dosages of the immunosuppressant<sup>[43]</sup>.

Also to assess the economic advantage of the genotypic determinations, a number of pharmacodynamics studies have been undertaken to define the overall impact of a more delayed optimization of the drug dosage on patient outcomes. Typically these studies evaluate the effects of sub- or supra-therapeutic calcineurin inhibitor drug levels on graft life, patient mortality and development of various drug toxicities. The calcineurin inhibitors are known to be endowed with a number of possible deleterious effects including seizures, tremors, nephrotoxicity, malignancy, hyperglycemia, hypertension, insomnia, hyperesthesia and hyperlipidemia<sup>[4]</sup>. They are also expensive and potentially life-long medications that can impose a heavy economic burden on patients and on the health care system in general. As a result, it would be beneficial to rapidly attain target blood trough drug levels in order to avoid side-effects, limit costs and assure appropriate level of immunosuppression.

## GENETIC POLYMORPHISMS

To date, a number of SNPs have been studied in relation to the dosing of tacrolimus. However, alleles relating to the following three genes have been the most frequently studied and shown to be the most promising.

### CYP3A4

CYP3A4, located in the liver, jejunum, colon, and pancreas, is polymorphically expressed, with at least 42 SNPs identified to date<sup>[44]</sup>. The most known CYP3A4 polymorphisms are CYP3A4\*1B (A392G)<sup>[45]</sup>, CYP3A4\*2 (Ser 222 Pro), and CYP3A4\*3 (Met 445 Thr)<sup>[46]</sup>.

The primary polymorphism implicated and studied in the metabolism of the calcineurin inhibitors occurs at position 392 and is an A>G substitution that produces a variant allele with diminished enzymatic activity, referred to as CYP3A4\*1B<sup>[47-51]</sup>. On the other hand, researchers have demonstrated that CYP3A4 expression is higher in carriers of the mutant allele due to reduced binding of a transcriptional repressor<sup>[52,53]</sup>. Consequently, the functional significance of this SNP is controversial and *in vivo* studies have generally failed to evidence an association between this polymorphism and the metabolism of various drugs<sup>[54-56]</sup>. This allele has been shown to occur in 2%-10% of Caucasians, 4.2%-11% of Hispanics, 35%-67% of

African-Americans, and about 0% of Asians<sup>[57-60]</sup>.

### CYP3A5

CYP3A5 in the liver, small intestine, stomach and kidney shows polymorphic expression, which is currently known to occur with at least 11 different SNPs. The most important polymorphism is that of the CYP3A5\*3, which, in homozygous condition, determines the absence of the enzyme, since the variant sequences A→G at nucleotide 6986 in intron 3 of the CYP3A5 gene cause alternative splicing and the formation of a truncated protein that is not functional<sup>[61,62]</sup>. On the contrary, the G6986A (CYP3A5\*1) allele is correlated with a high expression of the protein<sup>[63]</sup>. Consequently, individuals that exhibit homozygous expression of the variant allele CYP3A5\*3 are often referred to as “CYP3A5 non-expressers”. Patients with at least one CYP3A5\*1 wild type allele are able to produce functional CYP3A5 enzymes and are known as “CYP3A5 expressers”; they have a different pattern of metabolite formation compared with the non-expressers, resulting also in the belief that CYP3A5 expression in the kidney may play a protective role against the development of nephrotoxicity by limiting the exposure of the organ to toxic metabolites<sup>[64-66]</sup>. Several studies have also suggested a link between CYP3A4\*1B and the CYP3A5\*1 wild type allele, as these two allelic variants appear generally to be inherited together<sup>[59,61,67-69]</sup>. Again the CYP3A5\*1 wild type allele is differently distributed among the races and occurs in 5%-15% of Caucasians, 15%-35% of Asians, 25% of Mexicans, and 45%-73% of African-Americans<sup>[57]</sup>.

### ABCB-1 (MDR-1, P-gp)

The multidrug resistance-1 (MDR-1) gene, which encodes for the P-gp (ABCB-1) efflux pump in many organs and tissues (*e.g.*, liver, kidney, hematocerebral barrier, blood testis barrier, maternal side of the placenta, adrenal glands and small intestines), is also polymorphically expressed, with at least 50 currently known SNPs. Its name derives from the fact that it was first found in tumor cell lines where it enhanced the resistance to anti-neoplastic drugs<sup>[23,70-74]</sup>.

The most commonly studied ABCB1 polymorphisms include a C to T substitution at position 3435 on exon 26, a C to T substitution at position 1236 on exon 12, and a G to T/A substitution at position 2677 on exon 21<sup>[24]</sup>.

These three variant alleles have been shown to typically occur together, exhibiting a linkage disequilibrium that suggests that they may be further genetically linked<sup>[74-78]</sup>.

Several studies have also suggested that this haplotype results in diminished P-gp expression *in vivo* and, in turn, in lower drug efflux activity. Theoretically this could result in tacrolimus accumulation in the blood stream and nervous system and, as a result, in symptoms of neurotoxicity<sup>[79,80]</sup>.

In addition, recent data have suggested that 3435C>T may reduce MDR-1 mRNA stability in the liver<sup>[81]</sup> or affect the insertion and folding of P-gp into the membrane, resulting in an altered substrate specificity of the transporter<sup>[82]</sup>.

This haplotype occurs in 5% of African-Americans, 27%



**Table 1** Effect of *CYP3A4\*1B* single nucleotide polymorphism on tacrolimus pharmacokinetics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Cho <i>et al</i> <sup>[84]</sup>	70 Korean	Kidney recipients	No association between <i>CYP3A4*1B</i> genotype and tacrolimus dose requirements up to 6 mo after transplantation
Roy <i>et al</i> <sup>[85]</sup>	38 Caucasian, 4 Black, 2 Asian	Kidney recipients	No correlation between the <i>CYP3A4*1B</i> SNP and tacrolimus pharmacokinetic at first week and third month after transplantation
Hesselink <i>et al</i> <sup>[67]</sup>	37 Caucasian, 9 Black, 18 Asian	Kidney recipients	<i>CYP3A4*1B</i> allele carriers had lower tacrolimus dose-adjusted trough levels with respect to patients carrying the wild-type (*1/*1) genotype at third and 12 <sup>th</sup> month after transplantation This effect was not observed when analyzing only the Caucasian population.
Hesselink <i>et al</i> <sup>[87]</sup>	120 Caucasian, 7 Black, 8 Asian, 1 other	Kidney recipients	No significant correlation observed between <i>CYP3A4*1B</i> SNP and tacrolimus pharmacokinetics when <i>CYP3A5</i> and <i>ABCB1</i> SNPs were taken into account
Gervasini <i>et al</i> <sup>[33]</sup>	103 Spanish	Kidney recipients	Carriers of the <i>CYP3A4*1B</i> variant allele had 59% lower tacrolimus concentrations than those with <i>CYP3A4*1/*1</i> wild type genotype All <i>CYP3A4*1B</i> carriers were also carriers of <i>CYP3A5*1</i> allele (linkage disequilibrium)

of Asians, 32% of Caucasians and 35% of Mexicans<sup>[77]</sup>.

## GENETIC INFLUENCE ON TACROLIMUS PHARMACOKINETICS

A number of clinical studies have begun to evaluate the actual impact of the previously described polymorphisms on tacrolimus dosing, efficacy and toxicity. We will now review a number of these studies and summarize their findings before analyzing the potential clinical implications of their data.

### *CYP3A4\*1B*

Data regarding the influence of *CYP3A4* polymorphisms on tacrolimus pharmacokinetics are often inconsistent and confounded by the highly frequent linkage disequilibrium found between the *CYP3A4\*1B* variant allele and the *CYP3A5\*1* wild-type allele<sup>[59,61,67-69,83]</sup>. The overall impact of the *CYP3A4* genotype on tacrolimus dose requirements appears uncertain and should be further studied.

A study by Cho *et al*<sup>[84]</sup> on 70 Korean renal transplant patients found no association between *CYP3A4* genotype and tacrolimus dose requirements up to 6 mo after transplantation (Table 1).

Another study, by Roy *et al*<sup>[85]</sup>, confirmed these results, showing no correlation between the *CYP3A4\*1B* (392A>G) SNP and tacrolimus pharmacokinetics (Table 1). However, as other authors have pointed out, due to the limited data available it is not possible to understand if these results were influenced by the ethnicity or by a genetic linkage with the *CYP3A5* 6986A>G SNP<sup>[86]</sup>.

In a study on 64 kidney transplant patients, Hesselink *et al*<sup>[67]</sup> showed that patients carrying the *CYP3A4\*1B* allele had lower tacrolimus dose-adjusted trough levels with respect to patients carrying two copies of the wild-type \*1 allele. This effect was not observed when the analysis was made only in the Caucasian population (Table 1).

However, in a further study carried out in a more con-

sistent population composed of 136 renal transplant patients the same authors found that there was no significant correlation between the *CYP3A4\*1B* (392A>G) SNP and tacrolimus pharmacokinetics (dose and C<sub>0</sub>/Dose) when the influences of the *CYP3A5* 6986A>G SNP and *ABCB1* polymorphisms were taken into account<sup>[87]</sup> (Table 1).

In another study on 103 Spanish renal transplant patients, Gervasini *et al*<sup>[33]</sup> found that carriers of the *CYP3A4\*1B* variant allele displayed tacrolimus concentrations that were on average 59% lower than those of patients with the *CYP3A4\*1/\*1* genotype. The dose-adjusted trough levels observed were 145.59, 86.89, and 58.21 ng/mL per mg/kg per day for the 3A4\*1-3A5\*3, 3A4\*1-3A5\*1 and 3A4\*1B-3A5\*1 haplotypes, respectively, suggesting that the *CYP3A4\*1B*-*CYP3A5\*1* haplotype may have a more profound impact on tacrolimus pharmacokinetics than the *CYP3A5\*1* allele alone (Table 1). However, because of the linkage disequilibrium between the *CYP3A4* and *CYP3A5* polymorphisms, all *CYP3A4\*1B* carriers were also carriers of the *CYP3A5\*1* allele.

### *CYP3A5\*3*

Many studies have confirmed that *CYP3A5* polymorphisms have a major influence on the pharmacokinetics of tacrolimus. Consistently, patients homozygous for the *CYP3A5\*3* allele have shown lower dose requirements and higher whole blood trough levels of tacrolimus after transplantation, as well as clearances of the drug 25%-45% lower than patients expressing the *CYP3A5\*1* allele. In liver transplant patients, donor genotype has also generally been shown to have more important consequences on tacrolimus pharmacokinetics and dose requirements than recipient genetics<sup>[38,63,88-93]</sup>. However, it still remains to be seen whether these alterations in the drug pharmacokinetics correlate or not to the patient clinical outcomes.

A study by Barrera-Pulido *et al*<sup>[94]</sup> on 53 liver transplant recipients found that recipients with the *CYP3A5\*1/\*3* genotype receiving organs from \*1/\*3 donors failed to

achieve minimum blood tacrolimus levels at one month post-transplant (Table 2). Between days 30 and 60 post-transplant  $*3/*3$  recipients from  $*1/*3$  donors also had significantly greater tacrolimus dose requirements than recipients from  $*3/*3$  donors.

These results also occurred in a study on 24 Native American kidney transplant recipients where it was observed that after 1 mo from transplant the patients required a significantly lower daily tacrolimus dose than a control group of Caucasian kidney transplant patients (0.03 mg/kg per day *vs* 0.5 mg/kg per day). To explain these data, many of these Native Americans, but not the Caucasians, were found to express the *CYP3A5* $*3/*3$  genotype, associated with diminished CYP3A5 enzymatic activity (Table 2). However, despite the differences in tacrolimus dose requirements, there were no differences in the drug trough levels or the incidence of nephropathy between the two study groups<sup>[95]</sup>.

A study on 32 Caucasian liver transplant patients by Provenzani *et al*<sup>[91]</sup> found that dose requirements were significantly higher in patients receiving a liver with the *CYP3A5* $*1$  allele compared with donors who were homozygous for the  $*3$  polymorphism (0.111 mg/kg per day *vs* 0.057 mg/kg per day). In the organ recipients, the *CYP3A5* $*1$  genotype tended to increase tacrolimus doses, though not to a statistically significant degree (Table 2).

In a case report, the same research group found that a 53-year-old Caucasian male who was homozygous for the *CYP3A5* $*3$  allele and had received a liver from a donor expressing the *CYP3A5* $*1/*1$  genotype required a dose two-fold higher than that reported in the literature for adult liver transplant patients. During the first, second and third week of therapy the patient received tacrolimus doses of 0.219, 0.287, and 0.273 mg/kg per day, respectively, while the trough drug levels obtained remained below the target of 10–12 ng/mL (4.6, 5.6 and 6.1 ng/mL at the first, second and third week of therapy, respectively). The patient reached a target level of 10.4 ng/mL only after one month of therapy. This corroborates that the *CYP3A5* $*1$  allele may be associated with increased hepatic metabolic capacity for tacrolimus and, consequently, delayed response to drug therapy<sup>[93]</sup>.

The authors further confirmed these results when they looked at 51 Caucasian liver and 50 Caucasian kidney transplant recipients at 1, 3, and 6 mo post-transplant and again found that the presence of the *CYP3A5* $*1$  allele in liver donors, but not in recipients, had a statistically significant effect of decrease on the tacrolimus dose-adjusted trough levels. A similar result was also observed in the kidney transplant recipients, where the dose required to achieve and maintain target trough blood levels at 1, 3, and 6 mo was statistically lower in patients homozygous for the *CYP3A5* $*3$  allele compared with the patients expressing at least one copy of the wild type allele *CYP3A5* $*1$ <sup>[92]</sup> (Table 2).

Another study by Cho *et al*<sup>[84]</sup> on 70 Korean renal transplant patients found that patients expressing either the

*CYP3A5* $*1/*3$  or *CYP3A5* $*1/*1$  genotype, and thus a functional CYP3A5 protein, had tacrolimus dose requirements up to 80% greater than patients homozygous for the  $*3$  allele up to 6 mo post-transplant (Table 2).

Glowacki *et al*<sup>[96]</sup> in a study on 209 French kidney transplant patients, also found that patients with at least one *CYP3A5* $*1$  allele had significantly higher tacrolimus dose requirements and lower trough drug levels than  $*3$  homozygotes. However, these pharmacokinetic findings appeared to have no influence on the incidence of biopsy-proven acute rejection or on delayed graft function (Table 2). Patients were followed for a mean period of 21.8 mo, with no data suggesting that alterations in tacrolimus pharmacokinetics might have any significant impact on long-term clinical outcomes.

Another study, in 181 Japanese liver transplant recipients and 114 donors, showed that the level of CYP3A5 mRNA was significantly reduced in patients with livers carrying the *CYP3A5* $*3/*3$  genotype (0.41 amol/ $\mu$ g total RNA) *vs* the  $*1/*1$  and  $*1/*3$  genotypes (4.85 and 2.99 amol/ $\mu$ g total RNA, respectively). As a result, the dose-adjusted tacrolimus trough levels were significantly decreased, due to increased metabolism, in patients receiving a liver carrying the *CYP3A5* $*1/*1$  genotype<sup>[63]</sup> (Table 2).

Wei-lin *et al*<sup>[88]</sup>, in a study on 50 Chinese liver transplant donors as well as recipients, found again that at one month after transplantation, recipients who received organs from *CYP3A5* $*3/*3$  donors had significantly higher dose-adjusted tacrolimus trough levels than the patients receiving livers from *CYP3A5* $*1$  expressers (Table 2). However, neither the donors' ABCB1 genotype nor the recipients' CYP3A5 genotype had any impact on the recipients' tacrolimus pharmacokinetic profile, suggesting once more that in liver transplantation the donors' CYP3A5 genetics, rather than that of the recipient, has a more important effect on tacrolimus dosing.

López-Montenegro Soria *et al*<sup>[97]</sup> studied 35 kidney transplant patients and found that during the first six weeks after transplant the tacrolimus concentration/dose ratios were remarkably lower for patients expressing at least one *CYP3A5* $*1$  allele compared with those homozygous for the *CYP3A5* $*3$  genotype (0.65 *vs* 1.45), due to higher drug clearances in *CYP3A5* $*1$  expressers (Table 2).

Another trial, by Shi *et al*<sup>[66]</sup>, involving 216 Chinese liver transplant recipients concluded that daily tacrolimus dose requirements were higher for recipients with the *CYP3A5* $*1/*1$  genotype than patients expressing the  $*3/*3$  genotype (3.0 mg per day *vs* 2.0 mg per day). Dose-adjusted tacrolimus trough levels were also lower in the  $*1/*1$  genotype than  $*1/*3$  expressers and in the  $*3$  homozygotes (97.5, 124.8, and 144.4, respectively), suggesting in particular that CYP3A5 enzymatic activity is increased proportionally by the presence of one or two copies of the  $*1$  allele (Table 2).

These results were supported by Jun *et al*<sup>[98]</sup> in a study of 506 Korean solid organ transplant recipients and 62 corresponding liver transplant donors, which concluded that the blood tacrolimus concentrations per adjusted

**Table 2** Effect of *CYP3A5* \*3 single nucleotide polymorphism on tacrolimus pharmacokinetics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Barrera-Pulido <i>et al</i> <sup>[94]</sup>	53 Caucasian	Liver recipients and donors	<i>CYP3A5</i> *1/*3 recipients with *1/*3 donor livers had lower than minimum required blood tacrolimus levels at 1 mo after transplantation *3/*3 recipients with *1/*3 donors had significantly greater tacrolimus dose requirements at 1 and 2 mo after transplantation
Chakkerla <i>et al</i> <sup>[95]</sup>	24 native American and Caucasian control group	Kidney recipients	Native Americans had lower tacrolimus dose requirements than Caucasians at 1 mo after transplantation Native Americans more commonly expressed <i>CYP3A5</i> *3/*3 No difference in blood trough levels or nephropathy between the two groups
Provenzani <i>et al</i> <sup>[91]</sup>	32 Caucasian	Liver recipients and donors	Dose requirements significantly higher in the case of donors with the <i>CYP3A5</i> *1 allele at 1, 3 and 6 mo after transplantation No statistically significant difference in dose requirements considering recipient's genotypes
Provenzani <i>et al</i> <sup>[92]</sup>	101 Caucasian	Kidney ( <i>n</i> = 50, recipients) and liver ( <i>n</i> = 51, recipients and donors)	<i>CYP3A5</i> *1 allele in liver donors ( <i>n</i> = 51) had a significant effect of decrease on tacrolimus dose-adjusted trough levels at 1, 3 and 6 mo after transplantation. No statistically significant difference in dose requirements considering recipient's genotype Tacrolimus dose in kidney recipients ( <i>n</i> = 50) with <i>CYP3A5</i> *3/*3 genotype was significantly lower than in patients with at least one copy of the wild type allele
Cho <i>et al</i> <sup>[84]</sup>	70 Korean	Kidney recipients	Those patients who had <i>CYP3A5</i> *1/*3 or *1/*1 genotypes had 80% higher tacrolimus dose requirements than patients homozygotes for *3 allele (up to 6 mo after transplantation)
Glowacki <i>et al</i> <sup>[96]</sup>	209 French	Kidney recipients	Patients with at least one copy of the <i>CYP3A5</i> *1 allele had significantly higher dose requirements and lower blood trough levels than patients homozygous for the *3 allele No influence of this SNP on rejection or graft dysfunction rates.
Goto <i>et al</i> <sup>[63]</sup>	181 Japanese	Liver recipients and donors	Patients with the <i>CYP3A5</i> *3/*3 genotype had reduced levels of <i>CYP3A5</i> mRNA Dose-adjusted tacrolimus trough levels decreased in patients receiving a liver with the *1/*1 genotype
Wei-Lin <i>et al</i> <sup>[88]</sup>	50 Chinese	Liver recipients and donors	Those patients receiving a liver with the *3/*3 genotype had, at first month after transplantation, significantly higher tacrolimus dose-adjusted trough levels than those with at least one copy of the *1 allele
López-Montenegro Soria <i>et al</i> <sup>[97]</sup>	35 Spanish	Kidney recipients	Concentration/dose ratios were remarkably lower in patients with at least one copy of the *1 allele than in patients homozygous for the *3 allele
Shi <i>et al</i> <sup>[66]</sup>	216 Chinese	Liver recipients	Recipients with *1/*1 genotype had higher dosage requirements than those with *3/*3 genotype The study suggested also that <i>CYP3A5</i> enzymatic activity is increased proportionally by the presence of the *1 allele
Jun <i>et al</i> <sup>[98]</sup>	568 Korean	Kidney and liver recipients ( <i>n</i> = 506), and liver donors ( <i>n</i> = 62)	Patients with the *3 alleles had higher tacrolimus dose-adjusted trough levels than patients with the *1 allele *1/*1 patients may be more rapid metabolizers than *1 heterozygous patients
Elens <i>et al</i> <sup>[99]</sup>	150 Belgian	Liver donors	Those patients with at least one *1 allele had at least 67% higher tacrolimus dose requirements No influence of <i>CYP3A5</i> expression on tacrolimus hepatic concentrations
Macphee <i>et al</i> <sup>[100]</sup>	119 White, 23 Black, 26 South Asian, 12 Middle Eastern	Kidney recipients	Patients with at least one copy of the wild-type *1 allele achieved twofold lower dose-normalized tacrolimus blood concentrations compared with <i>CYP3A5</i> *3/*3 homozygote patients
Thervet <i>et al</i> <sup>[101]</sup>	168 Caucasian, 8 Black, 12 other	Kidney recipients	Pre-transplant dose adaptation, according to <i>CYP3A5</i> genotype, is associated with improved achievement of the target blood trough levels
Spierings <i>et al</i> <sup>[102]</sup>	81 Caucasian, 12 Black, 20 South Asian, 5 other	Kidney recipients	Tacrolimus dose requirements were significantly higher in patients expressing the wild type <i>CYP3A5</i> genotype Intra-patient variability of tacrolimus clearance was not associated with the same genotype
Chen <i>et al</i> <sup>[103]</sup>	120 Chinese	Kidney recipients	<i>CYP3A5</i> expressers not receiving diltiazem required significantly higher tacrolimus doses than those who received the CYP inhibitor. In non-expressers, no significant difference in tacrolimus dose requirements was observed between the subjects treated with diltiazem and those who were not

dose ratio was significantly higher in recipients with the \*1/\*3 genotype than in those with the \*1/\*1 one, and again higher in \*3/\*3 patients rather than in heterozygous \*1/\*3 recipients, suggesting that \*1 homozygous patients may be even more rapid metabolizers than heterozygous patients expressing only one \*1 allele (Table 2).

A study by Elens *et al.*<sup>[99]</sup> on 150 liver donors found that tacrolimus dose requirements were at least 67% higher among patients with at least one *CYP3A5*\*1 allele and expressing hepatic *CYP3A5* (Table 2). However, though hepatic *CYP3A5* expression reduced blood tacrolimus levels and increased dose requirements, it failed to influence hepatic tacrolimus concentrations, which may be better related to liver graft outcome<sup>[99]</sup>.

Another study, by Macphee *et al.*<sup>[100]</sup>, in white and South Asian renal transplant patients, suggested that patients with at least one copy of the wild-type \*1 allele achieved twofold lower dose-normalized tacrolimus blood concentrations compared with *CYP3A5*\*3/\*3 homozygote patients (Table 2).

In a prospective study involving 280 kidney transplant patients, Thervet *et al.*<sup>[101]</sup> found that a pre-transplant tacrolimus dose adaptation according to the *CYP3A5* genotype is associated with fewer successive dose modifications and with a rapid achievement of target trough levels (Table 2).

In a more recent study by the Macphee's group on 118 renal transplant patients, Spierings *et al.*<sup>[102]</sup> confirmed that the tacrolimus dose requirements were significantly higher in patients with the wild type *CYP3A5* genotype (Table 2). However, they also found that intra-patient variability of tacrolimus clearance was not associated with the wild type *CYP3A5* genotype.

Finally, in a 42-mo, prospective, randomized, parallel-controlled, open-label, single-center study, 62 Chinese *CYP3A5* expressers and 58 non-expressers who had received kidney transplants were randomized to receive 30 mg of diltiazem (a known CYP inhibitor) three times daily in order to assess the efficacy of the drug as a calcineurin sparing agent. Patients who were known to be *CYP3A5* expressers and did not receive diltiazem required significantly higher tacrolimus doses than the other groups ( $P = 0.017$ ). Among the *CYP3A5* non-expressers, there was not a significant difference in tacrolimus dose requirements between the subjects treated with diltiazem and those who were not. This was expected, as the proposed mechanism for diltiazem as a calcineurin sparing agent involves the inhibition of the metabolism of tacrolimus through the *CYP3A5* pathway (Table 2). This suggests that *CYP3A5* expressers are more susceptible to diltiazem-induced tacrolimus dose reductions and may possibly provide the prescribers with a mechanism able to limit the cost of immunosuppressive therapy as well as to treat concomitant hypertension in transplant patients<sup>[103]</sup>.

### ABCB1

Data showing a link between a patient's *ABCB1* genotype and tacrolimus pharmacokinetics have been inconsistent. Though most studies have failed to find any

association, some clinical trials have found a significant relation between the *ABCB1* genotype and tacrolimus dosing. These results are often confounded by the linkage disequilibrium expressed among genetic variants, underscoring the need for further research on *ABCB1* genetics before a definitive conclusion can be reached.

Provenzani *et al.*<sup>[91]</sup>, in a study on 32 Caucasian liver transplant patients, found no influence of the 3435C>T and 2677G>T SNPs on tacrolimus dose requirements (Table 3). A study by Cho *et al.*<sup>[84]</sup> on 70 Korean renal transplant patients also found no association between the *ABCB1* genotype and tacrolimus dose requirements up to 6 mo after transplantation (Table 3).

Further supporting these results, Shi *et al.*<sup>[66]</sup> found that in 216 Chinese liver transplant patients, there was no significant association between any of the *ABCB1* polymorphisms and daily tacrolimus dose requirements or trough levels (Table 3).

This was again confirmed by Jun *et al.*<sup>[98]</sup>, who studied 506 Korean solid organ transplant recipients and 62 corresponding liver transplant donors. They found no correlation between the *ABCB1* patient genotype and tacrolimus concentration to adjusted dose ratios (Table 3).

Gervasini *et al.*<sup>[33]</sup> also found that, in 103 renal transplant patients, none of the *ABCB1* polymorphisms were associated with altered dose-adjusted trough levels or increased dose requirements. This study also found no association between the *ABCB1* genotype and tacrolimus-induced toxicity (Table 3).

Another study by Kuypers *et al.*<sup>[104]</sup> found that in 304 kidney transplant patients the *ABCB1* genotype had no significant impact on tacrolimus exposure parameters or dosing requirements (Table 3).

A study by Provenzani *et al.*<sup>[92]</sup> on 51 liver and 50 kidney transplant patients found no association between the *ABCB1* polymorphisms and tacrolimus dosing among liver transplant patients, but did observe that kidney transplant patients carrying the 2677T/A allele required a significantly higher daily tacrolimus dose than patients homozygous for the wild type allele (Table 3).

Another study on 181 liver transplant recipients and 114 donors found that, in the first week post-transplantation, the recipients who displayed the wild type *MDR-1* allele and thus high *ABCB-1* activity in the intestine, had lower dose-adjusted tacrolimus trough levels than patients who displayed *MDR-1* variant alleles and were low *ABCB-1* expressers, even among patients with the same liver *CYP3A5* genotype. However, this difference was not observed after two weeks, suggesting that *MDR-1* expression in the intestine may contribute to tacrolimus trough levels in the first week post-transplantation; afterwards the transplanted liver would achieve a greater metabolic capacity and becomes the main organ that influences tacrolimus pharmacokinetics<sup>[63]</sup> (Table 3).

This was supported by Herrero *et al.*<sup>[43]</sup> in a study on 71 renal transplant patients, in which it was found that patients with the wild type *ABCB1* genotype tended to have more stable tacrolimus concentrations within the



**Table 3** Effect of *ABCB1* single nucleotide polymorphism on tacrolimus pharmacokinetics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Provenzani <i>et al</i> <sup>[91]</sup>	32 Caucasian	Liver recipients and donors	No influence of 3435C>T and 2677G>T SNPs on tacrolimus dose requirements
Cho <i>et al</i> <sup>[84]</sup>	70 Korean	Kidney recipients	No association between <i>ABCB1</i> genotype and tacrolimus dose requirements
Shi <i>et al</i> <sup>[66]</sup>	216 Chinese	Liver recipients	No association between any <i>ABCB1</i> SNPs and tacrolimus dose requirements or blood trough levels
Jun <i>et al</i> <sup>[98]</sup>	568 Korean	Kidney and liver recipients ( <i>n</i> = 506), and liver donors ( <i>n</i> = 62)	No correlation between <i>ABCB1</i> genotype and tacrolimus dose-adjusted blood trough levels
Gervasini <i>et al</i> <sup>[33]</sup>	103 Spanish	Kidney recipients	None of the <i>ABCB1</i> polymorphisms were associated with changes in dose-adjusted blood trough levels and in dose requirements No association between <i>ABCB1</i> genotype and tacrolimus-induced toxicity
Kuypers <i>et al</i> <sup>[104]</sup>	304 Belgian	Kidney recipients	No significant impact of <i>ABCB1</i> genotype on tacrolimus exposure parameters or dosing requirements
Provenzani <i>et al</i> <sup>[92]</sup>	101 Caucasian	Kidney ( <i>n</i> = 50) and liver ( <i>n</i> = 51, recipients and donors)	No <i>ABCB1</i> influence on dosing in liver transplant patients Those patients receiving kidney transplant carrying the 2677T/A allele required significantly higher doses than those patients with the wild type allele
Goto <i>et al</i> <sup>[63]</sup>	181 Japanese	Liver recipients and donors	In the first week after transplantation, the recipients with wild type <i>ABCB1</i> allele had lower tacrolimus dose-adjusted blood trough levels No difference observed after 2 wk
Herrero <i>et al</i> <sup>[43]</sup>	71 Spanish	Kidney recipients	Patients with wild type <i>ABCB1</i> alleles had more stable tacrolimus concentrations within the therapeutic range during the first 3 mo On the contrary, patients carrying the polymorphic <i>ABCB1</i> alleles showed a mean increase in tacrolimus blood concentration of more than 60%
Wei-Lin <i>et al</i> <sup>[88]</sup>	50 Chinese	Liver recipients and donors	Recipients with the wild type <i>ABCB1</i> -3435CC allele had significantly higher tacrolimus dose requirements than those with C3435T at 1 and 2 wk and 1 mo after transplantation
López-Montenegro Soria <i>et al</i> <sup>[97]</sup>	35 Spanish	Kidney recipients	Wild type <i>ABCB1</i> 3435CC patients had 40% lower concentration/dose ratios than those patients with variant alleles
Elens <i>et al</i> <sup>[99]</sup>	150 Belgian	Liver donors	<i>ABCB1</i> genetic polymorphisms significantly influence tacrolimus hepatic concentrations, but have no effect on tacrolimus blood levels Patients with <i>ABCB1</i> 1236C>T polymorphism showed significantly better liver functions and lower Banff scores with respect to patients with the wild-type allele

therapeutic range during the first 3 mo after transplantation, while patients expressing polymorphic *ABCB1* alleles showed a mean increase in the drug blood concentrations greater than 60% due to a diminished elimination capacity by the body (Table 3).

A study on 50 Chinese liver transplant donors and recipients also evidenced that daily tacrolimus dose requirements were significantly higher in recipients carrying the wild type *ABCB1*-3435CC rather than the C3435T allele at the weeks 1 and 2 and at 1 mo post-transplantation (Table 3). These data suggested that in Chinese people the *ABCB1* genotype plays a dominant role in the intestinal tacrolimus pharmacokinetics<sup>[88]</sup>: in fact patients with the wild type *MDR-1* genotype are more likely to extrude tacrolimus from enterocytes and therefore need a higher daily dose to achieve adequate blood tacrolimus levels.

López-Montenegro Soria *et al*<sup>[97]</sup>, in a study on 35 renal transplant patients, also found that patients expressing the wild type *ABCB1*-3435CC genotype showed up to 40% lower concentration/dose ratios compared with patients carrying variant alleles (Table 3).

Finally, a study by Elens *et al*<sup>[99]</sup> on 150 liver transplant patients found that *ABCB1* genetic polymorphisms in the donors significantly influenced tacrolimus concentrations in the liver, but failed to influence the drug mean blood levels. The *ABCB1*-1236C>T polymorphism was also associated with improved liver function and significantly lower Banff scores compared with the situation of patients with the wild type allele (Table 3). These data suggest that *ABCB1* polymorphisms may be important in liver transplant patients due to their effects on tacrolimus levels in the liver, which, as already said, may be a good marker to predict the liver graft rejection.

## INFLUENCE OF GENETICS ON TACROLIMUS PHARMACODYNAMICS

Despite many studies have demonstrated a strong association between *CYP3A5* genotype and alterations in tacrolimus pharmacokinetics, the results do not provide consistent evidence of organ rejection or drug-related toxicity as a consequence of genotype-related sub- or supra-therapeutic immunosuppression. This is likely due to the fact that the

patients are closely monitored in the first period following transplantation and undergo dose adjustments to more rapidly achieve target trough drug levels. However, different clinical trials have begun to explore the practical pharmacodynamics implications of genetic alterations in tacrolimus pharmacokinetics, and some of them have found clinically significant results.

Jun *et al.*<sup>[98]</sup> found no significant difference in the incidence of organ rejection in 506 Korean solid organ transplant recipients and 62 liver transplant donors after comparing both patients' genotypes and mean tacrolimus concentration per an adjusted dose ratios (Table 4).

Another study by Chen *et al.*<sup>[103]</sup> on 120 Chinese kidney transplant patients who were a mix of CYP3A5 expressers and non-expressers, found that patients who received genotype-guided initial tacrolimus dosing achieved target drug levels more rapidly than the patients who received a standard protocol dose of tacrolimus (90.9% *vs* 27.3% of patients in target range, respectively). However, no differences were observed between the two groups with respect to the incidence of leukocytopenia, nephropathy, abnormal liver function, hyperlipidemia, diarrhea or hyperglycemia (Table 4).

Jacobson *et al.*<sup>[105]</sup>, in a prospective study on 945 kidney transplant patients, found that every increase in tacrolimus trough level of 1 ng/mL increased the hazard of early calcineurin-inhibitor-associated nephrotoxicity by 22%, even after adjusting for clinical factors. Nine SNPs of the *XPC*, *CYP2C9*, *PAX4*, *MTRR* and *GAN* genes exhibited an association with cyclosporine, but not with tacrolimus, nephrotoxicity (Table 4).

In a prospective, open-label, observational cohort study, Kuypers *et al.*<sup>[106]</sup> found that among 304 kidney transplant patients, the proportion of patients who developed new-onset diabetes after transplant (NODAT) was significantly higher in patients with delayed graft function and who displayed trough tacrolimus levels greater than 15 ng/mL on the first day post-transplantation. In this study, the presence of the *CYP3A5\*1* allele and a functional *CYP3A5* enzyme appeared to attenuate the effects of delayed graft function on initial tacrolimus exposure and dose requirements, suggesting that CYP3A5 expressers may be at lower risk of NODAT following kidney transplantation due to diminished exposure to potentially toxic levels of tacrolimus (Table 4).

In a separate study, but in the same population of 304 kidney transplant patients, Kuypers *et al.*<sup>[104]</sup> found that calcineurin-inhibitor-associated nephrotoxicity (CNIT) was more common in patients carrying the *CYP3A5\*1* allele than in patients who did not (32.4% *vs* 15.2%). Additionally, these researchers observed that CNIT developed in 25% of patients with dose requirements exceeding 0.2 mg/kg per day, 16.2% of patients with doses between 0.1-0.2 mg/kg per day and 4.5% of patients needing less than 0.1 mg/kg per day; the carriers of the *CYP3A5\*1* allele predominantly comprised the higher tacrolimus dose ranges. These results suggest that patients expressing the *CYP3A5\*1* allele and a functional CYP3A5 enzyme may

be predisposed to developing CNIT following transplantation due to greater daily tacrolimus dose requirements. This was observed especially in patients who continued corticosteroid therapy (Table 4). However, the incidence of delayed graft function and post-transplant diabetes mellitus was not different between CYP3A5 expressers and non-expressers.

In a more recent study, on 319 Hispanic kidney transplant patients, other authors found that the SNPs in the cytoplasmic nuclear factor of activated T cells 4 (NFATc4) gene, which is expressed in pancreatic islets, may confer a certain protection or also a predisposition with regard to NODAT; in particular, the patients carrying the SNP (rs10141896) T allele (T-T-T-T-G haplotype) showed a protection from NODAT, while patients homozygous for the C-C-C-G-G haplotype were associated with increased risk of NODAT. Furthermore, the authors found that the use of sirolimus and tacrolimus and a more advanced age (> 45 years) were also possibly correlated to the development of NODAT<sup>[107]</sup> (Table 4).

Cho *et al.*<sup>[84]</sup> found that in 70 Korean renal transplant patients tacrolimus toxicity was more frequent in the subjects with *CYP3A5\*1* alleles, who had significantly higher dose requirements of the drug than patients expressing the \*3 polymorphism (Table 4). Despite these findings, the study found no difference in the rate of graft survival between the various genotype-differentiated study groups.

A study by Barrera-Pulido *et al.*<sup>[94]</sup> on 53 liver transplant recipients found that patients with the *CYP3A5\*3*/*\*3* genotype receiving the organs from donors with an *ABCB1* polymorphism had a lower frequency of renal dysfunction, the same rejection rate and a higher rate of diabetes than the other groups studied (Table 4).

However, Shi *et al.*<sup>[66]</sup> found that in 216 Chinese liver transplant patients, carriers of the *CYP3A5\*3* allele had an increased risk of early renal injury compared with expressers of the *CYP3A5\*1* allele, possibly due to decreased enzymatic activity and higher dose-adjusted trough concentrations (Table 4).

## CONSIDERATIONS FOR FURTHER GENETIC RESEARCH

In addition to the previously discussed genetic polymorphisms, a number of other variants that may potentially influence the pharmacokinetics and pharmacodynamics of tacrolimus and transplant outcomes have been proposed for further study.

### *P450 oxidoreductase\*28*

Cytochrome P450 oxidoreductase (POR) is essential for the electron donation in the microsomal-CYP450-mediated mono-oxygenation that catalyzes the metabolism of approximately 85%-90% of therapeutic drugs. More than 40 SNPs have been identified in the *POR* gene, and it has been suggested that several of these mutations, specifically the *POR\*28-C>T* polymorphism, can increase this

**Table 4** Effect of various single nucleotide polymorphisms on tacrolimus pharmacodynamics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Jun <i>et al</i> <sup>[96]</sup>	568 Korean	Kidney and liver recipients ( <i>n</i> = 506), and liver donors ( <i>n</i> = 62)	No difference in incidence of organ rejection between different genotypes
Chen <i>et al</i> <sup>[103]</sup>	120 Chinese	Kidney recipients	Patients that received genotype-guided initial tacrolimus dosing vs standard protocol dose were more likely to achieve target drug levels No influence on incidence of adverse effects between CYP3A5 expressers and non-expressers
Jacobson <i>et al</i> <sup>[105]</sup>	945 (different ethnicities)	Kidney recipients	Every increase in tacrolimus blood trough level of 1 ng/mL increased the risk of early tacrolimus nephrotoxicity by 22% Polymorphism was not associated with an increased or decreased risk of tacrolimus-related nephrotoxicity
Kuypers <i>et al</i> <sup>[106]</sup>	273 White, 3 Hispanic, 24 North African, 2 African, 2 Asian	Kidney recipients	Delayed graft function was associated with higher initial mean tacrolimus blood trough levels and lower tacrolimus daily dose requirements, especially in CYP3A5 non-expressers CYP3A5 expressers may be at lower risk of new-onset diabetes after transplant (NODAT) due to diminished exposure to potentially toxic tacrolimus levels
Kuypers <i>et al</i> <sup>[104]</sup>	273 White, 3 Hispanic, 24 North African, 2 African, 2 Asian	Kidney recipients	Patients expressing the CYP3A5*1 allele and a functional CYP3A5 enzyme may be predisposed to developing calcineurin-inhibitor-associated nephrotoxicity (CNIT) following transplantation due to greater daily tacrolimus dose requirements This was observed especially in patients continuing corticosteroid therapy The incidence of delayed graft function and post-transplant diabetes mellitus was not different between CYP3A5 expressers and non-expressers
Chen <i>et al</i> <sup>[107]</sup>	319 Hispanic	Kidney recipients	SNPs in the cytoplasmic NFATc4 gene may confer a certain protection or also predisposition for NODAT. Patients carrying the T allele and the T-T-T-T-G haplotype showed a trend of protection from NODAT while patients with the C-C-C-G-G haplotype were associated with an increased risk of NODAT The use of sirolimus and tacrolimus and advanced age were also possibly correlated in development of NODAT
Cho <i>et al</i> <sup>[84]</sup>	70 Korean	Kidney recipients	Higher drug-related toxicity in patients with the CYP3A5*1 allele than in those with the CYP3A5*3 SNP No difference in graft survival between the two genotypes
Barrera-Pulido <i>et al</i> <sup>[94]</sup>	53 Spanish	Liver recipients and donors	Patients with CYP3A5*3/*3 allele receiving livers with an ABCB1 SNP had lower frequency of renal dysfunction, same rejection rate and higher diabetes rate
Shi <i>et al</i> <sup>[66]</sup>	216 Chinese	Liver recipients	Patients with the CYP3A5*3 allele had greater risk of early renal injury than the patients with the *1 allele

activity and alter the baseline metabolic capacity of several CYP isoforms. The \*28 allelic variant has been found to be expressed in 19.1% of African-Americans, 26.4% of Caucasian Americans, 31.0% of Mexican Americans, and 36.7% of Chinese Americans<sup>[108]</sup>.

A study by Zhang *et al*<sup>[109]</sup> on 71 healthy Chinese volunteers found that the mean tacrolimus AUC<sub>(0-24)</sub> and C<sub>max</sub> (71.5 and 17.6 ng/mL, respectively) for patients who were CYP3A5 expressers as well as carriers of the wild type CC POR genotype were 1.53 and 1.57 fold higher than those (46.7 and 11.2 ng/mL) observed in patients carrying POR allelic variants. No significant differences were observed between POR\*28-CC homozygotes and POR\*28-T carriers in CYP3A5 non-expressers, suggesting that the POR genotype is important in altering tacrolimus metabolism only in CYP3A5 expressing patients.

These results were supported by a cohort study of de Jonge *et al*<sup>[110]</sup> on 298 renal transplant recipients, which it was found that in CYP3A5 expressers, POR\*28T allele carriers had lower trough tacrolimus levels in the first three

days post-transplant and took longer to reach the target trough levels when compared with POR\*28CC homozygous patients. These patients with the variant POR genotype ultimately had 25% higher tacrolimus dose requirements than patients expressing the wild type allele. Again, POR\*28 polymorphisms were found to have no influence on tacrolimus pharmacokinetics in CYP3A5 non-expressers, and no differences in transplant outcomes were observed between the study groups.

### CYP3A7

Previously thought to be confined to the fetal liver, CYP3A7 has been found to be expressed in up to 54-88% of adult livers, but with a diminished metabolic capacity compared with that observed in children<sup>[111,112]</sup>. The role of CYP3A7 in the biotransformation of the CYP3A substrates in the adult liver and intestine is unknown. However, it was observed that CYP3A7 expression in the adult liver and intestine is increased in the carriers of the CYP3A7\*1C allele<sup>[112,113]</sup>. This allele has a very low frequency (3%) both

in Caucasians and African-Americans<sup>[61]</sup>.

Although tacrolimus is believed to be a substrate for the CYP3A7 enzyme, the influence of CYP3A7 metabolism on the pharmacokinetics of tacrolimus requires further study, especially in pediatric patients<sup>[99]</sup>.

### CYP3A4\*18B

This *CYP3A4* polymorphism appears only in Asian (25%-30%)<sup>[114]</sup>, primarily Korean, populations, but has been linked with a potentially increased metabolic capacity of the CYP3A4 enzyme. It has also been shown that carriers of the *CYP3A5\*1* allele are more likely to possess the *CYP3A4\*18B* allele. As a result, further study is required to determine whether linkage disequilibrium with the *CYP3A5\*1* allele may confound the observed metabolic effects of the *CYP3A4\*18B* polymorphism. One study, by Jun *et al*<sup>[98]</sup>, found no correlation between the *CYP3A4\*18B* allele and tacrolimus concentration to adjusted dose ratios in 506 Korean solid organ transplant recipients. A study of 22 healthy Chinese people showed a higher tacrolimus clearance in patients carrying the *CYP3A4\*18B* allele with respect to those carrying the *CYP3A4\*1* allele<sup>[115]</sup>. A more recent study, by Li *et al*<sup>[116]</sup>, on 83 Chinese renal transplant recipients confirmed the results of the previous study. It found that the tacrolimus-dose-adjusted trough concentration was significantly lower in patients carrying the *CYP3A4\*18B* allele compared with patients with the *CYP3A4\*1* allele.

### CYP3A4\*22

A new *CYP3A4* allele (*CYP3A4\*22*; rs35599367 C>T in intron 6) was recently discovered and also investigated in transplant patients<sup>[117,118]</sup>.

In particular, a study on 185 renal transplant patients, mostly Caucasians, evaluated the impact of this new SNP on tacrolimus pharmacokinetics. It showed that in the first year after transplantation, patients carrying one or two T alleles required significantly lower tacrolimus doses (33%) compared with patients homozygous for the wild-type C allele<sup>[118]</sup>. The authors attributed the result to the fact that this *CYP3A4\*22* SNP is significantly linked to reductions in *CYP3A4* mRNA production and enzyme activity in human livers<sup>[118-120]</sup>. This SNP is relatively frequent in Caucasians (2.5%-6.9%). The authors also suggested that, though further studies are necessary, that pre-transplant genotyping of the *CYP3A4* C>T could reduce the risk of achieving supra-therapeutic tacrolimus levels<sup>[118]</sup>.

However, in a study done on Brazilian renal transplant patients, *CYP3A4\*22* was not associated with changes in tacrolimus dose requirements<sup>[121]</sup>.

### CYP2C8 and CYP2J2

These enzymes, which are polymorphically expressed in the kidney, are involved in the synthesis of epoxyeicosatrienoic acids that play a protective role against acute rejection and toxicity by acting as vasodilators to maintain adequate renal perfusion and limit hypertension.

In a study on 163 liver transplant patients the authors

found that patients with the *CYP2C8\*3* variant genotype appeared to be at higher risk of tacrolimus-induced kidney disease, possibly because of reduced formation of the kidney protecting epoxyeicosatrienoic acids<sup>[122]</sup>.

In another study, on 103 renal transplant patients, the authors found a higher incidence of delayed graft function and nephrotoxicity in patients homozygous for the *CYP2C8\*3* genotype, associated with reduced epoxyeicosatrienoic acid production and, consequently, less vasodilator activity<sup>[33]</sup>.

In a more recent study the same research group could associate both *CYP2C8\*3* and donor age (> 48 years) with a higher incidence of delayed graft function and poorer creatinine clearance<sup>[123]</sup>.

### SLC01B1

This gene is responsible for expressing the organic anion transporting polypeptides *OATP1B1* and *OATP1B3*. These transporters play a role in the transport of multiple compounds from the portal vein to hepatocytes and in the biliary excretion of many drugs. Recently, Elens *et al*<sup>[99]</sup> found that the 388A>G and 521T>C polymorphisms in the *SLC01B1* gene influenced tacrolimus trough blood concentrations after the administration of the first dose in 150 liver transplant patients. In this study, patients expressing the 388 polymorphism showed a lower mean tacrolimus blood level, while alterations of the 521 allele resulted in significantly greater trough drug levels. It was also recently demonstrated that cyclosporine and tacrolimus are inhibitors of the organic anion transporters, so that one cannot exclude the possibility that these drugs may be substrates of *OATP1B1* and *OATP1B3* as well.

### Angiotensinogen C3889T (rs4762) gene polymorphism

It is well known that tacrolimus has a negative effect on pancreatic beta islet cells and can cause glucose intolerance and diabetes mellitus<sup>[124]</sup>. However, new studies have suggested that post-transplant diabetes mellitus can also be related to other factors and, consequently, not only to tacrolimus administration<sup>[124,125]</sup>. Angiotensinogen (AGT) is the initial component of the renin-angiotensin system (RAS) and a precursor of both angiotensin I and II. In a study on 302 subjects, the authors found that the *AGT* gene polymorphism (rs4762) is associated with post-transplant diabetes mellitus, due to insulin resistance, in Korean renal transplant patients<sup>[126]</sup>. Molecular and genetic studies demonstrate a relationship between variants of the *AGT* gene, *AGT* gene expression and plasma AGT levels<sup>[127,128]</sup>. However, the association between this gene and glucose metabolism remain controversial.

## DISCUSSION

As clinical trials continue to evaluate the influence of genetics on drug dosing and response, the challenge now becomes to assess the potential clinical implications of this research for medical practice. Sufficient data has been accumulated to be certain that the liver donors and renal re-



ipients *CYP3A5* genotype has important influences on tacrolimus dosing and on its blood through levels. However, it remains the question whether genotyping should become a standard practice in transplantation.

This question is difficult to answer because of the multi-factorial approach needed to assess the pharmacokinetic profile of a drug. Wide variability of tacrolimus dosing requirements to reach target blood levels has been observed even among patients carrying the same genotype. This underlies the fact that genetic polymorphisms are only one of the possible factors that can influence tacrolimus pharmacokinetics. Patient age, race, metabolic level, concomitant medications and a variety of other environmental factors appear to play an even more significant role than genotype in altering drug pharmacokinetics. Specifically in liver transplant patients, time after transplantation also plays a critical role in altering drug metabolism and distribution. The intestine may play a more important role soon after liver transplantation, before the liver recovers from the trauma of surgery and resumes a higher level of metabolic capacity. As liver function improves, hepatic synthesis of albumin also increases, which, in turn, decreases the unbound fraction of tacrolimus and lowers drug clearance. This is just one example of the many considerations that can ultimately impact the pharmacokinetics of an agent and highlights the difficulties in basing drug dosing on just one parameter.

To further complicate the issue, studies have yet to demonstrate a clear association between tacrolimus blood trough levels, genotype and transplant outcomes. Organ rejection and drug toxicities have been seen to develop in patients without any notable difference in tacrolimus blood concentration, making difficult to predict the optimal trough drug targets in relationship to the characteristics of the individual patient. Toxicities associated with tacrolimus are also often difficult to study because of their insidious onset. Hypertension, hyperlipidemia and NO-DAT develop slowly over a period of many years, making the length of a trial an issue when one wants to monitor these chronic medication effects. The mechanisms of such adverse effects are, again, not fully understood and require further research to determine the need to genotype patients, not only as a way of lowering the incidence of organ rejection, but also of preventing drug toxicity after transplantation.

Studies in transplantation are also often difficult to conduct because of the limited patient population. Many studies involve fewer than 100 patients, which may help explain some of the variable results. A number of these studies also differ in their pharmacokinetic methods, dosing strategies, times when blood drug concentrations are assessed and patient's characteristics. Differences between donor and recipient organ genotypes may also have confounded the results of some studies, as the genetics of both the recipient and of the donor were not always taken into account.

Genotyping is an attractive option for starting the dosing of tacrolimus; also, unlike phenotypic tests, the results

of which may vary with environmental factors, the genotype is a stable characteristic that needs to be determined only once for any given gene. However, to ultimately prove the usefulness of genotyping, prospective clinical studies must show that genotype determination before transplantation allows the better use of a given drug and improves the safety and clinical efficacy of that medication. Currently Amplichip, a genetic test manufactured by Roche Pharmaceuticals, can determine a patient's *CYP2D6* and *CYP2C19* polymorphisms for between United States \$350 and \$400, not including the mark up and other costs associated with the test. As a result, to offset the cost of genetic testing, genotypic analyses must demonstrate the ability to significantly improve transplant patient outcomes, in particular, graft life and patient survival, and show a cost saving for patients and for the health care system as a whole.

## CONCLUSION

At present, research has been able to reliably show that the *CYP3A5*, but not the *CYP3A4* or *ABCB1*, genotype modifies the pharmacokinetics of tacrolimus. However, it has not been possible to incontrovertibly show that the corresponding changes in the pharmacokinetic profile are linked with different patient outcomes regarding tacrolimus efficacy and toxicity. Additionally, given the high cost of genotypic tests and the wide availability and utility of therapeutic drug monitoring, genotyping all transplant patients is not convenient for many individuals or Institutions. This may change in the near future as further studies on pharmacogenetics will produce new data and the improvements in the genotyping analyses will drive down the costs associated with this type of tests. For these reasons, pharmacogenetics and individualized medicine remain a fascinating area for further study and may ultimately become the face of future medical practice and drug dosing.

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## WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

# Liver transplantation for hepatocellular carcinoma: Role of inflammatory and immunological state on recurrence and prognosis

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mor growth. Among these markers, the neutrophil-to-lymphocyte ratio appears to be the most promising and easily available serum parameter able to predict HCC recurrence after LT and following other types of treatment, although the exact mechanisms determining its elevation have not been clarified. Post-LT immunosuppression may impact on cancer control, and the exposure to high levels of calcineurin inhibitors or other immunosuppressants has recently emerged as a negative prognostic factor for HCC recurrence and patient survival. Despite the absence of prospective randomized trials, inhibitors of the mammalian target of rapamycin have been shown to be associated with lower rates of tumor recurrence compared to other immunosuppressors, suggesting their use especially in patients with HCC exceeding the conventional indication criteria for LT.

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**Key words:** Liver transplantation; Hepatocellular carcinoma; Inflammation; Immunosuppression; Recurrence

## Abstract

Criteria for liver transplantation (LT) for hepatocellular carcinoma (HCC) and post-LT indicators of prognosis are historically based on the measurement of the tumor mass. Recently, high throughput technologies have increased the prediction of recurrence, but these tools are not yet routinely available. The interaction between HCC and the immune system has revealed an imbalance of lymphocyte phenotypes in the peritumoral tissue, and the increase of regulatory T cells with respect to cytotoxic lymphocytes has been linked to a higher rate of post-LT HCC recurrence. Moreover, some inflammatory markers have shown good reliability in predicting cancer reappearance after surgery, as a result of either a systemic inflammatory response or a decreased capacity of the organism to control the tu-

**Core tip:** This review focuses on inflammatory markers recently emerged as indicators of tumor biological behavior and on immune state of patients submitted to liver transplantation for hepatocellular carcinoma (HCC), with a particular reference to the role of neutrophil-to-lymphocyte ratio. The impact of post-transplant immunosuppression on HCC recurrence is also analyzed according to the most relevant evidences published so far, which outline the importance of minimization of the use of calcineurin inhibitors and the protective role of inhibitors of the mammalian target of rapamycin.

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Pinna AD. Liver transplantation for hepatocellular carcinoma: Role of inflammatory and immunological state on recurrence and prognosis. *World J Gastroenterol* 2013; 19(48): 9174-9182 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9174.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9174>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and its incidence is increasing in Western countries<sup>[1]</sup>. For patients with HCC and cirrhosis, liver transplantation (LT) represents the treatment of choice and provides excellent oncological results and a cure for cirrhosis.

Prognostic factors for tumor recurrence and patient outcome have mainly been recognized as an expression of tumor burden and of its biological aggressiveness. Among these factors, the number and size of HCC nodules, the degree of differentiation, the presence of hepatic vascular invasion and elevated serum levels of alpha-fetoprotein (AFP) are the ones most widely utilized to define the indications for LT and to predict the outcome<sup>[2-8]</sup>. Since it is often difficult to safely and/or reliably obtain histological parameters before LT<sup>[9,10]</sup>, radiological tumor criteria and AFP levels are the main preoperative indicators of prognosis.

The role of markers of inflammation and of the patient's immunological state have recently emerged as predictors of outcome, providing information on the environment in which the tumor grows and on the systemic response to its expansion<sup>[11-20]</sup>. These markers are often correlated with dimensional and histological factors determining a high risk of recurrence, but the mechanisms by which they are expressed are still largely unexplored. While waiting for more precise molecular markers<sup>[21-23]</sup> to become of routine use in defining the indications for and the prognosis of LT, the above parameters of inflammation may help to predict the biological behavior of HCC.

Since post-LT pharmacological immunosuppression can ideally impact on the ability to control tumor reappearance, the type, duration and total load of immunosuppressors have also been investigated in recent years as predictors of HCC recurrence<sup>[7,8,24-33]</sup>.

The role of inflammatory markers and of post-LT immunosuppression on tumor recurrence and patient prognosis after LT for HCC are the subject of the present review. For this purpose, an extensive review of the English literature using the PubMed database was performed independently by two authors (Cescon M, Bertuzzo VR), separately selecting papers pertinent to the key terms "liver transplantation", "hepatocellular carcinoma", "recurrence" and "inflammation" for the investigation of the impact of inflammatory markers, and to the terms "liver transplantation", "hepatocellular carcinoma", "recurrence" and "immunosuppression" to assess the post-LT impact of pharmacological immunosuppression.

## RELATIONSHIP BETWEEN INFLAMMATORY AND IMMUNOLOGICAL MARKERS, AND OUTCOME AFTER LIVER TRANSPLANTATION FOR HCC

In the last two decades, Virchow's hypothesis, which postulates that a relationship exists between inflammation and cancer, has permitted new insights into the phenomenon of carcinogenesis<sup>[34]</sup>. Given the importance of the peritumoral (micro)-environment, researchers have focused on markers that could be an expression of the relationship between liver cancer and surrounding tissue, with a possible consequent change of systemic inflammatory response.

Infiltration of pro-inflammatory macrophages, cytokines and chemokines in the tumor microenvironment has been shown to enhance tumor growth, invasion and metastases<sup>[34-36]</sup>, allowing the use of inflammation parameters as tumor markers<sup>[37,38]</sup> and the development of new therapeutic strategies<sup>[35,36]</sup>.

C-reactive protein (CRP)<sup>[37-41]</sup> and erythrocyte sedimentation rate (ESR)<sup>[42-45]</sup> were the first serum inflammation indicators used as tumor markers. Elevated preoperative CRP, an acute-phase reactant synthesized by hepatocytes in response to systemic inflammation, has been recognized as a risk factor for incidental colorectal cancer<sup>[39]</sup> and as an adverse prognostic factor in patients undergoing hepatectomy for HCC<sup>[40]</sup>, whereas ESR has been identified as an indicator of poor prognosis in patients with clear cell renal cell carcinoma and in children with Hodgkin's lymphoma<sup>[42,45]</sup>.

Inflammatory cytokines such as interleukin-6 (IL-6) and IL-1b are linked to transcriptional signaling pathways associated with carcinogenesis, tumor growth, and invasion<sup>[36,46]</sup>. IL-6 is known as one of the main regulators of CRP production.

The neutrophil-to-lymphocyte ratio (NLR) is another inflammation index that has been evaluated as a tumor marker<sup>[47-53]</sup>. Originally used as a systemic inflammatory response index in critically ill patients, it is obtained by dividing the absolute neutrophil count by the absolute lymphocyte count. According to published literature, an  $NLR \geq 5$  can be considered a valid cut-off<sup>[48,50,51]</sup>.

Some studies have demonstrated the relationship between NLR and tumor progression in patients with colon cancer, liver metastases from colorectal cancer, pancreatic cancer, breast cancer, esophageal cancer, cholangiocarcinoma, and HCC; in addition, a higher incidence of HCC recurrence has been observed in patients with high NLR and undergoing hepatic resection<sup>[47-53]</sup>.

An elevation of NLR could be related to a relative increase of neutrophils - as a consequence of some sort of inflammatory response - to a decrease of lymphocyte count - reflecting a lower immunological control of tumor growth - or to both phenomena, with several studies supporting each of these hypotheses.

LT for HCC represents a particular field of investiga-



tion of inflammatory markers and local immunological activation as possible expressions of tumor invasiveness and biological behavior. Although the visible tumor mass is usually treated preoperatively with neoadjuvant treatments, and then entirely removed with hepatectomy, some parameters detected in the serum may help in recognizing a systemic response to cancer relapse due to viable cancer cells still in the patient's circulation or in remote organs, at any time during the waiting time to LT, and following the procedure.

The role of CRP has been analyzed for prediction of post-LT outcomes of HCC patients<sup>[14]</sup>. In a series of 85 patients, those with high CRP levels ( $\geq 1$  mg/dL) at the time of LT had higher total bilirubin levels, Child-Pugh grade, Model for End-Stage Liver Disease score, maximal tumor size, and frequency of intrahepatic metastasis compared to patients with low CRP levels ( $< 1$  mg/dL).

By multivariate analyses, HCC beyond the Milan criteria, a high CRP level, and microvascular invasion were associated with tumor recurrence, while a high CRP level and microvascular invasion were related to lower overall survival. In addition, high CRP level was an independent factor for predicting poor outcomes in patients with HCC beyond the Milan criteria, but not in patients with HCC within the criteria<sup>[14]</sup>. Taken together, these findings suggest that CRP is related to poor liver function and higher tumor invasiveness, but the precise molecular mechanisms for its increase in such circumstances are not clarified. Moreover, another study<sup>[16]</sup> failed to detect any relationship between CRP (and ESR) and post-LT HCC recurrence.

Unitt *et al.*<sup>[11]</sup> studied the tumor CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup> and Foxp3<sup>+</sup> lymphocyte infiltrate in the explant tissue of 69 patients transplanted due to HCC. On multivariate analysis, CD4:CD8 ratio, vascular invasion, tumor size, and reduced lymphocyte infiltration were significant independent predictors of recurrence. The presence of regulatory T cells (Tregs; CD4<sup>+</sup>, CD25<sup>+</sup>, Foxp3<sup>+</sup> T-lymphocytes) was not predictive of recurrence, but was associated with tumor vascular invasion. These data suggest that a reduced immunological response against cancer expressed as prevalence of Tregs and a lower expression of cytotoxic lymphocytes is associated with poor prognosis.

The above findings were partly supported by another study by Mathai *et al.*<sup>[12]</sup>, who assessed the phenotype of tumor-infiltrating lymphocytes in 131 histology sections of patients undergoing LT or liver resection for HCC. An increased Foxp3:CD3 ratio was associated with poorly differentiated HCC and higher Edmonson-Steiner nuclear grade. An increased Foxp3:CD8 ratio was also associated with poorer differentiation, higher Edmonson-Steiner nuclear grade, tumor recurrence, decreased overall survival, and decreased disease-free survival.

Although not focused on LT recipients, other studies showed that patients with HCC have increased numbers of CD4<sup>+</sup> CD25<sup>+</sup> Tregs not only among tumor-infiltrating lymphocytes, but also in the peripheral blood; furthermore, the abundance of this cell population correlated with tumor progression. These cells were anergic toward

T-cell receptor stimulation and, when cocultured with activated CD4<sup>+</sup> CD25<sup>+</sup> cells, potently suppressed their proliferation and cytokine secretion. Concomitantly, the expression of granzyme A, granzyme B, and perforin was decreased dramatically in tumor-infiltrating CD8(+) T cells, confirming their inefficacy in controlling tumor expansion<sup>[54,55]</sup>.

In summary, an imbalance between Tregs and CD8 lymphocytes, with a prevalence of the former and a defective function of the latter, does reflect an aggressive behavior of HCC and the inability of the organism to control the disease. While these findings potentially pave the way to new treatments, they cannot be unequivocally correlated with markers easily available by means of common lab tests, such as NLR (see below).

Nevertheless, novel methods for assessing the immune function of transplanted patients could be useful in the future. The Immu-Know assay, which measures the amount of adenosine triphosphate (ATP) produced by activated CD4<sup>+</sup> T cells, has been used to evaluate the global immune status, and thus the tendency to develop rejection or, on the contrary, post-LT infections<sup>[56]</sup>.

This tool has also proven to be reliable in predicting post-LT HCC recurrence, with recipients diagnosed with recurrent tumors having significantly lower values of ATP compared to those without recurrence<sup>[13]</sup>. This refined measurement of the immune state of LT recipients could replace the more indirect evaluation allowed by systemic exposure to immunosuppressive agents.

Several studies have demonstrated that an increased NLR is an independent factor for lower recurrence-free survival and/or overall survival in LT HCC patients<sup>[15-20]</sup>. These studies are reported in Table 1. A total of 892 patients were included. The chosen cutoff value of NLR ranged from 3 to 5, with most studies using the value of 5<sup>[15,16,18]</sup>, while others identified lower values<sup>[17,19,20]</sup>.

In the groups of patients with NLR above the selected risk thresholds, overall survival ranged between 14% and 57%, and recurrence-free survival was between 6% and 42%. Only one study reported both the NLR at diagnosis of HCC and NLR at transplant, showing that this variable had a similar negative impact on outcome at the two chosen time points<sup>[18]</sup>.

High NLR was an independent predictor of outcome in all studies, in most cases together with other commonly recognized risk factors. Interestingly, in two studies NLR was not correlated with histological, serological and dimensional features with a recognized, negative impact on recurrence<sup>[15,18]</sup>.

In the above reports, different explanations for the alteration of NLR were provided but, though reasonable, most of them were speculative. Only one group, which produced two different analyses on this topic, investigated the correlation between NLR and the alterations of phenotype/function of leucocytes or other cells in tissues surrounding neoplastic nodules<sup>[19]</sup>. Interestingly, the Authors found that serum and peritumoral IL-17 levels were significantly higher in patients with high NLR, and that the density of peritumoral CD163-positive tumor

**Table 1 Studies reporting the negative impact of increased neutrophil-to-lymphocyte ratio measured at transplant on the outcome of liver transplantation for hepatocellular carcinoma**

Ref.	Patients (n)	Type of LT	NLR cut-off level for poor prognosis	Other factors associated with worse outcome	5-yr RFS with high vs low NLR	5-yr OS with high vs low NLR	Parameters positively correlated with increased NLR
Halazun <i>et al</i> <sup>[15]</sup>	150	NA	5	Tumor size AFP	25% vs 75% <sup>1</sup>	28% vs 64%	None
Bertuzzo <i>et al</i> <sup>[16]</sup>	219	DDLT	5	Microvascular invasion	6% vs 89%	14% vs 73%	Micro/macro vascular invasion Tumor grading AFP CRP Outside MC
Wang <i>et al</i> <sup>[17]</sup>	101	DDLT	3	Tumor number Macrovascular invasion	28% vs 65% <sup>1</sup>	19% vs 62%	Macrovascular invasion AFP Tumor size Outside MC Outside UCSF criteria Outside Hangzhou criteria None
Limaye <i>et al</i> <sup>[18]</sup>	160	NA	5	Microvascular invasion AFP	27% vs 79%	38% vs 68%	Serum/peritumoral IL-17 Density of peritumoral CD163 CRP Tacrolimus vs cyclosporine Microvascular invasion Tumor grading
Motomura <i>et al</i> <sup>[19]</sup>	158	LDLT	4	Outside MC	30% vs 89%	57% vs 84%	
Yoshizumi <i>et al</i> <sup>[20]</sup>	104	LDLT	4	Nodule size + number ≥ 8.0	42% vs 86%	Not reported	

<sup>1</sup>In these studies, disease-free survival instead of recurrence-free survival rates were reported (and displayed in the present table); <sup>2</sup>This study was performed by the same authors as the previous one<sup>[19]</sup>, and included only patients with surgical and/or locoregional treatment preceding living donor liver transplantation (LDLT). Thus, the patient population is probably at least partly included in the population of the previous study from the same Institution. NLR: Neutrophil-to-lymphocyte ratio; LT: Liver transplantation; HCC: Hepatocellular carcinoma; RFS: Recurrence-free survival; OS: Overall survival; NA: Not assessable; AFP: Alpha-fetoprotein; CRP: C-reactive protein; MC: Milan criteria; DDLT: Deceased donor liver transplantation; UCSF: University of California at San Francisco.

associated macrophages (TAM) was both correlated with the density of peritumoral IL-17-producing cells, and significantly higher in subjects with elevated NLR. Conversely, tumor, peritumoral and serum expression of vascular endothelial growth factor (VEGF) and of IL-8, *i.e.*, two recognized angiogenesis and tumor growth factors, was similar between high and low NLR groups. Tumor expression of IL-17, CD68, and CD163 was also comparable in patients with elevated or normal NLR.

A positive correlation between CRP and NLR, the absence of correlation between NLR and tumor markers, number and size of nodules, and microvascular invasion, the association between high NLR and an increased serum neutrophil count, and the absence of correlation between NLR and total serum lymphocytes were other important findings<sup>[19]</sup>.

Consistently with previous studies<sup>[57-61]</sup>, the authors came to the following conclusions: (1) contrary to other investigations, the elevation of NLR seems correlated with an increase of neutrophil number rather than of lymphocytes, suggesting a dependence of tumor relapse on the inflammatory state rather than on an impaired host immune response; (2) elevated neutrophils are thought to be a reservoir of VEGF, but the expression of VEGF and of IL-8 did not have any impact on NLR, suggesting that NLR elevation is not directly responsible for augmented HCC-related neo-angiogenesis; (3) IL-17 is a pro-inflammatory cytokine that promotes HCC

growth and neutrophil recruitment, thus it could be a key molecule in the relationship between NLR (which is supposed to increase due to expansion of neutrophils following recruitment) and HCC recurrence; and (4) the authors' results are consistent with the demonstrated relationship between IL-7-producing T cells and TAMs. IL-7-producing T cells promote the differentiation of tissue macrophages in peritumoral tissue into TAMs, which in turn promote tumor proliferation and angiogenesis. In fact, monocytes are recruited from the circulation into local tissue or malignant sites, where they are recognized by CD68-positive residential macrophages. Under the effect of inflammatory cytokines released by tumors, some of these macrophages differentiate into CD163-positive TAMs that, contrary to CD68<sup>+</sup> macrophages, are suppressors of the anti-tumor immune response.

IL-17-producing cells interact with TAMs in patients with HCC, and both IL-17-producing cells and CD163<sup>+</sup> TAMs generate the same family of chemokines promoting the recruitment of monocytes and neutrophils<sup>[19,57-61]</sup>.

Finally, it should be considered that in the authors' series splenectomy was performed during LT in patients with hepatitis C virus-positive or significant portal hypertension, and splenectomy itself could have had a role in the balance between neutrophil and lymphocyte count. Moreover, TAMs have been demonstrated to originate from splenic monocytes. However, splenectomy itself was not associated with HCC recurrence in this study,

**Table 2** Studies reporting the effect of different basal immunosuppression schedules on the outcome of liver transplantation for hepatocellular carcinoma

Ref.	Evaluated immunosuppressor	Evaluated parameter	Patients (n)	Overall recurrence rate	Outcome parameters	P value
Vivarelli <i>et al</i> <sup>[24]</sup>	CsA cumulative dosage 1 <sup>st</sup> yr	Low dosage 1 <sup>st</sup> yr <i>vs</i> high dosage 1 <sup>st</sup> yr	39 <i>vs</i> 30	12.20%	5 yr RFS: 93% <i>vs</i> 5 yr RFS: 76%	0.0100
Kneteman <i>et al</i> <sup>[25]</sup>	SRL	in MC <i>vs</i> out MC	19 <i>vs</i> 21	12.50%	4 yr RFS: 81.1% <i>vs</i> 4 yr RFS: 76.8%	0.4800
Vivarelli <i>et al</i> <sup>[26]</sup>	CsA	Low exposure <i>vs</i> high exposure	49 <i>vs</i> 21	10.00%	RR: 0% <i>vs</i> RR: 33.3%	< 0.0010
Decaens <i>et al</i> <sup>[27]</sup>	CNI	CsA <i>vs</i> TAC	264 <i>vs</i> 119	31.80%	5 yr RFS: 52.5% <i>vs</i> 5 yr RFS: 70.8%	0.0030
Decaens <i>et al</i> <sup>[27]</sup>	ATG/OKT3	Not administered <i>vs</i> administered	356 <i>vs</i> 55	31.80%	5 yr RFS: 58.8% <i>vs</i> 5 yr RFS: 45.4%	0.0200
Vivarelli <i>et al</i> <sup>[7]</sup>	TAC	Low exposure <i>vs</i> high exposure	44 <i>vs</i> 16	20.00%	RR: 9.1% <i>vs</i> RR: 50%	0.0010
Zhou <i>et al</i> <sup>[28]</sup>	TAC and SRL	TAC <i>vs</i> SRL	46 <i>vs</i> 27	27.40%	2 yr OS: 50.9% <i>vs</i> 2 yr OS: 80.6%	0.0110
Zimmerman <i>et al</i> <sup>[29]</sup>	in patients outMC					
	TAC and SRL	TAC + MMF <i>vs</i> TAC + SRL	52 <i>vs</i> 45	12.40%	5 yr RFS: 54.0% <i>vs</i> 5 yr RFS: 78.8%	-
Chinnakotla <i>et al</i> <sup>[8]</sup>	TAC and SRL	TAC + MMF <i>vs</i> SRL	106 <i>vs</i> 121	11.00%	5 yr RFS: 60% <i>vs</i> 5 yr RFS: 80%	0.0001
Vivarelli <i>et al</i> <sup>[30]</sup>	TAC and SRL	TAC <i>vs</i> TAC + SRL	31 <i>vs</i> 31	25.80%	3 yr RFS: 56% <i>vs</i> 3 yr RFS: 86%	0.0400
Toso <i>et al</i> <sup>[31]</sup>	SRL	Not administered <i>vs</i> administered	2382 <i>vs</i> 109	-	5 yr OS: 68.7% <i>vs</i> 5 yr OS: 83.1%	≤ 0.0500
Xing <i>et al</i> <sup>[32]</sup>	Basiliximab and steroids in patients in MC	TAC + MMF + basiliximab <i>vs</i> TAC + MMF + steroids	28 <i>vs</i> 36	-	5 yr OS: 88.9% <i>vs</i> 5 yr OS: 57.4%	0.0220
Rodríguez-Perálvarez <i>et al</i> <sup>[33]</sup>	CNI	Low exposure 1 <sup>st</sup> mo <i>vs</i> high exposure 1 <sup>st</sup> mo	171 <i>vs</i> 48	16.40%	5 yr RR: 14.7% <i>vs</i> 5 yr RR: 27%	0.0070

LT: Liver transplantation; HCC: Hepatocellular carcinoma; CsA: Cyclosporine A; RFS: Recurrence free survival; SRL: Sirolimus; MC: Milan criteria; RR: Recurrence rate; CNI: Calcineurin inhibitors; TAC: Tacrolimus; ATG: Anti-thymocyte globulins; OS: Overall survival; MMF: Mycophenolate mofetil.

even though in the group of patients with elevated NLR, splenectomy led to significantly better recurrence-free survival than the abstention from this procedure, suggesting the supply of splenic TAMs with high IL-17 concentrations after LT<sup>[19]</sup>.

The same authors confirmed the relevant role of NLR on HCC recurrence in patients undergoing living donor liver transplantation for tumor recurrence after surgical resection and/or locoregional treatment<sup>[20]</sup>, and in those submitted to liver resection<sup>[62]</sup>.

By evaluating 958 patients who underwent hepatectomy without preoperative therapy for HCC, multivariate analysis showed that NLR was an independent prognostic factor of lower overall and recurrence-free survival, the best cutoff being 2.81. Again, CD163-positive cell counts were significantly higher in tumors of patients with high NLR than in those with low NLR<sup>[62]</sup>.

Finally, one of the advantages of an easily obtainable serum marker is to assess the response to pre-LT treatments of HCC and the probability of dropout from the waiting list. NLR has been shown to be a good predictor of the risk of dropout, while platelet-to-lymphocyte ratio has been related to post-LT HCC recurrence<sup>[63]</sup>. On the other hand, since multimodal treatments are usually adopted while on the waiting list for LT, it has also been shown that NLR, or NLR postoperative changes, correlate with HCC recurrence and patient outcome after radiofrequency ablation<sup>[64,65]</sup>.

## EFFECT OF IMMUNOSUPPRESSION ON HCC RECURRENCE AFTER LIVER TRANSPLANTATION

At present, there is a general consensus on the negative

impact of pharmacological immunosuppression on the outcome of LT for HCC<sup>[7,8,24-33]</sup>. Specifically, two clinical pieces of evidence have emerged: (1) the higher the exposure to calcineurin inhibitors (CNI), *i.e.*, cyclosporine and tacrolimus, the higher the risk of post-LT HCC recurrence; and (2) one specific class of immunosuppressors, *i.e.*, inhibitors of the mammalian target of rapamycin (mTORi), have a favorable effect in reducing the incidence of post-LT HCC recurrence compared to standard immunosuppressors (CNI). Everolimus and sirolimus, the two mTORi currently in use in solid organ transplantation, interfere with hepatocarcinogenesis through the inhibition of the PI3K/Akt/mTOR pathway, which is a key regulator of cellular proliferation and angiogenesis<sup>[66,67]</sup>.

Several studies led to the above conclusions<sup>[7,8,24-33]</sup>, although it is of relevance that none of these is a prospective, randomized trial. Table 2 depicts the retrospective clinical studies published so far on this topic, with the exclusion of reports with less than 20 patients and previous reviews or meta-analyses.

Overall recurrence rates ranged between 12% and 32%. Four out of 13 reported studies showed that among patients immunosuppressed with CNI, those exposed to higher dosages had unfavorable outcomes, with significantly higher HCC recurrence rates or lower recurrence-free survival rates compared to patients receiving lower dosages<sup>[7,24,26,33]</sup>. One study reported a lower recurrence-free survival in patients treated with cyclosporine *vs* those treated with tacrolimus<sup>[27]</sup>.

In 5 studies, patients treated with sirolimus (most frequently in combination with low dosages of tacrolimus) showed higher overall or recurrence-free survival rates compared to patients receiving standard CNI-based immunosuppression<sup>[8,28-31]</sup>. In one study<sup>[25]</sup>, patients treated

with sirolimus had similar recurrence-free survival rates, irrespective of fulfillment of the Milan criteria.

One study showed a detrimental effect of the use of monoclonal antibodies (anti-thymocyte globulins or OKT3), with a lower recurrence-free survival in patients receiving these drugs compared to those not administered them<sup>[27]</sup>. Another study revealed that the use of steroids *vs* basiliximab led to significantly lower overall survival rates<sup>[32]</sup>.

A definitive validation of the benefit of mTORi in LT for HCC is expected to be provided in 2014 by an international multicenter, prospective, randomized trial comparing the outcomes of patients administered or not administered sirolimus following post-LT histological confirmation of HCC<sup>[68]</sup>. However, at present the use of mTORi in LT for HCC seems justified on the basis of the above reported results and according to a recent meta-analysis conducted on 5 studies and 474 patients, which showed a lower recurrence rate, longer recurrence-free survival and overall survival, and lower recurrence-related mortality in sirolimus-treated patients in comparison with CNI-treated patients<sup>[69]</sup>.

## CONCLUSION

Recent insights into the interactions between tumor, peritumoral tissue, and systemic inflammatory and immune response have offered new indicators for prognosis of patients with HCC undergoing various types of treatment, including LT. NLR has proven to be a reliable and easily available inflammatory marker of tumor biological aggressiveness, making its use advisable along with common dimensional indexes in assessing the response to treatments and the indication for LT, and to predict the outcomes. Although recent reports provided a reasonable molecular basis for the alteration of NLR and, more in general, for the tumor-related imbalance between immune cells in terms of number and function, much remains to be explored to expand targeted diagnostic and therapeutic tools. On the other hand, despite the lack of prospective, randomized studies, there is sufficient evidence for the minimization of immunosuppression and for the use of mTORi in LT for HCC, especially in the case of extended indications for transplant.

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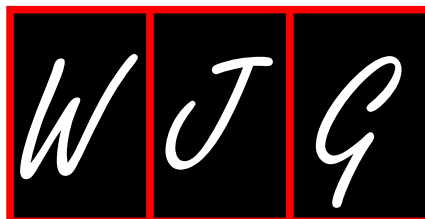
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## Transplant benefit for patients with hepatocellular carcinoma

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### Abstract

Although liver transplantation is theoretically the best treatment for hepatocellular carcinoma (HCC), it is limited by the realities of perioperative complications, and the shortage of donor organs. Furthermore, in many cases there are available alternative treatments such as resection or locoregional therapy. Deciding upon the best option for a patient with HCC is complicated, involving numerous ethical principles including: urgency, utility, intention-to-treat survival, transplant benefit, harm to candidates on waiting list, and harm to living donors. The potential contrast between different principles is particularly relevant for patients with HCC for several reasons: (1) HCC candidates to liver transplantation are increasing; (2) the great prognostic heterogeneity within the HCC population; (3) in HCC patients tumor progression before liver transplantation may significantly impair post transplant outcome; and (4) effective alternative therapies are often available for

HCC candidates to liver transplantation. In this paper we suggest that allocating organs by transplant benefit could help balance these competing principles, and also introduce equity between patients with HCC and non-malignant liver disease. We also propose a triangular equipoise model to help decide between deceased donor liver transplantation, living donor liver transplantation, or alternative therapies.

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**Key words:** Hepatocellular carcinoma; Deceased donor liver transplantation; Living donor liver transplantation; Transplant benefit; Utility; Urgency; Intention-to-treat survival; Harm

**Core tip:** Deciding upon the best option for a patient with hepatocellular carcinoma is complicated, involving numerous ethical principles including: urgency, utility, intention-to-treat survival, transplant benefit, harm to candidates on waiting list, and harm to living donors. In this paper we suggest that allocating organs by transplant benefit could help balance these competing principles, and also introduce equity between patients with hepatocellular carcinoma and those with nonmalignant liver disease. We also propose a triangular equipoise model to help decide between deceased donor liver transplantation, living donor liver transplantation, or alternative therapies.

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## GENERAL PRINCIPLES REGULATING PATIENT SELECTION AND ORGAN ALLOCATION IN LIVER TRANSPLANTATION

### *Urgency, utility, and equity*

Liver transplantation (LT) is theoretically the best treatment for patients with end-stage liver disease but its effectiveness is limited by intrinsic characteristics with important ethical implications: (1) LT remains a technical demanding procedure with a well-established short-term mortality and morbidity<sup>[1]</sup>; (2) a persistent shortage of deceased donors corresponds to an increasing demand of deceased donor liver transplantation (DDLT)<sup>[2]</sup>; and (3) the application of living donor liver transplantation (LDLT) is limited by ethical and legal issues related to the risk of harming the living donor<sup>[3]</sup>. Specific selection policies have consequently been developed over the last two decades to identify good candidates for this complex therapeutic option<sup>[4,5]</sup>.

For patients with non-malignant (NM) liver cirrhosis, scores have been developed to measure disease severity, such as the Child Pugh and the model for end-stage liver disease (MELD) scores<sup>[4]</sup>, which support a selection policy based on the urgency principle. Under a medical urgency-based selection system, patients with worse outcomes while on the waiting list (WL) are given higher priority for transplantation<sup>[6]</sup>. Use of the MELD score, for example, has significantly reduced waiting list times and in the United States system in recent years<sup>[4,7]</sup>. If we consider the development of hepatocellular carcinoma (HCC) as a complication of liver cirrhosis, and therefore as a sign of disease severity, assigning a high priority to HCC patients would also comply with this principle of urgency. This viewpoint is reflected in the United Network for Organ Sharing (UNOS) allocation system, where an arbitrary high MELD score is assigned to patients with T2-HCC<sup>[7]</sup>.

The limit of this approach is that it fails to consider the extremely relevant prognostic heterogeneity of patients with HCC and the potential effectiveness of alternative therapies<sup>[8]</sup>. It is also only reasonable to consider HCC as a complication of liver cirrhosis if this condition is maintained within certain proportions of candidates on the WL (*e.g.*, < 20%), as in the US<sup>[9]</sup>. In some geographical LT settings, however, there has been a significant increase in the proportion of LT candidates on the WL with liver tumors in recent years and this has given rise to similar proportions of liver transplants for HCC and NM disease<sup>[10]</sup>. In these modern LT realities, it is probably more reasonable to consider HCC patients as a separate LT population and analyze the prognostic heterogeneity of this particular medical condition more deeply<sup>[11]</sup>.

If we observe the issues of patient selection and organ allocation from the HCC population point of view, therefore, current LT selection policies for HCC patients (*e.g.*, the UNOS allocation system) appear to be based

mainly on a utility principle for two main reasons. First, a utility-based system is one that gives priority according to expected post-transplant outcomes<sup>[6]</sup>. For patients with HCC, the poor results achieved in early experiences with patients transplanted for advanced tumors have favored the introduction of strict selection criteria focusing mainly on post-LT outcome<sup>[5]</sup>. Therefore, patients beyond Milan criteria have limited probability of receiving a transplant.

Second, in the current system all T2 HCC patients receive the same priority regardless of their likelihood of death on the waiting list.

If we consider LT candidates with and without cancer as two separate populations, therefore, apparently opposite allocation principles are currently used at the majority of LT centers around the world. This diversity in patient selection policy intrinsically creates an ethical paradox, in that donated organs are allocated to the “sickest patient first” among the candidates with NM hepatic disease, but to the “earliest patient first” among candidates for LT who have HCC, irrespective of their survival prospects with therapies other than transplantation.

Aristotle defined justice as “treating equal cases equally, and unequal cases unequally”. One of the fundamental challenges of organ allocation science is maintaining equity among the heterogeneous groups of patients on the waiting list. In the specific organ allocation context, equity means treating all patients according to a common endpoint. From this perspective, the principle of equity is hierarchically more important than all others, whether we decide to favour urgency, or utility or benefit as endpoints for our allocation system.

Based on these considerations (*i.e.*, the increasing proportion of HCC patients enlisted, and an excess of priority for HCC patients with a low urgency for LT), recent proposals have tried to resolve the unbalance in the access to transplantation between HCC and non-HCC patients. One attempt involved developing risk models within the HCC population for 3-mo drop-out risk as common urgency endpoint<sup>[12,13]</sup>. However, this approach (*i.e.*, to equate the drop-out risk of different patients) carries the risk of prioritizing HCC patients with higher biological aggressiveness in terms of nodule size and AFP levels, and consequently dramatically increasing the risk of post-LT tumour recurrence or death<sup>[14]</sup>. Thus, methods are needed which balance the principles of urgency and utility when attempting to reach equity between HCC and non-HCC patients.

### *Intention-to-treat survival*

To describe the effect of long waiting times on the effectiveness of LT as curative therapy for HCC<sup>[15]</sup>, some years ago the concept of intention-to-treat (ITT) survival was introduced. Interestingly, analyzing the survival figures of HCC patients from the day of enlisting and not from that of transplant, the overall results of LT for HCC became worse than resection<sup>[16]</sup> due to the high dropout rate of HCC patients from the WL for tumor progression.

However, ITT survival is strongly related to the specific local/regional WL characteristics and in particular to the patient median waiting time: assuming as a constant the post-LT outcome, the lower the pre-LT mortality, the higher the intention-to-treat survival. For this reason, in a clinical scenario where HCC patients receive high priority for LT (*i.e.*, low waiting time and low risk of dropout) the intention-to-treat survival of LT for HCC patients may exceed that of liver resection<sup>[17]</sup>.

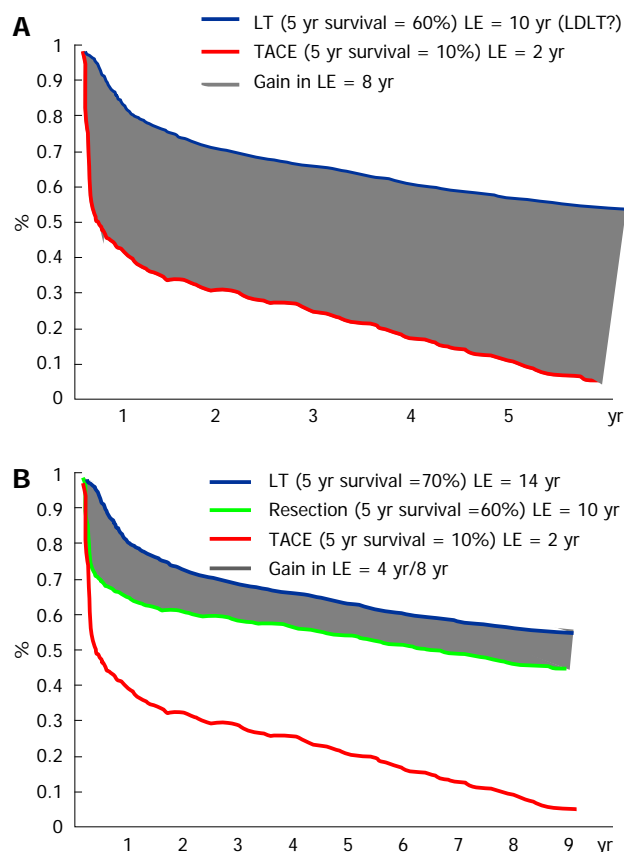
For these reasons, survival analysis in LT should use the ITT principle because it accounts for all the complex LT processes from the day that LT is first considered.

### Transplant benefit

The concept of transplant benefit expresses the survival gain offered by LT by comparison with the best alternative therapy. Transplant benefit can be calculated from the time of transplant, or from the time a patient is first evaluated for transplant—the latter would make it an ITT endpoint. On an individual basis, the main advantage of this principle is that it covers the overall LT process, simultaneously considering post- and pre-LT outcome. The transplant benefit principle applied to the individual LT candidate thus has the potential to create an ideal balance between the concepts of urgency and utility. As suggested by Schaubel *et al.*<sup>[6]</sup>, moreover, by prioritizing patients based on life-years gained thanks to transplantation, the transplant benefit principle performs better than urgency and utility schemes from a population perspective too. This is because an urgency-based system would assign donor organs to patients who are most likely to die while on the WL, but this approach may be to the detriment of utility because patients at the greatest risk of death while on the WL may also be patients with the highest post-LT mortality risk. A utility-based allocation system would ensure that transplanted organs go to patients with the lowest post-LT mortality risk, but patients with the best post-LT outcomes may also have the best outcomes while on the WL. The transplant benefit principle is consequently the one best able to maximize the total life-years gained by the patient population.

In recent years, the transplant benefit principle has been proposed for LT candidates based on studies using data from the Scientific Registry of Transplant Recipients (SRTR)<sup>[2,6,18]</sup>, but these studies did not consider the transplant benefit for the HCC population of LT candidates, because they either focused only on NM candidates<sup>[2,11]</sup> or they considered HCC as a complication<sup>[6]</sup> and not as a separate, prognostically heterogeneous medical condition.

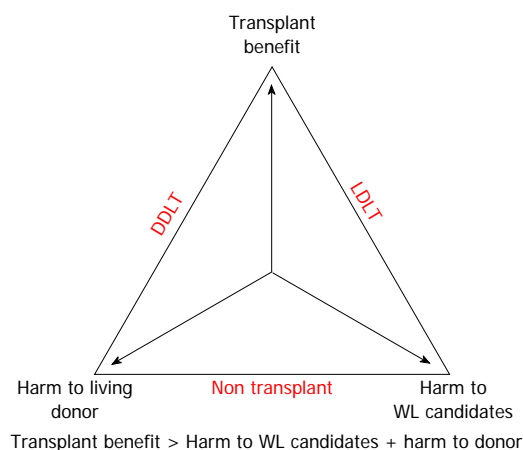
The concept of transplant benefit has the intrinsic potential for being especially useful for HCC patients since a particular feature of the approach lies in that it is calculated by subtracting the area under the survival curve after alternative therapies from the area under the survival curve after transplantation<sup>[9]</sup>, a definition that coincides with the gain in life expectancy (LE). This gives a relevant weight not only to the crude post-LT outcome, but also to the alternative therapies available and to the patient's



**Figure 1** Clinical examples of the transplant benefit principle applied to hepatocellular carcinoma patients. A: Man 40-year-old, HBV with 2 HCC nodules, the largest of 6 cm, Child B (Milan out, University of California San Francisco out); B: Man 65-year-old, HCV, with 1 HCC (diameter = 4 cm), Child A. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

age, which are extremely important prognostic variables for HCC patients<sup>[19]</sup>. Figure 1 shows two different clinical scenarios. The first (Figure 1A) concerns the case of a young patient (40 years old) with a tumor beyond the Milan criteria (calculated 5-year post-transplant survival = 60%). The lack of any effective alternative therapies makes the benefit of LT extremely high (8 years). The second scenario (Figure 1B) considers an older patient (65-year-old) within the accepted indications for LT (5-year post-transplant survival = 70%), but with an effective alternative treatment option, *i.e.*, liver resection, which makes the benefit of LT much lower (4 years) than in the first case, although the post-LT outcome would be better.

The recent publication of important studies on the survival prospects of patients with more advanced tumors after LT<sup>[20]</sup> and other therapies<sup>[21,22]</sup> makes it potentially feasible now to evaluate transplant benefit across different stages of HCC disease. This could be extremely important because, from a utility perspective, adopting extended criteria for HCC patients would mean allocating more donated organs to HCC patients than to NM patients<sup>[16]</sup>; taking a transplant benefit perspective, on the other hand, would mean reallocating the same number of organs to different groups of patients with a greater



**Figure 2** Ethical equipoise between benefit and harm of deceased-donor liver transplantation and living donor liver transplantation. WL: Waiting list; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation.

benefit. In other words, the transplant benefit principle would be able to maximize the total life-years of both the HCC and NM population.

These concepts have been recently incorporated in three papers<sup>[23-25]</sup> evaluating the transplant benefit principle in the HCC population. These studies underline three main points: (1) Liver transplantation results in the highest survival benefit for HCC patients with advanced liver cirrhosis (BCLC stage D); (2) Patients with intermediate tumours (BCLC stages B-C) without effective alternative therapies receive a relevant benefit from LT, regardless of the nodule number-size criteria (*i.e.*, Milan criteria), provided that macroscopic vascular invasion and extra-hepatic disease are absent; and (3) Patients with early tumors and compensated cirrhosis have the lowest benefit from LT when effective alternative therapies are available<sup>[23-25]</sup>.

### Harm-benefit to other patients on the waiting list

When patients on a given WL receive an organ, they harm the rest of the candidates on the WL because it is as if they were taking that organ away from other potential candidates. The entity of this harm depends on the extra time the other patients on the WL have to wait for another organ. We can also see this concept from the opposite point of view: if we find an alternative treatment for a patient on a WL for LT (*e.g.*, if we perform a LDLT or a liver resection), we create a benefit for the people on said WL that can be calculated from the further waiting time they spare. Knowing the characteristics of a WL in detail (death probabilities according to disease severity, median waiting time for LT, mean number of organs per year, patient stratification according to MELD score and HCC stage), we can calculate this harm/benefit to candidates on the WL<sup>[9,26,27]</sup>. This is very important because it is the only allocation principle that takes the characteristics of a specific WL into account (WL size, donor resources and proportions of patients with severe disease).

### Harm to the living donor

The crucial element limiting the general applicability of LDLT is the risk of harming a healthy living donor. In the literature, the overall mortality attributed to living donor procedures is lower than 1%, but the risk of morbidity is significant, being around 38% in some experiences as a whole, and < 10% when severe complications are considered alone<sup>[28]</sup>.

A recent worldwide survey<sup>[29]</sup> has brought more evidence about this field. Overall donor morbidity rate was 24%, but only 0.2% of them died, and 0.04% required transplantation. If harm to donors is only considered in terms of mortality, its impact on the therapeutic decision (between LDLT, DDLT, or no LT) would be minimal compared to the recipient's risk of death on the WL<sup>[30]</sup>. Quantifying morbidity could be done by determining the impact of complications on quality of life, but limited data is currently available to derive such estimates.

Furthermore, it is controversial whether donor morbidity and mortality should be weighted equally to that of the recipient<sup>[31]</sup>. Currently the transplant community takes a protective approach (paternalist principle) to the living donor, and tends to assign greater ethical weight to the donor's risk of death than to the recipient's risk of death. This approach, however, comes at the expense of donor autonomy. Further thought is needed on this subject, including input from donors themselves.

One interesting proposal is to define a cut-off for acceptable morbidity and mortality from the perspective of the donor<sup>[32,33]</sup>.

## REPRESENTATION OF THE POTENTIAL EQUIPOISE BETWEEN BENEFITS AND HARMS OF TRANSPLANTATION FOR HCC PATIENTS

An ideal selection/allocation process for patients with HCC should consider all aspects of the benefits and harms of LT, and the aim of allocation systems should be to reach a balance between the different principles involved in the selection process.

We have represented this equipoise using a triangle containing vectors (Figure 2): the transplant benefit (life expectancy with LT minus life expectancy without LT) is at the top vertex and the potential harm to the rest of the WL and to the living donor at the bottom vertices. According to this model, transplantation is generally indicated when the transplant benefit exceeds the harm. Then, according to the relative weights of the harm to the WL and donor, the decision will be oriented towards LDLT or DDLT.

The first advantage of this conceptual model is that it includes all ethical principles involved in the LT decision process. The use of transplant benefit satisfies both utility and urgency principles, while the relationship between benefit and harm to the waiting list satisfies equity-the first principle aims to maximize the need of the single



patient, while the second maximizes population total life years<sup>[26]</sup>.

The second advantage of this model is that it considers as different therapeutic procedures DDLT and LDLT. Whenever we used urgency, utility or benefit, these principles taken alone do not distinguish between LDLT and DDLT, so they cannot be used to decide between these different strategies. The indication for LDLT is therefore inevitably the same as for DDLT<sup>[32,33]</sup>, so choosing between the two is difficult. This may partially explain why LDLT has had a limited development in Western countries, especially since the introduction of the MELD<sup>[33]</sup>.

Some authors<sup>[32]</sup> have recently stressed the possibility to consider different indications between LDLT and DDLT based on the consideration that living donor recipients don't compete with other patients on the WL. The same authors proposed a sort of double equipoise model specific for LDLT to balance the donor risk and the recipient benefit<sup>[32]</sup>. Our model has the advantage to be used for both DDLT and LDLT. LDLT has a potentially relevant advantage over DDLT because it only minimally harms the other candidates on the WL: this harm is limited to the risk of the patient needing re-LT after LDLT, which is estimated to be approximately 7%<sup>[34]</sup>, while the risk of liver failure requiring transplantation of the donor is estimated to be 0.04%<sup>[29]</sup>.

This model helps the selection of HCC patients for LT and the choice of the more appropriate transplant procedure (DDLT *vs* LDLT). However, it can not consider some crucial aspects. First of all, in some countries religiosity or cultural aspects are barriers to DDLT<sup>[31]</sup>. As second point, in some recipients of a partial liver from a living donor insufficient liver volume can not be avoided to maintain an adequate donor safety. A small-for-size graft easily causes perioperative complications and results in poor outcomes<sup>[31]</sup>. In summary, although LT is theoretically the best treatment for HCC, it is limited by the realities of perioperative complications, and the shortage of donor organs. Furthermore, the benefit of transplantation is not uniform among patients with HCC; rather, it depends upon the severity of liver disease and the available alternative treatment options. Current systems allocate organs to HCC patients primarily based upon the utility principle, as opposed to the urgency principle which governs allocation to patients with nonmalignant liver disease. Allocating organs by transplant benefit could introduce equity between these patient groups. We propose a triangular equipoise model to help decide between DDLT, LDLT, or alternative therapies.

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## WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

# Review of the pharmacological management of hepatitis B viral infection before and after liver transplantation

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## Abstract

The progress in treatment against hepatitis B virus (HBV) with the development of effective and well tolerated nucleotide analogues (NAs) has improved the outcome of patients with HBV decompensated cirrhosis and has prevented post-transplant HBV recurrence. This review summarizes updated issues related to the management of patients with HBV infection before and after liver transplantation (LT). A literature search using the PubMed/Medline databases and consensus documents was performed. Pre-transplant therapy has been initially based on lamivudine, but entecavir and tenofovir represent the currently recommended first-line NAs for the treatment of patients with HBV decompensated cirrhosis. After LT, the combination of HBV immunoglobulin (HBIG) and NA is considered as the standard of care for prophylaxis against HBV recurrence. The combination of HBIG and lamivudine is related to higher rates of HBV recurrence, compared

to the HBIG and entecavir or tenofovir combination. In HBIG-free prophylactic regimens, entecavir and tenofovir should be the first-line options. The choice of treatment for HBV recurrence depends on prior prophylactic therapy, but entecavir and tenofovir seem to be the most attractive options. Finally, liver grafts from hepatitis B core antibody (anti-HBc) positive donors can be safely used in hepatitis B surface antigen negative, preferentially anti-HBc/anti-hepatitis B surface antibody positive recipients.

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**Key words:** Hepatitis B virus; Liver transplantation; Hepatitis B virus immunoglobulin; Antivirals; Lamivudine; Adefovir; Entecavir; Tenofovir; Telbivudine; Resistance

**Core tip:** In the present review the current knowledge on the management of hepatitis B virus (HBV) infection before and after liver transplantation is updated. There is no doubt that all HBV patients with decompensated cirrhosis should be treated with potent anti-HBV agents with high genetic barrier (*i.e.*, entecavir or tenofovir). After liver transplantation, the combination of HBV immunoglobulin (HBIG) (at least for a certain period) and entecavir or tenofovir currently appears to be the most reasonable approach, while HBIG-free antiviral prophylaxis cannot be excluded in the future, particularly in patients with low risk of recurrence.

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## INTRODUCTION

The development of effective, well tolerated and relatively safe oral antiviral agents [nucleos(t)ide analogues (NAs)] has offered the opportunity for successful management of hepatitis B virus (HBV) related chronic liver disease. However, chronic hepatitis B (CHB) is still associated with increased morbidity and mortality. Currently, it is estimated that more than half a million people die every year due to complications of liver decompensation and hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. Liver transplantation (LT) remains the only hope for many patients with complications of end-stage CHB, mostly HCC<sup>[2,3]</sup>.

The introduction of passive immunoprophylaxis using long-term hepatitis B immune globulin (HBIG) in early 1990s significantly decreased the rates of post-LT HBV recurrence<sup>[4]</sup>. During the last 15 years, the use of NAs has decreased the need for LT due to HBV decompensated cirrhosis and has further improved the outcome of HBV transplant patients<sup>[5]</sup>. NAs have been used either in combination with HBIG or as monotherapy in an effort to further improve the rates of HBV recurrence after LT and/or reduce the need for expensive HBIG preparations<sup>[5]</sup>. The management of hepatitis B surface antigen (HBsAg) positive transplant patients can be divided into the pre-transplant, prophylactic post-transplant and therapeutic post-transplant approach<sup>[6]</sup>. HBV prophylaxis is also required for recipients who receive grafts from anti-hepatitis B core (HBc) positive donors, as they are at risk for *de novo* HBV infection.

## PRE-TRANSPLANT APPROACH

### *Anti-HBV therapy in HBV decompensated cirrhosis*

The aim of antiviral therapy is to reverse or delay complications of cirrhosis and the need for LT, and to decrease the risk of HBV re-infection in those who eventually undergo LT. Currently, there are five oral NAs that have been licensed for the treatment of CHB: three nucleoside (lamivudine, telbivudine, entecavir) and two nucleotide (adefovir dipivoxil and tenofovir disoproxil fumarate) analogues<sup>[7-9]</sup>. NAs target the reverse transcriptase of HBV and achieve inhibition of HBV replication via their incorporation in viral HBV DNA causing DNA chain termination<sup>[7-9]</sup>. Antiviral therapy should be started immediately in patients with HBV decompensated cirrhosis and any level of detectable serum HBV DNA regardless of ALT activity.

Lamivudine was the first NA approved for treatment of CHB and probably remains the most widely used NA worldwide due to its low cost. Its efficacy, at a daily dose of 100 mg, has been confirmed in randomized controlled trials and cohort studies showing stabilization or even improvement of liver function and reduction in the incidence of HCC<sup>[10]</sup> and the need for LT<sup>[11-13]</sup>. However, long-term lamivudine monotherapy is associated with progressively increasing rates of viral resistance due to YMDD mutations (15%-25% at year 1, 65%-80% at year 5), which can lead to clinical deterioration with development of liver failure and even death<sup>[13-15]</sup>. Importantly,

patients with detectable HBV DNA at LT have increased rates of post-transplant recurrence of HBV<sup>[16,17]</sup> and even of pre-existing HCC<sup>[18]</sup>. Thus, lamivudine monotherapy is not currently recommended for patients with HBV decompensated cirrhosis<sup>[7-9]</sup>.

Adefovir was the second NA approved for the treatment of CHB. It is effective against both wild type and lamivudine resistant HBV strains<sup>[3]</sup>. Adefovir at the daily licensed dose of 10 mg improves liver function in patients with HBV decompensated cirrhosis<sup>[19]</sup>. However, its weak potency<sup>[20]</sup>, the moderate risk of resistance during long-term therapy in naive patients (29% at year 5)<sup>[21-23]</sup> and its higher cost have resulted in its replacement by the newer, more effective and cheaper nucleotide analogue, tenofovir, in all countries with tenofovir availability<sup>[19,21]</sup>. Finally, adefovir has been associated with renal adverse events including decline of glomerular filtration rate (GFR) and proximal tubular dysfunction resulting occasionally in Fanconi syndrome<sup>[24,25]</sup>. The potential nephrotoxicity, which seems to be dose dependent<sup>[26]</sup>, is of particular concern in difficult-to-manage patients with decompensated cirrhosis.

Telbivudine is a potent nucleoside analogue<sup>[27]</sup> which achieves satisfactory virological remission rates in CHB patients with undetectable HBV DNA at 24 wk of therapy<sup>[28]</sup>. However, it also selects for mutations in the YMDD motif, but at a lower rate compared to lamivudine [25% vs 40% after 2 years of treatment in hepatitis B e antigen (HBeAg) positive CHB patients]<sup>[3,8,9]</sup>. In a recent randomized trial<sup>[29]</sup> including 232 naive patients with HBV decompensated cirrhosis, telbivudine was well tolerated. In addition, telbivudine, compared to lamivudine, achieved greater viral suppression, similar stabilization of liver function and significant improvement in the estimated GFR<sup>[29]</sup>. The place of telbivudine monotherapy in the treatment of patients with HBV decompensated cirrhosis is unclear due to its unfavourable resistance profile, compared to the newer NAs with high genetic barrier [*i.e.*, entecavir (ETV) and tenofovir (TDF)]. However, its use in a combined regimen may need further evaluation in patients with HBV decompensated cirrhosis due to the potentially favourable effect of telbivudine on renal function<sup>[30]</sup>.

ETV (0.5 mg daily) is a selective anti-HBV agent with potent activity against wild type HBV<sup>[7,31]</sup>. ETV has a high genetic barrier to resistance in naive patients (< 1.5% cumulative rate of viral resistance after 6 years of treatment)<sup>[32,33]</sup> including those with advanced fibrosis or histological cirrhosis<sup>[34]</sup>. Regarding safety, lactic acidosis has been occasionally reported in small cohorts patients with severe liver dysfunction receiving ETV<sup>[35]</sup>. However, its true incidence is unclear, since studies with larger cohorts did not confirm this lethal complication<sup>[2,31]</sup>. In any case, close monitoring is advised for ETV and perhaps any NA treated patient with MELD score  $\geq 20$ . The high efficacy and the minimal resistance rates combined with the lack of significant nephrotoxicity make ETV a first-line option for the treatment of naive patients with HBV decompensated cirrhosis<sup>[36]</sup>. On the other hand, ETV monotherapy even at the licensed dosage of 1 mg daily

**Table 1** Studies of nucleos(tide) analogues in patients with hepatitis B related decompensated cirrhosis

Ref.	Fontana <i>et al</i> <sup>[47]</sup>	Schiff <i>et al</i> <sup>[19]</sup>	Shim <i>et al</i> <sup>[31]</sup>	Liaw <i>et al</i> <sup>[44]</sup>	Chan <i>et al</i> <sup>[29]</sup>	Hyun <i>et al</i> <sup>[49]</sup>
Number of patients	154	226	70	45/45/22	114/114	45/41
NA(s) used	LAM	ADV	ETV	TDF/TDF + FTC/ETV	LdT/LAM	ETV/LAM
Baseline data						
LAM resistance (%)	0	100	0	18/22/14	0/0	0/0
CTP score	9	NR	8.4	7/7/7	8.1/8.5	9.6/9.5
MELD score	NR	NR	11.5	11/13/10.5	14.7/15.5	16.7/16.1
1-yr data						
↓ CTP score ≥ 2 (%)	NR	NR	49	26/48/42	32/39	NR/NR
MELD score ↓	NR	-2	-2.2	-2/-2/-2	-1.0/-2.0	-4.9/-3.7
1-yr survival (%)	84	86	87	96/96/91	94/88	90.7/92.4
Prognostic factors of the outcome	Serum bilirubin and creatinine levels at baseline	NR	NR	NR	NR	Baseline CTP and MELD at 3 mo

ADV: Adefovir; CTP: Child-Turcotte-Pugh; ETV: Entecavir; TDF: Tenofovir; FTC: Emtricitabine; LAM: Lamivudine; LdT: Telbivudine; MELD: Model for end stage liver disease; NR: Not reported; NAs: Nucleos(tide) analogues.

taken ≥ 2 h away from food is not a good option for patients with lamivudine resistance, as HBV resistance develops in approximately 50% of lamivudine resistant patients after five years of ETV treatment<sup>[37,38]</sup>.

TDF is the most recently approved agent for the treatment of CHB. Although it is structurally similar to adefovir, it is more potent with activity against both wild type and nucleoside-resistant HBV strains<sup>[21,39-41]</sup>. It is also active in patients with primary non-response to adefovir<sup>[2]</sup>. To date, there has been no confirmed case of drug resistance in CHB patients treated with TDF for 6 years, although most patients remaining viremic after 72 wk and being therefore at the highest risk for drug resistance received additional treatment with emtricitabine<sup>[42]</sup>. Due to its great potency and high genetic barrier, TDF has a beneficial effect on regression of advanced liver fibrosis<sup>[43]</sup>. Although TDF may be potentially nephrotoxic, similar rates of renal adverse events were observed after one year of therapy with TDF, TDF plus emtricitabine or ETV in patients with HBV decompensated cirrhosis<sup>[44]</sup>.

In conclusion, ETV and TDF are potent antiviral agents with a minimal or even no risk of resistance and therefore they represent the currently recommended first-line NAs for the treatment of patients with HBV decompensated cirrhosis<sup>[5]</sup>. In addition, TDF is the preferred option for patients with lamivudine, ETV or telbivudine resistance, while the use of ETV (even at a higher daily dose of 1.0 mg) is a less attractive option for the long-term treatment of patients with known lamivudine resistant strains<sup>[9]</sup>. Whether a combination of antivirals could offer additional benefits is unknown. Given the current cost of anti-HBV agents, the combination that might have a reasonable cost is that of TDF plus lamivudine or emtricitabine<sup>[5]</sup>. The combination of TDF with emtricitabine was reported not to be significantly superior to TDF or ETV monotherapy<sup>[44]</sup>, but the small numbers of patients in each group of this study cannot allow strong conclusions. Thus, whether any NA combination therapy would confer benefits in patients with impaired renal function who need NA dose reductions or in patients

with very high baseline viral load has not been completely clarified yet. Telbivudine (alone or in a combined regimen) with its potentially favorable effect on glomerular filtration seems to be an attractive option in patients with HBV decompensated cirrhosis and renal dysfunction<sup>[30]</sup>.

### Referral for liver transplantation

Patients with HBV decompensated cirrhosis should be referred for LT, since the relevant criteria are fulfilled in most of these patients with hepatic dysfunction (Child-Pugh score ≥ 7 or MELD score ≥ 10) and/or at least one major complication (ascites, variceal bleeding, hepatic encephalopathy)<sup>[45]</sup>. While waiting for LT, the patients should be monitored carefully at least every 3 mo for virologic response and possible virologic breakthrough in order to achieve serum HBV DNA undetectability using a sensitive polymerase chain reaction assay<sup>[36,46]</sup>. Interestingly, the liver function of patients with HBV decompensated cirrhosis may substantially improve under effective antiviral therapy and LT candidates may be eventually withdrawn from the transplant lists<sup>[47,48]</sup> (Table 1). However, the most important parameters affecting the outcome of patients with HBV decompensated cirrhosis under antiviral agents have not been completely elucidated.

Previous studies using lamivudine monotherapy showed that baseline HBV DNA levels are independently associated with the outcome<sup>[47]</sup>, but in a recent study using a quantitative PCR technique, neither HBV DNA at baseline nor its changes from baseline to 3 mo of treatment were associated with death or LT<sup>[49]</sup>. Most of the studies including patients with HBV decompensated cirrhosis under oral antivirals have shown that the baseline severity of liver disease, expressed by the Child-Pugh score or the baseline bilirubin and creatinine levels, are critical for the outcome<sup>[47,49]</sup> (Table 1). In a prospective multicenter study<sup>[47]</sup> including 154 lamivudine treated patients with HBV decompensated cirrhosis, most of the deaths (78%) occurred within the first 6 mo suggesting that lamivudine may not be able to reduce the short-term mortality or the need for LT in patients with very



advanced liver failure. In contrast, initiation of antiviral therapy at earlier stages is associated with better chances of liver function recovery, since clinical benefit may take 3-6 mo. Whether these results are still valid with the current more potent anti-HBV agents is not clear, but this might be still the case as patients with very advanced liver failure may not benefit from antiviral therapy regardless of the rapidity of the inhibition of viral replication<sup>[2]</sup>. Nevertheless, further well designed large studies with longer follow-up are needed for final conclusions (Table 1).

## PROPHYLACTIC POST-TRANSPLANT APPROACH

### *Hepatitis B immune globulin*

HBIG is a polyclonal antibody to HBsAg derived from pooled human plasma<sup>[50]</sup>. Its mechanism of action is not completely understood, but it possibly acts by binding with circulating viral particles preventing hepatocyte infection<sup>[50]</sup>. It also seems to undergo endocytosis by hepatocytes decreasing HBsAg secretion<sup>[50]</sup>. HBIG was introduced in the early nineties leading to reduction in the rates of post-transplant HBV recurrence<sup>[4]</sup>. In the landmark study by Samuel *et al*<sup>[4]</sup> in 1991, it was shown that HBV recurrence could be prevented in 80% of transplant patients treated with HBIG. Prior to the availability of NAs, the initial anti-HBV prophylaxis included administration of high dosage HBIG monoprophyllaxis at the anhepatic phase followed by daily doses and then monthly at a fixed dose or according to anti-HBs titers (usually aiming to maintain anti-HBs titers > 100-500 IU/L)<sup>[51-53]</sup>. However, protocols that use high doses of HBIG are expensive (estimated cost at least \$50000-70000 for the first year and \$25-40000 for each additional year post-transplant)<sup>[54]</sup>. Additional limitations of HBIG include the unreliable supply, the parenteral administration, the local or systemic side effects and the risk of infection from HBV mutants that escaped from neutralization<sup>[50]</sup>.

The use of HBIG monoprophyllaxis was abandoned after the introduction of lamivudine and the more recent and potent NAs<sup>[55]</sup>. Nowadays, the most commonly used protocol includes the combination of a NA with a low dose of HBIG<sup>[5,55]</sup>. Several efforts have tried to reduce the cost using HBIG in lower dosage or preparations for intramuscular administration, which have similar pharmacokinetic properties with intravenous preparations<sup>[56]</sup>, or subcutaneous HBIG<sup>[57]</sup>. Another strategy has been the substitution of HBIG with HBV vaccination. However, results on the efficacy of active vaccination using new vaccines and adjuvants are rather conflicting<sup>[58-60]</sup>, and therefore, further studies with greater numbers of patients and longer follow-up periods are required before definite conclusions can be drawn.

### *Prophylactic post-transplant combined approach*

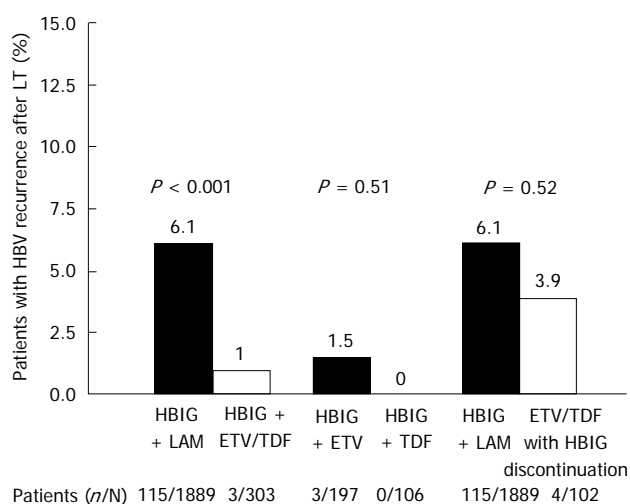
Currently, the combination of HBIG and NA is considered the standard of care against HBV recurrence after LT<sup>[5]</sup>. This combined regimen relies on the complimen-

tary mechanisms of action of HBIG and NA<sup>[55]</sup>. A recent meta-analysis of 6 studies showed that HBIG plus lamivudine, compared to HBIG alone, was associated with 12-fold, 12-fold and 5-fold reduction of HBV recurrence, HBV-related death and all-cause post-transplant mortality, respectively<sup>[61]</sup>. A second meta-analysis also showed that the combination of HBIG and lamivudine was superior in preventing only serum HBsAg re-appearance, compared to lamivudine alone<sup>[62]</sup>. However, lamivudine is not considered an optimal first-line option because of the progressively increasing rates of viral resistance<sup>[5,63]</sup>. This was confirmed in our systematic review<sup>[55]</sup> including 2162 HBV liver transplant recipients from 46 studies. In this review, we found that the patients under HBIG and lamivudine, compared to those under HBIG and adefovir (with or without lamivudine) had HBV recurrence more frequently (6.1% *vs* 2%,  $P = 0.024$ ), although they had detectable HBV DNA less frequently at the time of LT (39% *vs* 70%,  $P < 0.001$ ).

Although several questions about the ideal duration, dosage, frequency and mode of HBIG administration remain unanswered<sup>[55]</sup>, we found that patients under HBIG and lamivudine who received high ( $\geq 10000$  IU/d) dosage of HBIG, compared to those who received low HBIG dosage ( $< 10000$  IU/d) during the 1<sup>st</sup> wk post-LT, had significantly less frequent HBV recurrences (3.3% *vs* 6.5%,  $P = 0.016$ ). On the other hand, HBIG administration had no impact on HBV recurrence in patients under HBIG and adefovir. Based on these findings<sup>[55]</sup>, we concluded that the patients under HBIG and lamivudine combination prophylaxis should receive high HBIG dosage (10000 IU IV) for the first week after LT, while the characteristics of the HBIG protocol do not seem to have any impact on the efficacy of HBIG and adefovir combination prophylaxis against HBV recurrence.

Adefovir has several drawbacks in the post-transplant setting including high cost, relatively low potency in the licensed 10 mg daily dose, risk of viral resistance and risk of nephrotoxicity<sup>[5]</sup>. The latter is of particular concern in liver transplant recipients because most of them receive nephrotoxic calcineurin inhibitors as part of an immunosuppressive regimen and frequently suffer from diabetes mellitus and arterial hypertension.

Newer and more potent NAs with a higher genetic barrier, such as ETV and TDF, are currently used in the post-transplant period in many transplant centers, mainly in an effort to increase the efficacy of post-LT prophylaxis and/or reduce the need for the expensive HBIG preparations at least after the initial post-operative period<sup>[5]</sup>. The efficacy of ETV and TDF was evaluated in our recently published systematic review including 519 HBV liver transplant recipients from 17 studies<sup>[64]</sup>. We found that patients under HBIG and lamivudine developed HBV recurrence significantly more frequently, compared to patients under HBIG and ETV or TDF combination (6.1% *vs* 1.0%,  $P < 0.001$ ) (Figure 1), although they received a more intense HBIG protocol after LT<sup>[64]</sup>. In addition, ETV and TDF had similar antiviral efficacy when



**Figure 1** Risk of recurrence of hepatitis B virus infection after liver transplantation in relation to the type of post-transplant hepatitis B virus prophylaxis<sup>[64]</sup>. HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine; ETV: Entecavir; TDF: Tenofovir; LT: Liver transplantation.

they combined with HBIG (1.5% *vs* 0%, respectively,  $P > 0.05$ )<sup>[64]</sup> (Figure 1).

Given the several limitations of HBIG and the fact that waiting list patients are more likely to undergo LT with undetectable HBV DNA, one relatively recent strategy has been the use of HBIG for a limited post-transplant period followed by long-term NA therapy alone<sup>[64]</sup>. The first results published with lamivudine monoprophyllaxis after HBIG withdrawal were encouraging<sup>[65,66]</sup>, but longer follow-up showed that 20% of patients eventually experienced recurrence of HBV<sup>[65,67]</sup>. ETV and TDF, however, may allow early and safe discontinuation of HBIG. Strong data are not available, but our systematic review<sup>[64]</sup> showed that ETV or TDF monoprophyllaxis after HBIG discontinuation does not seem to be inferior to the combination of a newer NA with HBIG or the combination of HBIG plus lamivudine (3.9% *vs* 1.0%, 3.9% *vs* 6.1%,  $P > 0.05$ ) (Figure 1). Although larger studies with longer follow-up are needed for definitive conclusions, this approach has been already used in several transplant centres, particularly in patients with relatively low risk of HBV recurrence<sup>[68]</sup>.

### Prophylactic post-transplant monotherapy with nucleos(t)ides analogues

The high efficacy of antiviral prophylaxis using a shorter course of HBIG with continuation of NA without HBIG, and the availability of NAs without cross-resistance in cases of prophylaxis failure, led to the consideration of HBIG-free prophylactic regimens. This approach, which is challenging and controversial, started with lamivudine, but the unacceptably high rates of HBV recurrence (up to 35%-50% of cases at 2 years post-transplant)<sup>[69-74]</sup> has rendered this approach suboptimal. However, recent studies have renewed the interest in HBIG-free prophylactic regimens using the more potent regimens with high genetic barrier ETV and TDF.

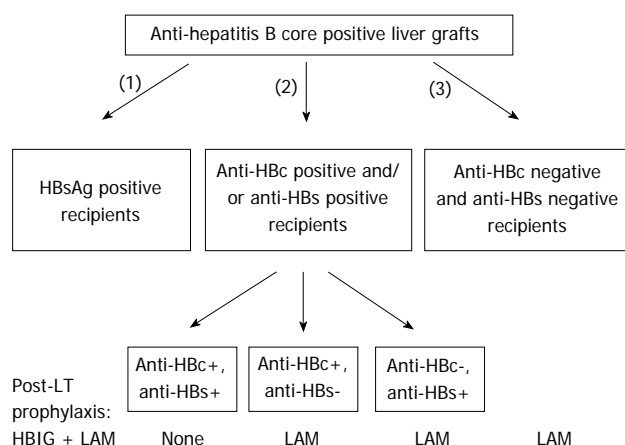
Recently, Fung *et al.*<sup>[75]</sup> evaluated 80 consecutive patients transplanted for HBV-related liver disease. Fifty nine (74%) of the patients had detectable HBV DNA at the time of LT, and all patients received ETV monoprophyllaxis without HBIG at any time point after LT. After a median follow-up of 26 mo, 18 (22.5%) patients were HBsAg positive, but only one of them had detectable HBV DNA<sup>[75]</sup>. In their subsequent study<sup>[76]</sup> including 362 transplant recipients under HBIG-free prophylaxis, none of the patients who receive ETV had HBV recurrence, compared to 17% of those who received lamivudine, highlighting the importance of using potent regimens with a high genetic barrier (ETV or TDF) in HBIG-free prophylaxis protocols.

In our recent systematic review<sup>[64]</sup>, HBV recurrence was observed significantly more frequently in patients who received ETV or TDF HBIG-free prophylaxis, compared to patients under combination of HBIG and lamivudine prophylaxis, if the definition of HBV recurrence was based on HBsAg positivity (26% *vs* 5.9%,  $P < 0.0001$ ). However, if the definition of HBV recurrence was based on HBV DNA detectability, the rates of HBV recurrence were similar between the two groups (0.9% *vs* 3.8%,  $P = 0.11$ )<sup>[64]</sup>. Given the current availability of potent NAs with negligible risk of long-term viral resistance, the clinical significance of HBsAg seropositivity in HBV transplant patients is unclear<sup>[68]</sup>. The prognosis of non-transplant CHB patients who maintain HBV DNA undetectability under NA(s) is excellent, particularly if they had not developed cirrhosis before treatment<sup>[77]</sup>, but the long-term outcome of HBsAg-positive, HBV DNA negative transplant patients under NAs needs further evaluation. In a recent study<sup>[78]</sup>, 5 (20%) of 25 HBV transplant patients who discontinued anti-HBV prophylaxis became HBsAg-positive, but none of them experienced any clinically relevant event and three eventually cleared HBsAg and achieved seroconversion to anti-HBs without any therapeutic intervention.

Currently, ETV and TDF should be the first-line options for HBIG-free prophylaxis. ETV may be avoided in patients with previous lamivudine resistance, who should be preferably treated with TDF. Compliance is always an issue with long-term oral antiviral therapy, particularly in prophylaxis after LT when patients feel well but remain at life-long risk of HBV recurrence<sup>[64]</sup>. Until well designed studies determine the optimal monoprophyllaxis approach, the combination of HBIG (at least for a short period) and one nucleos(t)ide appears to be the most reasonable post-transplant approach. Monoprophyllaxis with the new nucleos(t)ides analogues cannot be excluded in the future, particularly in patients with low risk of recurrence<sup>[68]</sup>.

## THERAPEUTIC POST-TRANSPLANT APPROACH

Recurrence of HBV infection after LT is usually characterized by reappearance of serum HBsAg and/or serum



**Figure 2** Proposed algorithm for allocation and management of anti-hepatitis B core positive liver grafts. Such grafts should be first offered to hepatitis B surface antigen positive, then to anti-hepatitis B core (HBc) and/or anti-hepatitis B surface (HBs) positive and lastly to hepatitis B virus naive (both anti-HBc and anti-HBs negative) recipients<sup>[79]</sup>. LT: Liver transplantation; HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine.

HBV DNA, which is frequently accompanied with biochemical or clinical evidence of recurrent liver disease. As mentioned before, particularly in patients under HBIG-free post-LT HBV prophylaxis, the definition of HBV recurrence might be reconsidered, as HBsAg seropositivity, usually in low titers, with undetectable HBV DNA, normal liver enzymes and no clinical manifestations of HBV recurrence may not have any clinical impact on the long-term graft and patient survival.

The choice of treatment for HBV recurrence depends on prior prophylactic therapy. In general, the principles of treatment in post-transplant HBV recurrence resemble those in the pre-transplant setting. ETV may be preferred in NA-naïve patients because of the lack of nephrotoxicity, although in a recent study there was no difference in renal complications between ETV and TDF in liver transplant recipients<sup>[68]</sup>. In patients with prior lamivudine resistance, TDF is the best choice<sup>[37]</sup>. Little is known about the efficacy and safety of the combination of TDF and ETV which might be used in patients with multidrug resistant HBV strains.

## ANTI-HBC POSITIVE DONORS

The current efforts to overcome the organ shortage include the use of marginal liver grafts, such as those from anti-HBc positive donors. This source of organs can be of particular importance in countries with high prevalence of HBV infection, such as the Mediterranean area and Asia. HBsAg positive liver patients are the optimal recipients to receive liver grafts from anti-HBc positive donors. Unfortunately, the “occult” HBV infection in the donor liver may be reactivated in the HBsAg negative recipient due to post-LT immunosuppressive therapy leading to *de novo* HBV infection. In our systematic review<sup>[79]</sup> including 903 recipients of anti-HBc positive liver grafts, *de novo* HBV infection developed in 19% of HBsAg nega-

tive recipients being less frequent in anti-HBc/anti-HBs positive than HBV naive cases without prophylaxis (15% *vs* 48%,  $P < 0.001$ ). Anti-HBV prophylaxis reduced *de novo* infection rates in both anti-HBc/anti-HBs positive (3%) and HBV naive recipients (12%)<sup>[79]</sup>. *De novo* HBV infection rates were 19%, 2.6% and 2.8% in HBsAg-negative recipients under HBIG, lamivudine and their combination, respectively. Based on these findings<sup>[79]</sup>, we concluded that liver grafts from anti-HBc positive donors can be safely used in HBsAg negative recipients, preferentially in anti-HBc/anti-HBs positive recipients who may need no prophylaxis at all, while the anti-HBc and/or anti-HBs negative recipients should receive long-term prophylaxis with lamivudine (Figure 2).

## CONCLUSION

Over the last two decade, the progress in anti-HBV therapy has led to great improvements in the management of HBV patients before and after LT. There is no doubt that all HBV patients with decompensated cirrhosis should be treated with a potent antiviral agent with minimal or no risk of resistance, *i.e.* ETV or TDF. In addition, TDF is the preferred option for patients with prior lamivudine, ETV or telbivudine resistance. An effective pre-transplant anti-HBV therapy often stabilizes or even improves the underlying liver disease resulting sometimes in withdrawals from the transplant list. In addition, achievement of serum HBV DNA undetectability prevents post-transplant HBV recurrence. After LT, the combination of HBIG (at least for a certain period) and one NA (ETV or TDF) currently appears to be the most reasonable prophylaxis, while monoprophyllaxis with ETV or TDF cannot be excluded in the future, particularly in patients with low risk of recurrence. Depending on previous drug exposure and possible pre-existing resistance mutations, ETV or TDF seem to be the most attractive options for post-LT HBV recurrence as well. Finally, liver grafts from anti-HBc positive donors can be safely used in HBsAg negative, preferentially anti-HBc/anti-HBs positive recipients.

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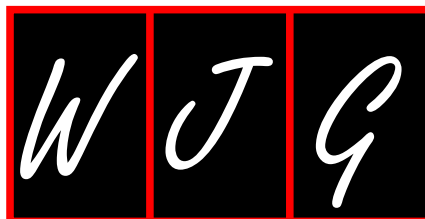


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## WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

# Long-term survival after liver transplantation for alcoholic liver disease

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## Abstract

Currently, alcoholic cirrhosis is the second leading indication for liver transplantation in the United States and Europe. The quality of life and survival after a liver transplantation (LT) in patients with alcoholic liver disease (ALD) are similar to those in patients with other cirrhosis etiologies. The alcoholic relapse rate after a LT varies from 10%-50%, and these relapse patients are the ones who present a reduced long-term survival, mainly due to cardiovascular diseases and the onset of *de novo* neoplasms, including lung and upper aerodigestive tract. Nearly 40% of ALD recipients resume smoking and resume it early post-LT. Therefore, our pre-and post-LT follow-up efforts regarding ALD should be focused not only on alcoholic relapse but also on treating and avoiding other modifiable risk factors such as tobacco. The psychiatric and psychosocial pre-LT evaluation and the post-LT follow-up with physicians, psychiatrists and addiction specialists are important for reversing these problems because these professionals help to identify patients at risk for relapse as well as those patients who have relapsed, thus enabling responsive actions.

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**Key words:** Alcoholic liver disease; Alcohol recidivism; Alcohol relapse prevention; Long term survival; Liver transplantation

**Core tip:** Transplanted alcoholic liver disease (ALD) patients who relapse have an increased long-term mortality due to cardiovascular pathologies and the onset of *de novo* neoplasms, including lung and upper aerodigestive tract cancer. Nearly 40% of ALD recipients resume smoking and resume it early post-liver transplantation (LT). Therefore, our pre-and post-LT follow-up efforts regarding alcoholic liver disease should be focused not only on alcoholic relapse but also on treating and avoiding other modifiable risk factors such as tobacco. The psychiatric and psychosocial pre-LT evaluation and the post-LT follow-up with physicians, psychiatrists and addiction specialists are important for reversing these problems.

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## INTRODUCTION

Excessive alcohol consumption causes approximately 2.5 million deaths per year and is responsible for almost 4% of mortality worldwide. Alcohol has been associated with nearly 60 types of diseases and is the third leading risk factor for disease and disability worldwide. Furthermore, excessive alcohol consumption contributes to multiple social problems, including violence, child neglect and ab-



**Table 1 Primary indication for liver transplantation in Europe and the corresponding survival**

Indication for LT	Patients (n)	From 1988 to 2009		
		1 yr	5 yr	10 yr
Alcoholic cirrhosis	15019	86%	73%	59%
Acute hepatic failure	6507	70%	64%	58%
Cirrhosis virus C	10753	80%	65%	53%
Cirrhosis viral C and alcoholic	1790	85%	69%	54%
Cirrhosis virus B	4187	83%	74%	68%
Hepatocarcinoma and cirrhosis	9122	83%	62%	49%
Cholestatic disease	9114	87%	78%	70%
Autoimmune cirrhosis	1892	85%	76%	67%
Hemochromatosis	468	76%	66%	53%

Adapted from the European Liver Transplant Registry<sup>[3]</sup>. LT: Liver transplantation.

sentecism<sup>[1]</sup>.

Alcoholic liver disease (ALD) is the main cause of cirrhosis in Western countries and contributes to one third of the mortality associated with liver cirrhosis. Furthermore, ALD is the second most common indication for liver transplantation (LT) in the United States and Western Europe<sup>[2,7]</sup>, accounting for about 40% of transplants in Europe and 20% of transplants in the United States<sup>[2,7]</sup>.

If we analyze the medium- and long-term survival of a transplant patient, there is no doubt that the recipients with an alcoholic etiology have great results, with a European global 5-year survival rate of 73% and a 10-year survival rate of 59%<sup>[2,3]</sup>, rates that are superior to those for recipients with other etiologies (Table 1)<sup>[3]</sup>. Therefore, we can infer that ALD is a good indication for LT<sup>[7]</sup>. However, these excellent results are diminished when a harmful alcohol consumption relapse occurs. These relapses and their possible consequences on the transplant and on the survival and quality of life of the patient, as well as possible actions to avoid relapses, are discussed below.

An evidence-based approach was used for this review. MEDLINE search was performed to September 2013 using the following MeSH terms: liver transplantation, alcohol-related disorders, alcohol-induced disorders, drug abuse, substance abuse, tobacco, and neoplasm. Searches were limited to English language articles. References of suitable articles were searched for other appropriate articles.

## QUALITY OF LIFE AFTER LT

Quality of life involves physical, mental and social well-being, including working life, and is considered a survival indicator that even surpasses traditional indicators<sup>[8]</sup>. In the cirrhotic patient, a decrease in physical, psychological and intellectual capabilities occurs alongside liver function impairment<sup>[9]</sup>. Therefore, it is logical to believe that those patients with advanced liver disease might have a significant reduction in their quality of life. Therefore, the question we might ask is the following: does LT im-

prove the quality of life in these patients? To answer this question, numerous studies that evaluated the quality of life after LT have been performed<sup>[10-17]</sup>. However, it is not easy to extrapolate the results of these studies due to the heterogeneity of the post-transplantation follow-up times and the instruments that were used to evaluate the different spheres comprising the quality of life<sup>[10-17]</sup>. In general, studies reveal a significant short-term improvement in the quality of life with no differences observed between ALD and non-alcoholic liver disease<sup>[10-18]</sup>. Notably, although ALD patients seem less likely to be involved in structured social activities during the post-LT phase than the patients who were transplanted as a result of other etiologies, the ALD patients return to society to lead active and productive lives<sup>[19]</sup>. Few studies analyzed the quality of life long-term. As a representative study, the study by Ruppert *et al*<sup>[17]</sup>, that included a 12-year follow-up after LT does not show a progressive loss of quality of life in these patients after the first year of LT<sup>[17]</sup>.

Regarding job reinsertion, the age at the time of the LT, the duration of the pre-transplant disability and the physical and general health status of the patient are the factors that correlate more with employment<sup>[14,20-22]</sup>. Globally, approximately half of the LT patients return to work<sup>[20-22]</sup>, with no differences between the ALD patients and those with the remaining etiologies<sup>[15,23]</sup>.

## ALCOHOLIC RELAPSE/RECIDIVISM AFTER LT

We must recall that, although LT effectively restores the physiological function of the liver and reverses the complications of portal hypertension, LT does not treat the underlying alcoholism. Alcoholism is a life-long disease that is often characterized by episodes of a relapsing-remitting pattern of alcohol use despite the physical, psychological and social consequences, wherein the probability of long-term sobriety becomes robust only after 5 years of sustained abstinence<sup>[24-26]</sup>.

### Dimension of the problem

Addiction specialists define relapse as the prolonged resumption of heavy alcohol intake and distinguish this harmful drinking behavior from so-called slips, which are defined as sporadic drinking episodes followed by the reestablishment of abstinence<sup>[27]</sup>. This definition of alcoholic relapse is in contrast to that by most transplant centers that consider any alcohol consumption after LT to be unacceptable and define recidivism as any use of alcohol after LT<sup>[28]</sup>. Most of these episodes of alcohol abuse are effectively diagnosed with interviews and validated self-reporting questionnaires<sup>[29,30]</sup>.

Reviews summarizing the post-transplantation alcoholic relapse rates note differences across studies ranging from 10%-95%, likely due to several factors, including variations in the study methodology, the definition and assessment of relapse and the duration of the follow-up (Table 2)<sup>[28,31-60]</sup>. In general, the risk that alcoholic



**Table 2** Alcohol relapse (any use) after liver transplantation for alcohol liver disease

Ref.	Study design	Patients (n)	Year	Follow-up median or mean (mo)	Relapse rate
Bird <i>et al</i> <sup>[34]</sup>	Retrospective	18	1990	84	17%
Kumar <i>et al</i> <sup>[48]</sup>	Retrospective	52	1990	25	12%
Gish <i>et al</i> <sup>[42]</sup>	Prospective	29	1993	24	24%
Knechtle <i>et al</i> <sup>[38]</sup>	Retrospective	32	1993	Not stated	13%
Berlakovich <i>et al</i> <sup>[22]</sup>	Retrospective	44	1994	78	32%
Howard <i>et al</i> <sup>[45]</sup>	Retrospective	20	1994	43	95%
Krom <i>et al</i> <sup>[48]</sup>	Retrospective	30	1994	Not stated	13%
Osorio <i>et al</i> <sup>[51]</sup>	Retrospective	43	1994	21	19%
Gerhardt <i>et al</i> <sup>[41]</sup>	Retrospective	41	1996	47	49%
Tringali <i>et al</i> <sup>[56]</sup>	Retrospective	58	1996	27	21%
Zibari <i>et al</i> <sup>[58]</sup>	Retrospective	29	1996	Not stated	7%
Coffman <i>et al</i> <sup>[84]</sup>	Prospective	91	1997	Not stated	20%
Anand <i>et al</i> <sup>[31]</sup>	Retrospective	39	1997	25	13%
Everson <i>et al</i> <sup>[38]</sup>	Retrospective	42	1997	Not stated	17%
Foster <i>et al</i> <sup>[40]</sup>	Retrospective	63	1997	49	21%
Lucey <i>et al</i> <sup>[50]</sup>	Retrospective	50	1997	63	34%
Stefanini <i>et al</i> <sup>[53]</sup>	Retrospective	18	1997	Not stated	27%
Fabrega <i>et al</i> <sup>[39]</sup>	Prospective	44	1998	40	18%
Tang <i>et al</i> <sup>[54]</sup>	Retrospective	56	1998	24	50%
Yates <i>et al</i> <sup>[57]</sup>	Retrospective	43	1998	21	19%
Gledhill <i>et al</i> <sup>[44]</sup>	Retrospective	24	1999	14	25%
Pageaux <i>et al</i> <sup>[75]</sup>	Retrospective	53	1999	32	42%
Pereira <i>et al</i> <sup>[13]</sup>	Retrospective	56	2000	30	50%
Burra <i>et al</i> <sup>[73]</sup>	Prospective	34	2000	40	33%
Jain <i>et al</i> <sup>[29]</sup>	Retrospective	185	2000	94	20%
Dimartini <i>et al</i> <sup>[37]</sup>	Prospective	36	2001	12	38%
Gish <i>et al</i> <sup>[43]</sup>	Prospective	61	2001	83	20%
Mackie <i>et al</i> <sup>[28]</sup>	Retrospective	46	2001	25	53%
Bellamy <i>et al</i> <sup>[32]</sup>	Retrospective	123	2001	84	13%
Karman <i>et al</i> <sup>[57]</sup>	Retrospective	49	2001	36	21%
Bravata <i>et al</i> <sup>[93]</sup>	Retrospective	313	2001	Not stated	32%
Pageaux <i>et al</i> <sup>[52]</sup>	Retrospective	128	2003	54	31%
Jauhar <i>et al</i> <sup>[86]</sup>	Retrospective	111	2004	44	15%
Cuadrado <i>et al</i> <sup>[36]</sup>	Retrospective	54	2005	99	26%
Bjornsson <i>et al</i> <sup>[35]</sup>	Retrospective	103	2005	31	33%
Kelly <i>et al</i> <sup>[85]</sup>	Retrospective	90	2006	67	31%
Pfizman <i>et al</i> <sup>[83]</sup>	Retrospective	300	2007	89	19%
Karim <i>et al</i> <sup>[91]</sup>	Retrospective	80	2010	Not stated	10%
Schmeding <i>et al</i> <sup>[60]</sup>	Retrospective	300	2011	84	27%
Rice <i>et al</i> <sup>[74]</sup>	Retrospective	300	2013	78	16%

recipients return to any alcohol use after LT is between 10%-50% with 8-year follow-ups<sup>[28,31-60]</sup>. More specifically, between 20 and 50% of the patients who received a liver transplant for end-stage ALD acknowledge some alcohol use in the first 5 years after LT, and 10%-15% will resume heavy drinking<sup>[28,55,59]</sup>. This finding compares favorably to post-treatment relapse rates as high as 80%-95% in treatment studies of alcoholics without ALD<sup>[24]</sup>.

In a meta-analysis performed in 2008 on the risk of recurrence of substance use after solid organ transplantation that included 54 studies, 50 of which were on LT, it was concluded that the relapse rate of alcohol consumption after LT was 5.6 cases per 100 patients/year, and the relapse rate of excessive consumption was 2.5 cases per 100 patients/year<sup>[27]</sup>. Additionally, the authors concluded that it was possible that these cumulative incidence rates would become stable at some point that could not be established because few of the studies had a post-transplant

follow-up over 7-8 years<sup>[27]</sup>.

Being able to determine the threshold of initiation of alcohol consumption after a liver transplant would be of great clinical and therapeutic utility because this knowledge would allow us to plan specific interventions more accurately. DiMartini *et al*<sup>[61]</sup> have described four different patterns of alcohol consumption depending on the starting date, quantity and duration as follows: (1) Minimum consumption over a long period; (2) Early consumption that progresses rapidly to moderate consumption; (3) Early consumption that progresses continuously to a harmful consumption; and (4) Moderate consumption with a late start. These results indicate that we should maintain surveillance after the first year post-LT, despite the fact that the rates for the initiation of consumption generally attenuate over time post-transplantation, probably due to the increase in the stability of sobriety over time<sup>[30]</sup>.

### Consequences

The impact of alcohol use on the patient is not entirely clear. The available literature suggests that abusive drinking leads to a decrease in both graft and patient survival and may also lead to the lack of therapeutic compliance.

**Adherence to immunosuppressant medication:** In LT, adherence to the immunosuppressant treatment and any other drugs medically prescribed is crucial for positive short- and long-term results in the transplanted patients because non-adherence to these measures might lead to graft rejection and failure<sup>[62]</sup>. Reviews summarizing the nonadherence rates post-transplantation note differences across studies ranging from 3%-47%, probably due to several factors, including variations in study methodology, definitions and the small number of patients included in the studies<sup>[63-65]</sup>.

Therefore, we question whether the consumption or abuse of substances pre-LT increases the risk of non-adherence to immunosuppressant treatment<sup>[63,66]</sup> and whether an alcoholic relapse is associated with pre-LT alcohol use<sup>[36,62,63]</sup>. Berlakovich *et al*<sup>[63]</sup> studied the effect of alcohol consumption on adherence and found that the patients who relapsed (15 of the 118 transplanted ALD patients) had a non-adherence rate that was no different from that of the patients who did not relapse. This finding was also demonstrated in a study performed in our hospital, where there was no association between the adherence to drug treatment and the presence or absence of alcoholic relapse in a series of transplanted ALD patients<sup>[36]</sup>. We believe that this concept is endorsed by the meta-analysis of Dew *et al*<sup>[27]</sup>, which showed a lack of association. Specifically, these authors observed that European studies presented a lower non-adherence rate to immunosuppressant treatment compared to the North American studies, despite presenting significantly higher relapse rates of harmful alcohol consumption<sup>[27]</sup>.

Thus, the lack of adherence seems to be linked to the personality of the patient, the acknowledgement of their

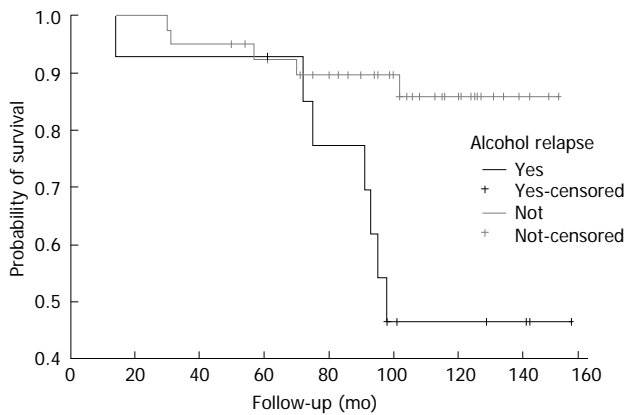


Figure 1 Kaplan-Meier survival curves from patients with alcoholic liver diseases, with or without alcohol recidivism<sup>[36]</sup>.

disease, the complexity of the medical prescriptions, the presence of family support and the doctor-patient relationship, more so more than to alcohol consumption<sup>[67,68]</sup>.

**Liver graft:** Resuming alcohol consumption after LT may damage the graft because of poor compliance with immunosuppressive drug treatment and alcohol-related liver injury. Graft loss from recurrent disease related to alcohol use is rare<sup>[69,70]</sup>. Globally, graft dysfunction related to relapse ranges from 0%-17%, although deaths related to relapse range from 0%-5%<sup>[52,71]</sup>. There are few studies on the severity of the liver lesions associated with alcohol consumption after LT on ALD patients<sup>[60,72-74]</sup>. Rice *et al*<sup>[74]</sup> found that alcohol relapse is associated with advanced fibrosis on biopsy. In contrast, our histologic study revealed only mild hepatic changes directly attributable to alcohol<sup>[36]</sup>. As reported by Pageaux *et al*<sup>[52,75]</sup>, fatty changes and pericellular fibrosis represented the most relevant histological findings in patients who resumed heavy alcohol intake.

In contrast, several studies have shown that ALD LT patients have a lower rejection risk compared to other LT indications, suggesting an inhibitory effect of alcohol over some components of the immune response<sup>[2,76-78]</sup>. We have corroborated this finding and observed a lower incidence of acute rejection among patients who relapse to alcohol consumption compared to abstemious patients<sup>[36]</sup>.

**Long-term survival:** Jain *et al*<sup>[79]</sup> observed that the 5-year post-transplantation survival rate was significantly lower for transplanted ALD patients compared to transplanted non-alcoholic liver disease patients, mainly due to cardiovascular events and *de novo* neoplasms, especially of the aerodigestive tract, which suggests that immunosuppression by itself is not an initiation factor for malignant changes<sup>[80,81]</sup>. We reached a similar conclusion after we evaluated the alcoholic relapse risk in a series of transplanted ALD patients and the influence of a relapse on survival<sup>[35]</sup>. In our case, the 5-year survival rate was similar between relapsers and non-relapsers (92.9% *vs* 92.4%, respectively), but after 10 years, the survival rate decreased

significantly in the relapse patients (45.1% *vs* 85.5%), with malignant tumors and cardiovascular events the main cause of death in these patients (Figure 1)<sup>[36]</sup>. In addition, tobacco consumption was observed in all the patients with an alcoholic relapse and in only one quarter of the abstemious patients, which might explain the higher mortality rate due to cardiovascular events and neoplasms in these patients; this finding has been observed in other studies, as will be discussed later. However, the transplanted ALD patients are potentially affected not only by alcohol consumption but also by liver diseases with other etiologies. This finding has been shown in a recent study in which excessive alcohol consumption had a negative impact on long-term survival after LT regardless of the indication<sup>[82]</sup>.

Despite these results, it is important to distinguish from among the relapsers those who are “slip” drinkers (mild alcohol consumption that is usually isolated or self-limited) and those who are “heavy” drinkers (a long period of alcohol consumption with a loss of control) because the former have a better survival rate compared to the latter<sup>[83]</sup>.

## IDENTIFICATION OF THESE PATIENTS

Because of the above findings, it is important to identify relapse patients, but it is more important to prevent this relapse by identifying the patients at risk.

In order to predict the post-LT alcoholic relapse risk with a high degree of accuracy, it is necessary to acknowledge the risk factors that have a strong correlation, which has not yet been achieved. In this regard, numerous studies have identified factors related to the risk of post-LT alcoholic relapse, such as alcohol dependence, an age less than 40 years at the time of the transplantation, a lack of family and social support, a family history of alcoholism, personality or psychiatric disorders, previous abstinence or substance abuse failures, younger age at LT, and the refusal of further rehabilitation before the LT<sup>[30,37,40,42,43,58,78,83-87]</sup>. However, this association has not yet been corroborated in other studies<sup>[26,30,37,54,75,84-86]</sup>. For this reason, Kotlyar *et al*<sup>[88]</sup> decided to perform a critical review of the literature on LT in ALD candidates and concluded that patients with a lack of social support, active smoking, psychotic or personality disorders or a pattern of nonadherence should be listed only with reservation, and those who have a diagnosis of alcohol abuse as opposed to alcohol dependence may make better transplant candidates. Finally, the most controversial among these risk factors is the 6-mo pre-LT period of abstinence, about which many studies have reported a high predictive power regarding relapse<sup>[27,30,34,89-91]</sup>, while others have not found such a correlation<sup>[40,79,85,86,88,92]</sup>.

Most LT centers in Europe and the United States require a minimum of 6 mo of abstinence before being included in the waiting list. This common practice is based on two points: first, the possibility of improving liver function and possibly avoiding the LT, and second,

the higher alcoholic relapse rate reported in patients with a period of abstinence less than 6 mo<sup>[51,93]</sup>. Both points have been discussed. Veldt *et al*<sup>[94]</sup> demonstrated that those with irreversible ALD were identified with 3 mo of abstinence; out of 74 patients with a Child-Pugh C liver function, the percentage of patients with improvement after 1, 2 and 3 mo of abstinence was 23%, 40% and 66%, respectively, and the remaining 33% did not show improvement at a 1-year follow-up. Furthermore, it has not been proven that this 6-mo abstinence period improves survival after LT<sup>[95]</sup>. Considering all of this, and although improved post-LT abstinence rates have been documented with a longer pre-LT abstinence period, a cut-off point has not yet been established<sup>[30,40]</sup>. Therefore, the pre-LT alcohol abstinence period could be shortened for some patients because this factor by itself is a poor indicator of post-LT relapse. In addition, some patients, especially those with a high Model for End-Stage Liver Disease score, have a considerable risk of mortality during the 6 mo abstinence period<sup>[96,97]</sup>.

In conclusion, a thorough assessment by a trained alcoholism and addiction professional, rather than defined sobriety periods, should be the tool used to assess the future risk of alcoholic relapse in the alcoholic patient.

## ALCOHOL AND TOBACCO

Patients who undergo LT have an unexpectedly high rate of *de novo* extrahepatic cancer<sup>[98,99]</sup>, including lung and upper aerodigestive tract cancer<sup>[98,100,101]</sup>, and studies have reported that patients with ALD are particularly affected<sup>[79,99,102]</sup>. Indeed, these tumors are known to be associated with alcohol intake and smoking because the carcinogenic or co-carcinogenic effects of smoking and drinking might be enhanced by the post-LT immunosuppressive therapy<sup>[103]</sup>. The purported mechanisms of alcohol-mediated oncogenesis are poorly understood, but these pathways may involve the carcinogenic properties of acetaldehyde and/or the inhibition of DNA methylation *via* the alteration of retinoid processing<sup>[104,105]</sup>. Saigal *et al*<sup>[106]</sup> found that patients who underwent LT for ALD appeared to have an increased risk of developing post-transplantation malignancies compared with those who underwent LT for other liver diseases. These authors hypothesized that a tumorigenic action mediated by the immunosuppressive effect of alcohol on natural killer cells could explain this observation. In fact, in the non-immunosuppressed population, alcoholism is associated with an increased risk for several malignancies, including liver and alimentary tract tumors<sup>[107,108]</sup>. Jain *et al*<sup>[79]</sup> observed a higher rate of *de novo* oropharyngeal and pulmonary neoplasms in transplanted ALD patients than in those with a non-alcoholic disease, similar to Duvoux *et al*<sup>[102]</sup>, suggesting the presence of other initiators of malignant changes in addition to immunosuppression. Among the identified risk factors, alcohol and tobacco consumption were highlighted<sup>[98,99,109]</sup>, data also obtained in our study<sup>[36]</sup>.

Regarding tobacco, nearly 90% of alcoholics smoke<sup>[110]</sup>,

compared to 26.7% of the general population of the United States<sup>[111]</sup>. Regarding the candidates for LT, approximately 60% are smokers<sup>[112,113]</sup> and 15%-40% continue to smoke after the LT<sup>[112,114]</sup>. DiMartini *et al*<sup>[114]</sup> found that nearly 40% of ALD recipients resume smoking and resume it early post-LT, increase their consumption over time and quickly become tobacco dependent. In a recent meta-analysis, active smoking was revealed as one of the major risk cofactors, independent of alcoholic relapse, of long-term morbidity and mortality in transplant recipients, either from cardiovascular complications or from *de novo* neoplasms<sup>[88]</sup>. This data was confirmed in numerous studies<sup>[36,109,114-121]</sup>. In an interesting study from a conceptual point of view, Herrero *et al*<sup>[122]</sup> showed that smoking withdrawal after LT may have a protective effect against the development of neoplasia. In particular, these researchers observed that patients with a smoking history who continued smoking after the LT, presented a hazard ratio of approximately 20 for the development of neoplasms associated with tobacco (head and neck, lung, esophagus, kidney and urinary tract carcinomas), while the risk of developing these neoplasms was reduced significantly in ex-smokers<sup>[122]</sup>. Furthermore, as noted earlier, smoking is also a risk for cardiovascular disease, and this is one of the most frequent causes of late mortality after LT<sup>[36,123]</sup>. Pungpapong *et al*<sup>[113]</sup> found a higher rate of vascular complications in LT recipients who had a history of smoking. Those who quit smoking 2 years prior to the transplantation reduced the incidence of vascular complications by 58%. Therefore, our pre- and post-LT follow-up efforts regarding ALD should be focused not only on alcoholic relapse but also on treating and avoiding other modifiable risk factors such as tobacco, not simply because of what was discussed earlier, but because we now acknowledge that tobacco is a risk factor for alcohol abuse. In a recent study on mice, it was observed that the rodents exposed to nicotine tended to ingest alcohol more frequently than those that were not administered such a substance due to a reduction in the dopamine response of the reward-response system in the brain, which thus decreased the pleasurable response to alcohol<sup>[124]</sup>. Given all the above-mentioned observations, the establishment of control programs and post-LT interventions could perhaps reduce the mortality in these patients, as will be discussed below.

## POST-LT FOLLOW-UP IN ALD

Several approaches have been evaluated to reduce alcohol recidivism in alcoholic patients after LT, but there is no standardized approach, and the available data are few and often controversial. In some liver transplant centers, alcoholic patients are encouraged to attend support groups, even if the data demonstrating the efficacy of such treatment in this cluster of patients are currently lacking. In a pilot study, Georgiou *et al*<sup>[125]</sup> reported that psychological interventions could be a valid approach to enhance motivation in these patients. However, this study was con-

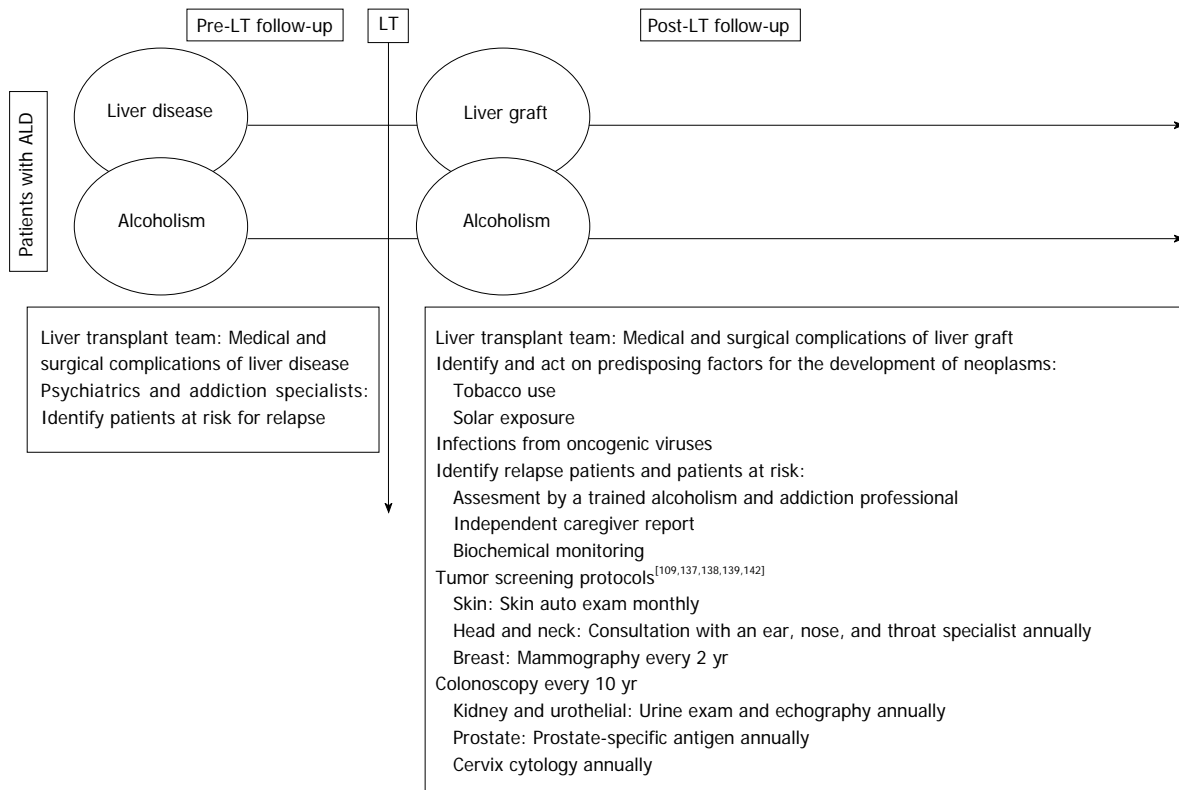


Figure 2 Proposed pre- and post-liver transplantation follow-up in alcohol liver disease. LT: Liver transplantation; ALD: Alcohol liver disease.

ducted on a limited number of patients, and the efficacy of this intervention on alcohol recidivism after LT was not evaluated. Björnsson *et al.*<sup>[35]</sup> evaluated the impact of the management of alcoholic patients by addiction psychiatrists, social workers and tutors in the period before LT and reported a 22% prevalence of alcohol recidivism in the treated group *vs* 48% in the untreated group. The presence of an alcohol addiction unit within a liver transplant center is not usual, but the study of Addolorato *et al.*<sup>[126]</sup> suggests that it could represent a useful approach to reducing alcohol recidivism after LT. However, objective and accurate indicators of abstinence are required<sup>[127]</sup>. Direct detection in the blood or breath only assesses alcohol intake within the preceding 10-12 h<sup>[39,128]</sup>. Carbohydrate-deficient transferrin (CDT) is an indirect marker that reflects alcohol intake in the previous 1-4 wk<sup>[129,130]</sup>; however, the daily consumption of 60-89 g of ethanol for a period of at least 7-10 d is required for a positive result. Therefore, CDT is inappropriate for the detection of low-to-moderate alcohol intake. Furthermore, wide ranges in sensitivity and specificity of 46%-73% and 70%-100%, respectively, have been reported<sup>[131]</sup>. Nevertheless, a high rate of false-positives with the CDT test has been reported, particularly in patients with severe liver damage<sup>[131]</sup>.

Currently, the determination of ethyl glucuronide (EtG), a metabolic product of alcohol, either in the urine or in the hair of patients offers a new, reliable possibility for the detection of alcohol intake<sup>[132-135]</sup>. Urinary EtG (uEtG) remains positive for up to 80 h after alcohol

consumption and allows for the detection of very small amounts of ethanol (uptake of < 5 g)<sup>[133,135]</sup> with a sensitivity and specificity of 89% and 99%, respectively<sup>[131]</sup>. However, positive uEtG tests may occur after the accidental consumption of foods containing alcohol, such as chocolate, cake and others. To reduce this problem in the transplant setting, a higher cut-off level for uEtG than what is routinely used (> 0.5 mg/L instead of > 0.1 mg/L) is recommended<sup>[132]</sup>.

Furthermore, the detection of EtG in the scalp hair of patients is a powerful tool for monitoring abstinence over a retrospective period of up to 6 mo. Each hair segment of 1 cm in length reflects alcohol consumption over a period of approximately 1 mo. The test has been validated for a maximal hair length of 6 cm<sup>[135]</sup>.

Thus, based on the above, we can infer that regular monitoring after LT is critical for determining the ongoing abstinence from tobacco and alcohol and for providing treatment assistance when tobacco or alcohol use are identified. Using a combination of methods (patient interviews by a trained alcoholism and addiction professional connected to the transplant team, independent caregiver reports and biochemical monitoring) provides the greatest yield because every method can add to the number of identified cases<sup>[27]</sup>.

As we have discussed previously, patients who undergo LT have an unexpectedly high rate of *de novo* extrahepatic cancer<sup>[98,99]</sup>, including lung and upper aerodigestive tract cancer<sup>[100,102]</sup>, and studies have reported that patients with ALD are particularly affected<sup>[79,99,102]</sup>. Therefore,



apart from identifying and acting on predisposing factors for the development of neoplasms (such as tobacco use, solar exposure and infections from oncogenic viruses), intensive tumor screening protocols have been suggested for these patients. Herrero *et al.*<sup>[109]</sup> concluded that ALD transplant patients, smokers or ex-smokers, should have a further follow-up, including a low-radiation-dose thorax computed tomography (CT) scan, a consultation with an ear, nose and throat specialist and a urine exam, as suggested by Benlloch *et al.*<sup>[136]</sup>, who recommend an annual head and neck cancer screening due to the high risk of this type of cancer. However, only two studies have shown that intensive screening protocols increase survival<sup>[137,138]</sup>. Therefore, at the present time, patient education, mainly to avoid smoking and sun exposure, and periodic clinical follow-ups continue to be the standard of care regarding treatment<sup>[98]</sup>.

## CONCLUSION

In the last 10 years, ALD is the LT indication that has seen the greatest increase in prevalence<sup>[3]</sup> as well as in post-LT survival rate compared with other causes of liver disease, although concerns over alcoholic relapse remain. Even though less than 5% of grafts are rejected at 5 years post-LT due to a direct or indirect consequence of alcohol consumption<sup>[139]</sup>, transplanted ALD patients who relapse have an increased long-term mortality due to cardiovascular pathologies and the onset of *de novo* neoplasms.

Much has been discussed regarding the risk factors for relapse, and the most controversial has been, and continues to be, the pre-LT abstinence period. In view of the foregoing, we can say that this period by itself should not be a determining factor to include a patient on the list because many other factors exist; therefore, a good psychiatric and psychosocial evaluation that identifies and addresses such factors before and after the LT is important<sup>[140,141]</sup>.

The major incidence of *de novo* neoplasms in this type of patient could be remedied with the detection of and action on the predisposing factors for the development of neoplasm in addition to the development of more intensive programs for the detection of neoplasm; however, the efficacy of this approach must be demonstrated. What we conclude is that the pre-LT evaluation and the post-LT follow-up in ALD patients should be a multidisciplinary task that includes transplant specialists, psychiatrists and addiction treatment specialists (Figure 2).

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## WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

# Liver transplantation for hilar cholangiocarcinoma

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**Core tip:** The most appropriate treatment for Klatskin tumor (KT) with a curative intention is multimodal therapy based on achieving R0 resection combined with other types of neoadjuvant or adjuvant treatment. In irresectable non-disseminated KT patients, using liver transplantation without neoadjuvant treatment, the 5-year survival rate increase to 38%, reaching 50% survival in early stage. In selected cases, with liver transplantation and neoadjuvant treatment (chemotherapy and radiotherapy), the actuarial survival rate is 65% at 5 years and 59% at 10 years. In conclusion, correct staging, neoadjuvant treatment, living donor and priority on the LT waiting list may lead to improved results.

## Abstract

The most appropriate treatment for Klatskin tumor (KT) with a curative intention is multimodal therapy based on achieving resection with tumour-free margins (R0 resections) combined with other types of neoadjuvant or adjuvant treatment (the most important factor affecting KT survival is the possibility of R0 resections, achieving 5-year survival rate of 40%-50%). Thirty to forty percent of patients with KT are inoperable and present a 5-year survival rate of 0%. In irresectable non-disseminated KT patients, using liver transplantation without neoadjuvant treatment, the 5-year survival rate increase to 38%, reaching 50% survival in early stage. In selected cases, with liver transplantation and neoadjuvant treatment (chemotherapy and radiotherapy), the actuarial survival rate is 65% at 5 years and 59% at 10 years. In conclusion, correct staging, neoadjuvant treatment, living donor and priority on the liver transplant waiting list may lead to improved results.

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## INTRODUCTION

The most appropriate treatment for cholangiocarcinoma (CC) with a curative intention is multimodal therapy based on achieving R0 resection combined with other types of neoadjuvant or adjuvant treatment such as external radiation therapy or brachytherapy, systemic or arterial chemotherapy, chemoradiation therapy and photodynamic therapy<sup>[1-10]</sup>.

Thirty to forty per cent of patients with Klatskin tumour (KT) are inoperable and present a 5-year survival rate of 0%. The other 60%-70% of patients may be eligible for surgery, although some 15%-50% are non-resectable and have an identical prognosis to inoperable

patients. In these non-resectable cases (due to spread to the second-generation intrahepatic radicals), and providing there is no lymph node dissemination, liver metastases or extrahepatic spread, some authors classically have proposed liver transplant (LT)<sup>[9]</sup>. The most important factor affecting KT survival is the possibility of tumour resection with tumour-free margins (R0 resections)<sup>[9,11-15]</sup>. The rate of resectability has increased over the last 20 years<sup>[16-22]</sup> as a result of several factors: (1) extending tumour resection to the hepatic parenchyma, especially caudate lobe resection<sup>[16-18]</sup>, since bile drainage occurs at the bifurcation of the hepatic ducts, performing extended right-sided resections also increases resectability<sup>[9]</sup> and it is often necessary to increase the residual liver volume with portal vein<sup>[23,24]</sup> or arterial<sup>[16]</sup> embolization; (2) extending tumour resection to the pancreatic head, associating a cephalic duodenopancreatectomy (CDP) to the liver resection<sup>[16,25-30]</sup>. This technique has been used when performing both liver resection and LT<sup>[16,25-30]</sup>. Most authors generally only consider CDP for KT when there is invasion of the lower biliary resection margin; (3) performing vascular resections has led to higher rates of morbidity and mortality, although it increases the possibility of resection and also of performing R0 resections<sup>[16]</sup>. Portal vein resection does seem to increase the 5-year survival rate, whereas hepatic artery resection does not appear to increase survival but does increase postoperative morbidity and mortality; and (4) performing a lymphadenectomy appears to be fundamental for achieving an R0 resection by removing the lymphatic pathways of dissemination and eliminating the frequent perineural dissemination through the periduodenal and peripancreatic elements of the hepatic hilum<sup>[31]</sup>.

## RESULTS OF LIVER TRANSPLANTATION WITHOUT NEOADJUVANCY

The indications for LT in KT patients were not well established due to the poor results reported in the literature, and each case needs to be analysed separately. When the tumour was non-resectable and not disseminated, palliative treatment obtains a zero 5-year survival rate but LT may achieve complete R0 resection of the tumour<sup>[16]</sup>. Some authors associated a CDP to the LT<sup>[16,25-30]</sup> and Starzl *et al.*<sup>[32]</sup> and Alessiani *et al.*<sup>[33]</sup> even extended the resection to neighbouring organs (cluster transplantation). The drawback with LT is immunosuppression, which favours the dissemination of tumour remains that might have gone unnoticed, which is why the fundamental cause of death following LT is usually abdominal tumour recurrence (occurring in 56%-96% of cases)<sup>[34-39]</sup>. The 5-year survival rate in LT series for KT is 0%-38%<sup>[34-39]</sup> and did not exceed 38% when it was more aggressive (cluster transplantation)<sup>[32]</sup>. An example of these poor results was published by the Cincinnati Transplant Tumour Registry in 2000<sup>[36]</sup> in an analysis of 207 cases of LT for CC, with a 23% 5-year survival rate, a 51% rate of early tumour recurrence and a 10% rate of postoperative mortality.

The survival rate was lower than with LT for other indications, and because of the scarcity of organs many centres considered LT contraindicated for KT. These poor results have been related to three factors: (1) poor patient selection, LT being indicated in patients with non-resectable tumours with biliary, portal and arterial invasion; (2) not performing a preoperative exploratory laparotomy and therefore many patients undergoing transplantation with disseminated peritoneal disease (16% of the cases) and affected regional lymph nodes; and (3) none of the patients receiving neoadjuvant therapy.

The Spanish series<sup>[40]</sup> reported 36 LTs for KT over a period of 18 years [3 of them associated with primary sclerosing cholangitis (PSC)], with 52.7% recurrence and 8.3% postoperative mortality and 30% and 18% survival at 5 and 10 years, respectively. In the early stages (stages I - II) the survival rate was suitable for indicating LT (47% at 5 years), whereas in very advanced stages (III-IV) it was only 15%. Despite the bad results (30% survival at 5 years) we showed that a small group of patients undergoing LT with negative lymph nodes had prolonged survival rates and that LT might therefore be an option in carefully selected cases. Subsequently Kaiser *et al.*<sup>[41]</sup>, in a similar study, presented their experience in Germany and reported 47 patients undergoing transplantation for KT, with a higher postoperative mortality rate (20%) and a 5-year survival rate of 22%. As with the Spanish series, when the selection criteria were strict (from 1998 onwards) the 5-year survival rate of the 15 transplant patients was 48% ( $P < 0.014$ ). Friman *et al.*<sup>[42]</sup> have reported the Scandinavian experience with LT for CC and 20 of the 53 patients in the series were KT. The same results were reported as for the Spanish and German series, with a 48% 5-year survival rate in patients with tumor node metastasis (TNM) stages  $\leq 2$  who received transplantation from 1995 onwards (the rate of PSC in this series was 64%, compared to 8% in the Spanish series). As Friman *et al.*<sup>[42]</sup> state, this survival rate was similar to that obtained in some series with LT for hepatic cirrhosis secondary to C virus.

Some authors have compared resection with LT without neoadjuvant. Hidalgo *et al.*<sup>[43]</sup> have reported 106 patients with KT, managing resection in 44 cases and performing LT in 12. There were no differences between resection and LT for sex, early stages, tumour size, lymph node invasion (7 from the LT group were N1), differentiation grade, perineural invasion and vascular invasion. There were also no differences for 5-year survival (28% with resection *vs* 20% with LT). As in our series, the patients undergoing LT were much younger than those with resection ( $P < 0.012$ ). Factors of poor prognosis were stages III-IV, R1-2 resections, presence of lymph node invasion and liver metastases, undifferentiated tumours and vascular invasion. The Mayo Clinic in Rochester, in a study published in 2005<sup>[44]</sup>, compared 38 LTs selected among 71 patients with the neoadjuvant protocol and 26 patients with liver resection from a total of 54 patients in whom it was attempted (48% resectability). The authors



concluded that transplantation may be the ideal treatment for KT due to a better 5-year survival rate (82% *vs* 21%) and lower rates of recurrence (13% *vs* 27% with resection), but the drawback with the study was that the two series were not homogeneous. The age of the LT group was 48 years, compared to 63 years in the resection group, and they were all stages I - II whereas 14% of the resection group had hepatic metastases, 39% vascular invasion, 25% positive lymph nodes in the hepatic hilum and 18% peritoneal metastases. They also performed just 38% of caudate lobe resections in the resection group, a technique which all authors currently claimed to be fundamental for preventing KT recurrence. They also selected PSC patients with an early diagnosis for the transplant group, as 58% of the transplanted patients had a Klatskin tumour besides PSC, compared to 8% of the resected patients. These results were in contrast to those published by Iwatsuki *et al.*<sup>[45]</sup>, who found no differences between LT and resection, in this case without neoadjuvant. When we have compared<sup>[46]</sup> 11 LTs and 29 KT resections without neoadjuvant, we also found no differences for 5-year survival (38% with LT and 36% with resection), and in no case was PSC associated.

## RESULTS OF LIVER TRANSPLANTATION WITH NEOADJUVANCY

In 1987 the University of Nebraska initiated a protocol of brachytherapy and chemotherapy with fluorouracil (5-FU) up until transplantation and in 2002<sup>[47]</sup> published a series of 11 patients showing prolonged survival rates for a select group of patients with non-resectable KT who received neoadjuvant internal radiation therapy alone or with chemotherapy with 5-FU (external radiation therapy was associated in 2 patients). Of these, 45% were alive and disease-free between 2.8 and 15.5 years after the transplant. The authors reported a high postoperative mortality rate of 27% and a low recurrence rate of 18%.

Subsequently in 1993 the Mayo Clinic initiated a protocol<sup>[26,44,48-53]</sup> of neoadjuvant treatment with external radiation therapy, chemotherapy with 5-FU for three days and internal radiation therapy, followed by capecitabine up until transplantation. All the patients were considered non-resectable by an experienced group of hepatobiliary surgeons and all the patients had to belong to stages I and II of the TNM classification<sup>[54]</sup>. For this they established an exhaustive selection process, performing exploratory laparotomy 2 mo after the end of radiation therapy and excluding the patient from the study if the tumour was disseminated. In 2008<sup>[50]</sup> they reported 148 patients (90 having completed neoadjuvant and LT), of whom 71 were alive, 19 died (8 due to tumour recurrence), 19 were awaiting LT and 39 failed to complete neoadjuvant due to progression of the disease. The 5-year survival rate in the group was 55%, and 71% among the transplant patients. The good results with this protocol were related to several factors: external and internal radiation therapy (useful for controlling wall and perineural

invasion); strict patient selection, as all were stages I - II, unlike other series in which stages III-IV exceed 40%, and most were young patients; and lastly the significant rate of PSC (65%). The neoadjuvant treatment was so effective that no tumour was found in the explanted liver (even though cytology prior to LT had been positive). Factors of poor prognosis in their series were age > 45 years, carbohydrate antigen [carbohydrate antigen (CA) 19-9] < 100, previous cholecystectomy, residual tumour of > 2 cm, perineural invasion, and waiting time > 100 d, hence the importance of living donor LT and application of a scoring system besides the Model for End-Stage Liver Disease (MELD) system. The drawback of this protocol were a higher rate of late vascular complications and a greater need for the use of grafts<sup>[55]</sup>, especially when living donor LT was used. This greater difficulty was related to the significant fibrosis encountered during the transplant as a result of radiation therapy, although it does not affect patient or organ survival.

### Results after acknowledgement of LT for KT by UNOS

The good results reported by the Mayo Clinic in Rochester lead to United Network Organ Sharing (UNOS) adopting this protocol on 17 November 2009 and beginning to allow priority MELD exception scores for CC patients who have completed the neoadjuvant chemoradiation protocol and for whom staging laparotomy was negative<sup>[26,50]</sup>. Darwish Murad *et al.*<sup>[26]</sup> re-published the results of the Mayo Clinic in Rochester, including 199 patients in the protocol, both intrahepatic CCs and KTs. Twenty patients did not reach the staging laparotomy, due in 15 cases to progression of the disease and to 4 dying from causes unrelated to the disease and 1 from intolerance to the treatment. An exploratory laparotomy was performed in 179 at the end of the protocol and 42 patients were excluded: 36 for metastases, 40 for progression of the disease and 2 who died without progression prior to transplantation. One hundred and thirty-seven patients underwent transplantation: 131 in their hospital (66%) and 6 in other hospitals. Thirty-six patients died (27%): 24 due to recurrence and 12 from other causes. The actuarial 5-year survival rate was 71%, with tumour recurrence in 26 patients, of whom 24 died as a result.

Darwish Murad *et al.*<sup>[26]</sup> analysed pre-LT dropout factors and found that 62 of the 199 patients (31%) abandon the waiting list, their mean survival being just 3.6 mo. Statistically significant factors of poor prognosis in the univariate analysis were presentation with painless jaundice, weight loss, visible tumour mass of  $\geq 3$  cm, positive or suspicious intraluminal brushing or biopsy, high CA 19-9 (> 500) and higher MELD score. Statistically significant in the multivariate analysis were mass size of  $\geq 3$  cm, positive or suspicious intraluminal brushing or biopsy, high CA 19-9 and higher MELD score.

Darwish Murad *et al.*<sup>[26]</sup> also analysed the prognostic factors related to tumour recurrence following liver transplantation. Statistically significant in the univariate analysis were age over 50 years, size  $\geq 3$  cm, CA 19-9 over



500 and vascular encasement. Statistically significant in the multivariate analysis were high CA 19-9 and complete portal vein encasement, perineural invasion and tumour persistence in the explant. In the multivariate study tumour persistence in the explanted liver was exclusively significant. It is worth noting in this series that the patients undergoing transplantation for KT associated with PSC had a lower risk of recurrence than the patients with the novo KT.

In 2012, after approving the neoadjuvant protocol in 2009 in the United States, Darwish Murad *et al.*<sup>[30]</sup> send a survey to 50 American centres to collect their experience in LT for cholangiocarcinoma between 1993 and July 2010 and received 30 responses (8 of the 20 non-respondents were because they did not apply the neoadjuvant protocol). Selection for transplantation from the waiting list was done with a MELD score of 22 points and with the same criteria as with hepatocarcinoma: every 3 mo 10% drop off the waiting list. The objectives have been: (1) to assess the efficacy of neoadjuvant for KT; (2) to analyse the intercentre impact of neoadjuvant; and (3) to evaluate whether the MELD system applied is appropriate. They included 287 patients, of whom 22 were excluded (16 due to progression, 3 who died of causes unrelated to cancer, and 3 who did not tolerate the treatment). Staging laparotomy was performed in 229 patients and extrahepatic disease detected in 40, who were also excluded. Nine patients were excluded after the laparotomy (7 for tumour progression and 2 who died of non-tumour-related causes). Transplantation was done in 184 cases following staging and in another 30 who underwent transplantation without staging after neoadjuvant (214 liver transplants in total).

Of the 287 patients included in the study 193 belonged to the Mayo Clinic in Rochester and 94 to other centres (between 2 and 12 transplants). A CDP was associated in 22 cases. One hundred and twenty-two patients died: 60 prior to liver transplantation and 62 after transplantation (22%). There was a post-transplant recurrence in 43 patients (20%), of whom 40 died. The actuarial survival rate was 65% at 5 years and 59% at 10 years. They analysed the prognostic factors and found no differences between living and deceased donor transplantation or between KT associated with PSC and the novo KT; there were also no differences between patients with and without exploratory laparotomy: 36/184 recurrences (20%) *vs* 7/30 recurrences (23%), respectively. A poorer prognosis was shown by patients who do not fulfil UNOS criteria for MELD exception: existence of a mass of > 3 cm (21 patients), with a 5-year survival rate of 32%, *vs* 69% for < 3 cm masses; those with liver metastases (4 patients); and when a percutaneous biopsy was done for tumour diagnosis (16 cases). As in previous series there was a group of patients with no preoperative biopsy for diagnosis, no tumour in the explanted specimen and whose deaths were not tumour-related. Of 87 patients with no reliable preoperative tumour diagnosis 55 did have a tumour in the explant and another 17 presented with tumour recur-

rence during evolution. The remaining 15 cases (5%) had no tumour in the preoperative period or in the explant and the authors claim that even if they had been excluded the 5-year survival rate was 50% in the other 272. They concluded that neoadjuvant was effective, there were no inter-centre differences and that the MELD system was valid for waiting list selection.

### Validation of the results of other centres

These results show that LT is a valid therapeutic option for both hilar and intrahepatic cholangiocarcinoma, especially when neoadjuvant treatment is used. However, there are doubts in the literature, as not all centres reproduce these results, something which, as also claimed by Friman *et al.*<sup>[56]</sup>, may be related to the high % of patients with PSC, strict criteria for a preoperative diagnosis of malignancy and especially<sup>[55]</sup> strict criteria for selection (performing a staging laparotomy after performing neoadjuvant). Other American centres have recently reported their results for LT for cholangiocarcinoma. Panjala *et al.*<sup>[27]</sup>, from the Mayo Clinic in Florida, reported 22 patients in whom the protocol of the Mayo Clinic in Rochester was applied between 2001 and 2008. Seventeen cases (77%) were associated with PSC and 5 were the novo KTs. They did not perform an exploratory laparotomy and staging was done during LT with 2 recipients. The preoperative diagnosis was certainty in 12 cases and suspicion in 10. During transplantation 3 of the 12 patients with a preoperative diagnosis of certainty presented with liver metastases in 2 cases and an intestinal implant in 1 case. Overall survival was 63%, similar to that reported by the Rochester group. As with the Rochester group there were patients with no tumour in the explant, which influenced the survival rate: there was still tumour in 77% (17 cases) but no tumour remains could be identified in 5 cases (23%), a factor with which survival was related, such that patients with tumour in the explant had a survival rate of 52% at 3 years and those with no tumour in the explant had a survival rate of 100%. Nine patients (41%) died as a result of recurrence and 3 for other non-tumour-related causes, the recurrence rate being 27% at one year, 4.5% the second year and 4.5% the third year. When the association with PSC was analysed, these patients made up 93% of the group of patients with no tumour recurrence, whereas it constituted 50% of the patients who did have tumour recurrence, which implies that patients with *de novo* KT carry a higher risk of recurrence than those associated with PSC: of the 17 with PSC there were 4 recurrences and 1 with visceral metastases, whereas in the 5 *de novo* KTs there were 4 recurrences and 2 visceral metastases.

Of a total of 132 cholangiocarcinomas, the UCLA University<sup>[57,58]</sup> selected 57 for surgery and perform LT in 38 of them<sup>[57]</sup>. In a subsequent publication they reported 40 liver transplants for CC, of which 14 were for KT<sup>[58]</sup>. Only 13 patients received neoadjuvant treatment with chemotherapy plus radiation therapy<sup>[58]</sup> and the 5-year survival rate was 47% when neoadjuvant was applied, compared to 20% without neoadjuvant treatment and

33% when adjuvant treatment was administered. These results were lower than those reported by the Rochester group and similar to those reported for European groups without neoadjuvant on selected early cases<sup>[40-42]</sup>.

In 2012 the Anderson Cancer Center reported the efficacy of neoadjuvant treatment in patients with resection of tumours of the bile duct. Of 157 patients 94 were cholangiocarcinomas<sup>[59]</sup>. Forty-eight point seven per cent received adjuvant chemotherapy, 17.8% had neoadjuvant chemotherapy and 15.8% had chemotherapy plus neoadjuvant radiation therapy (the latter treatment delayed surgery by 6.8 mo). The 5-year survival rate was 30.4%, and when immediate tumour resection was achieved without neoadjuvant with a margin of at least 1 cm the survival rate was 52.4%. Thus, immediate tumour resection increased survival from 42.3 to 53.5 mo. This protocol was applied to patients considered initially resectable, and not as occurs in CC patients considered non-resectable to whom the Rochester protocol was applied before transplantation.

The results for other non-American groups<sup>[60-62]</sup> has been contradictory, with favourable<sup>[60]</sup> and unfavourable<sup>[61]</sup> cases, very small series and an absence of multicentric prospective studies. Wu *et al.*<sup>[62]</sup> achieved good results with the protocol. They reported 6 patients with PSC and cholangiocarcinoma, with a similar early detection protocol to that of the Mayo Clinic in Rochester; the patients only received neoadjuvant radiation therapy before LT with CDP and only 1 died from a non-tumour-related cause, the other 5 having survived more than 5 years.

In conclusion, R0 resection is the most accepted treatment of KT. In non-disseminated unresectable tumours, liver transplantation in early stages have an acceptable survival (50% at 5 years). In these same patients (KT and early stages), treatment with neoadjuvant chemoradiotherapy and very strict selection criteria achieves a 5-year survival rate of over 65%. The series with neoadjuvant treatment are not homogeneous and most tumours are associated with PSC as compared to other series where most are the novo KT. Therefore, some authors consider it necessary prospective randomized studies, comparing KT associated to PSC and the novo KT, to discover the proper role of neoadjuvant chemoradiation<sup>[63]</sup>. Staging correct, the priority in the waiting list LT (MELD) and living donor LT may lead to better results.

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## Current management of fecal incontinence: Choosing amongst treatment options to optimize outcomes

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### Abstract

The severity of fecal incontinence widely varies and can have dramatic devastating impacts on a person's life. Fecal incontinence is common, though it is often under-reported by patients. In addition to standard treatment options, new treatments have been developed during the past decade to attempt to effectively treat fecal incontinence with minimal morbidity. Non-operative treatments include dietary modifications, medications, and biofeedback therapy. Currently used surgical treatments include repair (sphincteroplasty), stimulation (sacral nerve stimulation or posterior tibial nerve stimulation), replacement (artificial bowel sphincter or muscle transposition) and diversion (stoma formation). Newer augmentation treatments such as radiofrequency energy delivery and injectable materials, are minimally invasive tools that may be good options before proceeding to surgery in some patients with mild fecal incontinence. In general, more invasive surgical treatments are now reserved for moderate to severe fecal incontinence. Functional and quality of life related outcomes, as well

as potential complications of the treatment must be considered and the treatment of fecal incontinence must be individualized to the patient. General indications, techniques, and outcomes profiles for the various treatments of fecal incontinence are discussed in detail. Choosing the most effective treatment for the individual patient is essential to achieve optimal outcomes in the treatment of fecal incontinence.

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**Key words:** Fecal incontinence; Treatment; Sacral nerve stimulation; Sphincteroplasty; Artificial bowel Sphincter; Biofeedback

**Core tip:** An increasing number of treatment options for the management of fecal incontinence have been developed. In addition to traditional options such as sphincteroplasty and colostomy, non-surgical options such as biofeedback and dietary modification may be considered for mild incontinence. Injectable materials and radiofrequency energy delivery are two newer treatments for mild incontinence. Surgical options for moderate to severe incontinence include sacral nerve stimulation, artificial bowel sphincter implantation, muscle transposition, antegrade continence enemas, sphincteroplasty, and colostomy formation. Treatment for fecal incontinence (repair, stimulation, replacement, augmentation, or diversion) must be individualized to the patient, considering the underlying cause and impact on quality of life of the fecal incontinence.

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## INTRODUCTION

Fecal incontinence is a common problem; one that is likely underreported in the general population. The prevalence of fecal incontinence varies in the literature, with one study of over 4000 surveyed American adults finding a prevalence of 8.3%<sup>[1]</sup>. The much larger and more recent Mature Women's Health Study of over 5800 American women found an even higher incidence of accidental bowel leakage of almost 20%<sup>[2]</sup>. Incontinence to liquid or solid stool, mucous, or flatus occurs with varying frequency and can have a range of impact on daily function<sup>[1]</sup>. The Mature Women's Health Study found that nearly 40% of women with accidental bowel leakage have severe symptoms impacting their quality of life, even though less than one third of women sought medical care for their bowel leakage<sup>[3,4]</sup>. While there can be many etiologic factors contributing to its development, there are some common risk factors. Age, diarrhea or frequent bowel movements, nocturnal bowel movements, other bowel disorders, and the presence of urinary incontinence are commonly associated with fecal incontinence<sup>[1,4,5]</sup>. In women, internal sphincter injury and reduced perineal descent related to obstetrical trauma independently predict the development of fecal incontinence<sup>[6]</sup>. Other risk factors include neurological disorders, congenital anorectal malformations, trauma, iatrogenic injury during anorectal procedures, and chronic diseases such as diabetes<sup>[6-9]</sup>.

It is necessary to complete a physiological and anatomical assessment of the pelvis and colon in order to choose the most appropriate treatment option for a patient's fecal incontinence. This caveat is especially important since many women with fecal incontinence have associated genital and urinary anatomical or functional problems<sup>[10]</sup>. A rectal examination may identify a sphincter defect or decreased rectal tone. This finding may be helpful to identify potential etiologies and treatments for a patient's fecal incontinence. Though not all investigations are required for every patient, options include anal or pelvic ultrasound, anal manometry, defecography, magnetic resonance imaging, and electromyography with pudendal nerve terminal motor latency testing. Anatomical imaging can help identify sphincter defects and associated pelvic floor disorders such as rectocele or prolapse, which may be contributing to the severity of incontinence<sup>[11,12]</sup>. A physiology lab is helpful for the assessment of incontinence and other pelvic floor disorders.

The impact of fecal incontinence varies and can greatly alter a person's ability to perform daily activities. One may alter timing of meals or eating habits, and possibly avoid all social occasions for fear of embarrassment<sup>[8]</sup>. While fecal incontinence is not a normal part of aging it may be perceived as such, and older people may not seek treatment until symptoms are severe. Treatment options for fecal incontinence range from dietary modification and physical therapy to major surgery, such as colostomy formation. In recent decades, many new treatments for fecal incontinence have been developed with

good success, adding to traditional options of sphincteroplasty and ostomy formation. These alternatives include biofeedback, radiofrequency, injectable materials, and surgical approaches such as sacral nerve stimulation, the artificial bowel sphincter, and muscle transposition. A recent Cochrane review concluded that there is insufficient evidence to allow for quality comparisons to be made among the various surgical approaches to fecal incontinence<sup>[13]</sup>. The decision among these options is multifactorial and the severity of the incontinence, patient anatomy, and patient wishes must all be carefully considered. The aim of this article is to review current options for the management of fecal incontinence, their indications, and reported outcomes. The treatments most commonly offered by the authors, from the five available categories of repair, stimulation, replacement, augmentation, and diversion, are discussed.

## DIETARY MODIFICATION AND MEDICATION

Modifiable diet and lifestyle factors may be identified which can provide simple interventions to try to improve symptoms. Smoking and sedentary lifestyle are associated with fecal incontinence<sup>[14]</sup>. Weight loss has been shown to improve fecal incontinence in obese women<sup>[15]</sup>. Medications should be reviewed with the help of a pharmacist to identify potentially incriminating medications. Low fiber and high fat diets may be contributory to loose stools. Loose stools and diarrhea often precipitate symptoms of fecal incontinence and may be improved with dietary and medication alterations. Other factors may be identified that may suggest the need for further testing or anatomical causes of fecal incontinence. For example, cholecystectomy may lead to persistent diarrhea and flatulence which may amplify symptoms of fecal incontinence; cholestyramine may help relieve these symptoms<sup>[16,17]</sup>.

The addition of a daily fiber supplement should be advocated in fecal incontinence. It acts as a bulking agent to allow for more solid stool and adds little to no morbidity to the patient. A randomized, blinded, placebo controlled study found that fiber improved fecal incontinence and stool consistency within 1 mo in the community living population<sup>[18]</sup>. In addition to fiber, medications with a constipating effect may be useful for patients with fecal incontinence with loose stools. These pharmacologic agents include loperamide, diphenoxylate and atropine, and codeine. Loperamide is most commonly used and may also have beneficial effects on anal sphincter resting tone<sup>[19]</sup>. Unfortunately, studies comparing various medications are lacking and trials of medications for the treatment of fecal incontinence include very heterogeneous populations and treatments<sup>[20]</sup>. A Cochrane review conducted in 2013 concluded that there is insufficient evidence to guide the decision between medications for the treatment of incontinence in various clinical situations<sup>[20]</sup>. Clearly, no medication will cure moderate to severe fecal incontinence, but it should certainly be utilized in mildly

**Table 1** Success of biofeedback for fecal incontinence

Ref.	Year	Patients (n)	Significant reduction in incontinence (percentage of patients)	Improvement in quality of life (percentage of patients)	Adjuncts to traditional biofeedback
Keck <i>et al</i> <sup>[33]</sup>	1994	15	73%	NR	None
Solomon <i>et al</i> <sup>[28]</sup>	2003	102	70%	69%	Anal manometry, transanal ultrasound
Terra <i>et al</i> <sup>[34]</sup>	2006	239	60%	NR	EMG, electrostimulation
Naimy <i>et al</i> <sup>[30]</sup>	2007	49	None	None	Electrostimulation
Byrne <i>et al</i> <sup>[32]</sup>	2007	385	70%	87%	None
Heymen <i>et al</i> <sup>[27]</sup>	2009	45	76%	NR	None
Schwandner <i>et al</i> <sup>[29]</sup>	2010	158	50%	NR	EMG, electrostimulation
Bartlett <i>et al</i> <sup>[26]</sup>	2011	72	86%	100%	None
Jodorkovsky <i>et al</i> <sup>[31]</sup>	2013	12	80%	NR	None

NR: Not reported; EMG: Electromyography.

symptomatic patients where indicated.

## BIOFEEDBACK

Biofeedback is a form of physical therapy and muscle re-training offered to patients refractory to medical treatment of fecal incontinence. There are numerous regimens, most of which involve many weeks of treatment lead by a physical therapist. Numerous studies have attempted to define the most effective regimen and most responsive patient population, but overall there are few high quality studies showing a definitive impact of biofeedback on fecal incontinence<sup>[21]</sup>. It has been suggested by some authors that biofeedback should be offered to all patients who have not responded to medical interventions of fecal incontinence because it is safe, inexpensive, and effective long term<sup>[22]</sup>. Older patients with normal defecation physiology appear to respond well<sup>[23]</sup>. Advanced anorectal physiology tests such as manometry, defecography, pelvic magnetic resonance imaging, and pudendal nerve terminal motor latency testing do not seem to predict who will respond best to biofeedback<sup>[24]</sup>. Patients with mild or moderate fecal incontinence who have not responded well to medical treatments are likely the best candidates for biofeedback<sup>[25]</sup>.

The technique of biofeedback may include monitored or home sessions, pelvic floor exercises, digital feedback, electrical stimulation, balloons, and manometric or ultrasound monitoring of response. Pelvic floor exercises alone have been shown to improve fecal incontinence scores and quality of life<sup>[26]</sup>. In one study of pelvic floor exercises, no differences in treatment effect were found between the different regimens, but symptoms improved in both groups<sup>[26]</sup>. The addition of biofeedback using manometry is more effective than pelvic floor exercises alone to improve fecal incontinence scores and achieve more physiologically normal defecation<sup>[27]</sup>. Biofeedback with digital feedback alone may be just as effective as manometry and ultrasound guided treatment, providing enough feedback to guide re-training, as found in a randomized controlled trial of different methods of biofeedback<sup>[28]</sup>. Some literature suggests that electrical stimulation leads to more effective results over biofeedback alone, while others have found that biofeedback alone is

adequate to improve patient symptoms<sup>[29,30]</sup>. A multicenter randomized and blinded trial found that the combination of electrical stimulation with extended treatment duration (longer than 3 mo) achieved the best results<sup>[29]</sup>. Such treatment regimens may not be available in many centers, but access to a trained biofeedback therapist who is aware of the various treatment modalities may be invaluable to the population with fecal incontinence.

Biofeedback requires the patient and therapist to commit to treatment for a number of weeks to months. One study found that only 44% of patients with fecal incontinence who were recommended to undergo biofeedback therapy completed the treatment<sup>[31]</sup>. This finding was largely due to lack of insurance coverage and distance to treatment centers<sup>[31]</sup>. It is important to note that in this study those patients who did undergo biofeedback reported an 80% positive response to the treatment<sup>[31]</sup>. Other studies have confirmed improvement in over 70% of patients when fecal incontinence scores and quality of life scores were assessed<sup>[32,33]</sup>. Table 1 summarizes the success of biofeedback. Physiologic parameters such as squeeze pressure and maximum tolerated volume have also been reported to improve with biofeedback<sup>[34]</sup>. Improvements in fecal incontinence scores are durable over at least 1 year, but some patients may require additional sessions to boost the effect<sup>[35]</sup>. Pelvic floor training with biofeedback is likely beneficial to many patients with fecal incontinence long term, but patients and therapist must be willing to devote the time to a complete set of sessions to see maximum benefit. In those able to do so, biofeedback may achieve improvement in symptoms without invasive procedures.

## REPAIR

### Sphincteroplasty

Sphincteroplasty has long been the standard of care of the management of fecal incontinence related to anal sphincter injury<sup>[36]</sup>. The vast majority of patients who undergo sphincteroplasty have a history of vaginal delivery<sup>[37]</sup>. However, only about one third of women who have had a known sphincter injury related to vaginal delivery develop fecal incontinence over time<sup>[36]</sup>. Pudendal nerve injury, failed prior sphincteroplasty, multiple

**Table 2 Success of overlapping sphincteroplasty**

Ref.	Year	No. of patients with follow-up	Mean follow-up (mo)	Success <sup>1</sup> (percentage of patients)
Karoui <i>et al</i> <sup>[52]</sup>	2000	74	40	28%
Halverson <i>et al</i> <sup>[40]</sup>	2002	49	69	46%
Bravo Gutierrez <i>et al</i> <sup>[39]</sup>	2004	130	120	41%
Barisic <i>et al</i> <sup>[49]</sup>	2006	65	80	48%
Maslekar <i>et al</i> <sup>[55]</sup>	2007	64	84	80%
Oom <i>et al</i> <sup>[50]</sup>	2009	120	111	60%
Mevik <i>et al</i> <sup>[51]</sup>	2009	25	84	53%
Zutshi <i>et al</i> <sup>[53]</sup>	2009	31	129	0%

<sup>1</sup>Success variably defined in studies. Good, excellent or complete continence included as success.

vaginal deliveries, history of third or fourth degree tear, and instrument-assisted vaginal deliveries are all factors which may predispose to fecal incontinence associated with sphincter defect and impact the success of sphincteroplasty<sup>[38]</sup>. It is important to note that the majority of recent studies indicate that pudendal nerve injury as demonstrated by prolonged pudendal nerve terminal motor latency does not independently predict the success of sphincteroplasty<sup>[39-41]</sup>. Many women who undergo sphincteroplasty have associated pelvic floor injuries, which do not seem to impact the success of sphincteroplasty<sup>[42]</sup>. In addition, the combination of internal and external anal sphincter defect repair can lead to successful and equivalent outcomes when compared to external anal sphincter defect repair, alone<sup>[43]</sup>.

While various techniques for sphincteroplasty have been described, the most commonly performed procedure is the anterior overlapping sphincteroplasty. A curvilinear incision is made on the perineum and dissection proceeds until the edges of the external anal sphincter are identified and isolated. Care is taken to not dissect too far laterally to avoid nerve injury. The ends are overlapped and sutured together, providing new bulk to the sphincter complex and an intact circumferential ring of sphincter. Separate attention to the imbrication of the internal anal sphincter does not seem to add to the overall durability of the sphincteroplasty if the internal sphincter is not injured<sup>[44]</sup>. Post-operative manometry shows significant increases in the length of the high pressure zone and resting and squeeze pressures<sup>[37]</sup>. A diverting stoma is not required to achieve optimal outcomes in early repair of third and fourth degree tears during vaginal delivery<sup>[45]</sup>. Delayed repair is associated with higher overall cost in this situation, but may still achieve good long term outcomes and may be the safer option depending on the clinical scenario<sup>[45,46]</sup>.

Posterior sphincter repair is rarely needed, given that most sphincter injuries are associated with traumatic vaginal delivery. However, posterior repair may be occasionally utilized for neurogenic fecal incontinence, multifocal sphincter defects, or after failed anterior sphincteroplasty in order to avoid any significant scar tissue in the area.

A similar technique is used as in the anterior technique, with a curvilinear posterior incision being used for access to the external anal sphincter. Some surgeons may proceed with a combined anterior and postanal approach, though this combination is not common. The success rate of the postanal approach is likely equivalent or less durable compared to anterior sphincteroplasty<sup>[47,48]</sup>. In the absence of a specific iatrogenic posterior sphincter injury or excessive anterior scar tissue, the anterior sphincteroplasty should be considered the preferred approach.

The long term functional outcomes following anal sphincteroplasty are not ideal. The Wexner fecal incontinence score is commonly used to assess for incontinence following sphincteroplasty. In the short term, good results are achieved in over 70% of patients and excellent results in over half of patients<sup>[49]</sup>. However, the long term outcomes which have been reported in numerous retrospective studies reveal a consistent decrease to 15% to 60% good long term continence<sup>[39-40,50-56]</sup>. Interestingly, there is poor correlation between long term quality of life scores and fecal incontinence scores, with one study reporting that 95% of patients were satisfied with their operation a mean of 7 years following sphincteroplasty<sup>[39,53]</sup>. A summary of long term outcomes is found in Table 2. Age has long been felt to be a predictor of success of sphincteroplasty, with many studies reporting that older patients do not have as durable long term outcomes compared to younger patients<sup>[39,53,56]</sup>. However, a recent large review of 321 women who underwent sphincteroplasty showed that age is not a predictor of long term incontinence scores<sup>[57]</sup>. A review of both sphincteroplasty and sacral nerve stimulation concluded that sphincteroplasty remains a good option for the management of incontinence due to sphincter defect, despite new technologies<sup>[58]</sup>. Patients must be chosen after appropriate pre-operative evaluation to achieve optimal outcomes.

## STIMULATION

### Sacral nerve stimulation

For many patients and practitioners, sacral nerve stimulation has revolutionized the treatment of moderate to severe fecal incontinence. Adapted from its use in urinary incontinence, it may provide effective relief from fecal incontinence without any direct intervention on the anal sphincter complex. Interestingly, one study found that the only positive predictors of successful treatment with sacral nerve stimulation were loose stools and low stimulation intensity during the test phase of the procedure<sup>[59]</sup>. Conversely, age, gender, etiology of fecal incontinence, and physiology study results did not impact the efficacy of sacral nerve stimulation<sup>[59]</sup>. Though sacral nerve stimulation and sphincteroplasty have not been directly compared in the literature, numerous studies have shown that patients with sphincter defects can have excellent results with sacral nerve stimulation<sup>[60-64]</sup>. The success of sacral nerve stimulation in these patients also does not appear to be correlated to the degree of sphincter defect<sup>[63]</sup>. Pa-



**Table 3 Studies of outcomes of sacral nerve stimulation**

Ref.	Year	Patients (n)	Significant reduction in incontinence scores and incontinent episodes	Significant increase in quality of life
Leroi <i>et al</i> <sup>[73]</sup>	2005	27	Y	Y
Boyle <i>et al</i> <sup>[63]</sup>	2009	15	Y	NR
Brouwer <i>et al</i> <sup>[64]</sup>	2010	55	Y	Y
Wexner <i>et al</i> <sup>[79]</sup>	2010	120	Y	Y
Hollingshead <i>et al</i> <sup>[76]</sup>	2011	18	Y	NR
Lim <i>et al</i> <sup>[78]</sup>	2011	41	Y	Y
Mellgren <i>et al</i> <sup>[81]</sup>	2011	83	Y	Y
George <i>et al</i> <sup>[77]</sup>	2012	23	Y	Y
Devroede <i>et al</i> <sup>[83]</sup>	2012	78	Y	Y
Hull <i>et al</i> <sup>[75]</sup>	2013	76	Y	Y
Damon <i>et al</i> <sup>[82]</sup>	2013	92	Y	Y

Y: Yes; NR: Not reported.

tients known to have pudendal nerve injuries or previous sphincteroplasty can have good responses to sacral nerve stimulation<sup>[64]</sup>.

The mechanism by which sacral nerve stimulation improves fecal incontinence is not well defined, as it is multifactorial. A systematic review found that sacral nerve stimulation likely works in 3 ways: stimulation of a somato-visceral reflex, direct effect on the anal sphincter complex, and afferent nerve modulation<sup>[65]</sup>. It is postulated that sacral nerve stimulation may induce a change in anal sphincter muscle type from fast to slow twitch, thus reducing muscle fatigue, though this has not been definitively demonstrated in the sacral nerve stimulation population<sup>[66]</sup>. Sensory changes include the sensation of rectal filling and urge to defecate at higher rectal volume<sup>[67]</sup>. Sacral nerve stimulation alters colonic transit by inducing retrograde colonic propagating sequences, activity which may slow transit in the setting of fecal incontinence<sup>[68]</sup>. In an animal model, sacral nerve stimulation was found to increase activity in the central cerebral cortex<sup>[69]</sup>. The effects of sacral nerve stimulation are well beyond local effect on the anal sphincter complex.

There are two approaches to the implantation of the sacral nerve stimulator. Some surgeons introduce a peripheral nerve stimulator wire in the office, guided by anatomical landmarks. The patient is tested for response for a period of 1-2 wk and if good response is achieved, the permanent tined lead and stimulator device are implanted in the same setting in the operating room. The authors' preferred approach is a two-stage operative technique. The first stage is the insertion of the tined lead into the S3 foramen in the operating room with careful fluoroscopic and patient-directed guidance. Local anesthetic injections and light sedation allow the patient to signal when stimulation is felt in the perianal, perineal, or saddle regions during lead electrostimulation. In addition, sphincter bellows and plantar flexion of the great toe on the side of lead placement are used to further indication stimulation of the sacral nerve. Once a good response

is achieved the lead is tunneled into position. A temporary device is used during a 2 wk test phase. If a good response is achieved during the test phase, the patient undergoes a second procedure to implant the permanent device which is attached to the tined lead. This approach is associated with very little lead migration during the test phase but does require two operations. A test phase is important in both approaches, as not all patients will have a good response to lead placement<sup>[70]</sup>. Each permanent device is programmed to the individual's response pattern. Successful strategies to prolong the durability of the device battery beyond the average of six years include cyclical stimulation and subsensory stimulation<sup>[71,72]</sup>.

Results of the first randomized multi-center study of sacral nerve stimulation were reported in 2005, showing that fecal incontinence was improved when the sacral nerve stimulator was activated<sup>[73]</sup>. Longer term results are now available. Compared to medical treatment of fecal incontinence, sacral nerve stimulation is significantly more effective<sup>[74]</sup>. A recent report from the SNS Study Group showed that in patients followed for at least 5 years, 89% have significant continued reduction in fecal incontinence and 36% had a complete response to sacral nerve stimulation<sup>[75]</sup>. Numerous other studies from around the world have demonstrated significant long term reduction in fecal incontinence scores<sup>[75-79]</sup>. Table 3 summarizes the results of studies of outcomes of sacral nerve stimulation. Furthermore, in women who have undergone sacral nerve stimulation for fecal incontinence; urinary, sexual, and vaginal symptoms also improve with a global benefit on pelvic floor health<sup>[80]</sup>. Quality of life scores are also improved in the short and long term after sacral nerve stimulation<sup>[79,81-84]</sup>.

There are potential morbidities with sacral nerve stimulation including a 5% risk of lead displacement associated with the percutaneous lead testing technique<sup>[85]</sup>. Pain at the surgical site and paresthesias are the most commonly reported complaints<sup>[81]</sup>. Infection of the permanent device or surgical site occurs in 10%, with about half of those infections requiring surgical management<sup>[81,85]</sup>. Overall, about one third of patients required surgical manipulation of the device in a study of long term outcomes<sup>[75]</sup>. Despite potential morbidity associated with the device, sacral nerve stimulation has been shown to be cost-effective in the treatment of fecal incontinence<sup>[86,87]</sup>. When balancing the effectiveness, morbidity profile, and cost-effectiveness of the technique, sacral nerve stimulation is a very valuable tool for the treatment of fecal incontinence, especially in its more severe forms.

## REPLACEMENT

### Artificial bowel sphincter

The artificial bowel sphincter is considered only for patients with severe fecal incontinence. It is an effective device, but requires long term follow up and a motivated patient. The use of an artificial bowel sphincter requires both manual dexterity and mental capacity to operate the device<sup>[88]</sup>. Due to the high incidence of adverse events,

**Table 4 Outcomes of artificial bowel sphincter**

Ref.	Year	Patients (n)	Explanted devices (n)	Success (percentage of patients), intention to treat	Complications
Lehur <i>et al</i> <sup>[93]</sup>	2000	24	7	83%	Obstructed defecation
Altomare <i>et al</i> <sup>[94]</sup>	2001	28	3	75%	Obstructed defecation, infection, device erosion
Devesa <i>et al</i> <sup>[95]</sup>	2002	53	10	65%	Perforation, infection, sepsis, device erosion, pain, impaction
Wong <i>et al</i> <sup>[97]</sup>	2002	112	41	53%	Infection, pain
Lehur <i>et al</i> <sup>[101]</sup>	2002	16	4	69%	Erosion
Parker <i>et al</i> <sup>[96]</sup>	2003	45	18	49%	Infection, pain
O'Brien <i>et al</i> <sup>[98]</sup>	2004	14	1	NR as percentage	Obstructed defecation, non-healing of wound
Melenhorst <i>et al</i> <sup>[103]</sup>	2008	33	7	NR as percentage	Pain, perforation, infection, obstructed defecation
Ruiz Carmona <i>et al</i> <sup>[99]</sup>	2009	17	11	53%	Infection, erosion
Wexner <i>et al</i> <sup>[90]</sup>	2009	51	31	NR as percentage	Infection, malfunction, erosion, pain
Wong <i>et al</i> <sup>[100]</sup>	2011	52	14	67%	Perforation, cuff leak

NR: Not reported.

other treatment options should be considered and attempted before proceeding to artificial bowel sphincter<sup>[89]</sup>. Contraindications include Crohn's disease, local sepsis, prior radiation, poor quality of the perineal tissues, severe constipation, and incontinence associated irritable bowel syndrome<sup>[89]</sup>. Disruption of the anal sphincter complex due to trauma, severe obstetrical injury, and imperforate anus are common indications<sup>[89,90]</sup>. Sacral nerve stimulation and the artificial bowel sphincter have largely replaced muscle transposition and dynamic graciloplasty for the treatment of severe fecal incontinence, with better functional outcomes and quality of life parameters<sup>[91,92]</sup>. Patients must be carefully selected and extensively counselled on the risks and benefits of the artificial bowel sphincter, as discussed below.

Meticulous sterile technique and thorough bowel preparation are essential to reduce the risk of infection associated with the artificial bowel sphincter. The 3 components of the artificial bowel sphincter are connected *via* tubing and compose the sphincter cuff, the reservoir balloon, and control pump. These components are inserted *via* perineal, Pfannenstiel, and labial or scrotal incisions, respectively. The cuff itself is chosen for size based on circumferential length around the rectum and width. It is inserted first and great care is taken to ensure there is adequate tissue bulk distal to the cuff, in an attempt to avoid device erosion and infection. The balloon holds approximately 40 mL of liquid and is left filled with the device deflated at the end of the procedure after testing the control pump. The device is not activated for four to six weeks to allow for complete healing. The patient is taught how to fill and empty the cuff by using the implanted control pump.

Patients who retain the artificial bowel sphincter long term have reported very good functional and qualitative results. Manometry results show that the artificial bowel sphincter achieves normal resting tone when the cuff is filled<sup>[93]</sup>. Improved continence is achieved in over 75% of patients, with one series reporting normal continence in two-thirds of patients<sup>[94,95]</sup>. Though adverse events are

significant, patients who retain the device have excellent responses to artificial bowel sphincter implantation based on incontinence scores<sup>[93-100]</sup>. Quality of life scores are also markedly improved after successful treatment of fecal incontinence with the artificial bowel sphincter<sup>[96,98,99,101]</sup>. A systematic review of the safety of the artificial bowel sphincter noted that functional outcomes and quality of life scores for those patients who do not retain a functioning device are not reported in the literature<sup>[102]</sup>.

Complications following artificial bowel sphincter implantation unfortunately remain high and often lead to device explantation, mitigating the overall population benefit in fecal incontinence. Unfortunately, these complications continue to accrue long term<sup>[90]</sup>. The rate of revision of the device has been reported to be up to 50%, with infection and device failure the most common reasons<sup>[100]</sup>. About 25%-40% of artificial bowel sphincters become infected over time<sup>[90,100,103]</sup>. Erosion of the cuff or control pump and post-operative constipation may also occur<sup>[92,104,105]</sup>. The outcomes and complications associated with the artificial bowel sphincter are included in Table 4. In summary, a balanced consideration of potential benefits and adverse events is important and artificial bowel sphincter may still be the optimal treatment consideration for select patients with severe fecal incontinence.

### Muscle transposition

Muscle transposition is a technique used to physically replace the sphincter with *in vivo* muscle bulk. It is most often used in the setting of a traumatic or iatrogenic disruption of the anal sphincters to recreate a wrap of muscle around the anus. A substantial congenital or post-traumatic defect is indicated to consider muscle transposition. The two muscles widely described in the literature for transposition are the gluteus maximus and gracilis muscles. These are useful because of their proximity to the anus, sizeable muscle bulk, and nerve locations which are amenable to preservation upon transposition. In addition, the gluteus maximus was thought to be a good

**Table 5 Outcomes of graciloplasty**

Ref.	Year	Type of graciloplasty	Patients (n)	Success (percentage of patients)
Kumar <i>et al</i> <sup>[114]</sup>	1995	Unstimulated	9	100%
Eccersley <i>et al</i> <sup>[113]</sup>	1999	Unstimulated	8	100%
Madoff <i>et al</i> <sup>[109]</sup>	1999	Stimulated	128	66%
Wexner <i>et al</i> <sup>[110]</sup>	2002	Stimulated	115	62%
Bresler <i>et al</i> <sup>[112]</sup>	2002	Stimulated	24	79%
Rongen <i>et al</i> <sup>[111]</sup>	2003	Stimulated	200	72%
Thornton <i>et al</i> <sup>[117]</sup>	2004	Stimulated	38	73%
Hassan <i>et al</i> <sup>[107]</sup>	2010	Stimulated	31	71%

choice for transposition given that involuntary gluteal contraction occurs with the strong urge to avoid involuntary defecation<sup>[106]</sup>.

The surgical technique of muscle transposition is complex and requires significant experience to gain expertise. Three main options exist: gluteoplasty, graciloplasty, and dynamic (or stimulated) graciloplasty. Gluteoplasty is performed with the patient in the prone position with the table flexed at the hips. Bilateral incisions over the gluteus are made and two tongues (one from each side) of the lower 10% of the muscle are raised with care taken to preserve the neurovascular bundles<sup>[106]</sup>. The mobilized muscle is then tunnelled and delivered through separate bilateral curvilinear incisions around the anus. The contralateral mobilized segments are sutured together to create a ring of muscle.

In a graciloplasty procedure, the patient is placed in the modified lithotomy position. Two or three incisions are made along the longitudinal access of the gracilis muscle on the chosen side to harvest the entire length of the gracilis. The neurovascular bundle is preserved through its identification during medial dissection. The muscle is released distally and tunneled medially. A perineal incision is made and the gracilis is wrapped circumferentially around the anus. In the dynamic graciloplasty technique, an electrode is placed in the gracilis muscle and an implantable device similar to that used for sacral nerve stimulation is implanted in the abdominal wall. Modified approaches to dynamic graciloplasty include temporary stimulation with an external stimulator for muscle retraining, similar to biofeedback<sup>[107]</sup>. It must be noted that the stimulator and leads for dynamic graciloplasty are not currently approved for use in North America.

Much like the artificial bowel sphincter, muscle transposition has fairly good functional outcomes but high rates of complications and re-operation; graciloplasty has largely replaced gluteoplasty. The largest and most recent study of gluteoplasty reported a good functional outcome in 59% of patients<sup>[108]</sup>. Successful functional outcomes for graciloplasty, dynamic and unstimulated, is consistently reported to be about 60%-75%, with earlier success of unstimulated graciloplasty being even higher<sup>[107-114]</sup>. Table 5 lists the published success rates of graciloplasty. If a patient has a stoma at the time of the graciloplasty, eventual outcomes are equivalent to those

who do not have a stoma, but are delayed in achieving them<sup>[110]</sup>. Complications of the procedure are common, and include surgical site infections, pain, rectal injury, and erosion of the device in the case of dynamic graciloplasty<sup>[112,115,116]</sup>. In addition, constipation due to obstructed defecation is commonly reported in as many as 50% of patients<sup>[115-117]</sup>. There are no studies directly comparing muscle transfer to other surgical treatments of fecal incontinence. Graciloplasty followed by artificial bowel sphincter implantation may be the best combination option for adult patients with fecal incontinence attributable to congenital imperforate anus<sup>[118]</sup>.

## DIVERSION

### Antegrade continence enema

The antegrade continence enema was first described by Malone *et al*<sup>[119]</sup> in 1990. It is used to control fecal soiling in both adults and children, but is most commonly used and reported in the pediatric population. Neurogenic conditions, such as spina bifida, resulting in neurogenic bowel and urinary symptoms are the most common indications in children. While the antegrade continence enema may be helpful in pure fecal incontinence, most often patients who undergo this procedure have the combination of constipation or colonic dysmotility with associated overflow fecal incontinence. Patients also commonly undergo urological procedures at the same time to control neurogenic bladder symptoms, with good results for these combined indications<sup>[120]</sup>. In adults, good functional outcomes are better in this setting, when compared to those patients who undergo the procedure for constipation alone<sup>[121]</sup>. While an antegrade continence enema does not alter anorectal physiology or anatomy, it provides a mechanism to empty the colon in a controlled fashion, allowing the patient to perform their daily activities with little worry of fecal soiling or incontinent episodes.

Since Malone's original description, various techniques have been described for the creation of an antegrade continence enema. The appendix, ileum, cecum, and left colon may be used successfully as the access point for irrigation<sup>[122-124]</sup>. The appendix is most commonly used, where it is inverted and fixated to the skin at the umbilicus or right lower quadrant. This can be performed open or laparoscopically with good results<sup>[124]</sup>. The access point is left intubated with a catheter for about 3 wk after the operation before intermittent intubations begin. Patients or their caregivers then intubate the bowel daily to every few days and perform colonic irrigation with tap water or an electrolyte or bowel cleansing solution. Both tap water and commercial products have good irrigation results, with solution irrigants achieving slightly better continence rates<sup>[125]</sup>. The volume of irrigation is gradually increased over time after the procedure and the timing and frequency of irrigation through the site may be largely patient directed. In the pediatric patient population, the operation is performed around the age of 10 years.

Few studies report on outcomes of antegrade con-

**Table 6 Outcomes of antegrade continence enema in incontinent adults**

Ref.	Year	Patients using antegrade continence enema on follow-up	Percentage of patients achieving continence	Complication rate
Gerharz <i>et al</i> <sup>[121]</sup>	1997	8	100%	44%
Teichman <i>et al</i> <sup>[120]</sup>	1998	7	86%	71%
Teichman <i>et al</i> <sup>[128]</sup>	2003	4	75%	67%
Lefevre <i>et al</i> <sup>[127]</sup>	2006	18	94%	33%
Poirier <i>et al</i> <sup>[126]</sup>	2007	14	78%	67%

tinence enemas in adults. Overall, functional results are very good, with about 75% of adults achieving continence with the procedure<sup>[126-128]</sup>. Quality of life improves in adult patients with antegrade continence enemas, although not all patients continue to use their antegrade continence enema in the long term<sup>[127,128]</sup>. See Table 6 for a summary of antegrade continence enema study results. In children, full continence is achieved in 65%-100% of patients<sup>[122,125,129-133]</sup>. Even though the amount of time devoted to bowel care may not significantly change, satisfaction and quality of life scores improve for most children and parents<sup>[131,134-137]</sup>. Persistent leakage, stoma stenosis, and surgical site infections are common complications, with one study quoting a 13% chance of requiring stoma revision due to stoma complications<sup>[130,131,138]</sup>. While the antegrade continence enema is not commonly performed in adults, the patients who have grown to adulthood require long term follow up and attention to these possible complications.

### Fecal diversion

The creation of a colostomy or ileostomy provides definitive control of fecal incontinence. An ileostomy may be considered in patients with colonic transit abnormalities but the colostomy is the standard ostomy utilized in the treatment of fecal incontinence. In many patients the ostomy can be created using a laparoscopic approach to improve recovery time. While a colostomy is not without short and long term risks, such as bleeding, anesthesia related cardiac or respiratory morbidities, and parastomal hernia, it is a safe and effective treatment of severe fecal incontinence. It is generally only offered if other treatment modalities have failed. Patients are usually understandably very resistant to the idea of a permanent colostomy, fearing it will be difficult to manage and have great impact on self-image and social interactions.

When patients who had undergone colostomy creation for fecal incontinence were surveyed, general quality of life and fecal incontinence quality of life scores were actually higher in the colostomy group when compared to other patients with fecal incontinence<sup>[139]</sup>. Another study found that patients generally reported high satisfaction levels with their stomas for fecal incontinence, with over 80% of patients stating that they would likely or definitely choose to undergo the procedure again<sup>[140]</sup>. Compared to other surgical treatments of severe incontinence (dynamic

**Table 7 Outcomes of radiofrequency energy treatments**

Ref.	Year	Patients (n)	Significant improvement in incontinence scores after treatment	Significant improvement in quality of life
Efron <i>et al</i> <sup>[143]</sup>	2003	50	Y	Y
Felt-Bersma <i>et al</i> <sup>[147]</sup>	2007	11	Y	NR
Takahashi-Monroy <i>et al</i> <sup>[142]</sup>	2008	19	Y	Y
Lefebure <i>et al</i> <sup>[144]</sup>	2008	15	Y	N
Kim <i>et al</i> <sup>[148]</sup>	2009	8	N	N
Ruiz <i>et al</i> <sup>[145]</sup>	2010	24	Y	Y
Abbas <i>et al</i> <sup>[146]</sup>	2012	27	Y	NR

Y: Yes; N: No; NR: Not reported.

graciloplasty and artificial bowel sphincters), a British study found colostomy to be most cost effective in terms of quality adjusted life years<sup>[92]</sup>. While fecal diversion is not required in the majority of patients presenting for treatment of fecal incontinence, it is a viable, definitive, and well-tolerated treatment which offers good quality of life.

## AUGMENTATION

### Radiofrequency energy

There is a gap between medical and surgical treatment options in fecal incontinence<sup>[141]</sup>. Radiofrequency energy delivery and injectable materials are becoming increasingly popular as minimally invasive procedural treatments that may bridge this gap. The delivery of radiofrequency energy to the internal anal sphincter, known as the SECCA<sup>®</sup> procedure, is proposed to induce local restructuring of collagen, leading to a more robust internal anal sphincter and better continence. It can be used for patients with mild or moderate fecal incontinence who are unwilling or not candidates to undergo surgical treatment after failing medical management. It may also be applied to patients with idiopathic or sphincter defect-associated fecal incontinence.

The technique of radiofrequency energy delivery is simple. It is done with conscious sedation and local anesthesia on an outpatient basis in endoscopy or the operating room. A commercial device is utilized and the procedure takes about 30 min. The device resembles a clear plastic anoscope with four retractable needles. The needles are electrodes which are deployed into the anorectal mucosa to deliver radiofrequency energy to the internal anal sphincter, starting just distal to the dentate line and moving proximally. The device delivers radiofrequency while simultaneously monitoring the temperature and impedance of the tissues to avoid burning. The device is activated four or five times per quadrant of the anorectum, moving 5 mm more proximal before each activation in a quadrant. The machine provides constant feedback on the contact with the tissues, temperature and impedance during the device activation, and the timing of each activation, giving visual and sound cues to the surgeon



**Table 8 Outcomes of dextranomer in hyaluronic acid gel for fecal incontinence**

Ref.	Year	Patients (n)	> 50% reduction in fecal incontinence episodes (percentage of patients)	Significant improvement quality of life
Dodi <i>et al</i> <sup>[150]</sup>	2010	115	64%	Yes
Graf <i>et al</i> <sup>[161]</sup>	2011	136	52%	Yes
Schwandner <i>et al</i> <sup>[159]</sup>	2011	21	56%	Yes
Danielson <i>et al</i> <sup>[160]</sup>	2012	34	76%	Yes
La Torre <i>et al</i> <sup>[156]</sup>	2013	83	63%	Yes

throughout the procedure.

Reports of the success of radiofrequency energy treatment are generally, though not universally, positive and are summarized in Table 7. Numerous studies have reported long term improvement in fecal incontinence scores<sup>[142-145]</sup>. The cohort with the longest reported follow-up showed a durable reduction in mean Wexner fecal incontinence scores from 14 to 8 and found that most participants had a greater than 50% improvement in symptoms after 5 years<sup>[142]</sup>. Similarly, patient satisfaction and quality of life scores show improvement after radiofrequency energy treatment<sup>[142-145]</sup>. Another study with a higher average baseline fecal incontinence score compared to other trials found that only 22% of patients had sustained treatment benefits at an average follow up of 40 mo<sup>[146]</sup>. Despite the overall favorable outcomes of radiofrequency energy delivery, anal manometry testing does not show any significant change in physiologic parameters<sup>[143,147,148]</sup>. No major adverse events have been reported following radiofrequency energy delivery, though there have been reports of infection, hematoma, minor bleeding, and anal pain<sup>[145,147,148]</sup>.

### Injectable materials

Various injectable materials have included trialed for local injection of the sphincter complex to treat fecal incontinence. Benefits of this approach are that it is an outpatient procedure with little discomfort that has low morbidity. The materials used have included collagen, silicone, autologous fat, glutaraldehyde, carbon-coated beads, dextranomer in hyaluronic acid gel, and others<sup>[149]</sup>. Dextranomer in hyaluronic acid gel (NASA/Dx) has received the most extensive recent investigation and attention in the literature. Injectables may be used in patients who have failed medical treatment and have fecal leakage or mild to moderate fecal incontinence<sup>[149]</sup>. The bulking effect may not be permanent and may require repeat injections at subsequent office visits.

The technique of injection is relatively simple. The open-label multicenter trial of NASA/Dx involved four quadrant injections of 1 mL of NASA/Dx into the deep submucosa of the anal canal<sup>[148]</sup>. This was performed through an anoscope and done with the patient in the prone jack-knife or lithotomy positions. The injections were placed at a 30 degree angle 5-10 mm proximal to the dentate line<sup>[150]</sup>. The needle was kept in place for

up to 30 s so that the gel would not leak from the site<sup>[150]</sup>. There are very few comparative trials amongst injectable materials. A small study of 40 patients found that silicone was more effective than carbon-coated beads to reduce incontinence<sup>[151]</sup>. No published studies have compared NASA/Dx with other injectables. One randomized controlled trials comparing NASA/Dx to biofeedback and found no significant difference in functional outcomes<sup>[152]</sup>. Biofeedback, however, certainly requires more dedication and long term commitment from the patient. The effect of injectables on manometry parameters are an increase in the length of the high pressure zone and asymmetry index<sup>[153]</sup>. The impact on resting pressure is variable in the literature, ranging from improvements in resting pressure to no effect<sup>[153,154]</sup>.

There are no long term outcomes reported yet for NASA/Dx, the most popular injectable. The longest reported outcomes are at 2 years<sup>[155,156]</sup>. A Cochrane review published in 2013 noted the absence of long term studies, making definitive conclusions about the utility of injectables difficult<sup>[157]</sup>. See Table 8 for a summary of cohort studies investigating the utility of NASA/Dx gel. A good response is considered a 50% reduction in the number of reported incontinence episodes, which is reported to occur in over 50% of patients who have been treated with injectables<sup>[150,154,156,158-161]</sup>. In addition, the majority of patients have good quality of life improvement, as reported on both global quality of life and fecal incontinence quality of life scores<sup>[150,155,156,158]</sup>. Morbidity from the use of injectables is low, with fever and proctalgia being the two most common adverse events and bleeding, abscess, and pain being other rare reported events<sup>[150,156,160,161]</sup>. Though many patients with fecal incontinence may be candidates for the use of injectables, the ideal candidate is one who has seepage or mild to moderate incontinence who has failed medical management but is not yet ready to pursue surgical treatment. Prior use of an injectable such as NASA/Dx does not preclude future surgical treatments such as sacral nerve stimulation, sphincteroplasty or artificial bowel sphincter.

## CONCLUSION

Successful treatment of fecal incontinence requires careful consideration of the individual patient's severity of incontinence. Treatments range from inexpensive medications and physical therapy to complex surgical procedures such as artificial bowel sphincter implantation and muscle transposition. In general, more invasive treatments are required for more severe incontinence or after less invasive treatments have failed. A careful history including obtaining an incontinence score, physical examination, bowel diary, and adjunctive anal physiology tests should be utilized to define the nature of the fecal incontinence. Minimally invasive approaches including biofeedback, radiofrequency energy, and injectables have moderate long term success. Sphincteroplasty remains an acceptable option for patients with documented sphincter defects. Be-

cause initially adequate functional outcomes decline over time, quality of life improvement after sphincteroplasty is not robust long-term. Sacral nerve stimulation is very effective in managing moderate to severe fecal incontinence and has had a great impact on the treatment of fecal incontinence. In the very long-term, patients will require additional procedures to change the battery of the sacral nerve stimulator but the procedure has excellent reproducible long term functional and quality of life outcomes. The artificial bowel sphincter has similar outcomes in those patients who retain the device, but further studies aimed at reducing infection, erosion, and device failure must be undertaken. Fecal diversion remains a good option for severe fecal incontinence and actually provides the patient with satisfying quality of life. Knowledge of these currently used treatments is essential to honest and thorough counseling of the patient with fecal incontinence to improve treatment success. Together with the patient, the surgeon can then best select treatment from the five available categories of repair, replacement, augmentation, stimulation, and diversion.

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## Sleep, immunity and inflammation in gastrointestinal disorders

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gastrointestinal diseases and discuss the interdependent relationship between sleep and these gastrointestinal disorders. Different physiologic processes including immune system and inflammatory cytokines help regulate the sleep. The inflammatory cytokines such as tumor necrosis factor, interleukin-1 (IL-1), and IL-6 have been shown to be a significant contributor of sleep disturbances. On the other hand, sleep disturbances such as sleep deprivation have been shown to up regulate these inflammatory cytokines. Alterations in these cytokine levels have been demonstrated in certain gastrointestinal diseases such as inflammatory bowel disease, gastro-esophageal reflux, liver disorders and colorectal cancer. In turn, abnormal sleep brought on by these diseases is shown to contribute to the severity of these same gastrointestinal diseases. Knowledge of these relationships will allow gastroenterologists a great opportunity to enhance the care of their patients.

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**Key words:** Sleep; Immune function; Immunity; Irritable bowel syndrome; Inflammatory bowel disease; Gastro-esophageal reflux disease; Liver disorders; Colon cancer; Circadian rhythm

### Abstract

Sleep disorders have become a global issue, and discovering their causes and consequences are the focus of many research endeavors. An estimated 70 million Americans suffer from some form of sleep disorder. Certain sleep disorders have been shown to cause neurocognitive impairment such as decreased cognitive ability, slower response times and performance detriments. Recent research suggests that individuals with sleep abnormalities are also at greater risk of serious adverse health, economic consequences, and most importantly increased all-cause mortality. Several research studies support the associations among sleep, immune function and inflammation. Here, we review the current research linking sleep, immune function, and gas-

**Core tip:** Sleep disorders have become a global issue, and discovering their causes and consequences are the focus of many research endeavors. Recent research suggests that individuals with sleep abnormalities are at greater risk of all-cause mortality and serious adverse health and economic consequences. Several studies support the associations among sleep, immune function and inflammation. We review the current research linking sleep, immune function, and gastrointestinal diseases and discuss the interdependent relationship between sleep, overall immune function with emphasis on inflammatory bowel disease, irritable bowel syndrome, gastro-esophageal reflux and colorectal cancer.



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## INTRODUCTION

Research into sleep and its associated health abnormalities has had a relatively recent surge, and sleep quality has been shown in many investigations to be an important, if not essential element of good health<sup>[1-3]</sup>. Sleep disorders can be primary, secondary or behavioral. Primary disorders are related to neurologic defects like narcolepsy and restless leg syndrome, breathing problems like obstructive sleep apnea and central sleep apnea, or circadian rhythm abnormalities like jet lag and delayed sleep phase syndrome. Secondary sleep disorders are secondary to primary diseases such as depression, chronic illness *etc.* Behavioral sleep problems such as insomnia or insufficient sleep are caused or perpetuated by poor sleep hygiene.

Sleep disorders have become a global issue. Sleep abnormalities occur in 17%-22% Japanese<sup>[4,5]</sup>, while sleep disorders are estimated to range from 7% to 50% in people living in Portugal and Finland<sup>[6-8]</sup>. In the United States, more than 70 million people suffer from a sleep disorder, and modern lifestyles have led to Americans sleeping approximately 2 h less per night than 100 years ago<sup>[4,7,9]</sup>. Abnormalities in the sleep cycle are linked with neurocognitive consequences ranging from performance decrements, slower response times, and decreased cognitive ability<sup>[10]</sup>.

Receiving fewer hours of sleep may also impact metabolism in a manner that contributes to obesity<sup>[10]</sup>. A strong association has been found between disruption in sleep and gastrointestinal disease. We will review the interdependent relationship of sleep dysfunction and gastrointestinal issues including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), gastro-esophageal reflux disease (GERD), liver disorders and colon cancer. Sleep abnormalities have been shown to worsen symptoms of IBS, IBD and GERD which, in turn, can worsen sleep abnormalities. Sleep disorders and circadian dysfunction have also been shown to increase the risk of colon cancer.

## HUMAN SLEEP

Sleep is classified based on polysomnographic data into two main categories known as rapid eye movement (REM) sleep and non-REM (NREM) sleep. NREM sleep is further divided into three stages based on increasing depths of sleep and increasing arousal thresholds. These sleep stages cycle through REM and NREM approximately every 90 min<sup>[11]</sup>. More time is spent in slow-wave delta sleep each cycle during the first half of the night, with increasing time in REM sleep in the later portions of the

night. Humans spend around 25% of total sleep time in REM sleep<sup>[12]</sup>. The exact biological purpose of sleep is unknown. However, slow-wave sleep is thought to be restorative, restful sleep, and REM sleep is associated with dream recall and memory consolidation<sup>[13]</sup>. Although the ideal quantity of sleep is different among individuals, most studies recommend seven to eight hours a night for adults as an optimal amount of sleep<sup>[14]</sup>. Alterations in normal sleep patterns are thought to be a significant contributor to a vast array of illness including depression, metabolic syndrome, inflammation, gastrointestinal diseases, and also cancer<sup>[15,16]</sup>.

## REGULATION OF SLEEP

Sleep regulation is often described by a two process model<sup>[17]</sup>. Process S, or the sleep homeostatic drive, linearly increases the longer an individual stays awake<sup>[18]</sup>. Process C, or the circadian alerting drive, oscillates with body temperature on an approximate 24-h cycle<sup>[15,18]</sup>. During the later hours of the day, Process C enters its decline in the circadian pattern, and Process S has accumulated approximately 16 h of continuous wakefulness. The combination of declining alertness and a sufficient amount of prior wakefulness facilitates the onset of sleep<sup>[18]</sup>. Biological clocks have evolved based on a 24-h cycle that allow organisms to anticipate and physiologically adjust to daily environmental changes and this circadian system provides a temporal organization of waking and sleep<sup>[15,19]</sup>. The circadian clock is entrained or synchronized to the specific day-night cycle (phase) of the environment through signals such as light, meals, and social interaction. These affect neuro hormonal pathways which influence the circadian clock. Light is the most important factor affecting the circadian rhythm. Light travels from the retina *via* the retinohypothalamic pathway to the suprachiasmatic nucleus (SCN), and then *via* a multi-synaptic pathway to the pineal gland where it suppresses melatonin production. Melatonin is a neurohormone that serves to synchronize circadian rhythms both with the environment and the human body as melatonin receptors are found in nearly all human tissue. Furthermore, the 24-h circadian rhythm is governed by a main circadian clock and a system of peripheral clocks located in multiple tissues including the pancreas, liver, and adipose tissue<sup>[20]</sup>. The SCN also serves as a “standard time” which synchronizes peripheral tissue clocks<sup>[21]</sup>. A series of “clock genes” help regulate the timing through both positive and negative feedback loops. CLOCK and Brain and Muscle Arnt-like protein (BMAL-1) form heterodimers that accumulate throughout the day. These heterodimers then bind to the promoter regions of the genes Period (PER) and Cryptochrome (CRY) to activate their transcription. PER and CRY proteins then accumulate and form heterodimers that inhibit transcription of CLOCK and BMAL-1 proteins<sup>[22]</sup>. Point mutations in these clock genes have been linked to altered circadian function and sleep abnormalities in mammals including familial ad-

vanced sleep phase syndrome and delayed sleep phase syndrome<sup>[23-25]</sup>.

Research has also focused on determining whether similar feedback-loop clock genes are present within the gastrointestinal tract. PER2 expression has been identified in the myenteric plexus and affects the rhythmic releases of acetylcholine and nitric oxide, ultimately regulating peristalsis<sup>[26,27]</sup>. Hypotheses on circadian rhythms affecting nutrient transport in the small intestine, gastric acid secretion, gut motility, and production of digestive enzymes have also been proposed<sup>[27]</sup>.

## IMMUNE ACTIVATION AND CYTOKINE EFFECTS ON SLEEP

Many immune and endocrine pathways exhibit a diurnal profile including cortisol and growth hormone. The onset of sleep corresponds with an increase in the serum levels of some cytokines, peaking at 2.5 h after sleep onset<sup>[28]</sup>. This surge of cytokines and their pro-inflammatory effects are suggested to be linked with nocturnal exacerbations of diseases like asthma and rheumatoid arthritis<sup>[11]</sup>. Increasing evidence supports a reciprocal relationship between sleep and the immune system. An activated immune system alters sleep and sleep abnormalities affect immune function<sup>[29,30]</sup>. Studies have also shown that an immune response elicits a pro-inflammatory cytokine response that helps to modulate sleep<sup>[22]</sup>. This was first illustrated in the 1970s<sup>[31]</sup> after the identification of a sleep-inducing muramyl peptide known as factor S was found to have both immune and sleep regulatory properties<sup>[18,32]</sup>. Although the diverse range of cytokines released in early inflammation limits our ability to isolate individual contributions<sup>[33]</sup>, tumor necrosis factor (TNF)- $\alpha$ , interleukin-1 (IL-1), and IL-6 have shown the strongest potential<sup>[30]</sup>. However, numerous other cytokines with at least partial sleep regulatory properties have been identified. In animal models, IL-1 and TNF- $\alpha$  elevations have correlated with increased time in NREM sleep. Furthermore, an inhibitory effect on both spontaneous sleep and sleep rebound (increased REM sleep after sleep deprivation) was produced when IL-1 was inhibited by anti-IL-1 specific antibodies<sup>[34]</sup>. In addition, high serum levels of TNF- $\alpha$  has been linked to sleepiness in patients with obstructive sleep apnea and rheumatoid arthritis<sup>[35,36]</sup>. IL-6 also plays a role in sleep modulation. Sleep deprivation can increase IL-6 levels leading to daytime fatigue<sup>[37]</sup>. In a human study, subjects received an injection of IL-6 that simulated the levels found in infection, and they experienced marked subjective fatigue, inhibition of REM sleep, and elevated CRP in 6.5 h<sup>[33]</sup>. The inhibition of REM and the promotion of NREM sleep appear to play key roles in the immune response. IL-1, IL-6 and TNF- $\alpha$  are at high levels at time of infection and correlated with increased duration of NREM, changes in core body temperatures, more shivering, and an overall greater capacity to fight off illness<sup>[32]</sup>. This was confirmed in several studies evalu-

ating the effect of infection with human immunodeficiency virus (HIV) on sleep. In early stages of HIV infection, polysomnographic data showed larger percentage of time spent in NREM than in REM and prolonged REM sleep latency<sup>[18,38]</sup>. Serotonin also is an integral component to IL-1 activity. Depletion of serotonin or inhibition of the serotonin receptor led to a reduction in the IL-1-induced increase in the amount of NREM sleep<sup>[39,40]</sup>. Thus, there appears to be an interaction of IL-1 and its ability to modulate sleep based on baseline levels of serotonin. Infection caused by viral, bacterial, fungal or even parasites was evidenced to increase the amount of time spent in NREMS and decrease the amount of time spent in REMS<sup>[41]</sup> based on severity of infection<sup>[12]</sup>.

## SLEEP EFFECTS ON THE IMMUNE RESPONSE

Both human and animal studies have shown that sleep has an overall protective role and that sleep deprivation is associated with an increased susceptibility to infection<sup>[18,22]</sup>. A study on infected rabbits showed that animals who had longer periods of sleep had less morbidity and mortality<sup>[42]</sup>. In humans, long-term sleep deprivation was shown to increase risk of septicemia<sup>[43,44]</sup>. Furthermore, decreased sleep has been linked to impaired antibody response to hepatitis A vaccine<sup>[29]</sup>, influenza<sup>[45]</sup>, and increased risk of getting a upper respiratory infection<sup>[46]</sup>. The timing of sleep is also important because most immune cells have their highest response to immune challenges during the night<sup>[12,18]</sup> and their lowest response in the morning<sup>[45]</sup>. This antibody impairment is very similar to the decrease in the immune response seen with human aging as both have a lowered T-cell response to antigens and impaired response to vaccinations<sup>[47]</sup>.

## GASTROESOPHAGEAL REFLUX DISEASE AND SLEEP

It is well established that gastroesophageal reflux and its most common symptoms, heartburn and regurgitation, is among the most frequently dealt with conditions encountered by gastroenterologists<sup>[48]</sup>.

Approximately, 10%-20% of the people in the United States have GERD<sup>[49]</sup>. One study found that approximately 74% of patients with GERD had nocturnal symptoms<sup>[50]</sup>. A Gallup survey revealed that approximately 63% of the people with nocturnal GERD felt it impaired their ability to sleep and 40% felt it impaired their ability the following day<sup>[51]</sup>. Several factors likely contribute to nocturnal GERD. Numerous studies now have documented that reflux during sleep presents physiologic issues not encountered during the waking state. For example there is a notable prolongation of acid clearance due to the suppression of swallowing and salivation during sleep. This results in enhanced back diffusion of hydrogen ions and subsequent mucosal damage.

These issues are discussed in detail in a review by Orr *et al*<sup>[51]</sup> in which he presents an argument for considering nighttime reflux and its clinical manifestations as a distinct clinical entity<sup>[52]</sup>. However, sleep and GERD have been shown to have a more interdependent relationship. A study by Dickman *et al*<sup>[52]</sup> noted that poor quality of sleep led to exacerbations of reflux the following day. They also found that longer durations of reflux events correlated with reduced sleep quality. This was supported by the Gallup survey, a higher frequency of reflux was associated with higher frequency of sleep difficulties<sup>[51]</sup>. A likely contributing factor is the hyperalgesia due to sleep disturbances<sup>[54,55]</sup>. This was first reported by Onen *et al*<sup>[53]</sup> who found that sleep deprivation led to a somatic hyperalgesia. This hyperalgesia was evidenced after loss of REM sleep or cumulative 2 d loss of non-REM sleep<sup>[54]</sup>. Recently, Schey *et al*<sup>[54]</sup> have documented a visceral hyperalgesia and increased sensitivity to reflux in GERD patients with documented poor sleep prior to undergoing an acid perfusion test<sup>[55]</sup>. Further research in this area is needed, but current studies indicate that discussion and treatment of sleep abnormalities in patients with GERD may lead to improved management.

## PEPTIC ULCER DISEASE

Patients with sleep apnea sustain cessation of breath during sleep, leading to intermittent hypoxia, systemic inflammation and sympathetic activation. These insults are not only be a threat to cardiovascular system but can also contribute to damage to the gastrointestinal mucosa and hence initiation or progression of peptic ulcers<sup>[56]</sup>. In a very large study of nearly 35000 patients from Taiwan, patients with sleep apnea experienced 2.4 fold higher risk for peptic ulcer bleeding<sup>[56]</sup>. This may warrant surveying for sleep apnea as a potential predisposing factor in patients with peptic ulcer bleeding and without any apparent risk factors.

## INFLAMMATORY BOWEL DISEASE AND SLEEP

IBD is characterized by a chronic immune mediated inflammation of the gastrointestinal tract. It is estimated that approximately 400/100000 Americans suffer from IBD<sup>[57]</sup>. The relationship between sleep and IBD has been a topic of more recent consideration. Ranjbaran *et al*<sup>[57]</sup> used the Pittsburgh Sleep Quality Index (PSQI) to show a relationship with sleep abnormalities and the quality of life in patients with IBD. They noted several sleep-related issues: more sleep latency, less day time energy, and increased sleeping pill use<sup>[57]</sup>.

Abnormal sleeping habits may also play a role on disease severity. One study noted both worsened severity of UC and higher mortality in phase-shifted mice than in unaltered circadian-phase mice<sup>[59]</sup>. They noted that chronic circadian phase shifts led to worsening mucosal inflammation and colitis likely secondary to altered in-

flammatory cascade regulation<sup>[59]</sup>. Another study found that occupations that have artificial working conditions (such as light) and irregular hours had higher odds ratio (1.6-1.7) for development for IBD<sup>[60,61]</sup>.

Patients with Crohn's disease (CD) and sleep loss may also have a greater risk for disease relapse. These patients had twice the risk of active disease in 6 mo than patients who did not have sleep abnormalities<sup>[62]</sup>. In fact, Tang *et al*<sup>[62]</sup> performed a study examining sleep deprivation on mice with colitis and noted both acute and chronic sleep deprivation led to worsening colitis likely secondary to heightened sensitivity to pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$ <sup>[9,30,61,63]</sup>. A large survey study looking at sleep disturbances in over 3100 participants found that CD patients in clinical remission and subjective sleep disturbances had a 2-fold increased risk of active disease at 6 mo. They discovered approximately 75% of patients with active disease have subjective sleep complaints compared to 48% inactive disease<sup>[62]</sup>.

Recently, we performed a prospective observational cohort study looking at the sleep disturbances of IBD patients. We discovered that 100% of patients with active disease had poor sleep while only 72% of patients with clinically inactive disease had poor sleep. The difference between sleep disturbances became even higher when histology was used to define the disease activity. We found 100% of those in histologically active group had poor sleep while only 54% in the histologically inactive group had poor sleep (OR = 6.0, 95%CI: 2.9-12.5,  $P < 0.0001$ ). An abnormal PSQI had a positive predictive value for histologic inflammatory activity of 83%<sup>[64]</sup>. These patients were prospectively followed for 6 mo, and the relapse rate in clinically inactive patients with poor sleep was found to be 67%. No patients with normal sleep patterns relapsed (RR = 3, 95%CI: 1.5-6.1,  $P = 0.03$ ). We detected a significant correlation between the baseline PSQI and disease activity at the 6-mo follow up (CD:  $r = 0.56$ ,  $P = 0.0046$ ; UC:  $r = 0.54$ ,  $P = 0.024$ )<sup>[65]</sup>. Although the study was limited by the small number of patients, the results are intriguing and hold very important therapeutic implication in the management of immune-mediated inflammatory diseases.

Melatonin has recently been investigated as a possible method of improving outcomes for patients with UC. Data from several animal models indicate that melatonin administration increased serum levels of IL-10 (an anti-inflammatory cytokine) and decreased serum levels of pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$ <sup>[66-69]</sup>. Patients with UC had abnormally high levels of pro-inflammatory cytokines, and melatonin may play a role in reducing the severity of UC by reducing these specific cytokines<sup>[69-73]</sup>.

## IRRITABLE BOWEL SYNDROME AND SLEEP

IBS is a chronic gastrointestinal syndrome that is associated with abdominal pain and distorted bowel behavior.



IBS is commonly diagnosed and there is an estimated 10%-15% of the North American population suffering from this syndrome<sup>[74]</sup>. IBS appears to have a significant association with anxiety, stress, and overall environment. Interestingly, sleep dysfunction also has similar associations. The study conducted by Kim *et al*<sup>[74]</sup> examined IBS occurrence among irregular-shift workers and traditional day-shift workers. They found that the prevalence of IBS in irregular-shift workers was significantly higher (32.7%) than in the day-shift workers (16.7%). They also found that many of the individuals that worked irregular shifts experienced less sleep quality, higher rates of daytime sleepiness, and higher levels of stress<sup>[75]</sup>. Chen *et al*<sup>[75]</sup> compared sleep patterns and rectal sensitivity using anorectal manometry among patients with IBS and healthy subjects. They noted that IBS patients with lower amounts of quality sleep were prone to lower thresholds for rectal sensitivity and altered anal sphincter function<sup>[76]</sup>. This rectal hyperalgesia in patients with sleep abnormalities and IBS is consistent with the visceral hyperalgesia noted in patients with sleep abnormalities and GERD<sup>[55]</sup>.

## COLON CANCER AND SLEEP

Colorectal cancer is the second most commonly diagnosed cancer in the world in women and the third most common in men<sup>[77]</sup>. Surgery is often the primary method of intervention while adjuvant chemotherapy and radiation therapy are often employed to improve survival or quality of life<sup>[78-80]</sup>. Several surveys noted that fatigue was one of the highest concerns for people with cancer<sup>[78,81,82]</sup>.

Animal studies indicate that both circadian disruption by nocturnal light exposure or sleep deprivation accelerated tumor formation<sup>[83-85]</sup>. A recent study by Thompson and colleagues evaluated sleep and colon cancer and noted that shorter duration of sleep (< 6 h) led to an almost 50% increase in the risk for colorectal adenomas<sup>[86]</sup>. Shift work, abnormal clock gene expression, and other causes of disruption of circadian rhythms are emerging as cancer risk factors<sup>[83,87]</sup>. A study by Schernhammer *et al*<sup>[87]</sup> found an increased risk for colon cancer in women who worked night shifts<sup>[88]</sup>. Several theories have been proposed to explain the relationship between sleep and colon cancer. Increased obesity is a known risk factor for cancer<sup>[89]</sup>. Sleep disorders are also known to alter metabolism and contribute to obesity<sup>[10]</sup>. Sleep disturbance may play an indirect role in increasing the risk for cancer by increasing adiposity<sup>[90]</sup>. Another theory suggests melatonin and its anti-carcinogenic properties are a key factor. Nocturnal light exposure suppresses melatonin production, and the lack of melatonin and its anti-proliferative effects may contribute to intestinal cancer formation<sup>[88,91]</sup>. Open discussion, evaluation, and treatment of lower-than-normal duration of sleep may be an under-appreciated method of colorectal cancer risk modification.

## SLEEP DYSFUNCTION AND THE LIVER

Sleep disturbances are seen in numerous types of liver

diseases. One study found 47.7% of cirrhotic patients had unsatisfactory sleep when compared to 4.5% seen in controls<sup>[92]</sup>. Elevated levels of ammonia seen in hepatic encephalopathy is also evidenced to induce sleep wake cycle reversal and progressive electroencephalography changes with triphasic wave changes in Stage I hepatic encephalopathy and eventually delta waves and comatose state in Stage IV<sup>[93]</sup>. Another study found that women with primary biliary cirrhosis slept nearly twice as much during the day when compared to controls<sup>[94]</sup>. Although the exact mechanism behind this is known, it is thought that elevated IL-6 plays a role<sup>[95]</sup>. Patients with hepatitis C also are at higher risk for sleep abnormalities with 60%-65% reporting abnormal sleep complaints<sup>[96]</sup>. In addition, patients undergoing treatment with interferon- $\alpha$  are also at increased risk for sleep abnormalities as 22%-24% of patients experience sleep disturbance as a side effect<sup>[97]</sup>.

Summa *et al*<sup>[97]</sup> study on mice found that circadian disorganization *via* Clock <sup>$\Delta 19/\Delta 19$</sup>  mutation led to elevated liver/body weight ratios and advanced alcohol induced steatohepatitis<sup>[98]</sup>. The etiology behind this connection is thought to rely on abnormal intestinal epithelial permeability. Ideally, the intestinal epithelial barrier serves to protect the body from unwanted luminal contents while also allowing a fraction of permeability to allow immune surveillance and regulation<sup>[99]</sup>. Summa *et al*<sup>[97]</sup> followed the absorption of sugars in the gastrointestinal tract in phase shifted mice and found increased permeability in the colon when compared to control. This evidence indicates that circadian dysfunction may be a separate risk factor for alcohol induced liver damage<sup>[98]</sup>.

Patients with sleep apnea sustain cessation of breath during sleep, leading to intermittent hypoxia, systemic inflammation, and sympathetic activation. These insults may contribute to initiation or progression of peptic ulcers<sup>[56]</sup>. In a very large study of nearly 35000 patients from Taiwan, patients with sleep apnea experienced 2.4 fold higher risk for peptic ulcer bleeding<sup>[56]</sup>.

## TREATMENT IMPLICATIONS

As the complexities regarding the association between sleep and gastrointestinal disorders continue to become better understood, it begs the question as to how the medical and psychiatric community should address comorbid sleep and gastrointestinal disorders. Though current clinical trials have not directly addressed this population, several small preliminary trials have investigated the efficacy of cognitive behavioral therapy for insomnia in patients with comorbid chronic pain<sup>[100-103]</sup>. Collectively, these studies suggest that insomnia can be effectively treated among patients with chronic pain and that improvement in sleep confers some clinical improvement in pain. Therefore, given the state of the current science, it seems prudent that medical providers would recommend the evaluation and treatment of sleep disorders in patients with gastrointestinal disorders. Treating both disorders in parallel may not only result in a better outcome



for the patient, but also allow the medical provider to use less invasive and expensive means to improve the patient's overall quality of life.

## CONCLUSION

Sleep abnormalities are a global issue and its effects on well-known pathologies is both an interesting and relevant field of research. Sleep abnormalities contribute to many gastrointestinal diseases and conversely, gastrointestinal diseases often lead to sleep abnormalities. This interdependent relationship represents a novel approach to treating GERD, IBS, IBD, liver disorders and colon cancer. The evaluation, discussion, and treatment of sleep abnormalities may play a key role in further preventing and improving many gastrointestinal disorders.

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## Pathophysiology of cerebral oedema in acute liver failure

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### Abstract

Cerebral oedema is a devastating consequence of acute liver failure (ALF) and may be associated with the development of intracranial hypertension and death. In ALF, some patients may develop cerebral oedema and increased intracranial pressure but progression to life-threatening intracranial hypertension is less frequent than previously described, complicating less than one third of cases who have proceeded to coma since the advent of improved clinical care. The rapid onset of encephalopathy may be dramatic with the development of asterixis, delirium, seizures and coma. Cytotoxic and vasogenic oedema mechanisms have been implicated with a preponderance of experimental data favouring a cytotoxic mechanism. Astrocyte swelling is the most consistent neuropathological finding in humans with ALF and ammonia plays a definitive role in the development of cytotoxic brain oedema. The mechanism(s) by which ammonia induces astrocyte swelling remains unclear but glutamine accumulation within astrocytes has

led to the osmolyte hypothesis. Current evidence also supports an alternate 'Trojan horse' hypothesis, with glutamine as a carrier of ammonia into mitochondria, where its accumulation results in oxidative stress, energy failure and ultimately astrocyte swelling. Although a complete breakdown of the blood-brain barrier is not evident in human ALF, increased permeation to water and other small molecules such as ammonia has been demonstrated resulting from subtle alterations in the protein composition of paracellular tight junctions. At present, there is no fully efficacious therapy for cerebral oedema other than liver transplantation and this reflects our incomplete knowledge of the precise mechanisms underlying this process which remain largely unknown.

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**Key words:** Cerebral oedema; Acute liver failure; Ammonia; Hepatic encephalopathy; Intracranial pressure; Intracranial hypertension; Cerebral blood flow

**Core tip:** Cytotoxic and vasogenic cerebral oedema have been implicated in acute liver failure (ALF) with a preponderance of experimental data favouring cytotoxic mechanisms. Astrocyte swelling is a consistent neuropathological finding in human ALF and ammonia plays a definitive role. The mechanism(s) by which ammonia induces astrocyte swelling remains unclear but glutamine plays a central role inducing oxidative stress, energy failure and ultimately astrocyte swelling. Although complete breakdown of the blood-brain barrier is not evident in human ALF, increased permeation to water and ammonia has been demonstrated. There is no efficacious therapy other than liver transplantation reflecting the incomplete knowledge base.

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## INTRODUCTION

Acute liver failure (ALF) is a complex clinical syndrome that results from a sudden and severe loss in hepatocyte function in a patient without pre-existing liver disease<sup>[1]</sup>. This rapid loss of function is the result of massive hepatocyte necrosis and is typically associated with hepatic encephalopathy (HE) and coagulopathy, the hallmark features of ALF. In many cases progressive multi-organ failure ensues. Although ALF is rare, with an incidence of one to six cases per million people every year in the United States and Western Europe, the mortality rate and the cost of treatment is high<sup>[2]</sup>. The majority of those affected are young adults.

ALF is sometimes referred to as fulminant hepatic failure (FHF), a term first used in 1970 by Trey and Davidson<sup>[3]</sup> who described a potentially reversible disorder resulting from severe hepatic injury, with an onset of encephalopathy within 8 wk of symptom appearance in the absence of chronic liver disease. Whilst the main features of this definition remain relevant today, O'Grady *et al*<sup>[4]</sup> proposed a new classification for adults with ALF, dividing them into three groups based on the time between the onset of jaundice to the development of encephalopathy: hyperacute (within 7 d), acute (8-28 d) and subacute (5-12 wk). This classification recognises that ALF complications and prognosis depend on the rate of evolution of the disorder. It has now gained wide acceptance in clinical and research studies. Those with a hyperacute presentation, such as following an acetaminophen overdose, are at highest risk of developing cerebral oedema.

The most reliable clinical signs of severe ALF include coagulopathy [international normalised ratio (INR)  $\geq$  1.5], which may become severe enough to cause spontaneous bleeding, and HE (any degree of altered mentation). HE presents with a rapid onset of initially subtle mental alterations such as minor confusion, disorientation and agitation, progressing to delirium, seizures and coma. When severe, HE is typically associated with the development of cerebral oedema<sup>[5]</sup>.

Historically, cerebral oedema was thought to occur in up to 80% of patients with ALF and be the most common cause of death<sup>[6]</sup>. However, recent data following a review of 3300 patients presenting to a single tertiary liver centre has shown that the proportion of patients with intracranial hypertension (ICH) fell from 76% in 1984-1988 to 20% in 2004-2008 ( $P < 0.0001$ ). In those who developed ICH, mortality fell from 95% to 55% ( $P < 0.0001$ ). This mirrored a fall in the admission markers of disease severity and most likely reflects earlier illness recognition, improved intensive care, and use of salvage liver transplantation<sup>[7]</sup>. A further study from Bernal and colleagues from King's College Hospital on 165 patients presenting with ALF and grade 3/4 HE found that only 29% showed evidence of ICH. However, only one third

had intracranial bolts inserted which raises the possibility that some of this cohort may have developed ICH without showing clinical sequelae. Whether the development of cerebral oedema is similar or higher in patients with ALF in developing countries remains to be determined. Nevertheless, along with sepsis and multi-organ failure, it is one of the leading causes of death in these patients<sup>[1,8]</sup>.

Patients with ALF are acutely ill and are best managed in intensive care units within tertiary liver transplant centres. The armamentarium of treatments available to alleviate cerebral oedema include mannitol, hyperventilation, hypertonic sodium chloride, induced hypothermia and barbiturates which aim to decrease the total fluid volume within the brain either by reducing the interstitial fluid and/or by reducing cerebral blood flow<sup>[8]</sup>. However, at present, no fully efficacious medical therapy for ALF is available and the only effective treatment is an emergency liver transplantation<sup>[1]</sup>. Nevertheless, liver transplantation is not always an option, with co-morbidities, sepsis, multi-organ failure and graft availability posing a major obstacle to a patient qualifying for a life-saving liver transplant.

The pathophysiological mechanisms underpinning the development of cerebral oedema are complex and remain to be fully unravelled. The central role of ammonia in the pathogenesis of cerebral oedema in ALF however, remains undisputed. Indeed, arterial ammonia concentrations greater than 100  $\mu\text{mol/L}$  have been shown to predict the onset of severe HE with 70% accuracy with ICH developing in 55% of patients with ALF with an arterial ammonia concentration  $> 200 \mu\text{mol/L}$ <sup>[9]</sup>. Furthermore, Clemmesen and colleagues have shown that blood ammonia levels in excess of 150  $\mu\text{mol/L}$  predicted a greater likelihood of dying from brain herniation<sup>[10]</sup>.

## CEREBRAL OEDEMA AND ACUTE LIVER FAILURE: AN OVERVIEW

Cerebral oedema is a net increase in total brain water content. The rigid skull bone protecting the brain limits the compliance of the brain and as a consequence a small increase in fluid can cause a significant rise in intracranial pressure. ICH can lead to a decrease in cerebral perfusion pressure and capillary blood flow, culminating in ischaemia<sup>[11]</sup>.

Cerebral oedema as a complication of massive hepatic necrosis was first described by Ware *et al*<sup>[12]</sup> in 1971 and was found to be present in 80% of comatose ALF patients on post-mortem examinations. Increased water content of brain tissue has been considered to be a cardinal feature of cerebral oedema in ALF. However, ICH caused by an increase in cerebral blood flow has also been demonstrated in experimental models of ALF<sup>[13]</sup> in addition to patients with ALF<sup>[14]</sup>. Impaired autoregulation of CBF is well documented in patients with ALF and can be explained by the presence of vasodilatation of cerebral arterioles resulting in increased intracranial blood volume (cerebral hyperemia or the so-called luxury perfu-

sion)<sup>[15-17]</sup>.

More recent studies have suggested that neuroinflammatory mediators, particularly pro-inflammatory cytokines such as the interleukins (IL)-1 $\beta$  and IL-6 and tumour necrosis factor-alpha (TNF- $\alpha$ ), play an important role in the development of ICH<sup>[18]</sup> and progression of HE<sup>[19,20]</sup>. The presence of an infection or systemic inflammation (also known as 'systemic inflammatory response syndrome' or 'SIRS') is common in ALF and has been shown to be a major prognosticator of both the progression of HE and mortality in patients with ALF<sup>[21,22]</sup>. Moreover, evidence suggests that this inflammatory response may not only be peripheral but may arise within the brain itself<sup>[18,23,24]</sup>. Neuroinflammation is now widely considered to result from a direct interaction between microglia and ammonia<sup>[25,26]</sup>. The released pro-inflammatory cytokines from activated microglial cells and ammonia appear to act synergistically to induce cerebral oedema<sup>[27]</sup>.

The neuropathological aspects of cerebral oedema were first described by Klatzo<sup>[28]</sup> in a presidential address classifying the underlying mechanisms of cerebral oedema into cytotoxic or vasogenic. This was further explored within the context of ALF by Ede *et al.*<sup>[29]</sup>. In cytotoxic oedema the BBB is intact and there is intracellular swelling<sup>[30]</sup>, whereas in vasogenic oedema there is breakdown of the BBB and water and plasma constituents accumulate in the extracellular space<sup>[31]</sup>.

## CYTOTOXIC OEDEMA AND ASTROCYTE SWELLING

The most prominent neuropathological finding from studies of brain autopsies of patients with ALF<sup>[32]</sup> and from animal models of cerebral oedema due to ALF is astrocyte swelling<sup>[30,33-35]</sup>. Astrocytes found within the gray matter are mainly affected and swelling of astrocytic foot processes rather than cell bodies is more commonly seen<sup>[32]</sup>.

Magnetic resonance imaging (MRI) studies using diffusion tensor imaging (DTI) in humans support the view that astrocyte swelling, *i.e.*, cytotoxic oedema, represents the major component of cerebral oedema in ALF<sup>[36]</sup>. A reduction in the apparent diffusion coefficient (ADC) has been demonstrated in patients with ALF, indicative of a reduction in the size of the extracellular space. This implies that the development of cerebral oedema in ALF results from the accumulation of intracellular fluid.

Approximately one third of the brain volume is made up of astrocytes. They have an important function supporting neurones and have many biochemical, neurochemical and regulatory roles. Swelling of astrocytes therefore impacts upon their function. Abnormal membrane depolarisation has been demonstrated which could affect the ability of astrocytes to maintain ionic gradients and regulate neurotransmitter uptake and processing<sup>[37,38]</sup>. Impairment of astrocytic function can have deleterious effects on the rest of the central nervous system (CNS) leading to impairment of neuronal excitability and func-

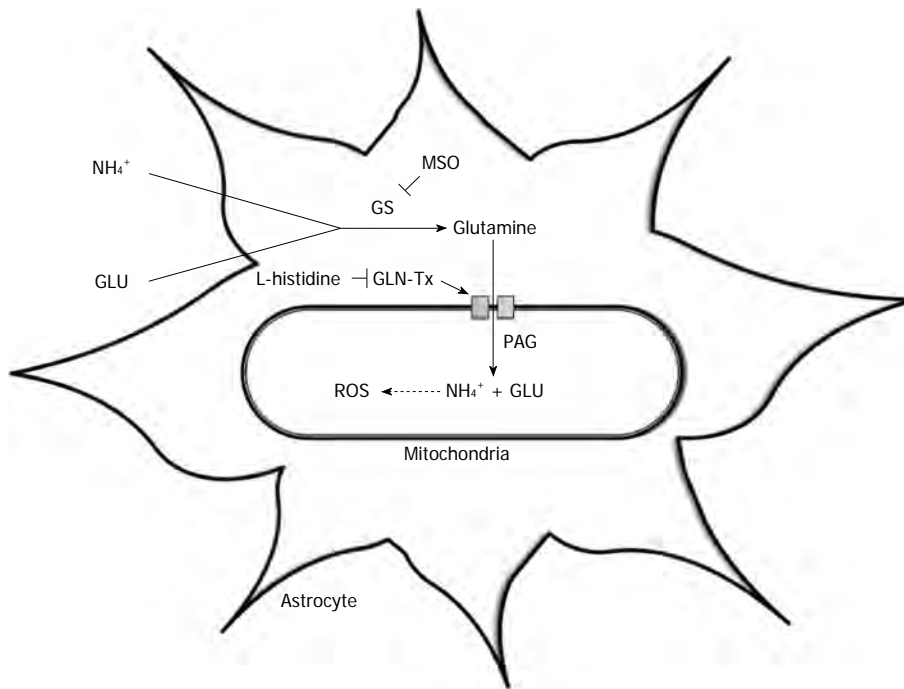
tion.

The precise mechanism by which astrocytes swell remains to be determined, although many factors have been implicated. The evidence is most compelling for a role for ammonia in the development of astrocyte swelling in ALF<sup>[35]</sup>. Whilst other factors, including cerebral blood flow, vaso paralysis, hyperthermia, hyponatremia, substances derived from the necrotic liver, infection, inflammatory cytokines, lactic acid and glutamate have all been implicated in astrocyte swelling, the data is insufficient and often conflicting<sup>[34,39]</sup>. These factors may all act synergistically to induce cytotoxic swelling with ammonia playing a central role<sup>[20]</sup>.

## AMMONIA-GLUTAMINE HYPOTHESIS

Ammonia is mainly produced in the small bowel by the enzyme glutaminase, which breaks down glutamine into ammonia and glutamate. Ammonia is metabolised to urea primarily by the liver and to a lesser extent by the kidneys. In ALF this detoxification pathway, known as the urea cycle, is impaired from the loss of hepatocytes and the concentration of ammonia in the blood rises. Arterial concentrations of ammonia have been shown to correlate with the development of intracranial hypertension<sup>[9]</sup> and cerebral herniation<sup>[10]</sup>. Numerous experimental models of ALF have unequivocally associated ammonia exposure with the induction of astrocyte swelling. Treatment of cultured astrocytes with ammonia has consistently caused astrocytes to swell<sup>[40]</sup>. *In vivo* animal models of hyperammonemia have also demonstrated the presence of astrocyte swelling<sup>[30,41]</sup>. Rose *et al.*<sup>[42]</sup> treated rats in ALF with *L*-ornithine-*L*-aspartate, an ammonia-lowering agent which acts by stimulating the urea cycle, and found a reduction in plasma ammonia concentrations and, more importantly, a reduction in cerebral oedema. Lastly, in the absence of liver pathology, patients with genetic disorders of urea cycle enzymes culminating in hyperammonemia develop cerebral oedema, suggesting that elevated levels of ammonia alone are sufficient to cause brain swelling<sup>[43]</sup>. The precise mechanisms underlying ammonia-induced astrocyte swelling are still poorly understood. Ammonia is able to enter the brain by diffusion<sup>[44,45]</sup> and its increased uptake from the circulation<sup>[46,47]</sup> leads to disturbances in astrocyte function<sup>[48-50]</sup>.

The exclusive localisation within astrocytes of glutamine synthetase<sup>[51]</sup>, a cytosolic enzyme which converts ammonia to glutamine, has led to the 'osmolyte' or 'ammonia-glutamine' hypothesis. Ammonia is detoxified to glutamine within the astrocyte, a precursor for the neurotransmitter glutamate. In addition to causing astrocyte swelling, ammonia has been shown to increase cerebral glutamine levels in the ALF setting<sup>[41]</sup>. Elevated glutamine levels have been found in brain tissue from animal models of HE<sup>[52]</sup> and in cerebrospinal fluid (CSF) and brain from patients with HE due to ALF<sup>[53,54]</sup>. These findings collectively suggest a potential role for glutamine in the development of astrocyte swelling, with hyperammonemia



**Figure 1** The 'Trojan Horse' hypothesis. This illustrates the synthesis of glutamine via the enzyme glutamine synthetase; its transport into mitochondria via the glutamine transporter (GLN-Tx); its hydrolysis by phosphate-activated glutaminase (PAG) resulting in glutamate (GLU) and ammonia ( $\text{NH}_4^+$ ) production and the subsequent generation of reactive oxygen species (ROS). MSO: L-methionine S-sulfoximine; GS: Glutamine synthetase.

mia causing increased synthesis and accumulation of glutamine in astrocytes, resulting in astrocyte swelling<sup>[55,56]</sup>.

Originally, it was thought that glutamine acted as an organic osmolyte increasing the intracellular osmolarity, resulting in an influx of water into the cell and culminating in astrocyte swelling and dysfunction. In order to verify whether glutamine accumulation induces astrocyte swelling in hyperammonemic states, studies utilising L-methionine S-sulfoximine (MSO), an irreversible inhibitor of glutamine synthetase, have been performed<sup>[57]</sup>. Firstly, MSO lowers glutamine in normal brains<sup>[58]</sup> and prevents cerebral oedema in ammonia-infused healthy rats<sup>[59]</sup>. Subsequently, it was found to significantly diminish astrocyte swelling both *in vivo*<sup>[41]</sup> and in cell culture<sup>[60]</sup>. Therefore, inhibition of glutamine synthesis may have a protective effect, preventing glutamine accumulation, astrocyte swelling and thus cerebral oedema.

Although these findings suggest that glutamine accumulation within astrocytes plays an important role in cerebral oedema, more recent studies have questioned the glutamine-osmolyte hypothesis. In rats with ALF, glutamine concentrations do not correlate well with the degree of encephalopathy and associated cerebral oedema<sup>[61]</sup>. In two experimental models of ALF, rats were cooled to reduce brain swelling. Although cerebral oedema was ameliorated by mild hypothermia, it was not accompanied by a similar decrease in glutamine level<sup>[62,63]</sup>. Jayakumar *et al*<sup>[64]</sup> further tested the hypothesis using cultured astrocytes exposed to ammonia. They found no direct correlation between astrocyte swelling and glutamine levels. More importantly, astrocyte swelling was absent when glutamine levels peaked and cell swelling was maximal when

glutamine levels were low. Furthermore, the duration and persistence of hyperammonemia, rather than its absolute level is most likely to determine brain glutamine levels and correlate with the development of cerebral oedema and raised intracranial pressure<sup>[65]</sup>. This delay in astrocyte swelling in relation to an increase in cellular glutamine content is not consistent with the concept of glutamine acting as an osmolyte in ALF and suggests that astrocyte swelling may not be the result of a direct osmotic effect of glutamine.

The 'Trojan horse' hypothesis has recently been proposed as an alternative theory by Albrecht *et al*<sup>[66]</sup> to explain the development of astrocyte swelling and brain oedema and suggests an important role for both ammonia and glutamine. The excess glutamine synthesised within astrocytes is transported into mitochondria where it is metabolised by phosphate-activated glutaminase (PAG) to ammonia and glutamate<sup>[67]</sup>. Glutamine, the "Trojan horse", thereby acts as a carrier of ammonia into mitochondria, where its accumulation can lead to oxidative stress and ultimately astrocyte swelling (Figure 1).

## OXIDATIVE STRESS, MITOCHONDRIAL PERMEABILITY TRANSITION AND ENERGY FAILURE

Oxidative stress has been implicated as an important factor in the pathophysiology of ammonia-induced neurotoxicity<sup>[68]</sup>. O'Connor *et al*<sup>[69]</sup> first suggested oxidative stress might play a role in the pathogenesis of HE when they found evidence of lipid peroxidation in hyperam-



monemic mice. Norenberg *et al*<sup>[70]</sup> subsequently described the concept that protein peroxidation as well as lipid peroxidation may occur in astrocytes treated with ammonia. Further studies revealed that ammonia was able to generate free radicals such as superoxide in cultured astrocytes<sup>[71]</sup> and *in vivo*<sup>[72]</sup>. Ammonia also increases mRNA levels of heme-oxygenase-1 (HO-1), which is considered to be one of the best markers of oxidative stress, in a portacaval shunt rat model of HE<sup>[73]</sup>. Finally, decreased activity of the antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase were described in rats exposed to ammonia toxicity adding to the burden of oxidative stress<sup>[72]</sup>.

Oxidative stress has been shown to be a key component of the cerebral oedema which develops in rats following hepatic devascularization<sup>[74]</sup>. Moreover, administration of antioxidants such as superoxide dismutase, catalase and vitamin E have been shown to inhibit the ammonia-induced astrocyte swelling<sup>[64]</sup>. Although most evidence supporting the development of oxidative stress in ALF comes from animal and cell culture studies, clinically, the antioxidant and anti-inflammatory agent *N*-acetylcysteine has proven to be beneficial in the management of patients with ALF<sup>[75-77]</sup>, and agents such as mannitol and sodium benzoate, which are occasionally used in the treatment of ALF, have also been shown to have antioxidant effects<sup>[78]</sup>.

Nitrosative stress is also considered to play an important role in ammonia-induced neurotoxicity. Data from experimental models of HE revealed increased nitric oxide synthase (NOS) gene expression and activity in the brain<sup>[79,80]</sup>. Inhibition of NOS by nitroarginine significantly reduced deaths in mice exposed to ammonia neurotoxicity<sup>[81]</sup>. In line with these findings, nitric oxide (NO), was shown to have increased in brains of portacaval-shunted rats given continuous ammonia infusions<sup>[82]</sup>. This animal model is a well-standardised paradigm of cerebral oedema which occurs in the absence of ALF.

Free radicals such as NO and superoxide can be categorised into reactive nitrogen and oxygen species (RNOS), respectively. In cultured astrocytes and in rat brain *in vivo*, ammonia triggers their formation through *N*-methyl-*D*-aspartate (NMDA)-receptor and calcium (Ca<sup>2+</sup>)-dependent mechanisms<sup>[71,83-86]</sup>. Activation of the NMDA receptor is thought to result from the depolarisation-induced removal of the magnesium blockade, which can be induced by ammonia and swelling of the cell itself. Ammonia induces glutamate release from cultured astrocytes<sup>[87]</sup> and NMDA receptor activity can be further amplified by subsequent Ca<sup>2+</sup>-dependent astroglial glutamate release and autocrine NMDA receptor stimulation<sup>[88]</sup>. There is a close relationship between oxidative stress and astrocyte swelling which makes it difficult to separate them temporally as both events are causally interlinked<sup>[85,89,90]</sup>. This suggests a self-amplifying cycle<sup>[91]</sup> whereby on the one hand, astrocyte swelling induces oxidative/nitrosative stress through NMDA receptor and Ca<sup>2+</sup>-dependent mechanisms, and on the other, NMDA receptor activa-

tion and oxidative stress trigger astrocyte swelling.

Exactly how ammonia-induced free radicals lead to cell swelling and cerebral oedema is not known. One possibility is that they cause direct damage to proteins and lipids in the membranes of cells and organelles such as mitochondria, thereby altering membrane permeability by affecting ion transport systems. In mitochondria, oxidative injury could lead to altered bioenergetics. Controlled ion transport systems and energy production are essential in maintaining normal cell volume, and alterations in their activity could lead to disturbed volume regulation.

One critical consequence of oxidative and nitrosative stress is induction of the mitochondrial permeability transition<sup>[92]</sup>. The MPT usually develops in response to an increase in mitochondrial Ca<sup>2+</sup> levels and results in a sudden opening of the permeability transition pore (PTP), a large non-selective permeability pore in the inner mitochondrial membrane. This leads to increased permeability of the inner mitochondrial membrane to protons, ions and other small solutes. As a result, the inner mitochondrial membrane potential dissipates causing mitochondrial dysfunction. The MPT is therefore associated with movement of metabolites across the inner mitochondrial membrane, swelling of the mitochondrial matrix, defective oxidative phosphorylation and adenosine triphosphate (ATP) production, and generation of free radicals<sup>[93]</sup>. Production of free radicals through MPT induction further aggravates the MPT, resulting in a vicious cycle. Induction of the MPT was described in cultured astrocytes exposed to ammonia<sup>[60]</sup>. The mechanism underlying MPT induction most likely involves oxidative stress, as antioxidants including superoxide dismutase, catalase and vitamin E were able to inhibit the development of the MPT by ammonia<sup>[94]</sup>.

Cyclosporine A (CsA) blocks ammonia-induced astrocyte swelling in culture during the evolution of swelling<sup>[95]</sup>. Nevertheless, the mechanism(s) by which the MPT mediates astrocyte swelling in hyperammonemia remains unclear. Interestingly, glutamine is capable of inducing the MPT in cultured astrocytes<sup>[55]</sup> as well as causing mitochondrial swelling in isolated rat cerebral mitochondria<sup>[96]</sup>. It is notable that, like ammonia, glutamine has been shown to induce oxidative stress by forming free radicals<sup>[97]</sup>. How glutamine acts to induce oxidative stress, the MPT and consequent astrocyte swelling, is less clear however although it has been suggested that glutamine mediates its deleterious effects through ammonia. Glutamine is hydrolysed in the mitochondria by PAG to yield high levels of ammonia which leads to oxidative stress and the MPT. In support of this concept is the finding that inhibition of PAG by 6-diazo-5-oxo-*L*-norleucine (DON) blocks free radical production<sup>[97]</sup>, MPT formation<sup>[98]</sup> as well as ammonia-induced astrocyte swelling<sup>[64]</sup>. In a further study, *L*-histidine, an inhibitor of mitochondrial glutamine transport, was further used to study the role of mitochondrial glutamine in a rat model of ALF<sup>[99]</sup>. *L*-histidine was found to inhibit HO-1 overexpression, the MPT and brain oedema, supporting the involvement

of glutamine in the development of oxidative stress. Taken together, the above data supports the key role of glutamine transport into mitochondria and subsequent metabolism to ammonia in the pathogenesis of cerebral oedema in ALF. Furthermore, these findings support the “Trojan horse” theory, which suggests that glutamine acts as a “stealth” carrier of ammonia in ammonia-induced neurotoxicity.

In terms of a timeline, it was shown that exposure of cultured rat astrocytes and mice brain slices to ammonia results in rapid ROS formation and astrocyte swelling<sup>[89,90]</sup>, whereas MPT-induction and glutamine accumulation occurs later<sup>[60,64]</sup> implying astrocyte swelling occurs primarily through oxidative/nitrosative stress and is then further aggravated by glutamine accumulation in astrocytes<sup>[100]</sup>.

Cell volume regulation is an energy-dependent process and involves ion homeostasis through ionic transporters and exchangers and extrusion of osmotically active amino acids<sup>[101]</sup>. In particular, the Na/K/Cl cotransporter-1 (NKCC1) was found to be implicated in astrocyte swelling. NKCC1 expression and activity was increased in cultured astrocytes exposed to ammonia and its activation appears to be mediated by oxidative/nitrosative stress<sup>[102]</sup>. Energy failure following MPT induction is another possible mechanism underlying cell swelling. Ammonia is thought to interfere with mitochondrial energy metabolism and several studies have reported depletion of ATP *in vitro* and *in vivo* models of ammonia neurotoxicity<sup>[103]</sup>. The implications of energy failure in ALF have largely been ignored despite the presence of higher lactate levels in patients with ALF, which is a consequence of energy failure<sup>[104,105]</sup>. Indeed, Zwingmann *et al.*<sup>[104]</sup> in an experimental ALF rodent model showed that in the early (pre-coma) stages of encephalopathy there was a significant 2 to 4.5-fold increase in total brain glutamine and lactate but in the severe (coma) stages of encephalopathy and brain oedema there was a further significant increase in brain lactate but no such increase in glutamine suggesting that impaired glucose oxidative pathways rather than intracellular glutamine accumulation *per se* may play a more dominant role<sup>[101]</sup>. This is supported by data by Bernal *et al.*<sup>[105]</sup> that unequivocally shows lactate to be an important prognostic marker in ALF<sup>[102]</sup> and data from Rose *et al.*<sup>[106]</sup> in a pig model of ALF which demonstrated using cerebral microdialysis that ALF animals had increased levels of lactate dehydrogenase activity and mitochondrial complex IV activity.

Mitogen-activated protein kinases (MAPKs) are activated by oxidative/nitrosative stress in cultured astrocytes exposed to ammonia and inhibition of MAPK phosphorylation abrogates astrocyte swelling<sup>[107]</sup>. Activation of MAPKs may therefore play an important protective role in cell volume regulation through phosphorylation of key proteins.

Water flow across cell membranes in astrocytes is largely dependent on aquaporin 4 (AQP4)<sup>[108]</sup>. Upregulation of AQP4 has been found to precede cell swelling in cultured astrocytes treated with ammonia and CsA can

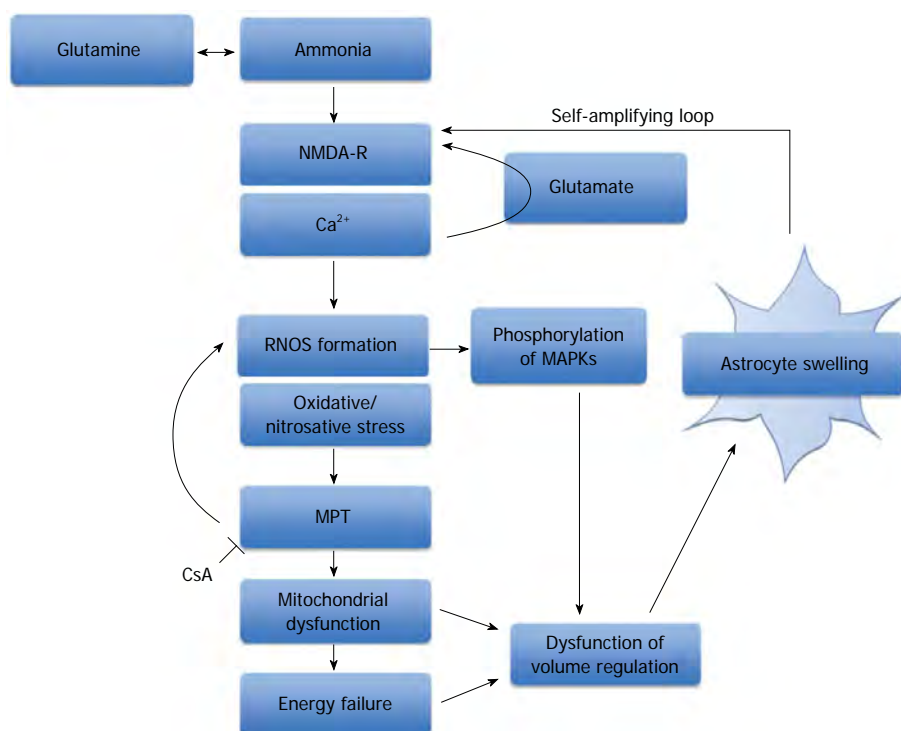
inhibit this upregulation, indicating that MPT induction is a key step in AQP4 upregulation in ammonia-induced astrocyte swelling<sup>[109]</sup>. Although it has been suggested that AQP4 is important in initiating signalling events associated with cerebral oedema<sup>[110]</sup>, Wright *et al.*<sup>[111]</sup> in a rat model of ALF could not find any association of the expression of AQP4 with the development of brain oedema, hyperammonemia or sepsis. The exact role of AQP4 in ALF therefore remains hotly debated.

In recent years, ammonia-induced and swelling-induced oxidative/nitrosative stress has been shown to result in multiple functional consequences. In addition to protein phosphorylation, oxidative/nitrosative stress can trigger protein tyrosine nitration, RNA oxidation and altered zinc metabolism, which can lead to changes in gene expression, intracellular signalling and synaptic plasticity<sup>[112]</sup>. Furthermore, nitration of glutamine synthetase inactivates the enzyme<sup>[113]</sup>, which suggests this regulatory mechanism leads to reduced glutamine production and therefore astrocyte swelling (Figure 2).

## VASOGENIC OEDEMA AND BLOOD-BRAIN BARRIER DYSFUNCTION

The BBB plays a critical role in establishing and maintaining homeostasis of the brain. It exerts tight control over any exchange of metabolites between the circulating blood and the central nervous system. The BBB consists of brain capillary endothelial cells, pericytes and the enveloping end foot processes of astrocytes. Together they form a neurovascular unit capable of regulating the special composition of the CNS fluid<sup>[114]</sup>. The main structural constituent of the BBB, and the first to come into direct contact with potentially toxic substances, is the endothelial cell. By spreading itself to cover the entire luminal surface of the capillary and sealing its two surface edges with junctional complexes known as tight junctions (TJ), the endothelial cell forms a physical barrier. These tight junctions consist of transmembrane proteins, including junctional adhesion molecules (JAM), occludin, claudins and intracellular proteins [zona occludin (ZO)-1, -2, and -3] linked to the cytoskeleton which control the stability and functioning of the TJ. Together with adherens junctions located in the basal region below the TJ, they prevent circulating compounds from freely entering the brain parenchyma and limit paracellular diffusion of small molecules. Transport of larger molecules into the brain occurs in a transcellular fashion utilising specific transport systems within endothelial cells. Water is able to diffuse through the bilayer of endothelial cell plasma membranes but can also enter the brain through water channels known as aquaporins, the predominant one in the brain being AQP4<sup>[108]</sup>.

Recent MRI studies of patients with ALF demonstrate evidence of interstitial brain oedema as well as cytotoxic oedema, implying there may be a vasogenic component to the cerebral oedema in ALF<sup>[115,116]</sup>. In an animal model of ALF, astrocyte swelling, extravascular



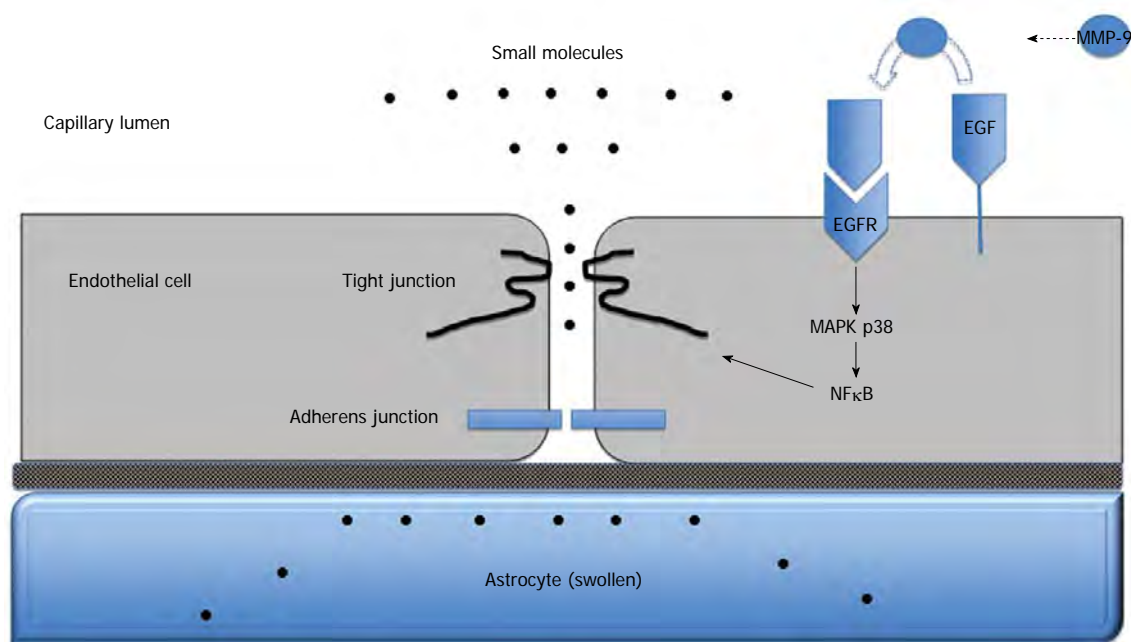
**Figure 2** The role of oxidative stress, mitochondrial permeability transition and energy failure in ammonia-induced neurotoxicity. A schematic representation of the central role that ammonia plays in the production of oxidative/nitrosative stress and astrocyte swelling. Ammonia-induced astrocyte swelling is mediated by oxidative and nitrosative stress resulting in the induction of the MPT, activation of intracellular signaling kinases and alterations in gene expression. Mitochondrial dysfunction and energy failure culminates in astrocytes failing to regulate their cell volume, thereby resulting in astrocyte swelling. NMDA-R: *N*-methyl-*D*-aspartate-receptor; RNOS: Reactive nitrogen and oxygen species; MPT: Mitochondrial permeability transition; MAPKs: Mitogen-activated protein kinases; CsA: Cyclosporine A.

and interstitial oedema have been described. However, brain capillary endothelial cells and their tight junctions appeared intact<sup>[30,117]</sup>. Similar findings were also reported in patients who died of ALF<sup>[132]</sup>. Apart from an increase in cytoplasmic vesicles, suggesting altered transcellular transport across the BBB, no gross structural damage was found in capillary endothelial cells. Similarly, Nguyen<sup>[118]</sup> has described physically intact tight junctions in ALF, but these were lengthened and tortuous in shape. Thus, electron microscopic examination of the BBB reveals only minimal ultrastructural changes in the brain capillaries of animals and humans with ALF.

Nevertheless, subtle increases in BBB transport of amino acids and energy metabolites have been widely described in the context of hyperammonemia<sup>[119]</sup>. Changes in BBB penetration of ammonia itself have also been reported in hyperammonemic states. However, the results of these reports, which used PET with <sup>13</sup>N-labeled ammonia to study BBB passage of ammonia, are inconsistent<sup>[47,120,121]</sup>. Nevertheless, in animal models of ALF, ammonia uptake into the brain is thought to increase<sup>[122]</sup>. Investigating possible changes in BBB permeability to ammonia has been hampered by the recent discovery that ammonia may be able to cross the BBB *via* two possible routes, and it is not known which of the two may be affected in hyperammonemic states. Circulating ammonia is largely present as a cation (NH<sub>4</sub><sup>+</sup>) and transport across the BBB was originally considered to occur *via* diffusion in its gaseous form (NH<sub>3</sub>), the amount of which is rather

small at physiological pH levels<sup>[45]</sup>. In ALF, due to the acidosis caused by lactic acid, the amount of NH<sub>3</sub> and hence its diffusion across the BBB would be expected to be reduced still further. The electric charge of the ionic form was thought to prevent ammonia transport across the BBB, but now an alternative, transcellular route, through potassium channels and transporters, has been suggested<sup>[123]</sup>. This transcellular transport of ammonia may be affected in ALF, resulting in increased ammonia concentrations within the CNS. Pathological increases in BBB permeability could also result in gaseous ammonia entering the brain *via* a paracellular route.

Although there has been little evidence for a complete BBB breakdown, findings from more recent studies suggest vasogenic oedema may still contribute to the development of cerebral oedema in ALF. Nguyen and colleagues used Evans blue dye, which binds to albumin and is normally unable to penetrate the BBB, and injected it into the circulation of mice with azoxymethane-induced ALF to assess brain extravasation. They found that BBB permeability to Evans blue dye and water was significantly increased in mice with experimentally-induced ALF. Under electron microscopy, they noted that the leakage of Evans blue dye, *i.e.*, extravasation, occurred mostly in the surrounding region of the brain capillaries. Consistent with previous findings, the BBB and tight junctions were found to be structurally intact<sup>[124]</sup>. Furthermore, they were able to demonstrate that BBB permeability and brain water was reduced in ALF mice given monoclonal



**Figure 3 Blood-brain barrier dysfunction in acute liver failure.** Anatomy of the blood-brain barrier (BBB) created by the brain capillary endothelial cell and its paracellular tight junction and adherens junction. In acute liver failure, activation of epidermal growth factor receptor (EGFR) and other signaling pathways results in a loss of BBB tight junction integrity. Tight junctional proteins are altered, resulting in increased permeability to small molecules, leading to astrocyte swelling. MMP-9: Matrix metalloproteinase-9; MAPK p38: Mitogen activated protein kinase p38; NFκB: Nuclear factor-κB.

antibodies specific for active matrix metalloproteinase-9 (MMP-9), a member of the matrix metalloproteinase (MMP) family of endopeptidase enzymes that degrade the extracellular matrix in normal and disease states. MMP-9 in particular, causes protein degradation of tight junctions and is upregulated in the liver of ALF mice. Increased blood concentrations of MMP-9 can also be found. These findings collectively show increased BBB permeation to water and plasma constituents in experimental ALF mice and suggest that BBB dysfunction is associated with protein deregulation in tight junctions but not necessarily with a structural breakdown. Circulating MMP-9 derived from the necrotic liver contributes to fine perturbation in BBB integrity and increased brain extravasation in mice with azoxymethane-induced ALF and inhibition of MMP-9 may be useful in preventing the development of brain oedema. Chen *et al.*<sup>[125]</sup> further demonstrated that MMP-9 induces significant degradation of the TJ proteins occludin and claudin-5 in brain endothelial cells *in vitro* and in mice with azoxymethane-induced ALF; these alterations in TJ proteins correlated with increased BBB permeability and were reversed by inhibiting MMP-9. Chen *et al.*<sup>[126]</sup> went on to demonstrate that MMP-9 induces activation of the epidermal growth factor receptor (EGFR) and p38 mitogen activated protein kinase/nuclear factor-κB (MAPK/NFκB) in brain endothelial cells. Activation of this pathway in turn leads to degradation of the TJ protein occludin and deregulation of the TJ. Taken together, these findings suggest that substances derived from the injured liver, such as MMP-9, reach the BBB and induce increased permeability through subtle changes in TJ composition (Figure 3).

An important role for a vasogenic mechanism in the development of cerebral oedema in ALF is thus supported by these studies.

Interestingly, activation of the p38 MAPK pathway as a result of oxidative/nitrosative stress is also thought to mediate ammonia-induced astrocyte swelling<sup>[107]</sup>. The p38 MAPK pathway and subsequent phosphorylation of key proteins appears to play an important role in the pathophysiology of cell swelling<sup>[127,128]</sup> and thus cerebral oedema, and therefore, this pathway may be a potential therapeutic target.

In recent years, there has been some controversy as to whether ALF *per se* causes the changes seen within the BBB integrity or whether these changes are due to secondary complications associated with ALF such as infection and sepsis<sup>[129]</sup>. Consistent with this viewpoint is the evidence that neurosteroid biosynthesis is increased in the brains of rats with ALF<sup>[130]</sup> and that these neurosteroids protect against BBB breakdown induced by ammonia<sup>[131]</sup>. Jayakumar *et al.*<sup>[132]</sup> have also reported neuroprotective effects of neurosteroids in some models of ALF but not in all suggesting that there may be differences in outcomes depending on which hepatotoxin-induced ALF model is used and that this may explain the inconsistent reports on BBB breakdown in ALF.

## TREATMENT OF CEREBRAL OEDEMA IN ACUTE LIVER FAILURE

### Management principles

In the absence of overt HE patients in the early stages of ALF may be observed and managed conservatively.



However such patients are susceptible to extrahepatic manifestations including the development of multiorgan dysfunction, acute kidney injury and infections<sup>[21]</sup> both of which can accelerate the development of advanced HE and brain oedema<sup>[8]</sup>. Frequent clinical and neurological examinations, concentrating on pupil size, coma grade, evidence of delirium and reflexes, are imperative to detect features which may herald the development of brain oedema. The development of grade 3/4 coma, indicative of impending raised intracranial pressure (ICP), typically necessitates intubation and ventilation<sup>[133]</sup>. ICH should be suspected in patients with sudden onset systemic hypertension, changes in pupillary reactivity, abnormal oculovestibular reflexes or decerebrate posturing. ICH becomes problematic when the ICP is above 20 mmHg due to the risk of compromising cerebral perfusion pressure. Ultimately severe ICH can result in brain stem compression causing ischaemia, haemorrhage and death<sup>[134]</sup>.

Transcranial doppler ultrasonography is a non-invasive device which can continuously measure middle cerebral artery blood flow velocity, producing a velocity-time waveform that indirectly monitors changes in cerebral hemodynamics, including ICP avoiding the complications associated with more invasive monitoring devices which include haemorrhage and infection. In a small retrospective study of 16 patients with ALF four features in the waveform were found to capture the cerebral hemodynamic state and potentially can be used to predict dynamic changes in ICP or CPP. This included the slope of the Windkessel upstroke, the slope of the Windkessel downstroke, the slope of the diastolic downstroke, and the angle between the end systolic downstroke and start diastolic upstroke<sup>[135]</sup>. ICP monitoring, involving intracranial bolt insertion, is used in patients who are at high risk for the development of ICH. ICP monitoring is indicated in a subset of patients with grade 3/4 coma<sup>[136]</sup> (Glasgow Coma Scale < 8) who also display a combination of the following features; fever and tachycardia, arterial ammonia > 150  $\mu\text{mol/L}$ , hyponatraemia, seizures or pupillary abnormalities, acute/hyperacute liver failure, vasopressor requirement, are less than age 40 or have jugular venous oxygen saturations or have middle cerebral artery doppler monitoring indicative of a very high or very low cerebral blood flow<sup>[8]</sup>. Additionally reverse jugular vein oxygen saturation should also be monitored, which gives an indication of cerebral oxygenation and metabolism which is often reduced as a result of the loss of CBF autoregulation in patients with ALF<sup>[15]</sup>. In terms of imaging the brain for evidence of cerebral oedema, computed tomography is only of benefit if cerebral herniation or intracranial bleeding is suspected and has no role in the routine surveillance. Electroencephalography is very useful for the detection of subclinical seizures and to measure brain activity in comatose patients, but due to its lack of specificity it is not employed routinely to diagnose encephalopathy or cerebral oedema<sup>[8]</sup>.

Metabolic changes contributing to the development of raised ICP in ALF can be monitored utilizing *in vivo*

cerebral microdialysis and have been documented in research settings in human ALF but this technique is currently only reserved for experimental studies and is not used in routine clinical settings<sup>[137]</sup>.

### Specific therapies

The treatment of cerebral oedema is aimed at preventing infection, reducing or controlling inflammation, ensuring sufficient sedation and correcting hypo-osmolality. The objective of ICH management is to maintain the ICP at less than 20 mmHg and to keep the cerebral perfusion pressure over 70 mmHg although this can be very difficult to practically achieve and the evidence base in human ALF to support such strategies is very limited. Patients are nursed in the 20°-30° head-up position favouring venous drainage to reduce ICP whilst maintaining cerebral perfusion pressure. Hypoxaemia should be avoided with target arterial oxygenation of above 95%. Patients with grade 3 encephalopathy and above should be intubated and ventilated. Propofol and other short acting sedatives are commonly utilised to ease mechanical ventilation and reduce seizure risk. Opiates, such as fentanyl, are often used for analgesia<sup>[133]</sup>. Most patients are normoventilated but hyperventilation is employed in those displaying signs indicative of imminent cerebral herniation, such as pupillary dilatation and extensor posturing. Hyperventilation results in reduced ICP by inducing hypocapnia which causes precapillary vasoconstriction decreasing CBF<sup>[17]</sup>.

Patients should be adequately fluid resuscitated. Plasma volume expansion results in a significant reduction in plasma ammonia concentration by increasing urinary ammonia excretion<sup>[138]</sup>. Hypertension can reduce cerebral perfusion pressure by increasing intracranial blood volume and is best avoided; sedation can help to combat this. Arterial hypotension, especially in the presence of reduced cerebral blood flow autoregulation, will also compromise cerebral perfusion pressure. Theoretically, diastolic blood pressure should be kept > 40 mmHg higher than the ICP in patients with severe cerebral oedema and ICH who have ICP bolt monitoring in situ to guarantee adequate CBF but again this is often hard to achieve in practice<sup>[139]</sup>. Vasopressors, commonly nor-adrenaline, may be necessary to maintain this.

Hyponatraemia should be corrected. Background hypertonic saline (30%) infusions are used to induce and maintain serum sodium levels between 145 and 150 mmol/L thus maintaining the BBB osmotic pressure gradient<sup>[140]</sup>. Hypertonic saline acts as a dehydrating agent reducing brain water content and subsequently lowers ICP. Mannitol may also be used for the same purpose but it may be more rational to use hypertonic saline instead of mannitol as the BBB has a reflection coefficient of 1 for sodium chloride *vs* 0.9 for mannitol making it more efficient to exclude saline from the brain. It is also recommended that serum osmolality be maintained at < 320 mOsm/L. Boluses of hypertonic saline or mannitol are used for sustained increases in ICP (> 25 mmHg) but resistant rises in ICP may be treated with indomethacin<sup>[141]</sup>.

or hypothermia<sup>[142]</sup>. Indomethacin (a non-selective cyclooxygenase inhibitor) induces cerebral vasoconstriction by inhibiting the endothelial cyclooxygenase pathway, reducing cerebral temperature and modifying extracellular pH. However, it has a number of adverse effects, including nephrotoxicity, platelet dysfunction and gastrointestinal bleeding, and therefore its use in ALF patients is limited to when all other management options to reduce ICP have been exhausted<sup>[8]</sup>. Moderate hypothermia (32–34 °C) may be useful in patients with resistant ICH awaiting liver transplantation by decreasing brain ammonia uptake and also through its role in reducing brain cytokine production, OS and CBF<sup>[143]</sup>. Barbiturates are postulated to reduce brain metabolism and consequently lead to a decrease in cerebral blood volume. Thiopental infusion has been shown to be efficacious in 14 patients with ALF as measured by extradural transducers with minimal side effects although additional data in the context of human ALF is scarce<sup>[144]</sup>. However, due to their hepatic metabolism and negative inotropic effects they are only used to reduce ICP surges as a last resort<sup>[8]</sup>.

ALF has many similarities to septic shock<sup>[145]</sup> and there is evidence that patients exhibiting a systemic inflammatory response progress more rapidly to severe encephalopathy<sup>[121]</sup>. Broad spectrum intravenous antibiotics and antifungals are therefore used empirically to reduce the risk of sepsis and development of severe encephalopathy.

Intravenous *N*-acetylcysteine (NAC) is now considered as standard of care in the treatment of acetaminophen-induced and non-acetaminophen induced ALF as it acts as both as an antioxidant and anti-inflammatory agent. Early administration of intravenous NAC after an overdose of acetaminophen replenishes glutathione stores and helps to alleviate hepatic necrosis<sup>[146]</sup>. NAC also has beneficial hemodynamic effects and has been shown to improve cerebral perfusion pressure<sup>[147]</sup> mediated by enhanced activity of the nitric oxide soluble cyclic GMP system<sup>[76]</sup>.

Ultimately emergency liver transplantation reverses cerebral oedema, although a variety of neurological manifestations including intracerebral haemorrhage and seizures, precipitated by cerebral hypoperfusion, coagulopathy and transfusion may still occur post-operatively. If the graft is functioning well ICH is expected to resolve 48 h post-transplant<sup>[148,149]</sup>.

Bernal *et al.*<sup>[7]</sup> reviewed 3305 patients with acute liver dysfunction from 1973–2008 and found a significant reduction in the proportion of patients with ICH (from 76% in 1984–1988 to 20% in 2004–2008 ( $P < 0.0001$ )). Furthermore, mortality of patients with ICH decreased from 95% to 55% ( $P < 0.0001$ ). The cause for this improvement is likely to be multifactorial. Patients now present and are diagnosed earlier and the prompt use of *N*-acetylcysteine, fluid resuscitation, empirical antibiotics and renal replacement therapy may have reduced the incidence of cerebral oedema and ICH by modulating principal contributory factors. Such approaches may also

limit hepatotoxicity, reduce plasma ammonia levels and prevent sepsis. The more timely use of emergency liver transplantation for those at greater risk may also have contributed to the reduction in ICH.

## NOVEL THERAPIES IN DEVELOPMENT

### *Minocycline*

Jiang *et al.*<sup>[74]</sup> studied the use of minocycline, a broad-spectrum tetracycline antibiotic which has been shown to attenuate lipopolysaccharide-induced neuroinflammation<sup>[150]</sup>, in an experimental model of ALF<sup>[24]</sup>. They were able to demonstrate that it delayed the progression of HE and brain oedema by exerting a potent inhibitory action on microglial activation independently of its antimicrobial properties.

### *NMDA receptor antagonists*

NMDA receptor antagonists have been shown to prevent the oxidative stress induced by acute ammonia intoxication<sup>[83]</sup>. This is likely to be the case as the production of ROS is mediated by NMDA-receptor activation in hyperammonemic states<sup>[151]</sup>. Memantine is a non-competitive NMDA-receptor antagonist and has been shown to improve EEG activity, clinical grading, ICP and brain water content in portocaval shunted rats infused with ammonia, and in rats with ALF induced by ischaemia which was independent of ammonia concentration<sup>[152]</sup>.

### *Endotoxin removal*

An albumin replacement system with a novel endotoxin ligation (ARSeNEL) function has been developed at University College London and tested in an ALF pig model. Early data have reported an improvement in survival, endotoxemia and ICP index which warrant further studies in clinical settings<sup>[153]</sup>.

### *Novel anti-inflammatory agents*

It make sense that if agents were used to reduce the systemic inflammation that is frequently seen in ALF and which sensitises the brain to the effects of ammonia, then we might be able to prevent the development of cerebral oedema. Unfortunately however, the proinflammatory response which develops in the wake of acute liver injury is also key in initiating liver repair and regeneration. One would postulate therefore that it may be detrimental to use agents and antibodies which target prominent pro-inflammatory mediators. Neutrophil malfunction, akin to that seen in septic shock is a consistent finding in patients with ALF and recent data support an intimate relationship with hyperammonemia<sup>[127,145]</sup>. Strategies that target innate and adaptive immune dysfunction in ALF including TLR expression and production of ROS would certainly be of therapeutic interest and warrant further study. Granulocyte colony stimulating factor (GCSF) has been shown in 2 small studies to improve neutrophil phagocytic capability in patients with ALF<sup>[154,155]</sup> and as such may have utility in the prevention of advanced HE.

### Plasmapheresis

Larsen *et al.*<sup>[156]</sup> have previously shown that high volume plasmapheresis can alleviate brain oedema in some patients with ALF with favourable changes in systemic hemodynamics despite increasing cerebral blood flow. Plasmapheresis may also have a positive impact on alleviating the systemic immune dysfunction and endothelial dysfunction that commonly develops. This was the stimulus for performing a randomised clinical trial of high volume plasmapheresis of which the preliminary analysed data suggests that it may improve survival in patients unsuitable for liver transplantation (verbal communication-Dr Finn Stolze Larsen).

## CONCLUSION

This detailed review has unequivocally presented the evidence to support the critical role of the neurotoxin ammonia in the development of astrocyte swelling and cytotoxic oedema in ALF. Although a generalised breakdown of the BBB cannot be demonstrated in patients with ALF, more recent studies have described a “leaky” BBB resulting from subtle changes in the integrity of the tight junctions, supporting a role of a vasogenic component in the pathophysiology of cerebral oedema in ALF.

Exactly how both cytotoxic and vasogenic mechanisms interact to bring about cerebral oedema in ALF, and the extent of their involvement, remains unknown. Moreover, the sequence of events is unclear. Is BBB dysfunction the result of a cytotoxic insult or is cytotoxic oedema a consequence of increased BBB permeability? It has been postulated that increased BBB permeability to small molecules such as water and ammonia arises as a result of BBB dysfunction as an initial event in the pathophysiology of brain oedema in ALF. Increased BBB permeability may then invoke vasogenic oedema. The subsequent development of ammonia neurotoxicity and cytotoxic oedema may then occur as a downstream manifestation. This sequence of events is supported by the observation that in rats with ALF an early increase in BBB permeability correlates with increased ICP and results in vasogenic oedema followed by a progressive increase in brain ammonia and glutamine levels<sup>[157]</sup>. However, it is difficult to determine to what extent these data definitively support a vasogenic mechanism in ALF. For example, the potency of mannitol in treating ICH in the context of cerebral oedema in patients with ALF supports the BBB being intact and the predominant mechanism being a cytotoxic one. Another possibility however, is that certain brain areas may behave respond differently to others. For example, Cauli *et al.*<sup>[157]</sup> were able to show in a rodent experimental ALF model that the mechanism and time course of the appearance of brain oedema differed between 12 different brain regions with the cerebellum showing predominantly vasogenic oedema whilst the frontal cortex exhibited cytotoxic oedema.

The syndrome of ALF arises in the context of various aetiological toxic insults to the liver and is frequently associated with the development of multiple organ

dysfunction and sepsis. It is not known how these manifestations independently impact on BBB integrity and function. Furthermore, the liver toxins utilised in the various animal ALF models could directly affect the BBB independently of ALF. Changes to the BBB in ALF are very different in nature to that seen in brain ischemia or traumatic brain injury, where complete BBB breakdown is commonly observed. The mechanisms underpinning cerebral oedema in ALF are therefore also different and therapeutic interventions that are beneficial in other types of brain injury may not be useful in the treatment of ALF.

It is clear that the development of more effective therapies in ALF will require further knowledge of the pathophysiology of cerebral oedema, which is a devastating and frequently fatal feature of ALF. Greater knowledge of the sequence of events and key mediators involved in the development of brain oedema will allow for specific targets to be identified.

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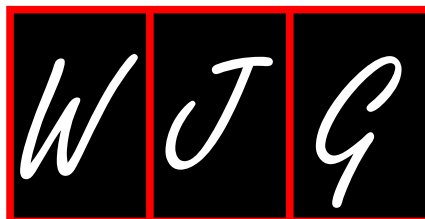
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## Therapeutic potential of curcumin in digestive diseases

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### Abstract

Curcumin is a low-molecular-weight hydrophobic polyphenol that is extracted from turmeric, which possesses a wide range of biological properties including anti-inflammatory, anti-oxidant, anti-proliferative and anti-microbial activities. Despite its diverse targets and substantial safety, clinical applications of this molecule for digestive disorders have been largely limited to case series or small clinical trials. The poor bioavailability of curcumin is likely the major hurdle for its more widespread use in humans. However, complexation of curcumin into phytosomes has recently helped to bypass this problem, as it has been demonstrated that this new lecithin formulation enables increased absorption to a level 29-fold higher than that of traditional curcuminoid products. This allows us to achieve much greater tissue substance delivery using significantly lower doses of curcumin than have been used in past clinical studies. As curcumin has already been shown to provide good therapeutic results in some small studies of both inflammatory and neoplastic bowel disorders, it is reasonable to anticipate an even greater efficacy with the advent of this new technology, which remarkably improves its bioavailability. These features are very promising and may represent a novel and effective therapeutic approach to both functional and organic digestive diseases.

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**Key words:** Curcumin; Curcumin-phytosome; Curcumin bioavailability; Digestive disorders

**Core tip:** Curcumin is a well-established molecule with multiple pharmacological activities, mainly anti-inflammatory and anti-proliferative. The major hurdle for a widespread clinical use has been represented by its poor bioavailability, which has been recently overcome by the development of a new formulation combining curcumin with phospholipids (curcumin-phytosome). This compound permits to improve markedly intestinal absorption of curcumin and guarantees a greater tissue delivery than the traditional curcuminoid mixtures. So, curcumin-phytosome has the potential to be exploited in many gastrointestinal diseases, both functional and organic.

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### INTRODUCTION

In recent years, we have witnessed a shortage of certain types of drugs synthesized from chemical laboratories and a growing interest in therapeutic substances derived from natural plants. Curcumin represents one of these compounds, and this nutraceutical has already undergone many experimental and clinical studies to assess its use in the treatment of various human diseases.

This polyphenol has been shown to possess anti-inflammatory, anti-oxidant, immuno-modulatory, wound-healing, anti-proliferative and antimicrobial activities. These diverse properties, together with the fact that curcumin is innocuous, inexpensive and easily available,

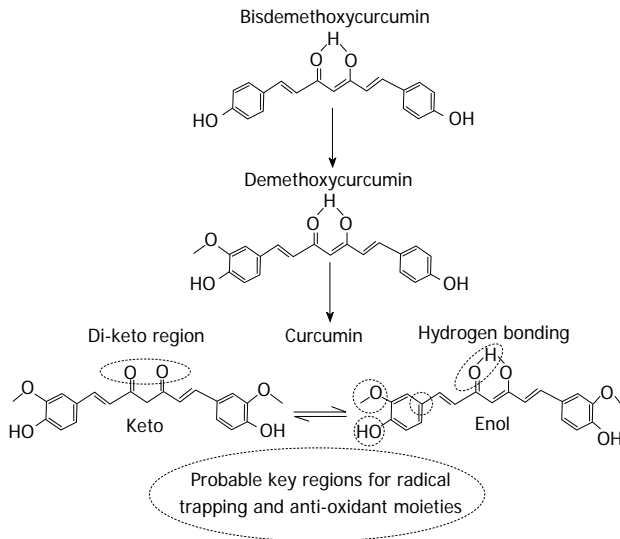


Figure 1 Proposed molecular pathway for the conversion of bisdemethoxycurcumin to demethoxycurcumin and finally to curcumin and the co-existence of keto and enol isomers of curcumin.

have sparked interest in its therapeutic application for several digestive disorders. Moreover, recent progress in the formulation of curcumin complexes with other substances, in particular with phospholipids, has remarkably increased the bioavailability of this compound, leading to greater absorption and a higher concentration in human tissues. This allows us to use lower dosages of curcumin than have been used in the past, which greatly reduces the number of tablets taken during the day while maintaining no adverse side effects.

Finally, distribution studies of curcumin in human tissues have shown that it preferentially accumulates in the intestine, colon and liver. This finding might be one major reason for the anticipation and observation of its most promising *in vivo* effects in gastrointestinal diseases when compared with other organ systems.

This review presents current knowledge of the physical and molecular properties of curcumin, its pharmacokinetics and metabolism, its mechanism of action and results of the few published clinical trials, as well as the potential therapeutic perspectives in patients with various digestive disorders.

Literature searches were performed in PubMed, Ovid, EMBASE and the Cochrane Library databases in accordance with published recommendations. We critically analyzed all full-text papers and reviews written in the English language and searched them using the terms curcumin, turmeric, colorectal cancer (CRC), inflammatory bowel diseases (IBD), functional digestive disorders, irritable bowel syndrome and liver diseases. Both animal and human studies were reviewed.

## PHYSICAL AND MOLECULAR PROPERTIES OF CURCUMIN

Turmeric (the common name for *Curcuma longa*) is an In-

dian spice derived from the rhizomes of the plant and has a long history of use in Ayurvedic medicine as a treatment for inflammatory conditions<sup>[1]</sup>.

The primary active constituent of turmeric, which is responsible for its vibrant yellow color, is curcumin, which was first identified in 1910 by Lampe and Milobedzka<sup>[2]</sup>. Curcumin exists as a bright yellow powder that provides the pigmentation of turmeric, which is used in the dye industry. Turmeric is composed of volatile oils (tumerone, atlantone, and zingiberone), sugars, proteins, resins and a group of the following three curcuminoids: about 75% curcumin (diferuloylmethane), about 16% demethoxycurcumin (DMC), about 8% bisdemethoxycurcumin (bDMC). DMC and bDMC possess similar molecular and biological properties. It is proposed that within natural pathways (Figure 1), bDMC is converted to DMC, which is then converted to curcumin<sup>[3]</sup>.

Curcumin (or diferuloylmethane) is a poly-phenolic molecule that exhibits keto-enol tautomerism and has a predominant keto form in acidic and neutral solutions and a stable enol form in alkaline medium<sup>[4]</sup>. The molecule is lipophilic and consists of two aromatic rings connected by two unsaturated carbonyl groups; therefore, it has poor solubility in water. The molecule is stabilized by hydrogen-bonding associated with the central OH group. This may be one of the important functional sites that is responsible for the array of molecular biological activities<sup>[5]</sup>. Curcumin is photosensitive, and precautions should be taken to avoid exposure and subsequent degradation.

## PHARMACOKINETICS AND METABOLISM OF CURCUMIN

### Absorption and systemic bioavailability

Over the past three decades, animal studies have shown that curcumin is hydrolytically unstable at intestinal pH, rapidly metabolized, conjugated in the liver, and excreted in the feces. Therefore, it has limited systemic bioavailability. The effects of reduced bioavailability of any agent within the body are low intrinsic activity, poor absorption, high rate of metabolism, inactivity of metabolic products and/or rapid elimination and clearance from the body. In this section, problems of limited curcumin bioavailability such as low serum levels, limited tissue distribution, apparent rapid metabolism and short half-life are described in detail.

### Serum concentration

One of the major observations from curcumin studies is very low serum levels. The first reported study to examine the uptake, distribution, and excretion of curcumin was by Wahlstrom and Blennow<sup>[6]</sup> in 1978 using Sprague-Dawley rats. Negligible amounts of curcumin in the blood plasma of rats after oral administration of 1 g/kg of curcumin showed that this molecule was poorly absorbed from the gut.

In 1980, Ravindranath *et al.*<sup>[7]</sup> showed that after oral administration of 400 mg of curcumin in rats, no curcumin

was found in the heart blood, whereas a trace amount (less than 5 µg/mL) was found in the portal blood from 15 min to 24 h after curcumin administration.

When curcumin was given orally at a dose of 2 g/kg in rats, a maximum serum concentration of  $1.35 \pm 0.23$  µg/mL was observed after 0.83 h, whereas in humans, the same dose of curcumin resulted in either undetectable or extremely low ( $0.006 \pm 0.005$  µg/mL at 1 h) serum levels<sup>[8]</sup>.

A phase I clinical trial<sup>[9]</sup> conducted among 25 patients with various precancerous lesions demonstrated that oral doses of 4, 6 and 8 g of curcumin administered daily for three months yielded serum curcumin concentrations of only  $0.51 \pm 0.11$ ,  $0.63 \pm 0.06$ , and  $1.77 \pm 1.87$  µm, respectively. This finding indicates that curcumin is poorly absorbed and may have limited systemic bioavailability. Serum levels peaked between one and two hours after administration and declined rapidly thereafter. This study did not identify curcumin metabolites, and urinary excretion of curcumin was undetectable.

Another phase I trial<sup>[10]</sup> involving 15 patients with advanced colorectal cancer administered curcumin at doses between 0.45 and 3.6 g daily for four months. In three of six patients who were given the 3.6 g dose, the mean plasma curcumin measured after one hour on day 1 was  $11.1 \pm 0.6$  nmol/L. This measurement remained relatively consistent at all-time points measured during the first month of curcumin therapy. The molecule was not detected in the plasma of patients taking lower doses.

A very recent study by Yang *et al.*<sup>[11]</sup> showed that 10 mg/kg of curcumin given *iv* in rats yielded a maximum serum curcumin level of  $0.36 \pm 0.05$  µg/mL, whereas a 50-fold higher curcumin dose administered orally yielded a maximum serum level of only  $0.06 \pm 0.01$  µg/mL.

These studies clearly suggest that the route of administration affects achievable serum levels of curcumin, and they further indicate that the serum levels of this compound in rats and in humans are not directly comparable.

### Tissue distribution

The uptake and distribution of curcumin in body tissues are obviously important factors determining its biological activity, yet a limited number of studies have addressed this issue.

Ravindranath *et al.*<sup>[7]</sup> showed that after oral administration of 400 mg of curcumin in rats, only traces of the unchanged molecule were found in the liver and kidney. At 30 min, 90% of the curcumin was found in the stomach and small intestine, but only 1% was present at 24 h.

Another study of the same group evaluated the tissue distribution of curcumin using a tritium-labeled molecule<sup>[12]</sup>. They found that radioactivity was detectable in the blood, liver, and kidney following doses of 40080, or 10 mg of (3H) curcumin. With 400 mg, considerable amounts of the radio-labeled products were present in tissues 12 d after dosing. The percentage of curcumin absorbed (60%-66% of the given dose) remained constant regardless of the dose, indicating that increased administration of the drug does not result in greater absorption.

Similarly, the concentrations of curcumin in normal and malignant colorectal tissue of patients receiving 3600 mg of the compound were  $12.7 \pm 5.7$  and  $7.7 \pm 1.8$  nmol/g, respectively, and these doses had pharmacological activity in the colorectum as measured by their effects on levels of M(1)G and cyclooxygenase-2 (COX-2) protein<sup>[13]</sup>. Another study by the same authors showed no curcumin in the liver tissue of patients with hepatic metastases from colorectal cancer who received 450-3600 mg of curcumin daily for 1 wk prior to surgery<sup>[14]</sup>.

### Metabolites

Various studies have evaluated the metabolism of curcumin in rodents and in humans. Once absorbed, curcumin is subjected to conjugations such as sulfation and glucuronidation at various tissue sites. The very first bio-distribution study reported the metabolism of the major part of curcumin orally administered in rats<sup>[6]</sup>. The liver was indicated as the major organ responsible for metabolism of this drug<sup>[15]</sup>.

Holder *et al.*<sup>[16]</sup> reported that the major biliary metabolites of curcumin in rats are glucuronides of tetrahydrocurcumin (THC) and hexahydrocurcumin. A minor biliary metabolite was dihydroferulic acid together with traces of ferulic acid. In addition to glucuronides, sulfate conjugates were found in the urine of curcumin-treated mice<sup>[13]</sup>.

Asai *et al.*<sup>[17]</sup> evaluated the absorption and metabolism of orally administered curcumin in rats. The enzymatic hydrolysis of plasma samples showed that the predominant metabolites in plasma following oral administration were glucuronides/sulfates of curcumin. The plasma concentrations of conjugated curcuminoids reached a maximum at 1 h after administration. The presence of conjugative enzyme activities for glucuronidation and sulfation of curcumin in the liver, kidney and intestinal mucosa suggests that orally administered curcumin is absorbed from the alimentary tract and is present in the general blood circulation after largely being metabolized to form glucuronide/sulfate conjugates.

Whether curcumin metabolites are as active as curcumin itself is not clear<sup>[18-20]</sup>. While most studies indicate that curcumin glucuronides and THC are less active than curcumin itself, other studies suggest that they may actually be more active than curcumin<sup>[19-21]</sup>.

### Half-life

Systemic elimination or clearance of curcumin from the body is another important factor that determines its relative biological activity. Wahlstrom and Blennow<sup>[6]</sup> reported that when 1 g/kg curcumin was given orally to rats, 75% of it was excreted in the feces, and negligible amounts were found in the urine. Intravenous (*iv*) and intraperitoneal (*ip*) administration of curcumin resulted in biliary excretion of the molecule from cannulated rats.

A clinical study of 15 patients receiving oral curcumin in doses between 36 and 180 mg daily for up to 4 mo found neither curcumin nor its metabolites in urine, but the drug was recovered from feces<sup>[22]</sup>. The absorption and

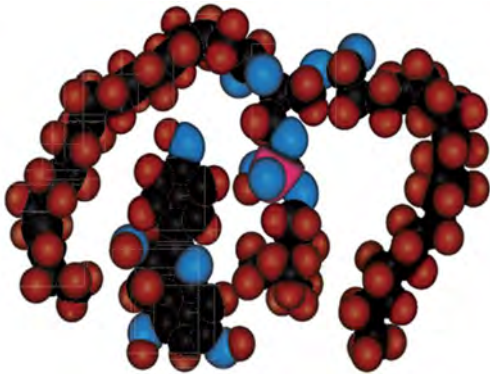


Figure 2 Phytosome molecular complex.

elimination half-lives of orally administered curcumin (2 g/kg) in rats were reported to be  $0.31 \pm 0.07$  and  $1.7 \pm 0.5$  h, respectively. However, in humans, the same dose of curcumin did not allow the calculation of these half-life values because the serum curcumin levels were below the detection limit at the majority of time points in most of the experimental subjects.

The existing evidence in the literature is not sufficient to make conclusions about the factors controlling the *in vivo* elimination half-life of curcumin, and future studies are warranted to address this issue.

## METHODS TO OVERCOME THE TRADITIONAL LOW BIOAVAILABILITY OF CURCUMIN

Because of the above-mentioned poor bioavailability, which limits the therapeutic usefulness of curcumin, many attempts have been made to improve oral absorption of the compound<sup>[23]</sup>. Among them, the complexation of curcumin with phospholipids using so-called phytosome technology has emerged as one of the most documented approaches from a preclinical and clinical standpoint.

Phytosome technology was developed in 1989 (Figure 2). Water-soluble phytosomes can be converted into a lipid-compatible molecular complex. Phytosomes are more available than uncomplexed products due to their enhanced capacity to cross the lipid biomembranes and to reach the systemic circulation<sup>[24]</sup>.

It is inferred that, at the intestinal level, the water-miscible phosphatidylcholine (PC) molecules enhance the dispersion of the poorly water-soluble polyphenol molecules into the water-soluble environment of the gastrointestinal lumen. PC further enhances transfer from the lumen into the lipid-soluble environment of the outer cell membrane of the epithelial absorptive cells (enterocytes). The enterocyte outer membrane has a lipid molecular bilayer that consists largely of PC. It is feasible that the PC in the phytosome merges into this PC domain of the enterocyte membrane, and by carrying the polyphenol with it, the PC “ushers” the polyphenol into the cell.

The bioavailability of the curcumin phytosome (CP)

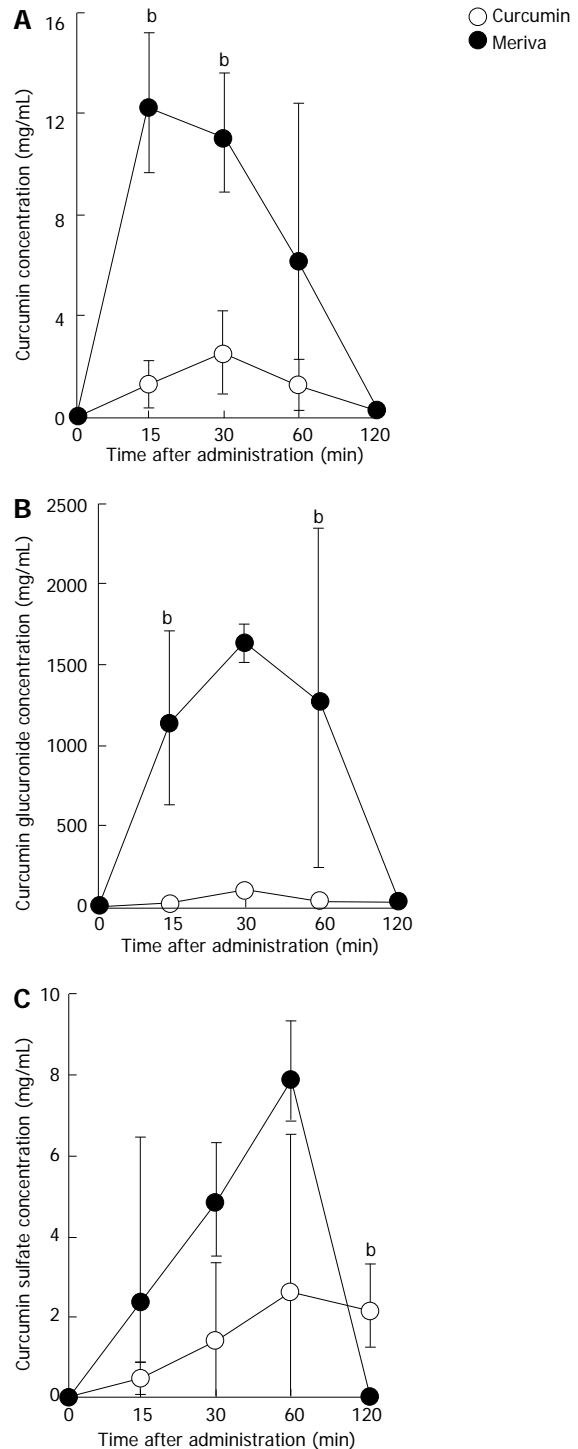


Figure 3 Plasma curcumin I in rats from curcumin phytosome or non-complexed curcumin. A: Curcumin concentration; B: Curcumin glucuronide concentration; C: Curcumin sulfate concentration. <sup>b</sup> $P < 0.01$  vs curcumin phytosome.

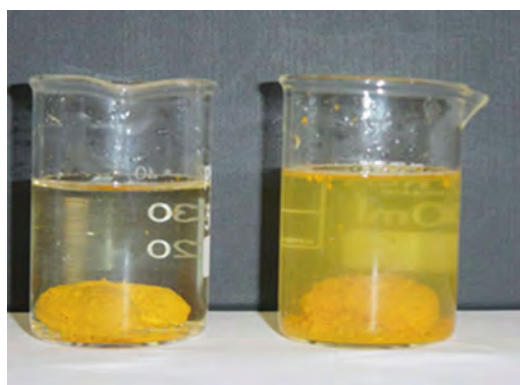
preparation (Meriva®, Indena Spa, Milan, Italy) has been tested against an equivalent non-phytosome curcumin extract by Marczylo *et al.*<sup>[25]</sup>. These authors administered equivalent dosages (340 mg of curcumin) of curcumin or curcumin phytosome preparation to rats and reported a dramatic increase in the bioavailability among the animals that received the curcumin phytosome preparation (Figure 3). Peak plasma levels of curcumin were approximately



**Table 1 Pharmacokinetics parameters in healthy volunteers after administration of curcuminphytosomes or unformulated curcumin**

Curcuminoid	Formulation	AUC (ng/mL)	C <sub>max</sub> (ng/mL)	t <sub>max</sub> (h)	Relative absorption <sup>2</sup>
Curcumin (1a)	Curcuminphytosome high	538.0 ± 130.7	50.3 ± 12.7	3.8 ± 0.6	19.2 <sup>1</sup>
	Curcuminphytosome low	272.6 ± 68.52	24.2 ± 5.9	4.2 ± 0.8	17.5 <sup>3</sup>
	Reference	122.5 ± 29.3	9.0 ± 2.8	6.9 ± 2.2	1
Demethoxycurcumin (1b)	Curcuminphytosome high	655.0 ± 195.7	134.6 ± 40.6	2.4 ± 0.3	68.3 <sup>4</sup>
	Curcuminphytosome low	297.4 ± 107.3	39.1 ± 11.4	3.1 ± 0.4	55.5 <sup>4</sup>
	Reference	55.8 ± 15.5	4.2 ± 1.1	4.4 ± 1.0	1
Bisdemethoxycurcumin (1c)	Curcuminphytosome high	142.2 ± 58.2	24.9 ± 8.1	2.2 ± 0.4	56.8 <sup>5</sup>
	Curcuminphytosome low	70.1 ± 34.3	8.8 ± 3.1	2.4 ± 0.6	53.1 <sup>5</sup>
	Reference	24.6 ± 10.3	2.1 ± 0.8	3.4 ± 1.2	1
Total curcuminoids	Curcuminphytosome high	1336.0 ± 357.1	206.9 ± 54.9	2.7 ± 0.3	31.5 <sup>6</sup>
	Curcuminphytosome low	640.2 ± 197.7	68.9 ± 16.9	3.3 ± 0.3	27.2 <sup>6</sup>
	Reference	202.8 ± 53.8	14.4 ± 4.2	6.9 ± 2.2	1

<sup>1</sup>Actual results not baseline subtracted, and errors are standard error of the mean ± SE; <sup>2</sup>Area under the curve (AUC) normalized; <sup>3</sup>Average: 18.3; <sup>4</sup>Average: 61.9; <sup>5</sup>Average: 54.1; <sup>6</sup>Average: 29.14.

**Figure 4** Image of Norflo® tablet in water after few seconds.

5-fold higher for CP than for traditional curcumin. Plasma levels of curcumin sulfate and curcumin glucuronide observed after the administration of CP were 3- to 20-fold higher, respectively, than those observed after the administration of uncomplexed curcumin. In the same study, significant amounts of curcumin were also measured at the tissue level and were found to have particular relevance for the liver and intestine.

More recently, Cuomo *et al.*<sup>[26]</sup> reported the results of a comparative pharmacokinetic study of healthy volunteers. In this randomized, double-blind, cross-over study, subjects received curcumin and the CP formulation at 2 dosage levels (209 and 376 total curcuminoids). The average dose-related absorption of curcumin following the 2 doses of CP was approximately 18-fold higher than the absorption of the reference curcumin. Moreover, the absorption of total curcuminoids was approximately 29-fold higher for CP in comparison with the unformulated reference, as the plasma concentration of demethoxycurcumin and bis-demethoxycurcumin from the former compound was approximately 50- to 60-fold higher than the concentration from the unformulated curcumin (Table 1).

CP is a powder that contains 20% curcumin, 40% microcrystalline cellulose and 40% phospholipids. It is utilized as an active ingredient in several food supplements in different markets. The product is available in various formulations including hard gel capsules and tablets. In

Italy, for example, the product has been developed as 500-mg tablets that combine CP with some dissolving substances (Curcusol) under the name Norflo® (Eyeepharm, Genoa, Italy). These tablets dissolve very rapidly in the first part of the intestine, favoring the formation of an emulsion with bile acids (Figure 4), which permits almost complete absorption of phospholipids (unpublished data). The use of this formulation overcomes the risk that undissolved tablets may pass through the entire intestine and be eliminated in the feces either intact or only partially dissolved.

CP has been widely documented in several health settings, but few studies have focused on gastrointestinal disorders, which, nevertheless, seem to be a very promising therapeutic area. From this perspective, a colon-targeted delivery preparation could further optimize the clinical effects.

## TOXICITY AND TOLERABILITY OF CURCUMIN

Curcumin has been reported to be safe in many human studies, and only minimal toxicity has been associated with this polyphenol<sup>[27]</sup>. In a dose escalation study among 34 healthy volunteers, in whom the doses of curcumin ranged from 500 to 12000 mg, safety was assessed after 72 h. Only 7 subjects complained of disturbances, which were mild and included headache, skin rash, diarrhea and yellow stool<sup>[9]</sup>. In another investigation lasting for 1-4 mo, escalating doses of curcumin from 0.45 to 3.6 g/d found rare instances of nausea and diarrhea, as well as an increase in alkaline phosphatase and LDH<sup>[10]</sup>. Some patients treated with doses as high as 8 g/d for 2 wk reported abdominal pain and complained about the bulky volume of the tablets<sup>[28]</sup>. As curcumin is particularly concentrated in the human liver, the risk of hepatotoxicity has been closely evaluated, but liver function tests have been shown to be unaffected with doses as high as 2-4 g/d<sup>[29]</sup>. As one of the most documented bioavailable curcumin formulations, the CP formulation has been widely employed in the clinical setting with a daily dosage rang-

ing between 1 and 2 g, and this preparation has shown good tolerability and compliance, even in medium-term trials. However, we must stress that studies of more than 6 mo of treatment are lacking, and it is not possible to draw any firm conclusions regarding the long-term safety profile of this compound.

## MECHANISMS OF ACTION AGAINST INFLAMMATORY AND NEOPLASTIC CONDITIONS

### Anti-inflammatory mechanisms

Curcumin is a highly pleiotropic molecule capable of interacting with numerous molecular targets involved in inflammation. It has been proposed that this compound modulates the inflammatory response by the following mechanisms<sup>[30,31]</sup>: (1) Down-regulation of COX-2, lipoxigenase, and inducible nitric oxide synthase (iNOS) enzymes; (2) Inhibition of the inflammatory cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, -2, -6, -8, and -12, monocyte chemoattractant protein, and migration inhibitory protein; and (3) Down-regulation of mitogen-activated and Janus kinases.

COX-2 inhibition and iNOS inhibition are likely achieved *via* curcumin suppression of nuclear factor kappa B (NF- $\kappa$ B) activation. NF- $\kappa$ B is a ubiquitous eukaryotic transcription factor involved in the regulation of inflammation, cellular proliferation, transformation, and tumorigenesis<sup>[32]</sup>. NF- $\kappa$ B is not a single gene but rather a family of interrelated transcription factors that include the following five genes: NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), RelA (p65), c-Rel, and RelB<sup>[33]</sup>. The member proteins form homo- or heterodimers, of which the p50/p65 heterodimer is the most abundant and is responsible for the majority of NF- $\kappa$ B canonical transcriptional activity. Generally, NF- $\kappa$ B dimers associate with an inhibitory- $\kappa$ B (I $\kappa$ B- $\alpha$ ) protein that keeps the dimer in the cytoplasm in an inactive state.

NF- $\kappa$ B activation begins with the activation of an I $\kappa$ B kinase (IKK) complex that consists of catalytic subunits IKK- $\alpha$  and IKK- $\beta$  and the scaffolding subunit IKK- $\gamma$  (the NF- $\kappa$ B essential modifier)<sup>[34]</sup>. Several mitogen-activated protein (MAP) kinases that also include NF- $\kappa$ B-inducing kinase (NIK) activate IKK through the phosphorylation of IKK- $\alpha$  and IKK- $\beta$ . IKK- $\beta$  has higher activity than IKK- $\alpha$  for I $\kappa$ B- $\alpha$  and is considered important in the canonical pathway.

In the canonical pathway, as shown in Figure 4, phosphorylation of I-kappa B kinase (I $\kappa$ B) kinase  $\alpha/\beta$  by mitogen-activated protein kinase (MAPK) is followed by phosphorylation of I $\kappa$ B- $\alpha$ , which occurs in an inactive complex with p50/p65. Phosphorylated I $\kappa$ B- $\alpha$  is released and degraded in the cytoplasm. The active heterodimer of p50/p65 enters the nucleus to regulate expression of multiple genes<sup>[35]</sup>.

Curcumin is thought to suppress NF- $\kappa$ B activation and proinflammatory gene expression by blocking phos-

phorylation of inhibitory factor I $\kappa$ B. Suppression of NF- $\kappa$ B activation subsequently down-regulates COX-2 and iNOS expression, thus inhibiting the inflammatory process and tumorigenesis<sup>[33]</sup>. In an animal model of inflammation, curcumin also inhibited arachidonic acid metabolism and inflammation in mouse skin epidermis *via* down-regulation of the cyclooxygenase and lipoxigenase pathways<sup>[36]</sup>.

*In vitro* studies indicate that curcumin inhibition of inflammatory cytokines is achieved through suppression of cytokine gene expression and down-regulation of intercellular signaling proteins, such as protein kinase C<sup>[36]</sup>.

### Curcumin anticancer effects

There has been some promising research concerning curcumin as a safe therapeutic agent for many cancers, including colorectal cancer. This has been shown through various studies in cell cultures, animal models, and humans<sup>[2,37]</sup>.

Carcinogenesis is a complex process mainly consisting of the following three phases: initiation, promotion, and progression<sup>[38]</sup>. There is suggestive evidence that inflammation may play a role in the three phases of carcinogenesis<sup>[39]</sup>. Cancer initiation is produced by oxidative stress and chronic inflammation<sup>[2]</sup>. Inflammation acts as a key regulator in the promotion of these initiated cells, possibly by providing them with proliferating signals and by preventing apoptosis<sup>[40]</sup>. The role of inflammation in tumor induction and subsequent malignant progression has also been investigated<sup>[41]</sup>. An inflammatory response produces cytokines, which act as growth and/or angiogenic factors, leading transformed cells to proliferate and undergo promotion. Leukocytes produce cytokines and angiogenic factors as well as matrix-degrading proteases that allow the tumor cells to proliferate, invade, and metastasize. Tumor-infiltrating lymphocytes secrete matrix-degrading proteinases such as matrix metallo-peptidase 9 (MMP-9) and thus promote neoplastic proliferation, angiogenesis, and invasion<sup>[42]</sup>.

These details demonstrate the role of inflammation in all three stages of carcinogenesis. Substantial evidence for the role of inflammation in cancer is provided by the frequent up-regulation of inflammatory mediators such as NF- $\kappa$ B. The pathways activated by NF- $\kappa$ B up-regulators are implicated not only in tumor growth and progression but also in the development of cancer cell resistance to anti-cancer drugs, radiation and death cytokines. NF- $\kappa$ B is an excellent target for anti-cancer therapy<sup>[43]</sup>.

### Effects on tumor initiation by curcumin

Curcumin has demonstrated a significant reduction in the levels of iNOS, which produces oxidative stress, which is itself one of the main causes of tumor initiation. Curcumin inhibits the induction of nitric oxide synthase and is a potent scavenger of free radicals such as nitric oxide<sup>[44]</sup>.

NF- $\kappa$ B has been implicated in the induction of iNOS. Curcumin prevents phosphorylation and degradation

of inhibitor  $\kappa$ B- $\alpha$  and thereby blocks NF- $\kappa$ B activation, which down-regulates iNOS gene transcription<sup>[45]</sup>. Curcumin was found to inhibit cell proliferation and cytokine production by inhibiting NF- $\kappa$ B target genes involved in this mitogen induction of T-cell proliferation, interleukin IL-2 production and nitric oxide generation. The over-expression of cytokines, such as IL-10, IL-6, and IL-18, is accompanied by NF- $\kappa$ B induction that is controlled and inhibited by curcumin<sup>[46]</sup>. Curcumin has been shown to increase expression of conjugation enzymes (phase II), which suppress ROS-mediated NF- $\kappa$ B, activator protein 1 (AP-1) and MAPK activation<sup>[47]</sup>.

### **Tumor proliferation and progression suppression by curcumin**

We have already mentioned that NF- $\kappa$ B has an important role in cancer initiation, promotion and progression. In addition to suppressing various cell survival and cell proliferative genes, including Bcl-2, cyclin D1, IL-6, COX-2, and MMP-9, curcumin induces apoptosis, as shown by caspase activation and poly (ADP-ribose) polymerase-cleavage<sup>[48-50]</sup>.

Curcumin is also able to block NF- $\kappa$ B signaling and inhibit IKK activation. The suppression of cell survival and cell proliferation genes, including Bcl-2, cyclin D1, IL-6, COX-2 and MMP, has also been noted<sup>[48,49]</sup>. It has been suggested that COX-2 induction is mediated by the NF- $\kappa$ B intracellular signaling pathway, and overexpression of COX-2 leads to malignant cell proliferation and invasion<sup>[51,52]</sup>. Curcumin inhibits COX-2 expression by repressing degradation of the inhibitory unit inhibitor  $\kappa$ B- $\alpha$  and hindering the nuclear translocation of the functionally active subunit of NF- $\kappa$ B, thereby blocking improper NF- $\kappa$ B activation<sup>[34]</sup>.

Curcumin has been found to reduce the invasion and subsequent metastasis of cancer cells. It suppresses MMP expression, which is believed to play a major role in mediating neovascularization and is increased during tumor progression<sup>[53]</sup>.

Curcumin down-regulates MMP-9 expression by inhibiting NF- $\kappa$ B and AP-1 binding to the DNA promoter region. MMP-9 is one of the two determinants of neovascularization that help to form new capillaries from preexisting blood vessels<sup>[54]</sup>.

Curcumin has been noted to cause significant inhibition of tumor necrosis factor  $\alpha$ -induced VCAM-1 expression, which is related to the activation of the MAPK NF- $\kappa$ B pathway<sup>[55,56]</sup>. Curcumin has been shown to reduce cell migration and invasion induced by osteopontin, an extracellular matrix protein, through the NF- $\kappa$ B pathway<sup>[57]</sup>.

Curcumin may inhibit cancer cell growth through down-regulation of IL-1- and IL-8-induced receptor internalization. It controls cancer progression by either blocking tumor growth or inhibiting its invasive and aggressive potential. In both cases, most of the effects are exerted by curcumin-induced NF- $\kappa$ B inhibition<sup>[57]</sup>.

However, curcumin has been found to arrest the cell

cycle and to induce apoptotic cell death through inhibition of the Janus family of kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway<sup>[58]</sup>.

The JAK and STAT comprise an important signaling pathway involved in dysregulation of cell growth, invasion, angiogenesis, metastasis and resistance to apoptosis<sup>[59,60]</sup>. The JAK-STAT system consists of the following three main components: (1) A receptor; (2) JAK; and (3) STAT.

The receptor is activated by a signal from interferon, IL-6, growth factors, or other chemical messengers<sup>[61]</sup>. This activates the kinase function of the JAKs (JAK1, JAK2, and JAK3), which autophosphorylation (phosphate groups act as "on" and "off" switches on proteins). The STAT protein then binds to the phosphorylated receptor, where STAT is phosphorylated by JAK. The phosphorylated STAT protein binds to another phosphorylated STAT protein (dimerizes) and translocates into the cell nucleus. In the nucleus, it binds to DNA and promotes transcription of genes responsive to STAT<sup>[62-63]</sup>. Studies have evaluated the regulators of cytokine signaling including protein tyrosine phosphatases (PTPases) such as Src homology 2 (SH2) domain-containing PTPases (SHP)-1 and SHP-2. Potential roles for SHP-1 and SHP-2 have been investigated for their use in the control of cytokine signaling through the dephosphorylation of JAKs and their receptors<sup>[64]</sup>.

Of the seven STAT proteins identified thus far, only activated STAT3 and STAT5 have been implicated in multiple myeloma, lymphomas, leukemias and several solid tumors<sup>[65]</sup>. Aberrant STAT3 signaling is an important process in the development and progression of cancer; thus, agents that block its activation have therapeutic potential. Rajasingh *et al.*<sup>[66]</sup> have demonstrated that *in vitro*, treatment with curcumin induced a dose-dependent decrease in JAK and STAT phosphorylation, resulting in the induction of growth-arrest and apoptosis in T cell leukemia. Curcumin reversibly inhibits STAT3 activation in human multiple myeloma cells and, by this mechanism, suppresses IL-6-induced cell proliferation<sup>[67-68]</sup>. It also inhibits STAT3 activation in five different human Hodgkin and Reed-Sternberg lymphoma cell lines<sup>[69]</sup>.

It has been shown that curcumin inhibits lysophosphatidic acid-induced IL-6 and IL-8 secretion and STAT3 phosphorylation in ovarian cancer cells<sup>[70]</sup>, and curcumin has also been shown to have a significant effect upon CRC by blocking STAT3-driven cancer cell growth<sup>[69]</sup>. In summary, the anti-inflammatory and anticancer effects of curcumin are listed in Table 2.

## **CLINICAL TRIALS EXPLORING THE THERAPEUTIC POTENTIAL OF CURCUMIN IN GASTROINTESTINAL DISEASES**

Because of its higher bioavailability in the gastrointestinal



**Table 2** Curcumin's anti-inflammatory and anticancer effects

Anti-inflammatory effects	Anticancer effects
Downregulation of NF- $\kappa$ B, Inhibition, <i>via</i> NF- $\kappa$ B, of COX-2, lipoxygenase, and iNOS enzymes Inhibition of the inflammatory cytokines, such as TNF- $\alpha$ , interleukin (IL)-1, -2, -6, -8, and -12, MCP, and migration inhibitory protein Inhibition of PPAR-g	Inhibition of carcinogen activation Stimulation of carcinogen detoxification Suppression of pro-inflammatory signaling  Inhibition of STAT Induction of cancer cell apoptosis cell cycle arrest Inhibition of angiogenesis and metastasis Modulation of oncogenes and tumor suppressor genes

TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; MCP: Monocyte chemoattractant protein; PPAR-g: Peroxisome proliferator-activated receptor-g; iNOS: Inducible nitric oxide synthase; STAT: Signal transducer and activator of transcription; NF- $\kappa$ B: Nuclear factor kappa B; COX-2: Cyclooxygenase-2.

tract than in other organs, the therapeutic potential of curcumin has been investigated in several studies of digestive diseases including IBD, CRC and hepatic fibrosis.

### Inflammatory bowel disease

Idiopathic IBD comprises the following two types of chronic intestinal disorders: Crohn's disease (CD) and ulcerative colitis (UC)<sup>[71-73]</sup>. Accumulating evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host<sup>[74]</sup>. Pathogen recognition by innate immune cells is coupled to the secretion of cytokines that inform the adaptive immune system about the nature of the pathogen and instruct naïve T cells to differentiate into the appropriate T cell subtypes required to clear the infection<sup>[75]</sup>. Thus, naïve T cells are induced to differentiate into Th1, Th2, Th17 and/or regulatory T cells (Treg) depending on the pathogen eliciting the response<sup>[76]</sup>. Recent studies reveal that IL-6/IL-12 family cytokines (IL-6, IL-12, IL-23, IL-27 and IL-35) play pivotal roles in these lymphocyte cell-fate decisions, and their influence on the T cell developmental program is mediated primarily through activation of an evolutionarily conserved family of latent cytoplasmic transcription factors called STATs<sup>[77,78]</sup>.

The progressive damage to the gut is characterized by an aberrant inflammatory response to components of the bacterial microflora, and Th17 cells are thought to contribute to the destruction of gut tissues by inducing secretion of the extracellular matrix-degrading enzymes MIP-3 $\alpha$  and IL-21. Autocrine secretion of IL-21, which perpetuates a cycle of elevated IL-21 secretion, and sustained STAT3 activation in the gut play important roles in exacerbating the disease<sup>[79]</sup>. In addition, pSTAT3 enhances survival of the pathogenic Th17 cells by up-regulating *Bcl-2*, *Bcl-xL*, and *Mcl-1* genes<sup>[80]</sup> and may thereby contribute to maintaining the chronic inflammatory process.

Very recently, the role of NF- $\kappa$ B in IBD has been elucidated<sup>[73]</sup>. Colon biopsies in IBD patients with active disease showed increased levels of NF- $\kappa$ B p65 protein, a member of the NF- $\kappa$ B family of proteins. The amount of NF- $\kappa$ B p65 in the tissue samples correlated with the severity of intestinal inflammation. This increased expression of NF- $\kappa$ B results in an increased ability to secrete inflammatory cytokines, such as TNF- $\alpha$ , IL-1, IL-6,

IL-12, and IL-23, the latter of which are directly responsible for mucosal damage in IBD. TNF- $\alpha$  is also able to up-regulate the production of NF- $\kappa$ B, which results in a cyclical feedback loop of inflammation<sup>[81]</sup>. Additionally, the findings that the degree of gut tissue inflammation correlates with the level of pSTAT3 in histological sections of IBD patients support a role of STAT3 and Th17 cells in IBD<sup>[82]</sup>.

Anti-inflammatory drugs, immunosuppressants, and TNF blockers are used to manage IBD. However, the high cost and adverse effects associated with these drugs encourage the use of alternative management options<sup>[83]</sup>.

Because curcumin plays a key role in the inhibition of both the activation of NF- $\kappa$ B pro-inflammatory cytokines and the IL-6/STAT3 signaling pathway, it could be proposed as a novel therapeutic agent in several inflammatory diseases, such as IBD<sup>[84]</sup>. However, to date, there have been only two human studies of curcumin in patients with IBD that have achieved encouraging results. Holt *et al.*<sup>[85]</sup> conducted a small, open-label, pilot study of curcumin in five patients with ulcerative colitis/proctitis and five patients with Crohn's disease. Patients with ulcerative proctitis, who were currently using 5-aminosalicylic acid (5-ASA) compounds and corticosteroids (four of five patients were on corticosteroids + 5-ASA compounds), were given 550 mg curcumin twice daily for one month and then 550 mg three times daily for the second month. Patients with CD were treated with 360 mg curcumin three times daily for 1 mo followed by 360 mg four times daily for another 2 mo. All patients were assessed at baseline and after two months of curcumin administration *via* hematological, biochemical, and inflammatory analysis (C-reactive protein and erythrocyte sedimentation rate) and by sigmoidoscopy and biopsy. Subjective analysis was performed *via* a self-reported symptom diary. In the ulcerative proctitis group, all five patients had significant improvement with reductions in concomitant medications in 4 patients. Although only four of five CD patients completed the study, they also improved, as evidenced by a lowered Crohn's Disease Activity Index. There was a mean reduction of 55 points and a mean reduction in the sedimentation rate of 10 mm/h. Based on the symptom diary ( $P < 0.02$ ), all patients improved from baseline after two months of ther-



apy, and the inflammatory markers decreased to normal limits.

Subsequently, Hanai *et al.*<sup>[86]</sup> evaluated the use of curcumin in 89 patients with quiescent UC in a randomized, double-blind, multicenter trial. After a four-week washout period, subjects were randomly assigned to a six-month regimen of either placebo ( $n = 44$ ) or curcumin. The treatments consisted of 1000 mg after breakfast and 1000 mg after dinner ( $n = 45$ ) in combination with sulfasalazine (SZ) (1-3 g/d; median 2 g/d) or mesalamine (1.5-3 g/d; median 2.25 g/d).

Patients were followed during treatment and for six months after the treatment ended; they received only SZ or mesalamine during the six-month follow-up period. Of 43 patients (2 patients violated the protocol) who received curcumin, 2 relapsed during the 6 mo of therapy (4.65%), compared to 8 of 39 patients (20.51%) in the placebo group ( $P = 0.040$ ).

Recurrence rates evaluated on the basis of intention to treat showed a significant difference between curcumin and placebo ( $P = 0.049$ ). Furthermore, curcumin improved both the clinical activity index (CAI) ( $P = 0.038$ ) and the endoscopic index (EI) ( $P = 0.0001$ ), measures that are used to evaluate the morbidity associated with UC. The authors drew the following three major conclusions: (1) Curcumin had better clinical efficacy over placebo in the prevention of relapse; (2) Curcumin significantly improved the CAI and EI; and (3) Curcumin was well-tolerated.

Based on these two studies, curcumin seems to be a promising and safe therapy for maintaining remission in patients with quiescent UC as well as for improving symptoms in patients with proctitis and CD. It is evident that further rigorous randomized controlled trials in larger samples of IBD patients are needed to validate the results of the above clinical studies. Considering its effect on multiple inflammatory pathways, curcumin also has the potential to be used as a steroid-sparing induction agent in mild to moderate colitis or as an adjunct to maintain remission in patients who are losing response to immunomodulators.

### Colorectal cancer

Currently, it appears that the anti-carcinogenic properties of curcumin are most likely due to its effects on multiple molecular targets, such as NF- $\kappa$ B factor and AP-1. These are both major transcription factors that regulate inflammation and thus affect cell proliferation, differentiation and even apoptosis.

We have already mentioned that curcumin has been shown to affect a variety of other key players involved in carcinogenesis, such as cyclooxygenase-2, matrix metalloproteinases 2 and 9 and tumor necrosis factor  $\alpha$ -induced vascular cell adhesion molecule.

Sharma *et al.*<sup>[10]</sup> conducted two separate clinical trials exploring the effect of curcumin on malignancies and tumor marker levels. In the first pilot study, the pharmacokinetics and pharmacodynamics of a standardized

Curcuma extract in capsule form (Phytopharm, United Kingdom) at doses ranging from 440 to 2200 mg/d, corresponding to 36-180 mg of curcumin, were evaluated. Fifteen patients with advanced CRC refractory to standard chemotherapies received Curcuma extract daily for up to 4 mo. In one patient, measurement of a serum tumor marker revealed a decrease in carcinoembryonic antigen levels from  $310 \pm 15$  to  $175 \pm 9$   $\mu$ g/L after two months of treatment with 440 mg Curcuma extract. Stable disease *via* computed tomography scan was observed in five of 15 patients. Oral Curcuma extract was well-tolerated, and dose-limiting toxicity was not observed.

In the second dose-escalation study<sup>[10]</sup>, 15 patients with advanced CRC refractory to standard chemotherapies consumed capsules compatible with curcumin doses of between 0.45 and 3.6 g/d for up to 4 mo. Levels of curcumin and its metabolites in plasma, urine, and feces were analyzed. Blood and imaging tests were performed at baseline and at various points throughout the trial. A daily dose of 3.6 g of curcumin caused decreases of 62% and 57% in inducible prostaglandin E2 (PGE2) production in blood samples taken 1 h after the dose was administered on days 1 and 29, respectively. PGE2 is an end product of cyclooxygenase that has been shown to stimulate the growth of human colorectal cancer cells.

Garcea *et al.*<sup>[14]</sup> studied curcumin levels in the colorectum and the pharmacodynamics of curcumin in 12 patients with confirmed CRC. The staging of patients was noted; 2 patients were Duke A, 3 patients were Duke B, and 7 patients were Duke C. Patients were assigned to 450, 1800 or 3600 mg of curcumin per day for 7 d prior to surgery. The recoveries of curcumin in normal and malignant colorectal tissues of patients receiving 3.6 g of curcumin were  $12.7 \pm 5.7$  and  $7.7 \pm 1.8$  nmol/g, respectively. Curcumin levels were highest in the normal tissue of the cecum and the ascending colon as opposed to the transverse colon, the splenic flexure and the descending colon, which suggests a local effect. The levels of M1G were also decreased by curcumin treatment in malignant colorectal tissue. COX-2 levels were undetectable in normal tissue but were detectable in malignant colorectal tissue. Curcumin was not found to modulate the expression of Cox-2 in malignant tissues. The study concluded that a daily dose of 3.6 g of curcumin is pharmacologically efficacious in CRC patients.

Curcumin has also demonstrated potential for the prevention and treatment of CRC in combination with other agents. Familial adenomatous polyposis (FAP) is an autosomal-dominant disorder characterized by hundreds of colorectal adenomas that eventually develop into CRC. One study<sup>[87]</sup> evaluated whether the combination of curcumin and quercetin could suppress adenomas in patients with FAP. Five patients with FAP received combinations of curcumin (480 mg) and quercetin (20 mg) orally three times a day, and the number and size of polyps were assessed at baseline and after therapy. Four patients had a retained rectum, and one had an ileoanal anastomosis. After 6 mo of combination treatment, all five patients had a

decrease in the number and size of polyps from baseline. Polyp number decreased by a mean of 60.4% ( $P < 0.05$ ), and polyp size decreased by a mean of 50.9% ( $P < 0.05$ ). This is the first human demonstration of the reduction in size and number of ileal and rectal polyps in patients with FAP by a curcumin-containing agent. Although the combinations seemed to reduce the adenomas, randomized controlled trials are needed to further validate these findings.

In a non-randomized, open-label clinical trial, Carroll *et al.*<sup>[28]</sup> assessed the effects of oral curcumin (2 or 4 g per day for 30 d) on PGE2 within abnormal crypt foci (ACF) as the primary endpoint using 5-hydroxyeicosatetraenoic acid (5-HETE), ACF number, and proliferation in 44 eligible smokers with eight or more ACF on screening colonoscopy. They assessed pre- and post-treatment concentrations of PGE2 and 5-HETE by liquid chromatography tandem mass spectroscopy in ACF and normal-tissue biopsies; ACF number *via* rectal endoscopy; proliferation by Ki-67 immunohistochemistry; and curcumin concentrations by high-performance liquid chromatography in serum and rectal mucosal samples. Forty-one subjects completed the study. A significant 40% reduction in ACF number occurred with the 4-g dose ( $P < 0.005$ ), whereas ACF were not reduced in the 2-g group. The ACF reduction in the 4-g group was associated with a significant, five-fold increase in post-treatment plasma curcumin/conjugate levels (*vs* pretreatment,  $P = 0.009$ ).

In summary, the above studies suggest that curcumin is safe and has bright prospects for the treatment of patients with CRC. In fact, curcumin has been shown to be beneficial in all 3 stages of carcinogenesis and in all multifactorial illnesses such as cancer. An agent that acts at a number of different cellular levels offers the potential for effective prophylaxis and treatment. It is hoped that larger and methodologically sound clinical trials in patients with CRC will lead to the consideration of curcumin as an anticancer agent.

### Liver disease

We are still remote from having available and effective drug therapies in hepatic diseases, with the exception of those with viral etiology. Especially in emerging liver diseases, such as non-alcoholic fatty liver disease (NAFLD), the only currently available therapies that have proven to be effective are those with nutritional agents such as vitamin E or those that are associated with antidiabetic drugs<sup>[88,89]</sup>. The only effective therapy for NAFLD/NASH remains non-pharmacological and involves a multidisciplinary treatment based not only on diet but also on frequent aerobic physical activity. In this scenario, curcumin appears to provide an opportunity to cure or improve liver pathologies. Curcumin has the following 4 basic effects on the hepatobiliary system<sup>[90]</sup>: (1) Choleric-cholagogue; (2) Antifibrotic; (3) Hepatoprotective; and (4) Antioxidant.

### Choleric-cholagogue effect

Experimental studies have shown large hepatoprotective

effects for curcumin against a variety of hepatotoxic endogenous (from cholestasis to fatty infiltration) and exogenous insults (alcohol to xenobiotics), a significant percentage of which may progress to cirrhosis or hepatocellular carcinoma<sup>[91,92]</sup>. In pharmacological terms, curcumin is a complete choleric-cholagogue. The cleavage products of curcumin (feluric and hydrofeluric acids) have cholelithkinetic properties because they squeeze the gallbladder, while another principle product, paratolilmethylcarbinol, has strong choleric activity<sup>[15]</sup>.

The choleric effect of curcumin increases bile production by approximately 62%. Its effect is not limited to the stimulation of contraction and is also expressed in the bile composition. Indeed, it has been reported that sodium curcumin increases the excretion of bile salts, cholesterol, and conjugate bilirubin, which increases the solubility and prevents the formation of stones in the gallbladder<sup>[93]</sup>.

### Antifibrotic effect

Curcumin may attenuate hepatic fibrosis induced experimentally by various pathogenetic mechanisms due to its protective effect on the inhibition of tissue growth factor TGF- $\beta$ <sup>[94]</sup>. In the development of liver fibrosis, this profibrogenic cytokine plays a key role by promoting the activation of stellate cells to myofibroblasts and through the production of extracellular matrix.

TGF- $\beta$  is one of the main targets of curcumin, which likely occurs through NF- $\kappa$ B<sup>[86]</sup>. A second target of the antifibrotic effect of curcumin is its effect on metalloproteinases, which are involved in remodeling the extracellular matrix. In an experimental model of cirrhosis, curcumin normalized some parameters (ALT, glutathione, glycogen), thus signifying a resumption of hepatic metabolism. Other parameters, such as fibrosis, were only attenuated, which is likely due to the activation of metalloproteinases by curcumin itself<sup>[95,96]</sup>.

### Hepatoprotective effect

Table 3 shows some examples of the hepatoprotective effects of curcumin against many hepatotoxic insults such as paracetamol, *Aspergillus aflatoxins*, or nitrosamines. The hepatoprotective effect derives mainly from its antioxidant activities, as well as its ability to reduce the formation of pro-inflammatory cytokines<sup>[97]</sup>.

Given its hepatoprotective effects, curcumin can be used in cases of insult by exogenous toxins derived from both the environment and lifestyle. It should be recalled that curcumin is able to induce the synthesis of phase II enzymes that protect cells from oxidative stress, such as glutathione transferase, heme-oxygenase and the NAPH-quinone reductase, which results in detoxification and reduced stress<sup>[98]</sup>.

### Antioxidant effect

Curcumin is characterized by its high antioxidant activity, which is comparable to, if not higher than, that of vitamin C and is more than ten times higher than the activity of the scavenger vitamin E<sup>[99]</sup>.

**Table 3** Substances and hepatic intoxication mechanisms contrasted by curcumin

Intoxications	Pathogenetic mechanisms	Curcumin effects
Iron (alcoholic liver disease; steatosis, viral hepatitis; anemia)	Fibrosis induced by oxidation	Anti-oxidant enzymatic activity
Alcohol (chronic or acute intoxication)	Phospholipase A2 activation	Phospholipase A2 inhibition by NF-κB
High-fat diet (lipid storage)	Focal degeneration, micronecrosis	Acil-CoA, cholesterol biliary acids; LDL peroxidation
Xenobiotics induced acute damage	ROS, lipid peroxidation, inflammation	Scavenger activity on NF-κB; anti-oxidant enzymatic activity
Xenobiotics induced chronic damage	Inflammation and hepatocellular necrosis	Hepatic fibrosis inhibition by NF-κB
Poisons (carbon tetrachloride)	Inflammatory self-maintenance	Hepatic inflammation inhibition by NF-κB

ROS: Reactive oxygen species; LDL: Low density lipoprotein; NF-κB: Nuclear factor kappa B.

Overall, the antioxidant action, especially towards cells subjected to increased oxidative stress such as hepatocytes, results in an increase of cellular resistance to oxidative damage for at least 18 h<sup>[99]</sup>. The antioxidant properties of curcumin reside in the same chemical structure. Numerous natural antioxidants can be classified into the following two types of compounds: phenolic (sesame extract) and β-diketones (extracts of eucalyptus).

Curcumin is one of the few antioxidants that possess both a phenolic group and one diketonic in the same molecule. This explains why curcumin possesses the ability to interrupt the chain that transmits the oxidation of biological structures until the oxidant energy is sufficient<sup>[99]</sup>.

In summary, the multiple positive effects of curcumin on both the biliary system and on liver structure and function encourage its clinical use, which needs to be validated in future controlled clinical trials.

### Functional digestive disorders

The mechanisms of symptom generation in patients with functional digestive disorders are poorly understood due to the lack of a mucosal injury that enables us to explain their troublesome disturbances<sup>[100]</sup>. Recent studies have shown that transient receptor potential vanilloid type 1 (TRPV1) receptors play a critical role in somatic and visceral nociceptive neural detection and transmission<sup>[101]</sup>, and they have been implicated in the induction of symptoms in these diseases. TRPV1 is a polymodal sensory transducer that can be activated by multiple noxious stimuli such as heat, low pH, and endogenous lipid derivatives such as anandamide as well as by exogenous substances that possess a vanilloid moiety such as capsaicin<sup>[102]</sup>. Of remarkable importance, the curcumin molecule has the same vanilloid ring moiety as capsaicin, making TRPV1 its likely target, and it has been shown in animals that curcumin blocks TRPV1 activation by capsaicin in a competitive manner<sup>[103]</sup>. It has been suggested that up-regulation of TRPV1 signaling may contribute to visceral hypersensitivity in functional gastrointestinal diseases, including esophageal hypersensitivity<sup>[104]</sup>. This condition can be found in more than 50% of patients with non-erosive reflux disease, which represents the most frequent form of gastro-esophageal reflux disease<sup>[105]</sup>. Recent epidemiological studies have shown that the rate of reflux patients with negative endoscopy can be as high as 75%<sup>[106]</sup>. This relevant population contains subgroups of

patients with hypersensitive esophagus to both acid and non-acid reflux or patients with functional heartburn, who are difficult to treat with antisecretory therapies and who therefore may benefit from drugs that are able to act on TRPV1 receptors. In fact, curcumin has been shown to antagonize the vanilloid receptors even at low dosages and thus has the potential to modulate the response of TRPV1 to various stimulants and to prevent the generation of symptoms in patients with hypersensitive esophagus and functional heartburn<sup>[103]</sup>.

Moreover, the TRPV1 receptors are widely expressed in the entire gastrointestinal tract and enteric nervous system, and there is evidence that curcumin can inhibit GI nociception and reverse gut hypersensitivity by acting on peripheral terminals. Taking into account this mechanism of action, it cannot be excluded that this molecule may be beneficial in treating patients with functional dyspepsia and irritable bowel syndrome, which are disorders that remain clinically challenging in the setting of current drugs and whose patients may benefit from the pharmacological properties of curcumin on TRPV1 as a novel pain modulator.

Finally, as it has been shown that low-grade inflammation of the intestinal mucosa is responsible for symptoms of irritable bowel syndrome<sup>[107]</sup>, we cannot exclude that the well-known anti-inflammatory effects of curcumin may also improve the quality of life of patients with this disease.

### CONCLUSION

In summary, curcumin is a well-known molecule with multiple pharmacological activities that have the potential to be used to treat many gastrointestinal diseases, both functional and organic. It appears to be a very promising therapeutic compound on the basis of thousands of pre-clinical studies, but its poor bioavailability has greatly hampered more widespread clinical use. However, the new formulation of curcumin with phospholipids has allowed us to overcome this problem by markedly improving intestinal absorption compared with the traditional unformulated curcuminoid mixtures. If curcumin is truly beneficial, as has been suggested by prior clinical trials using curcumin with limited bioavailability, we can expect to see greater therapeutic effectiveness from phospholipid-complexed curcumin, which enables increased absorption



and appropriate tissue delivery. These improved pharmacokinetic and pharmacodynamic properties are also able to significantly reduce the required dosages of curcumin and to increase the compliance of the product. Overall, these features make curcumin a very promising new therapeutic option for the treatment of gastrointestinal and hepatic diseases for which present therapies are largely unsatisfactory.

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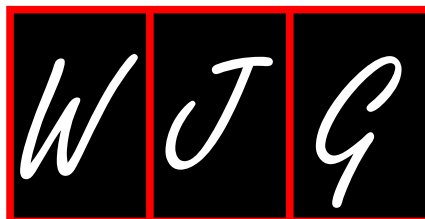
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## Early respiratory complications after liver transplantation

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### Abstract

The poor clinical conditions associated with end-stage cirrhosis, pre-existing pulmonary abnormalities, and high comorbidity rates in patients with high Model for End-Stage Liver Disease scores are all well-recognized factors that increase the risk of pulmonary complications after orthotopic liver transplantation (OLT) surgery. Many intraoperative and postoperative events, such as fluid overload, massive transfusion of blood products, hemodynamic instability, unexpected coagulation abnormalities, renal dysfunction, and serious adverse effects of reperfusion syndrome, are other factors that predispose an individual to postoperative respiratory disorders. Despite advances in surgical techniques and anesthesiological management, the lung may still suffer throughout the perioperative period from various types of injury and ventilatory impairment, with different clinical outcomes. Pulmonary complications after OLT can be classified as infectious or non-infectious. Pleural effusion, atelectasis, pulmonary edema, respiratory distress syndrome, and pneumonia may contribute considerably to early morbidity and mortality in liver transplant patients. It is of paramount importance to accurately identify lung disorders because infectious pulmonary complications warrant speedy and aggressive

treatment to prevent diffuse lung injury and the risk of evolution into multisystem organ failure. This review discusses the most common perioperative factors that predispose an individual to postoperative pulmonary complications and these complications' early clinical manifestations after OLT and influence on patient outcome.

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**Key words:** Respiratory complications; Postoperative respiratory failure; Liver transplantation; Postoperative edema; Post-transplant pneumonia

**Core tip:** This "minireview" underlines the most important perioperative factors that predispose to early post-liver transplant respiratory complications. Despite advances in surgical techniques and anesthesiological management the lung may still suffer throughout the perioperative period from various types of injury, with different ensuing ventilatory impairments, and different clinical outcomes. The incidence, etiology, pathophysiological features, clinical manifestations, preventing measures, and outcomes of post-operative respiratory disorders in this setting are also reported.

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### INTRODUCTION

Orthotopic liver transplantation (OLT) is currently the only definitive treatment for patients with acute liver failure and end-stage liver cirrhosis. Due to recipients' generally poor preoperative clinical conditions, the extensive surgical field, and lengthy operating times, postop-



erative respiratory disorders are very common after OLT and significantly contribute to the related morbidity and mortality, both in the acute postoperative stage and in the long term.

Several factors are involved in the onset of postoperative pulmonary complications (PPCs), and many preoperative and intraoperative variables have been associated with different degrees of severity of respiratory impairment after OLT.

Although refinements in surgical techniques, antimicrobial prophylaxis, immunosuppression, anesthesia, and intensive care management have most likely altered the frequency and overall spectrum of post-OLT respiratory disorders, it is still common for pulmonary infiltrates, atelectasis, pleural exudates, and other radiological abnormalities to be documented on chest X-ray at any time during a patient's stay at an intensive care unit (ICU).

All of these respiratory disorders can affect lung compliance and alveolar gas exchange and, when severe, may necessitate tracheal intubation and mechanical ventilation. In the early stages after transplantation, pulmonary complications may prolong intubation time and increase the risk of systemic infectious complications. Prolonged mechanical ventilation due to refractory respiratory failure is an extremely morbid event, as this event is a marker of poor recipient recovery, predisposes a recipient to long-term ventilator dependency, and predicts further complications.

This review focuses on the most common perioperative factors that predispose an individual to PPCs occurring early after OLT, along with these complications' clinical manifestations and contribution to outcome. The main strategies for preventing the development of post-OLT respiratory disorders are also mentioned.

## PREOPERATIVE RISK FACTORS FOR POST-OLT RESPIRATORY COMPLICATIONS

The most commonly identified risk factors for PPCs are detailed in Table 1 and relate to a recipient's age, the severity of liver dysfunction, cirrhotic encephalopathy, acute renal failure, smoking history, emphysema, high systolic pulmonary artery pressure, hypoxia, and hepatopulmonary syndrome. Pre-existing pulmonary abnormalities *per se* may also make a liver transplant recipient more vulnerable to pulmonary complications. Patients with chronic liver disease have pulmonary regional hemodynamic disturbances, with greater differences in alveolar-arterial oxygen tension, a weaker pulmonary vascular tone, and a poor hypoxic pulmonary vasoconstrictive response<sup>[1]</sup>.

Levesque *et al.*<sup>[2]</sup> reported that evidence of a preoperative restrictive pulmonary syndrome is one of the main risk factors for PPCs. An association between abnormal preoperative spirometry findings and a higher rate of PPCs was also mentioned by Bozbas *et al.*<sup>[3]</sup>.

The relationship between patients' Model for End-

**Table 1 Common preoperative risk factors for post-orthotopic liver transplantation pulmonary complications**

Recipient's age <sup>[2,8]</sup>
Female sex <sup>[5]</sup>
Smoking history <sup>[3]</sup>
Severity of liver dysfunction <sup>[2]</sup> (Child-Pugh class <sup>[5]</sup> , MELD score <sup>[12,43]</sup> )
Cirrhotic encephalopathy
Cerebral dysfunction <sup>[5]</sup>
Acute renal failure
Emphysema <sup>[3]</sup>
High systolic pulmonary artery pressure <sup>[3]</sup>
Hypoxia, orthodeoxia <sup>[3]</sup>
Hepatopulmonary syndrome
Pre-existing pulmonary abnormalities <sup>[1]</sup> :
Intrinsic cardiopulmonary disease: chronic obstructive pulmonary disease, congestive heart failure, pneumonia, asthma
Specific to liver disease: association with specific liver diseases (alpha-1 antitrypsin deficiency, primary biliary cirrhosis), fluid retention complicating portal hypertension (ascites, hepatic hydrothorax), pulmonary vascular abnormalities (hepatopulmonary syndrome, portopulmonary hypertension)
Evidence of a restrictive pulmonary syndrome <sup>[2]</sup>
Abnormal spirometry findings <sup>[3]</sup>
Preoperative ventilator support <sup>[6]</sup>
Severe preoperative respiratory failure requiring mechanical ventilation <sup>[8,9]</sup>
Higher value of INR <sup>[2]</sup>
Preexisting diabetes mellitus <sup>[6,7]</sup>
Impaired renal function <sup>[6]</sup>
Preoperative MARS use <sup>[6]</sup>
Deceased donor source of organ transplantation <sup>[6]</sup>

MELD: Model End Stage Liver Disease; INR: International normalised ratio; MARS: Molecular adsorbent re-circulating system.

Stage Liver Disease (MELD) scores and the incidence of PPCs has yet to be clearly elucidated, but liver transplant recipients with high MELD scores often have a higher incidence of pleural effusion, a need for more perioperative blood transfusions, a greater risk of fluid retention, severe restrictive pulmonary patterns, and muscle atrophy related to poor nutritional status. Given this higher rate of comorbidities in patients with higher MELD scores, cases of postoperative respiratory impairment or failure may be more common as well<sup>[4,5]</sup>.

In a retrospective study, Huang *et al.*<sup>[6]</sup> found that preoperative ventilator support, diabetes mellitus, impaired renal function, and OLT with grafts from deceased donors were the most significant preoperative predictors of the risk of postoperative respiratory failure (PRF). John *et al.*<sup>[7]</sup> demonstrated that patients suffering from diabetes mellitus prior to liver transplantation had a higher incidence of pulmonary complications afterward than did non-diabetic patients.

The main reasons why liver recipients given a graft from a deceased donor are at a higher risk of postoperative respiratory complications relate to the higher MELD scores of such recipients compared with patients receiving grafts from living donors, the "urgent" nature of the transplantation surgery, and the greater "marginality" of cadaveric grafts.

Severe preoperative respiratory failure requiring me-

**Table 2 Major intraoperative and common postoperative risk factors for post-orthotopic liver transplantation pulmonary complications**

Major intraoperative risk factors
Surgical procedure <sup>[2]</sup> (wide incision <sup>[19]</sup> )
Intraoperative fluid transfusion volume <sup>[2,8,12]</sup>
Intraoperative blood transfusion volume <sup>[6,12]</sup>
Perioperative fluid balance <sup>[12]</sup>
Intraoperative fluid retention <sup>[14]</sup>
Intraoperative bleeding volumes <sup>[14]</sup>
Common postoperative risk factors
Excessive perioperative fluid administration <sup>[2]</sup>
Postoperative duration of mechanical ventilation <sup>[2]</sup> (delayed removal of endotracheal tube <sup>[13,19]</sup> )
Acute rejection during the hospital stay <sup>[2]</sup>
Postoperative acute renal failure <sup>[5]</sup>
Postoperative hypoproteinemia
Onset of renal insufficiency
Poor postoperative myocardial function
Right hemidiaphragm paralysis <sup>[24]</sup>
Greater exposure to nosocomial agents <sup>[34]</sup>
Significant decline in the recipient's immune function <sup>[34]</sup>
Surgical complications <sup>[34]</sup>
Re-interventions or need for retransplantation <sup>[34]</sup>

chanical ventilation prior to OLT is one of the most serious events leading to the onset of PPC<sup>[8,9]</sup>, as the presence of an endotracheal tube is a well-recognized factor that predisposes an individual to lower respiratory tract infectious complications<sup>[10]</sup>.

## INTRAOPERATIVE RISK FACTORS FOR POST-OLT RESPIRATORY COMPLICATIONS

OLT is a lengthy procedure that may cause numerous physiological changes, such as mechanical derangement of the chest wall and diaphragm, hydrostatic and oncotic pressure abnormalities, increases in pulmonary vascular resistance and pulmonary artery pressure, abnormal pulmonary vascular permeability, and variable coagulopathies (Table 2).

Although the administration of fluids and blood products is adjusted in an effort to ensure hemodynamic stability and to correct unanticipated coagulation abnormalities and bleeding, a significant loss of blood and fluids during OLTx may be associated with excess fluid administration and a positive fluid balance.

At the end of the transplantation procedure, significantly lower respiratory compliance than before the operation is highly suggestive of increased extravascular pulmonary water content, as demonstrated by Tallegrén *et al.*<sup>[11]</sup>.

In a report by Lin *et al.*<sup>[12]</sup>, a MELD score  $\geq 25$  points, an intraoperative fluid transfusion volume  $>10$  L, and an intraoperative blood transfusion volume  $> 4$  L were all independent predictors of the risk of PPCs, whereas a fluid balance of  $\leq -300$  mL on the first two postoperative days appeared to be a protective factor.

Huang *et al.*<sup>[6]</sup> found that OLT recipients who developed PRF had significantly different intraoperative blood loss, *i.e.*, more patients in the non-PRF group completed the surgical procedure without needing any blood transfusions. This difference greatly influenced outcome, with patients who developed PRF staying longer in the ICU and exhibiting significantly higher morbidity and mortality rates.

Other clinical studies have demonstrated that intraoperative fluid overload is the strongest risk factor for PPCs<sup>[8-13]</sup>. Jiang *et al.*<sup>[14]</sup> investigated the link between intraoperative and postoperative fluid therapy and early PPCs, showing that patients with net intraoperative fluid retention volumes  $< 5000$  mL and intraoperative bleeding volumes  $< 800$  mL had fewer PPCs than did patients needing more fluid therapy. The group that was administered less fluid intraoperatively experienced a faster postoperative recovery, with shorter times to extubation and ICU stays.

Severe reperfusion syndrome is mainly characterized by prolonged hypotension, bradycardia, hyperkalemia, vasodilation, and pulmonary hypertension and may also trigger generalized endothelial injury, resulting in acute pulmonary edema and/or acute respiratory distress syndrome (ARDS)<sup>[13]</sup>. Liver ischemia-reperfusion may lead to an increase in the levels of multiple inflammatory mediators that become active in the lungs. Inflammatory lung-liver interactions, and the activation of nuclear factor  $\kappa$ B in particular, may be implicated in the pathogenesis of permeability-type pulmonary edema<sup>[16,17]</sup>.

A greater susceptibility to interstitial lung edema can seriously impair patients' postoperative oxygenation, worsen oxygen delivery to the newly transplanted organ, and increase the need for ventilation.

Preservation-related or graft-related factors, potentially contaminated preservation fluids, the amount of intraoperative blood transfusion, longer ischemia times, and poor initial graft function are other important factors that predispose an individual to postoperative infections that may also involve the respiratory tract<sup>[18,19]</sup>.

## POSTOPERATIVE RISK FACTORS FOR POST-OLT RESPIRATORY COMPLICATIONS

The most important factors involved in the development of PPCs following the transplantation procedure are reported in Table 2. After admission to the ICU, the residual effect of anesthetics, an excessive need for opioids for analgesia, and a high fluid input may all interfere with a patient's weaning from a ventilator in various ways. Inadequate deep inspiration due to a wide incision and the inhibitory effect of wound pain on coughing and mucus removal also predispose patients to various respiratory complications<sup>[20]</sup>.

As in other patients undergoing upper abdominal surgery, changes in respiratory pressures and chest wall

**Table 3** Major post-orthotopic liver transplantation pulmonary complications

Complication	Frequency
Pleural effusion <sup>[4,8,12,30]</sup>	32%-47%
Atelectasis <sup>[8,12,28,30]</sup>	5%-29%
Pulmonary edema <sup>[8,12-14,28,30]</sup>	4%-47%
Acute respiratory distress syndrome <sup>[4,8,9,12,14,28,30,55]</sup>	0.8%-42%
Pneumonia <sup>[2,4,8,12,14,28,30,36-39]</sup>	5%-38%

movement anomalies due to transection of the abdominal oblique muscles and rectus muscles and prolonged retraction of the right hemidiaphragm, which is associated with diaphragmatic dysfunction, may result in a 50%-60% reduction in vital capacity and a 30% reduction in functional residual capacity<sup>[21]</sup>.

Early weaning from mechanical ventilation is a primary goal for a favorable outcome, but primary graft dysfunction, the need for re-laparotomy, respiratory distress syndrome, the persistence of severe encephalopathy, or surgery-related emboligenic problems may delay removal of the endotracheal tube and correspondingly increase the risk of respiratory infections<sup>[14]</sup>.

One of the most severe, although rare, adverse effects of massive intraoperative transfusion is transfusion-related acute lung injury (TRALI), which has the potential to cause lung edema and severe postoperative respiratory distress. TRALI is particularly relevant in post-OLT patient care because this injury can lead to pulmonary infiltrates, hypoxia, and respiratory failure during or within 6 h after a blood transfusion, with no other apparent cause. According to the “two-hit” theory about the pathogenic mechanism of TRALI, a first event (*e.g.*, sepsis or trauma) could induce pulmonary endothelial activation, cytokine release, and “neutrophil priming”. Subsequent exposure to lipids, cytokines, or antibodies associated with massive transfusion would then prompt the activation of adherent neutrophils and a release of inflammatory mediators, thus leading to lung injury<sup>[22,23]</sup>. TRALI and acute lung injury (ALI) share the same pathophysiological pathway and clinical definition, except that TRALI is temporally and mechanistically related to the transfusion of blood or blood components. In both conditions, capillary permeability results in plasma moving into the alveolar space and causing pulmonary edema<sup>[24]</sup>.

Postoperative hypoproteinemia, the onset of renal insufficiency, and poor postoperative myocardial function can also set the stage for interstitial edema, reduce pulmonary compliance, increase the effort of breathing, and prolong the need for invasive ventilation.

Right hemidiaphragm paralysis after OLT is another complication responsible for the development of right lower lobe atelectasis. In an old study, McAlister *et al*<sup>[25]</sup> found that 79% of liver recipients had right phrenic nerve injury, and approximately half of these patients also had hemidiaphragm paralysis. Phrenic nerve conduction generally tends to recover within a few months, and most patients with phrenic nerve injury and right hemi-

diaphragm elevation rarely develop substantial respiratory dysfunction or need more prolonged mechanical ventilator support<sup>[25,26]</sup>.

A considerable risk of acute rejection invariably persists early after OLT, which is associated with a need for higher levels of immunosuppression. Acute allograft rejection demanding high-dose corticosteroids or cytolytic agents is known to raise the risk of systemic infection, which may also involve the respiratory tract.

## INCIDENCE AND PATHOLOGICAL FEATURES OF PPCS IN LIVER TRANSPLANT PATIENTS

Pulmonary complications after OLT can be classified as infectious and non-infectious (Table 3). Although uncommon in the first few days, the former complications later become an important cause of overall morbidity, whereas non-infectious complications account for most early problems but have less impact on patient outcome<sup>[27,28]</sup>.

In an old study conducted by the Pittsburgh group<sup>[29]</sup>, pulmonary infiltrates characterized as pulmonary edema occurred in 40% of patients; pneumonia, in 38%; atelectasis, in 10%; and ARDS, in 8%. Of the cases of infiltrates, 48% occurred within 30 d of transplantation. In total, 78% of the cases of pulmonary infiltrates and 87% of the cases of pneumonia diagnosed at the ICU involved mechanically ventilated patients.

Glanemann *et al*<sup>[30]</sup> reported that 11% of liver transplant patients required ventilatory support due to pulmonary complications, and 36.1% had to be reintubated. Among the patients who developed pulmonary complications and needed reintubation, 44.6% were intubated within 24 h after OLT.

Hong *et al*<sup>[31]</sup> reported that early post-OLT pulmonary infiltrates were detected in 68 of 131 liver recipients (42.7%), with pleural effusion in 50 patients (73.5%), pneumonia in 6 (8.8%), atelectasis in 6 (8.8%), pulmonary edema in 5 (7.4%), and ARDS in 1 (1.5%). Jiang *et al*<sup>[14]</sup> found that 29 of 62 patients (46.77%) had pulmonary complications after OLT, including pulmonary edema (4 cases, 13.79%), acute lung injury (7 cases, 24.14%), pneumonia (14 cases, 48.28%), and ARDS (4 cases, 13.79%).

In a series described by Bozbas *et al*<sup>[4]</sup>, pulmonary complications were detected in 42.1% of liver recipients; pneumonia, in 21.1%; and pleural effusion on early postoperative chest radiographs, in 32.5%. Right hemidiaphragm elevation was the most common disorder (25.4%).

## PLEURAL EFFUSIONS

Many patients undergoing OLT develop pleural effusions that usually mainly involve the right side, with variable amounts of fluid accumulation. The effusions are transudative and unrelated to primary cardiovascular disease. Pleural effusion after OLT is generally not a serious com-

plication, but if the effusion continues to expand beyond the first week or remains isolated to the left side, the fluid should be sampled to rule out other causes. Patients with large effusions may experience shortness of breath or a nonproductive cough.

Disruption of the diaphragmatic lymphatics during hepatectomy, along with diaphragmatic defects that allow the transfer of ascites developing in the abdominal cavity directly into the pleural space, are postulated to be the principal mechanisms behind fluid accumulation<sup>[26,32]</sup>. The negative intrathoracic pressure draws ascitic fluid into the pleural space, and analysis shows that this fluid has many of the same characteristics as abdominal ascites.

Pleural effusions may expand during the first post-operative week but frequently disappear in the following weeks. These effusions are usually asymptomatic and self-limiting, and thoracentesis or chest tube placement is rarely necessary.

Persistent pleural effusions may lead to respiratory dysfunction by causing atelectasis or may predispose patients to pneumonia and prolong recovery. Effusions may also recur at any time and are occasionally a sign heralding allograft rejection.

Postoperative atelectasis can also be the result of bronchial obstruction due to changes in bronchial secretions, a defective expulsion mechanism, or a reduced bronchial caliber.

A limited intraoperative production of surfactants reduces alveolar surface tension and thus prevents the lung from stabilizing at low volumes, predisposing the lung to collapse. The residual effects of anesthetics and postoperative narcotics can cause hypoventilation, ineffectual respiration, depression of the cough reflex, immobilization, and splinting.

## POST-LT PNEUMONIA

Early nosocomial pneumonia after OLT is nearly exclusively a perioperative complication and is characterized by the presence of pulmonary infiltrate, fever, leukocytosis, and new-onset respiratory symptoms (cough, sputum, and dyspnea). When the typical radiological picture of pneumonia is identified, it is important to isolate the responsible microorganism from deep tracheal aspirate or sputum cultures or bronchoalveolar lavage cultures to prescribe the appropriate, specific treatment.

The breakdown of the mucocutaneous defensive barriers that occurs after prolonged orotracheal intubation is a major risk factor for post-OLT pneumonia. Massive intraoperative bleeding during the transplantation procedure, the persistence of severe encephalopathy, diffuse pleural exudates, postoperative ALI/ARDS, and severe renal impairment are frequently associated with delayed weaning from mechanical ventilation and contribute to the development of infectious diseases<sup>[19,33]</sup>.

Other important risk factors for early pneumonia include a greater exposure to nosocomial agents, a significant decline in recipients' immune function, surgical

complications, re-interventions, and the need for retransplantation<sup>[34]</sup>.

Hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia are usually diagnosed in cases of early- or late-onset pneumonia, depending on whether the pneumonia occurs within or after the first 4-6 d of hospitalization, respectively<sup>[35]</sup>.

The incidence of post-LT pneumonia has been shown to vary from 5%-38%<sup>[8,36-39]</sup>. Pirat *et al*<sup>[8]</sup> reported an incidence of 22.7% and a mortality rate of 40%. These authors found that individuals who developed pneumonia had longer times to extubation and higher mortality. In a study by Xia *et al*<sup>[38]</sup>, the overall incidence of severe pneumonia was 18.2%, with an associated mortality rate of 37.5%. Bozbas *et al*<sup>[4]</sup> reported a higher rate of bacterial pneumonia (> 70%), with a 26% rate of fungal pneumonia; lung infections were noted in 21% of patients in their study and were responsible for 45.8% of deaths.

In a report by Weiss *et al*<sup>[39]</sup>, early HAP (within 6 d after OLTx) occurred in 15.5% of liver recipients. As in the above-mentioned reports, these cases of pneumonia were associated with prolonged postoperative mechanical ventilation, a long ICU stay, and a trend toward higher short- and long-term mortality rates.

Levesque *et al*<sup>[2]</sup> recently reported a 22% incidence of postoperative pneumonia, and 43% of their liver recipients who had pneumonia developed respiratory failure that required mechanical ventilation. Based on a univariate analysis, the researchers found that several preoperative factors and the number of intraoperative transfusions (units of blood and fresh frozen plasma) were associated with pneumonia. However, in a multivariate analysis, only a preoperative restrictive pulmonary pattern and the international normalized ratio measured prior to OLT were independent predictors of pneumonia after surgery. Ikegami *et al*<sup>[40]</sup> reported the prevalence and characteristics of bacterial pneumonia after living-donor liver transplantation (LDLT), stating that 50 of 346 patients (14.5%) experienced bacterial pneumonia after LDLT. The incidence of bacterial pneumonia was highest on postoperative day 6, whereas the incidence declined on postoperative days 8 and 9. Pneumonia was associated with a prolonged use of mechanical ventilation, a prolonged stay in the ICU, the creation of a tracheostomy, primary graft dysfunction, and a need for renal replacement therapy. The mortality rate of patients with early-onset pneumonia was 25.7%. Delayed-onset pneumonia (at least 10 d after liver transplantation) was significantly associated with graft dysfunction and resulted in a higher mortality rate (73.3%) than did early-onset pneumonia.

A wide variety of community-acquired and hospital-acquired microorganisms may be responsible for post-OLTx pneumonia, but Gram-negative pathogens dominate in the early post-transplant stages, as in the population undergoing general surgery. The Gram-negative bacteria that frequently colonize the oropharyngeal cavity are most often responsible for lower respiratory



tract infections. In liver recipients on prolonged mechanical ventilation, nosocomial pathogens, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* species, *Acinetobacter* species, and *Staphylococcus aureus* (including MRSA), are usually detected in bronchoalveolar lavage samples<sup>[41,42]</sup>. It is worth emphasizing, however, that the microbiological ecology may vary considerably from one ICU to another, and previous antimicrobial consumption may have a major influence on microbial ecology.

In a report by Weiss *et al*<sup>[39]</sup>, in a subgroup of patients with HAP occurring within the first 4 d after ICU admission, 61.5% of the causative pathogens were Gram-negative bacilli, and 38.5% were Gram-positive cocci. Of these microorganisms, 73% were classified as community acquired. More than 30% of liver recipients had a history of hospital stays and antibiotic treatments.

Given patients' obligatory immunosuppression, prompt isolation of the microorganisms causing post-OLT pneumonia and appropriate treatment are mandatory for a favorable outcome. An early diagnosis may not be achievable with "conventional" diagnostic techniques in certain patients, however, making it necessary to resort to more "invasive" methods. If pulmonary infiltrates persist or become worse, a histopathological diagnosis by bronchial brushing, telescope catheter culture, fiberoptic bronchoscopy with transbronchial biopsy, or even surgical pulmonary biopsy may be needed to rule out opportunistic infectious agents.

## POST-LT PULMONARY EDEMA

Severe pulmonary edema is unusual in the early postoperative period, unless the liver recipient experiences acute-onset, severe left ventricular dysfunction or acute fluid overload in the case of renal impairment. Despite a high incidence of postoperative radiological findings suggestive of acute pulmonary edema, most episodes are clinically easily overlooked, with only a mild deterioration in gaseous exchange.

In patients with fulminant hepatic failure, pulmonary edema is an ominous sign because it may predict evolving acute lung injury<sup>[43]</sup>.

Subclinical forms of interstitial/alveolar edema may prompt findings of a transient increase in pulmonary capillary hydrostatic pressure or hypoalbuminemia or nonspecific signs of mild or moderate lung endothelial injury. Additional causes of acute pulmonary edema include excessive amounts of fresh frozen plasma and total fluids being administered intraoperatively, postoperative changes in renal function (urine volume and serum creatinine), massive transfusions, large-volume thoracentesis, and reduced lymph flow. It has been speculated that the greater pulmonary vessel permeability associated with end-stage liver disease may be exacerbated by the systemic inflammatory reaction induced by liver transplantation<sup>[14]</sup>.

In a study by Aduen *et al*<sup>[44]</sup>, a worse preoperative MELD score could predict the risk of pulmonary edema

developing soon after the transplantation procedure. Preoperative right ventricular systolic pressure, as estimated by echocardiography, was also higher in patients who developed postoperative pulmonary edema, suggesting that elevated pulmonary pressures are associated with increased interstitial lung loading.

Chen *et al*<sup>[45]</sup> postulated that nitric oxide (NO) flow-mediated vasodilation is the pathogenic mechanism behind the high incidence of pulmonary edema after LDLT. In this study, the total volume of intraoperative fluid administered was higher in patients who developed pulmonary edema, but their net fluid retention did not significantly differ from that of the patients who did not experience this complication. Pulmonary edema did not prolong the hospital stay or increase the risk of infection and was overcome by administering diuretics.

In cirrhotic patients undergoing OLT, increased blood flow in the lung may increase shear stress on the endothelium, and this phenomenon is associated with an increased release of vasodilators, including NO, prostaglandins, and endothelium-derived hyperpolarizing factors<sup>[46,47]</sup>.

Pulmonary edema is diagnosed based on the strength of radiographic criteria, clinical symptoms, the PaO<sub>2</sub>/FIO<sub>2</sub> (PF) ratio (< 300), and hemodynamic data. According to the American-European Consensus Conference (AECC) on ARDS, permeability edema may be characterized by a pulmonary artery wedge pressure < 18 mmHg, whereas the hydrostatic type is usually associated with a wedge pressure > 18 mmHg<sup>[48]</sup>. Patients with persistent permeability-type edema may also have a higher mean pulmonary arterial pressure and a higher pulmonary vascular resistance, consistent with a resistance-dependent mechanism.

In a study by Snowden *et al*<sup>[13]</sup>, patients with pulmonary edema stayed longer in the ICU and were on mechanical ventilation for longer. Aduen *et al*<sup>[44]</sup> also found that the time on mechanical ventilation and in the ICU and hospital stays were longer in patients with persistent permeability-type edema. In contrast to the situation observed for the hydrostatic type, permeability-type pulmonary edema was associated with an increase in both mean pulmonary arterial pressure and pulmonary vessel resistance. In the series, 29% of patients with persistent permeability-type pulmonary edema died, as opposed to 7% of patients who never developed pulmonary edema and 0% of patients who developed hydrostatic-type pulmonary edema.

## POST-OLT ARDS

Post-OLT acute lung injury and even severe ARDS may develop within 24 h or the first few days after the procedure. Frequent causes of ARDS include crystalloid infusion overload, massive transfusion of blood or blood products, prolonged operating times, severe bleeding during liver removal, and severe ischemia-reperfusion syndrome. In the early postoperative course, serious systemic infections, gastric aspirations, disseminated intravascular

**Table 4 Major strategies to prevent postoperative pulmonary complications after orthotopic liver transplantation**

Preoperative strategies	Intraoperative strategies
Pulmonary rehabilitation prior to OLT	Reduction in the degree of surgical insult
	Reduction in the level of aggressiveness
	Reduction in the duration of procedure
	Reduction in the amount of blood lost
Postoperative ventilation <sup>[60,61,64,65]</sup>	Postoperative care <sup>[68]</sup>
Early extubation	Adequate postoperative pain relief
Lung expansion maneuvers	Optimal hemodynamic and fluid management
Deep breathing exercises	Improvement of general health and nutrition
Timely execution of bronchial toilette	
NIV	
Chest percussion and vibration	
Invasive mechanical ventilation: assisted modes with minimal sedation	

OLT: Orthotopic liver transplantation; NIV: Non-invasive ventilation.

coagulation, and other nonspecific generalized insults may also be involved.

The intraoperative transfusion of blood products, and platelets in particular, has been identified as a risk factor for a poor outcome after OLT. The negative impact cannot be explained simply by the activation of the coagulation system and platelet aggregation at the endothelium; the poor outcome most likely has to do with ischemia-related endothelial cell injury<sup>[49]</sup>. Platelets contain many cytokines and vasoactive and inflammatory mediators that are rapidly released and activated by various stimuli after reperfusion and that may affect the lung. Several other factors, such as the potential for viral transmission and bacterial contamination, the risk of alloimmunization, nonspecific immunosuppressive effects, and graft-versus-host disease, may also contribute to a worse outcome<sup>[50,51]</sup>.

Pereboom *et al.*<sup>[52]</sup> demonstrated that platelet transfusion during OLT is associated with higher postoperative mortality due to severe lung edema causing heaviness of the lungs, as described in the clinical diagnosis of TRALI or ARDS.

ARDS after OLT is a serious multifactorial complication associated with diffuse, bilateral pulmonary infiltrates of acute onset (and non-cardiogenic etiology), with a PF ratio of < 200. Based on an “old” concept, ALI was once defined as a milder form of ARDS and was distinguished by a PF ratio of between 200 and 300<sup>[48]</sup>. Currently, according to the Berlin definition, the term ALI is avoided and replaced by mutually exclusive subcategories of ARDS based on the degree of hypoxemia. ALI is now be called “mild ARDS” and applies to cases with a PF ratio of up to 201-300 mmHg, the upper limit for ALI according to the AECC definition<sup>[53]</sup>.

A poorly controlled systemic inflammatory response induced by severe reperfusion syndrome, along with transfusion related-adverse events, can substantially increase the risk of postoperative pulmonary injury. Inflam-

matory mediators cause damage to both the alveolar and the microvascular endothelia, and this damage alters the alveolar-capillary barrier, causing extravascular fluid accumulation. This pulmonary damage results in an increase in extravascular lung water, which is one of the hallmarks of mild ARDS and ARDS<sup>[54,55]</sup>.

Major clinical findings in ARDS include severely impaired pulmonary oxygen diffusion, with pulmonary edema developing in the presence of normal pulmonary capillary-filling pressures and in the absence of a marked reduction in oncotic pressure.

ARDS is an important cause of PRF after OLT. In an old study, the reported incidence of ARDS was in the range of 4.5%-15.7%, with a mortality rate nearing 80%<sup>[9]</sup>.

More than 10 years ago, Golfieri *et al.*<sup>[56]</sup> also reported that 4%-16% of patients who developed post-OLT lung injury deteriorated to severe ARDS, and the mortality rate of these patients was as high as 80%-100%.

Treatment for ARDS is primarily supportive, with fluid restriction, lung-protective mechanical ventilation, mild hypercapnia, and optimal PEEP<sup>[57]</sup>. The use of high PEEP has raised certain concerns, however<sup>[58]</sup>, because of the potentially reduced venous return in a newly engrafted liver. Given the still limited data available, the literature affords no definitive answers on the use of “permissive” PEEP in this setting.

When critical hypoxemia ensues in patients with severe ARDS, additional rescue therapies may be administered, such as inhaled NO and prostaglandins<sup>[59]</sup>.

## PREVENTING PPCS AFTER OLT

The period following transplantation surgery is marked by variable changes in the structure and function of the respiratory system, which can particularly affect severely debilitated patients. The normal activity of liver recipients is usually reduced due to a low physical performance status both before and after liver transplantation.

Similar to what is advisable after upper abdominal surgery, important strategies for PPC reduction may include early extubation associated with lung expansion maneuvers, which comprise incentive spirometry, deep breathing exercises, intermittent positive-pressure breathing, and continuous positive airway pressure (CPAP)<sup>[60]</sup>. Manual techniques, including chest percussion and vibration, are alternative treatment approaches if airway clearance is not sufficient (Table 4).

Early extubation is the key element to reduce PPCs and ICU stay and to speed patients' recovery. There is a substantial body of evidence proving that patients who undergo OLT can be extubated immediately after surgery, with few pulmonary complications, a lower risk of postoperative infection, and no effect on 1- or 3-year graft survival<sup>[61]</sup>.

The specific benefit of each chest physical therapy technique has not been fully evaluated, and even combining various methods does not seem to provide additional

risk reduction. However, CPAP is particularly useful for patients who cannot perform deep breathing or incentive spirometry exercises after extubation<sup>[62]</sup>.

In the case of reduced postoperative lung volumes, the elevation of both hemidiaphragms, and lower-lobe atelectasis, the work of breathing can be consistently augmented, making it difficult to achieve and maintain postoperative ventilatory autonomy. In liver recipients who remain under invasive mechanical ventilation, ventilator use may significantly influence the disuse of muscle dysfunction. Assisted modes of ventilation with minimal sedation should be favored over “controlled” modes, as complete diaphragm rest will rapidly lead to atrophy<sup>[63]</sup>.

Due to the important restrictive respiratory pattern of cirrhotic patients and abdominal hypertension, weaning from a ventilator after OLT can take longer because of unsatisfactory gas exchange during various T-piece trials. Rapid extubation followed by immediate noninvasive ventilation (NIV) application should be considered in this setting to shorten and accelerate the weaning process in those recipients who do not completely fulfill the criteria for safe extubation<sup>[64]</sup>. By resting and unloading the inspiratory muscles, NIV with pressure support enables both hypercapnic and hypoxic patients to improve faster and may prevent basal atelectasis induced by abdominal distension. Chest physical therapy associated with CPAP or NIV stimulates lung expansion and improves lung ventilation, thereby preventing or reducing the build-up of liquid in the pleural space.

The early and “prophylactic” use of NIV may also reduce the risk of reintubation<sup>[65]</sup>. Because NIV leaves the upper airways intact, this method can reduce not only bacterial colonization and nosocomially acquired infections but also hemorrhagic complications in cases of underlying coagulopathy.

Many respiratory disorders following OLT respond to specific treatments, such as hemofiltration, pleural drainage, bronchial toilette, and abdominal drainage, with expected improvements over a period of hours or days. Supporting the failing recipient with early NIV may reduce the work of breathing and maintain gas exchange while awaiting an improvement in spontaneous ventilation.

Adequate postoperative pain relief, optimal hemodynamic and fluid management, the timely execution of bronchial toilette, airway clearance maneuvers (assisted cough and expiratory airflow techniques), and trials of NIV in the case of respiratory fatigue are extremely useful to facilitate the rehabilitation process.

Postoperative pain is a major cause of shallow breathing and impaired coughing, resulting in retention of secretions, atelectasis, hypoxemia, hypercapnia, and respiratory failure, especially in patients with pre-existing lung disease. Adequate treatment of pain will prevent hypoventilation and reduce the respiratory rate. Paracetamol at reduced doses, along with rescue doses of tramadol, should be offered as a valid analgesic regimen<sup>[66]</sup>.

The improvement of sedation management, simple interventions aiming at actively mobilizing the recipient,

and increasing the amount of time out of bed are further advantageous for lung function.

Although the level of evidence for implementing multimodal preventive measures is relatively low<sup>[67]</sup>, and although many of the procedures believed to reduce the risk of PPCs are supported by a conventional “traditional” consensus, early respiratory disorders associated with cirrhosis and transplantation surgery undoubtedly benefit from an optimized patient care program with a multidisciplinary approach.

Promising new interventions may rely on more accurate preoperative respiratory assessment, *e.g.*, with maximal inspiratory pressure and maximal expiratory pressure measurements, quantification of the degree of respiratory muscle weakness, optimization of chronic inflammatory pulmonary disease, preoperative lung expansion maneuvers, inspiratory muscle training performed in a chest physical therapy outpatient setting or a pulmonary rehabilitation clinic in the hospital, and improvement of general health and nutritional status<sup>[68]</sup>.

It should be noted, however, that pulmonary rehabilitation prior to OLT may be of unpredictable value, as the variable waiting times before surgery, along with malnutrition and end-stage cirrhosis-related muscle weakness, may insufficiently affect exercise capacity and are thus unlikely to reduce postoperative risk.

Additional interventions of more expected benefit include an effort to reduce the degree of surgical insult, the level of aggressiveness, the duration of the procedure, and the amount of blood lost.

Better identification of patient- and procedure-related risks of pulmonary complications, the recognition of independent predictors of PRF, and the application of a local treatment protocol in high-risk patients, may favorably influence both the incidence and the outcome of PPCs.

## CONCLUSION

Many reports underscore that infectious and other PPCs are important contributors to early morbidity and mortality in liver transplant patients<sup>[4,8,55]</sup>. Despite advances in surgical techniques and anesthesiological management, the lung may still suffer throughout the perioperative period from various types of injury, with different ensuing ventilatory impairments and different clinical outcomes.

Postoperative respiratory complications are not always related to preoperative respiratory disorders, but rather may be the result of systemic inflammatory responses induced by surgical trauma, hemodynamic impairment, reperfusion syndrome, “distant” organ dysfunction, or early graft dysfunction. The severity of any PPC is also believed to depend on a recipient’s clinical condition at the time when the complication occurs<sup>[31]</sup>. The stress response to the surgery is maximal soon after OLT and is expressed by disrupted circulating hormone concentrations, with increased antidiuretic activity. Electrolyte abnormalities and water retention are also common at this



time due to the nephrotoxic effects of the immunosuppressants administered.

Pleural effusion, atelectasis, pneumonia, and ARDS may be severe enough to demand or prolong the need for tracheal intubation, resulting in a higher risk of nosocomial infections, longer stays at the ICU and/or in the hospital, and a worse clinical outcome<sup>[69]</sup>. The lungs are particularly vulnerable to infectious diseases after OLTx and represent the second most common site (after the abdominal cavity) of colonization by nosocomial pathogens. Infectious complications involving the respiratory tract are acknowledged to be an important cause of death in liver transplant recipients<sup>[18]</sup>. In certain old studies by Plevak *et al*<sup>[70]</sup> and Shieh *et al*<sup>[71]</sup>, patients who developed pneumonia in the early postoperative period and required prolonged mechanical ventilation had a mortality rate of 43%. Singh *et al*<sup>[29]</sup> also reported an overall mortality rate of 28% in transplant recipients with pulmonary infiltrates in the ICU and a mortality rate of 47% after 14 d among patients with pneumonia. Bozbas *et al*<sup>[4]</sup> found significantly lower survival rates for patients yielding microorganisms by deep tracheal aspirate culture. The early mortality rate was higher for patients whose thoracentesis cultures were positive.

Currently, the overall 1- and 5-year survival rates after OLT are approximately 85% and 68%, respectively, with a 10-year survival rate approaching 50%<sup>[72]</sup>. Judging from the multicenter-based prospective data collected by Watt *et al*<sup>[73]</sup>, post-transplant respiratory diseases now account for only 2.4% of all deaths. Among the deaths after OLT, those of an infectious nature (> 19%) occur earlier, and pneumonia is among the most important contributors to the overall morbidity and mortality rates.

In conclusion, numerous perioperative factors may be responsible for impaired respiratory function after OLT. It is of paramount importance to accurately identify any lung disorders because pulmonary infectious complications need to be treated rapidly and aggressively to prevent diffuse lung lesions and potential evolution into multisystem organ failure.

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## Refining pathological evaluation of neoadjuvant therapy for adenocarcinoma of the esophagus

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### Abstract

**AIM:** To assess tumour regression grade (TRG) and lymph node downstaging to help define patients who benefit from neoadjuvant chemotherapy.

**METHODS:** Two hundred and eighteen consecutive patients with adenocarcinoma of the esophagus or gas-

tro-esophageal junction treated with surgery alone or neoadjuvant chemotherapy and surgery between 2005 and 2011 at a single institution were reviewed. Triplet neoadjuvant chemotherapy consisting of platinum, fluoropyrimidine and anthracycline was considered for operable patients (World Health Organization performance status  $\leq 2$ ) with clinical stage T2-4 N0-1. Response to neoadjuvant chemotherapy (NAC) was assessed using TRG, as described by Mandard *et al.* In addition lymph node downstaging was also assessed. Lymph node downstaging was defined by cN1 at diagnosis: assessed radiologically (computed tomography, positron emission tomography, endoscopic ultrasonography), then pathologically recorded as N0 after surgery; ypN0 if NAC given prior to surgery, or pN0 if surgery alone. Patients were followed up for 5 years post surgery. Recurrence was defined radiologically, with or without pathological confirmation. An association was examined between t TRG and lymph node downstaging with disease free survival (DFS) and a comprehensive range of clinico-pathological characteristics.

**RESULTS:** Two hundred and eighteen patients underwent esophageal resection during the study interval with a mean follow up of 3 years (median follow up: 2.552, 95%CI: 2.022-3.081). There was a 1.8% ( $n = 4$ ) inpatient mortality rate. One hundred and thirty-six (62.4%) patients received NAC, with 74.3% ( $n = 101$ ) of patients demonstrating some signs of pathological tumour regression (TRG 1-4) and 5.9% ( $n = 8$ ) having a complete pathological response. Forty four point one percent ( $n = 60$ ) had downstaging of their nodal disease (cN1 to ypN0), compared to only 15.9% ( $n = 13$ ) that underwent surgery alone (pre-operatively overstaged: cN1 to pN0), ( $P < 0.0001$ ). Response to NAC was associated with significantly increased DFS (mean DFS; TRG 1-2: 5.1 years, 95%CI: 4.6-5.6 *vs* TRG 3-5: 2.8 years, 95%CI: 2.2-3.3,  $P < 0.0001$ ). Nodal down-staging conferred a significant DFS advantage for those patients with a poor primary tumour response to NAC (median DFS; TRG 3-5 and nodal down-staging: 5.533 years, 95%CI:



3.558-7.531 *vs* TRG 3-5 and no nodal down-staging: 1.114 years, 95%CI: 0.961-1.267,  $P < 0.0001$ ).

**CONCLUSION:** Response to NAC in the primary tumour and in the lymph nodes are both independently associated with improved DFS.

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**Key words:** Esophageal cancer; Gastro-esophageal cancer; Neoadjuvant; Regression

**Core tip:** Predictive markers of benefit from neoadjuvant chemotherapy (NAC) in esophageal adenocarcinoma are urgently required to provide a "personalised medicine" approach: directing treatment to those most likely to benefit. Before prospective studies can be initiated, retrospective series need to be interrogated to identify likely candidate markers of a positive response. In defining a positive response attention needs to be given to both response in the primary tumour and in the lymph nodes, as a previously unidentified group of patients who appear to have a poor tumoural response to NAC (tumour regression grade 3-5) do benefit from combination therapy by nodal downstaging.

Noble F, Nolan L, Bateman AC, Byrne JP, Kelly JJ, Bailey IS, Sharland DM, Rees CN, Iveson TJ, Underwood TJ, Bateman AR. Refining pathological evaluation of neoadjuvant therapy for adenocarcinoma of the esophagus. *World J Gastroenterol* 2013; 19(48): 9282-9293 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9282.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9282>

## INTRODUCTION

Neoadjuvant therapy followed by surgery is established as the gold standard in the management of patients with locally advanced adenocarcinoma of the esophagus/esophagogastric junction. In the United Kingdom neoadjuvant chemotherapy (NAC) in conjunction with transthoracic esophagogastric resection is the current standard of care for these patients<sup>[1]</sup>. The potential benefits of neoadjuvant therapy include: downstaging of the primary tumour<sup>[2]</sup> and lymph nodes<sup>[3]</sup>, an increase in the resectability of the tumour<sup>[4]</sup>, elimination of micrometastases<sup>[5]</sup> and improved survival<sup>[6]</sup>. A recently suggested advantage of neoadjuvant therapy and early assessment of response is the potential for assessing *in vivo* the chemosensitivity of the tumour and so providing information to tailor multimodal therapy<sup>[7]</sup>. Both NAC and surgery are associated with considerable morbidity and mortality<sup>[8]</sup> and evidence remains inconsistent for the survival benefit for patients who undergo NAC<sup>[4,8,9]</sup>. The most recent meta-analysis to compare NAC *vs* surgery alone in 2062 patients suggests a 5.1% survival advantage at 2 years for patients treated with NAC for adenocarcinoma<sup>[6]</sup>. Patients who have a significant pathological response to neoadjuvant therapy have consistently been shown to have

improved survival when compared to patients who have not had a significant response<sup>[10-13]</sup>. For those patients who do not have a significant pathological response, the consequences of delay to surgery and the benefits of neoadjuvant chemotherapy are not known. Furthermore, it is unclear which patients should be considered for tailored adjuvant systemic therapy or alternative neoadjuvant therapy.

The pathological response to chemotherapy is most widely assessed using Tumour Regression Grading (TRG)<sup>[11]</sup> as described by Mandard *et al*<sup>[14]</sup> although this has not gained universal acceptance<sup>[15]</sup>. This system is based on the amount of residual tumour and the degree of fibrosis at the primary tumour<sup>[14]</sup>. Other proposed pathological systems for measuring neoadjuvant treatment response include complete pathological response<sup>[16]</sup>, size of residual tumour<sup>[17]</sup>, number of residual tumour cells<sup>[15,18]</sup>, response classification system<sup>[19]</sup>, size based pathological response<sup>[17]</sup> and downstaging of cT and cN stage<sup>[10]</sup>. These grading systems have predominately been developed following chemoradiotherapy with heterogeneous histology with few studies assessing their utility following chemotherapy in patients with esophageal adenocarcinoma<sup>[2,20-23]</sup>. A number of clinically important questions could be addressed by a robust and universally accepted measure of response to neoadjuvant treatment including: the ability to accurately predict an individual patient's tumour response to preoperative therapy leading to non-responders proceeding directly to surgery or being considered for alternative neoadjuvant regimes; assessment of new neoadjuvant regimes, and identification of patients who are likely to benefit from adjuvant therapy.

We have therefore assessed pathological response to neoadjuvant chemotherapy by assessing the tumour response as well as the response in the lymph nodes in a large contemporary cohort of patients with esophagogastric adenocarcinoma managed with neoadjuvant platinum based triplet chemotherapy, and describe their associations with short- and long-term outcomes. In addition we suggest combining both local tumour and nodal responses to NAC.

## MATERIALS AND METHODS

### Patients

For this retrospective study, a prospectively collected database of consecutive patients undergoing esophagogastric resection treated at University Hospital Southampton National Health Service Foundation Trust (UHSFT) between January 2005 and December 2011 was reviewed. All patients were discussed at a specialist multidisciplinary team meeting (MDT). Standard staging investigations included endoscopic ultrasonography, high-resolution computed tomography, integrated fluorodeoxyglucose positron emission tomography/computed tomography (PET-CT) and staging laparoscopy, where indicated and were uniformly applied during the study interval. Patients considered suitable for potential surgical resection with tumours staged as T2N0M0 or above were considered for neoadjuvant chemotherapy.

Neoadjuvant chemotherapy consisted of three 21 d



**Table 1** Tumour regression scoring according to Mandard *et al*<sup>[14]</sup>

Grade	Definition
TRG 1	No residual cancer
TRG 2	Rare residual cancer cells
TRG 3	Fibrosis outgrowing residual cancer
TRG 4	Residual cancer outgrowing fibrosis
TRG 5	Absence of regressive changes

TRG: Tumour Regression Grade.

cycles of anthracycline, platinum and fluoropyrimidine: ECF (epirubicin 50 mg/m<sup>2</sup>, cisplatin 60 mg/m<sup>2</sup>, both intravenously on 1 d and protracted venous infusion 5-FU 200 mg/m<sup>2</sup> per day) or ECX (epirubicin 50 mg/m<sup>2</sup>, cisplatin 60 mg/m<sup>2</sup>, both intravenously on 1 d and capecitabine 625 mg/m<sup>2</sup> orally twice daily for 21 d) or EOX (epirubicin 50 mg/m<sup>2</sup> *iv* bolus and oxaliplatin 130 mg/m<sup>2</sup> *iv* infusion over 2 h on 1 d, capecitabine 625 mg/m<sup>2</sup> orally twice daily for 21 d).

Surgery was performed at UHSFT after initial staging or 4–6 wk following neoadjuvant chemotherapy. A repeat CT scan was performed, prior to surgery, for those who received chemotherapy to assess their response to chemotherapy and disease operability. Types of esophago-gastrectomies included Ivor Lewis, left thoracoabdominal with or without cervical anastomosis and transhiatal esophago-gastrectomy or minimally invasive esophago-gastrectomy (MIO) either 2 stage (MIO-2) or 3 stage (MIO-3) in accordance with recommendations arising from the consensus statement from the Association of Upper Gastrointestinal Surgeons and the Association of Laparoscopic Surgeons for introduction of MIO<sup>[24]</sup>.

Data recorded included demographics, tumour characteristics, resection type, estimated blood loss (calculated from suction bottles and weighed swabs) and histopathological analysis of the surgical specimen. TNM-7 (International Union Against Cancer TNM Classification 7<sup>th</sup> Edition) was used to report tumour stage after analysis of pathology reports<sup>[25]</sup>. Pathological tumour clearance (“R”-status) was determined according the Royal College of Pathologists’ guidance.

Postoperative complications were graded according to the Clavien-Dindo (CD) classification<sup>[26]</sup>. An AL was defined as a leak sufficient to cause symptoms and confirmed by radiology (contrast enhanced multi-detector CT scan with on-table oral contrast or water soluble contrast studies), endoscopy or during surgical exploration.

All patients were cared for by a specialist esophagogastric team who applied a similar perioperative regime to all patients. Patients were routinely followed-up for 5 years post surgery according to the following protocol: 2–4 wk post-discharge, 3 monthly for 1 year, 6 monthly for 2 years and yearly thereafter. Patients were also seen on an “as required” basis if symptomatic. Recurrence of disease during follow-up was defined as the first site or sites of recurrence with radiological or pathological confirmation. For assessment of disease free survival (DFS), recurrence was defined as time from operation to development of local, nodal (regional) and distant metastasis (whichever occurred first).

## Factors analysed

Pathological response to chemotherapy was assessed using the TRG system developed by Mandard *et al*<sup>[14]</sup> who scored regression based on the degree of fibrosis and residual cancer cells (TRG 1–5)<sup>[14,27]</sup>, see Table 1. All dissected lymph nodes were stained with hematoxylin and eosin and microscopically analysed for metastatic disease. TRG was scored by specialist gastrointestinal pathologists; initially by one pathologist (Bateman AC) prior to its introduction by all pathologists as part of routine pathological reporting.

## Statistical analysis

Descriptive data are represented as median and range unless indicated with Kruskal-Wallis, Mann Whitney *U*, *P* and  $\chi^2$  test, which were used as appropriate for comparison. Kaplan-Meier, univariate and multivariate cox logistic regression modelling were used to assess the relationship between pathological response grading systems with DFS. All factors that showed statistical significance on univariate analysis were entered to derive the final model. DFS curves of the patients were plotted by using the Kaplan-Meier method and analysed using the Log-rank test. Stratified analyses were performed based on receipt of neoadjuvant chemotherapy, nodal stage and response to chemotherapy. A *P* < 0.05 was considered statistically significant for all tests. Statistical analysis was performed with SPSS<sup>®</sup> version 19 (SPSS, Chicago, Illinois, United States).

## RESULTS

### Study patients

A total of 218 patients underwent esophageal resection during the study interval with a mean follow up of 3 years (median follow up: 2.552, 95%CI: 2.022–3.081). There was a 1.8% (*n* = 4) inpatient mortality rate. Detailed patient characteristics and clinical and pathological outcomes are summarised in Table 2, grouped by treatment.

Patients who underwent surgery alone (*n* = 82; 37.6%) were significantly older (*P* < 0.0001), had worse physiological status (ASA *P* = 0.005; performance status *P* = 0.001; O-POSSUM *P* < 0.0001) and lower preoperative staged disease (cT stage *P* < 0.0001; cN stage *P* < 0.0001) compared to patients that underwent multimodal therapy.

One hundred thirty-six (62.4%) patients received multimodal therapy, neoadjuvant chemotherapy and surgery, with 74.3% (*n* = 101) of patients demonstrating some signs of pathological tumour regression (TRG 1–4) with 5.9% (*n* = 8) having a complete pathological response. Forty four point one percent (*n* = 60) had downstaging of their nodal stage compared to only 15.9% (*n* = 13) whose lymph node status was cN1 on preoperative staging and pN0 following surgery alone (*P* < 0.0001).

There were no statistically significant differences in postoperative pathological tumour stage (yp or pT, *P* = 0.692); yp or pN *P* = 0.758), postoperative complications (CD maximum grade, *P* = 0.590) or completeness of resection (*P* = 0.772) in patients that underwent multimodal therapy *vs* surgery alone.

**Table 2 Clinical and pathological characteristics of the 218 patients operated on for esophageal and gastro-esophageal adenocarcinoma, according to treatment *n* (%)**

Characteristic		Surgery only 82 (37.6)	Neoadjuvant chemotherapy and surgery 136 (62.4)	P value
Preoperative status				
Age (range) <sup>1</sup> yr		74.32 (42.08-85.41)	63.76 (32.77-81.28)	< 0.0001
Sex ratio (M:F) <sup>1</sup>		68 (82.9):14 (17.1)	118 (86.8):18 (13.2)	0.439
cT stage	1	17 (20.7)	0 (0.0)	< 0.0001
	2	30 (36.6)	16 (16.0)	
	3	34 (41.5)	114 (84.0)	
	4	1 (1.2)	6 (4.4)	
cN stage	0	36 (43.9)	19 (14.0)	< 0.0001
	1	46 (56.1)	117 (86.0)	
cM stage	0	80 (97.6)	134 (98.5)	0.613
	1	1 (2.4)	2 (1.4)	
Performance status	0	8 (11.6)	35 (25.7)	0.001
	1	51 (73.9)	96 (70.6)	
	2	10 (14.5)	5 (3.7)	
ASA	1	3 (3.7)	11 (8.1)	0.005
	2	56 (68.3)	106 (78.5)	
	3	23 (28)	18 (13.3)	
O-POSSUM		18 (12-30)	16 (12-26)	< 0.0001
Tumour site	Middle 1/3	1 (1.2)	1 (0.7)	0.418
	Lower 1/3	32 (39)	57 (41.9)	
	GEJ-S1	19 (23.2)	23 (16.9)	
	GEJ-S2	18 (22.0)	34 (25.0)	
	GEJ-S3	12 (14.6)	20 (14.7)	
Operative outcomes				
Length of operation (min) <sup>1</sup>		255 (120-480)	261 (120-471)	0.409
Blood loss (mL) <sup>1</sup>		300 (0-2200)	318 (0-3000)	0.429
Clavien Dindo Max	0	26 (31.7)	53 (39.3)	0.59
	1	5 (6.1)	8 (5.9)	
	2	35 (42.7)	40 (29.6)	
	3	6 (7.3)	17 (12.6)	
	4	6 (7.3)	17 (12.6)	
	5	4 (4.9)	0 (0)	
Anastomotic leaks		8 (9.8)	9 (6.7)	0.413
Pathological outcomes				
pT or ypT	0	3 (3.6)	8 (5.9)	0.692
	1	23 (28)	23 (16.9)	
	2	17 (20.7)	34 (25)	
	3	34 (41.5)	66 (48.5)	
	4	5 (6.1)	5 (3.7)	
pN or ypN	0	40 (48.8)	73 (53.7)	0.758
	1	20 (24.4)	21 (15.4)	
	2	11 (13.4)	25 (18.4)	
	3	11 (13.4)	17 (12.5)	
pM or ypM	0	82 (100)	136 (100)	1.00
Tumour regression grade	1	-	8 (5.8)	n/a
	2	-	28 (20.6)	
	3	-	20 (14.7)	
	4	-	45 (33.1)	
	5	-	35 (25.7)	
Nodal downstaged (cN1 to p or ypN1)		13 (15.9)	60 (44.1)	< 0.0001
Positive nodes <sup>1</sup>		1 (0-21)	0 (0-24)	0.789
Nodal yield <sup>1</sup>		18 (4-49)	18 (3-53)	0.242
Resection clearance	R0	65 (79.3)	110 (80.9)	0.772
Vascular invasion		24 (29.3)	41 (30.1)	0.891
Lymphatic invasion		9 (11)	22 (16.2)	0.28
Perineural invasion		8 (9.8)	20 (14.7)	0.291
Maximum tumour diameter (mm) <sup>1</sup>		25 (0-90)	25 (0-155)	0.998
Morphology	Ulcer	48 (60)	96 (74.4)	0.029
	Polypoid	22 (27.5)	23 (17.8)	
	Fungating	2 (2.5)	3 (2.3)	
	Diffuse infiltrating	8 (10)	7 (5.4)	
Grade	G1	6 (7.3)	16 (11.8)	0.669
	G2	30 (36.6)	37 (27.2)	
	G3	46 (56.1)	82 (60.3)	
	G4	0 (0)	1 (0.7)	
Sites of recurrence	Local	3 (3.7)	8 (5.9)	0.461

Nodal	5 (6.1)	14 (10.4)	0.281
Distant	18 (22.0)	44 (32.6)	0.093

Values in parentheses are percentages unless indicated. <sup>1</sup>Values in parentheses are range. ASA: American society of anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity; GEJ-S1-3: Gastro-esophageal junction-Siewert type 1-3.

### **The relationship of tumour regression grade and clinicopathological characteristics**

The relationship between patient and tumour characteristics and response to neoadjuvant chemotherapy, as defined by tumour regression grade, are presented in Table 3.

Of the 136 patients that underwent NAC, 36 (26.5%) patients had a significant pathological response (TRG 1-2; responders) compared to 100 (73.5%) patients with no significant pathological response (TRG 3-5; non-responders). Responders and non-responders had similar preoperative clinical features (age, sex and physiological status) and clinical stage of disease (cT stage,  $P = 0.396$ ; cN stage,  $P = 0.987$ ; cM stage,  $P = 0.456$ ), yet responders had markedly reduced ypT stage ( $P < 0.0001$ ), maximal pathological tumour diameter ( $P < 0.0001$ ), and ypN stage ( $P < 0.0001$ ) and were more likely to have their nodal stage downstaged ( $P < 0.0001$ ) compared to non-responders (Table 3). In addition, responders had tumours that were more likely to be ulcers ( $P = 0.003$ ), showing less vascular ( $P = 0.004$ ), and perineural invasion ( $P = 0.072$ ) compared to non-responders. Complete resection (R0) was achieved in 97.2% ( $n = 35$ ) of responders compared with 75% ( $n = 75$ ) of non-responders ( $P = 0.04$ ). There was no significant difference in postoperative complications as classified by the Clavien Dindo system, nodal yield, blood loss or operative time between groups.

### **The relationship of TRG and lymph node downstaging with DFS**

There was a significant difference in survival between responders compared to non-responders, shown in Figure 1A [mean DFS; TRG 1-2: 5.064 years, 95%CI: 4.560-5.569 (median DFS: not reached) *vs* TRG 3-5: 2.759 years, 95%CI: 2.193-3.325 (median DFS: 1.613, 95%CI: 0.834-2.39),  $P < 0.0001$ ].

There was no statistically significant difference in survival between patients graded as TRG 1 compared to TRG 2 [mean DFS; TRG-1: 5.021, 95%CI: 4.069-5.973 *vs* TRG-2: 4.983, 95%CI: 4.069-5.973,  $P < 0.0001$  (median DFS's: not reached)].

Patients with lymph node downstaging following NAC had improved DFS *vs* patients without downstaging, Figure 1B [median DFS; lymph node (LN) downstaged: 5.316 years, 95%CI: 4.504-6.127 (median DFS: 5.544) *vs* LN not downstaged: 2.118 years, 95%CI: 1.594-2.643 (median DFS: 1.210, 95%CI: 1.026-1.394),  $P < 0.0001$ ].

### **Univariate and multivariate analysis for predicting DFS following neoadjuvant chemotherapy**

Univariate and multivariate analysis confirmed known predictors of DFS in esophageal adenocarcinoma (OAC) that are detailed in Table 4. Factors that retained significance for the prediction of worse DFS on multivariate analysis were: vascular invasion (HR = 1.929, 95%CI:

1.034-3.6,  $P = 0.039$ ), perineural invasion (HR = 2.766, 95%CI: 1.444-5.3,  $P = 0.002$ ), no significant response to NAC (HR = 6.315, 95%CI: 1.261-31.616,  $P = 0.025$ ) and the absence of lymph node downstaging (HR = 6.161, 95%CI: 1.683-22.554,  $P = 0.006$ ).

### **The relationship of lymph node downstaging and status with clinicopathological characteristics and DFS**

Patients with no pathological lymph node involvement were compared (pN<sub>0</sub> *vs* ypN<sub>0</sub>), grouped as those who had surgery alone (pN<sub>0</sub>) *vs* multimodal therapy (ypN<sub>0</sub>), with detailed clinical and pathological characteristics presented in Table 5 and DFS shown in Figure 1C.

For patients with no evidence of pathological lymph node involvement increased pre-operative clinical stage (cT stage,  $P < 0.0001$ ; cN stage,  $P < 0.0001$ ) of disease and increased nodal downstaging (NAC 83.6% *vs* surgery alone 37.5%,  $P < 0.0001$ ) was observed in patients who received multimodal therapy *vs* surgery alone despite pathological stage being similar (yp or pT stage,  $P = 0.224$ ; yp or pN stage,  $P = 1.00$ ).

Patients who underwent surgery alone (pN<sub>0</sub>) had increased DFS compared to patients who underwent NAC and surgery (ypN<sub>0</sub>) (mean DFS; pN<sub>0</sub>: 6.285 years, 95%CI: 5.647-6.923 *vs* ypN<sub>0</sub>: 5.102 years, 95%CI: 4.314-5.891 (median DFS's: not reached,  $P = 0.042$ ).

### **Evaluation of combined local tumour response grade and lymph node downstaging**

Eighty-three point three percent of responders' additionally demonstrated downstaging of their regional lymph nodes compared to only 30% of non-responders, spread across TRG 3-5, Figure 2.

The presence of lymph node downstaging in apparent non-responders was associated with significantly improved DFS (median DFS; TRG 3-5 and nodal downstaging: 5.544, 95%CI: 3.558-7.531 *vs* TRG 3-5 and LN not downstaged: 1.114, 95%CI: 0.961-1.267,  $P < 0.0001$ ), Figure 3.

## **DISCUSSION**

Neoadjuvant treatment for esophageal cancer is associated with increased survival. However, it is clear that not all patients (and their tumours) respond to neoadjuvant therapy in the same way. It is likely that improved outcomes will be observed by the tailoring of neoadjuvant and adjuvant therapy based on patient stratification according to tumour response.

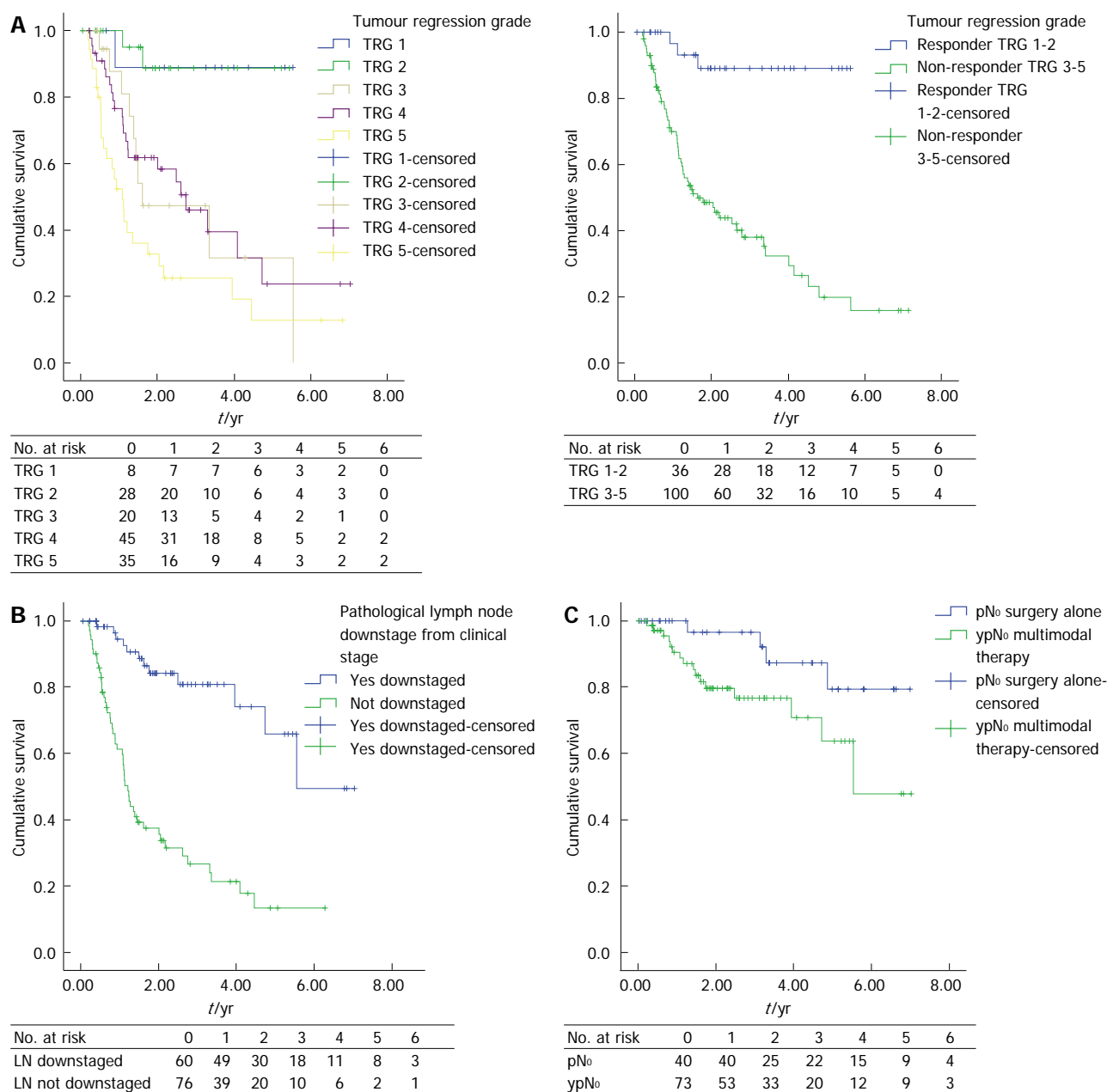
In this study we have analysed a consecutive cohort of patients with esophageal adenocarcinoma (OAC) undergoing treatment with curative intent to assess the primary tumour and regional lymph node response to NAC. We have described three main findings: firstly, we

**Table 3** Clinical and pathological characteristics of the 136 patients treated with neoadjuvant chemotherapy for esophageal and gastro-esophageal adenocarcinoma, classified as responders Tumour regression grade 1-2 or non-reponders tumour regression grade 3-5 *n* (%)

		TRG 1-2 36 (26.5)	TRG 3-5 100 (73.5)	P value
Preoperative status				
Age (range) yr <sup>1</sup>		65.27 (26.99-76.04)	63.51 (32.77-81.28)	0.410
Sex ratio (M:F) <sup>1</sup>		32 (88.9):4 (11.1)	86 (86):14 (14)	0.662
cT stage	1	0 (0)	0 (0)	0.396
	2	2 (5.6)	14 (14)	
	3	33 (91.7)	81 (81)	
	4	1 (2.8)	5 (5)	
cN stage	0	5 (13.9)	14 (14)	0.987
	1	31 (86.1)	86 (86)	
cM stage	0	35 (97.1)	99 (99)	0.456
	1	1 (2.8)	1 (1)	
Performance status	0	12 (33.3)	23 (23)	0.225
	1	23 (63.9)	73 (73)	
	2	1 (2.8)	4 (4.0)	
ASA	1	2 (5.6)	9 (9.1)	0.408
	2	32 (88.9)	74 (74.7)	
	3	2 (5.6)	16 (16.2)	
O-POSSUM		15 (12-23)	16 (12-26)	0.476
Tumour site	Middle 1/3	1 (2.8)	0 (0)	0.738
	Lower 1/3	15 (41.7)	42 (42)	
	GEJ-S1	7 (19.4)	16 (16)	
	GEJ-S2	9 (25)	25 (25)	
	GEJ-S3	4 (11.1)	16 (16)	
Operative outcomes				
Length of operation (min) <sup>1</sup>		262 (163-427)	260 (120-471)	0.513
Blood loss (mL) <sup>1</sup>		300 (0-3000)	325 (0-1700)	0.673
Clavien Dindo Max	0	14 (38.9)	39 (39.4)	0.531
	1	2 (5.6)	6 (6.1)	
	2	14 (38.9)	26 (26.3)	
	3	4 (11.1)	13 (13.1)	
	4	2 (5.6)	15 (15.2)	
	5	0 (0)	0 (0)	
Anastomotic leaks		1 (2.8)	8 (8.1)	0.276
Pathological outcomes				
yPT	0	8 (22.2)	0 (0)	< 0.0001
	1	11 (30.6)	12 (12)	
	2	9 (25)	25 (25)	
	3	8 (22.2)	58 (58)	
	4	0 (0)	5 (5)	
yPN	0	34 (94.4)	39 (39)	< 0.0001
	1	0 (0)	21 (21)	
	2	2 (5.6)	23 (23)	
	3	0 (0)	17 (17)	
yPM	0	36 (100)	100 (100)	0.579
Nodal downstaged (cN1 to ypN0)		30 (83.3)	30 (30)	< 0.0001
Positive nodes <sup>1</sup>		0 (0-5)	1 (0-24)	< 0.0001
Nodal yield <sup>1</sup>		18 (4-25)	18 (3-53)	0.984
Resection clearance	R0	35 (97.2)	75 (75)	0.004
Vascular invasion		4 (11.1)	37 (37)	0.004
Lymphatic invasion		4 (11.1)	18 (18)	0.338
Perineural invasion		2 (5.6)	18 (18)	0.072
Maximum tumour diameter <sup>1</sup>	(mm)	15 (0-110)	30 (0-155)	< 0.0001
Morphology	Ulcer	30 (93.8)	66 (68)	0.003
	Polypoid	2 (6.3)	21 (21.6)	
	Fungating	0 (0)	3 (3.1)	
	Diffuse infiltrating	0 (0)	7 (7.2)	
Grade	G1	8 (22.2)	8 (8)	0.104
	G2	9 (25)	28 (28)	
	G3	19 (52.8)	63 (63)	
	G4	0 (0)	1 (1)	
Sites of recurrence	Local	0 (0)	8 (8.1)	0.080
	Nodal	1 (2.8)	13 (13.1)	0.082
	Distant	2 (5.6)	42 (42.4)	< 0.0001

Values in parentheses are percentages unless indicated. <sup>1</sup>Values in parentheses are range. TRG: Tumour Regression Grade; ASA: American Society of Anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity; GEJ-S1-3: Gastro-esophageal junction-Siewert type 1-3.





**Figure 1** Kaplan-Meier curve of patients. A: Patients ( $n = 136$ ) received neoadjuvant chemotherapy grouped by tumour regression grade. Left: Tumour Regression Grade (TRG) 1-5 ( $P < 0.0001$ ); Right: TRG 1-2 vs TRG 3-5 ( $P < 0.0001$ ); B: Patients ( $n = 136$ ) received neoadjuvant chemotherapy grouped by presence or absence of lymph node downstaging ( $P < 0.0001$ ); C: Patients ( $n = 113$ ) with no pathological lymph node metastasis grouped by treatment ( $P = 0.042$ ).

have confirmed that a significant pathological response as described by Mandard *et al*<sup>[14]</sup> is associated with improved DFS; Secondly we have confirmed that lymph node downstaging leads to improved DFS<sup>[10]</sup>; Thirdly, and most importantly, we describe that when tumour and nodal response are combined, a group of patients who previously would have been classified as non-responders to NAC actually have significantly increased DFS.

There is considerable debate regarding the role of tumour regression in OAC. Conflicting opinions are evident, for what represents a significant tumour response, even within the TRG grading system. In our study TRG-3 tumours, despite representing tumours whose fibrosis outgrows the residual tumour, clearly grouped with TRG-4 and TRG-5 and not TRG-1 and TRG-2 tumours in terms

of DFS. This is in keeping with previous studies that have observed a significant increase in survival and/or metabolic response on serial PET imaging for TRG groups 1 and 2 compared to TRG groups 3 to 5<sup>[14,18,19,22,28,29]</sup>. In addition, we found there to be no significant difference in DFS between complete pathological responders (TRG-1) vs major responders (TRG-2) consistent with other studies<sup>[14,22]</sup>. As has been previously suggested this may reflect a type II error due to insufficient sample size or the intensity of pathological sampling<sup>[22]</sup>. The observed increase in DFS in patients with a significant tumour response to NAC in this study may also reflect the significantly increased resectability (R0 rate) of the primary tumour. It may also reflect the selection of tumours that are biologically more favourable as suggested by reduced vascular invasion ( $P = 0.004$ ), tu-

**Table 4** Univariate and multivariate Cox regression analyses of patient and tumour factors with disease free survival for patients undergoing neoadjuvant chemotherapy ( $n = 136$ )

		Univariate			Multivariate		
		HR	95%CI	P value	HR	95%CI	P value
Patient factors							
Age		0.972	(0.944-1.00)	0.054			
Sex	Female	1.000	Ref				
	Male	0.953	(0.453-2.005)	0.899			
ASA	1	1.000	Ref				
	2	0.696	(0.313-1.548)	0.374			
	3	0.947	(0.352-2.546)	0.914			
Performance status	0	1.000	Ref				
	1	1.016	(0.578-1.789)	0.955			
	2	0.950	(0.218-4.129)	0.945			
O-POSSUM							
Tumour response							
TRG	1	1.000	Ref				
	2	1.099	(0.099-12.148)	0.939			
	3	8.404	(1.071-65.929)	0.043			
	4	7.829	(1.054-58.163)	0.044			
	5	15.422	(2.083-114.189)	0.007			
TRG grouped	1-2	1.000	Ref		1.000	Ref	
	3-5	9.504	(2.973-30.380)	< 0.0001	6.315	(1.261-31.616)	0.025
Lymph node response							
Lymph nodes downstaged	Yes	1.000	Ref		1.000	Ref	
	No	5.784	(3.064-10.919)	< 0.0001	6.161	(1.683-22.554)	0.006
Tumour factors							
ypT stage	0	1.000	Ref		1.000	Ref	
	1	2.085	(0.232-18.711)	0.512	0.281	(0.020-3.928)	0.345
	2	5.214	(0.687-39.549)	0.110	0.286	(0.022-3.705)	0.338
	3	9.490	(1.293-69.635)	0.027	0.469	(0.034-6.460)	0.571
	4	52.907	(6.008-465.873)	< 0.0001	1.519	(0.087-26.389)	0.774
ypN stage	0	1.000	Ref		1.000	Ref	
	1	4.791	(2.434-9.431)	< 0.0001	0.476	(0.133-1.700)	0.253
	2	4.102	(2.005-8.392)	< 0.0001	0.254	(0.070-0.927)	0.038
	3	7.449	(3.522-15.756)	< 0.0001	0.476	(0.129-1.755)	0.265
ypM stage	0	1.000	Ref		1.000	Ref	
	1	3.172	(1.253-8.031)	0.015	2.693	(0.924-7.847)	0.069
Vascular invasion	No	1.000	Ref		1.000	Ref	
	Yes	3.444	(2.080-5.702)	< 0.0001	1.929	(1.034-3.600)	0.039
Lymphatic invasion	No	1.000	Ref		1.000	Ref	
	Yes	2.201	(1.268-3.821)	0.005	1.253	(0.637-2.462)	0.514
Perineural invasion	No	1.000	Ref		1.000	Ref	
	Yes	5.073	(2.896-8.886)	< 0.0001	2.766	(1.444-5.300)	0.002
Resection clearance	R0	1.000	Ref		1.000	Ref	
	R1	3.869	(2.272-6.588)	< 0.0001	1.805	(0.940-3.468)	0.076

TRG: Tumour Regression Grade; ASA: American Society of Anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity.

mour morphology ( $P = 0.003$ ) and increased lymph node downstaging ( $P < 0.0001$ ).

In this study we confirmed the association between lymph node downstaging after NAC and improved DFS<sup>[10]</sup>. Bollschweiler *et al*<sup>[3]</sup> showed regression in lymph nodes, such as central fibrosis, to predict improved survival and response to chemoradiotherapy<sup>[5]</sup>. This would require additional pathological time and expertise whereas downstaging can be more simply assessed from the data available to the multidisciplinary team (MDT) after surgery, to assess a patient's prognosis and potential for adjuvant therapies. The number of positive lymph nodes is consistently the most important prognostic factor associated with survival<sup>[30]</sup>. However, the clinical significance of downstaging is controversial due to the difficulties in evaluating preoperative status. This study

has the advantage of using contemporary and uniformly implemented clinical staging based on current United Kingdom practice. The comparison of nodal stage based on pre-operative staging assessment (cN) and post-operative pathology (pN) is open to the criticism that any downstaging simply reflects overdiagnosis of lymph node metastases on preoperative staging. To address this point we assessed the survival of patients with no positive lymph nodes in the pathological specimen, comparing NAC with surgery alone (ypN<sub>0</sub> *vs* pN<sub>0</sub>). We found that patients receiving NAC with ypN<sub>0</sub> disease had reduced DFS across all sites of recurrence compared to patients treated by surgery alone with pN<sub>0</sub> disease. This reached statistical significance when overall DFS was assessed ( $P = 0.042$ ). Whilst the patients that underwent multimodal or surgery only had comparable pathological

**Table 5 Clinical and pathological characteristics of the 113 patients with pathological N0 stage, according to treatment *n* (%)**

		pN0 Surgery alone 40 (35.4)	ypN0 Neoadjuvant chemotherapy and surgery 73 (64.6)	P value
Preoperative status				
Age (range) yr <sup>1</sup>		73.62 (56.73-85.41)	65.59 (32.77-78.43)	< 0.0001
Sex ratio (M:F) <sup>1</sup>		31 (77.5):9 (22.5)	66 (90.4):7 (9.8)	0.061
cT stage	1	13 (32.5)	0 (0)	< 0.0001
	2	17 (42.5)	9 (12.3)	
	3	10 (25)	61 (83.6)	
	4	0 (0)	3 (4.1)	
cN stage	0	27 (67.5)	13 (17.8)	< 0.0001
	1	13 (32.5)	60 (82.2)	
cM stage	0	40 (100)	71 (97.3)	0.293
	1	0 (0)	2 (2.8)	
Performance status	0	3 (9.4)	16 (21.9)	0.045
	1	25 (78.1)	54 (74)	
	2	4 (12.5)	3 (4.1)	
ASA	1	2 (5)	6 (8.2)	0.268
	2	31 (77.5)	59 (80.8)	
	3	7 (17.5)	8 (11)	
O-POSSUM <sup>1</sup>		17 (14-29)	16 (12-26)	0.015
Tumour site	Middle 1/3	0 (0)	1 (1.4)	0.190
	Lower 1/3	15 (37.5)	35 (47.9)	
	OGJ-S1	11 (27.5)	12 (16.4)	
	OGJ-S2	8 (20)	16 (21.9)	
	OGJ-S3	6 (15)	9 (12.3)	
Operative outcomes				
Length of operation (min) <sup>1</sup>		240 (120-360)	278 (120-471)	0.082
Blood loss (mL) <sup>1</sup>		200 (0-2200)	350 (0-3000)	0.167
Clavien Dindo Max	0	14 (35)	24 (32.9)	0.709
	1	1 (2.5)	3 (4.1)	
	2	17 (42.5)	27 (37)	
	3	4 (10)	10 (13.7)	
	4	2 (5)	9 (12.3)	
	5	2 (5)	0 (0)	
Anastomotic leaks		4 (10)	7 (9.6)	0.944
Pathological outcomes				
TRG 1-2		-	34 (46.6)	NA
TRG 3-5		-	39 (53.4)	
pT or ypT	0	2 (5)	11 (15.1)	0.224
	1	22 (55)	20 (27.4)	
	2	5 (12.5)	20 (27.4)	
	3	10 (25)	24 (32.9)	
	4	0 (0)	1 (1.4)	
Nodal Downstaged (cN1 to p or ypN0)		15 (37.5)	61 (83.6)	< 0.0001
Nodal yield <sup>1</sup>		16 (4-49)	18 (3-52)	0.150
Resection clearance	R0	35 (87.5)	69 (94.5)	0.189
Vascular invasion		7 (17.5)	10 (13.7)	0.590
Lymphatic invasion		2 (5)	6 (8.2)	0.525
Perineural invasion		2 (5)	5 (6.8)	0.698
Maximum tumour diameter (mm) <sup>1</sup>		24 (0-50)	24 (0-110)	0.324
Morphology	Ulcer	25 (65.8)	53 (79.1)	0.135
	Polypoid	10 (26.3)	11 (16.4)	
	Fungating	1 (2.6)	1 (1.5)	
	Diffuse infiltrating	2 (5.3)	2 (3)	
Grade	G1	4 (10)	13 (17.8)	0.811
	G2	17 (42.5)	20 (27.4)	
	G3	19 (47.5)	40 (54.8)	
	G4	0 (0)	0 (0)	
Site of recurrence	Local	0 (0)	2 (2.7)	0.293
	Nodal	1 (2.5)	4 (5.5)	0.463
	Distant	3 (7.5)	12 (16.4)	0.182

Values in parentheses are percentages unless indicated. <sup>1</sup>Values in parentheses are range. ASA: American society of anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity; NA: Not available.

staged disease they are different based on their clinical stage and survival. It is therefore unlikely that our clinical staging was inadequate and suggests that the majority

of patients with ypN<sub>0</sub> disease in fact had lymph node metastases prior to treatment.

The increased survival observed with lymph node

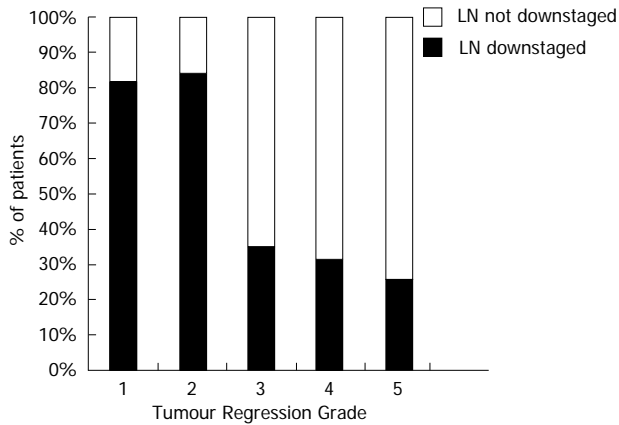
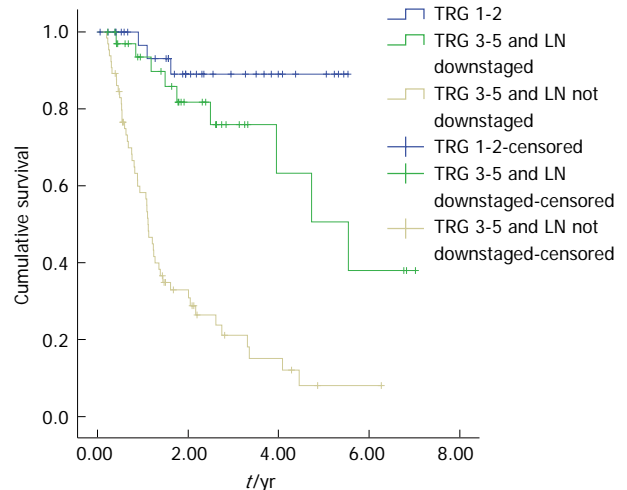


Figure 2 Percentage of patients who received neoadjuvant chemotherapy ( $n = 136$ ) having lymph node downstaging grouped by Tumour Regression Grade. LN: Lymph node.

downstaging has important implications for the staging of OAC as neoadjuvant therapy is increasingly used. Although the final pathological stage of disease may be similar between patients treated with either multimodal therapy or surgery alone we have demonstrated that the long-term DFS of these patients are different. This would suggest revisions for the staging system for OAC to take into account the differences in outcomes for patients who have similar pathologically staged disease after multimodal therapy compared to those treated by surgery alone. This hypothesis is further supported by the results of our multivariate analysis of factors independently related to outcome in neoadjuvant chemotherapy for OAC. This showed that nodal downstaging and TRG were independent predictors of DFS but that the classical markers of disease burden, PT stage and PN stage, were only statistically significant on univariate analysis. Similar observations and suggestions have been made for patients who have undergone neoadjuvant chemoradiotherapy followed by surgery when compared to patients who underwent surgery alone<sup>[31]</sup>.

There are several advantages of our study compared to other published series. This study consists of a large number of consecutive patients ( $n = 218$ ) of uniform histological type, with consistent clinical and pathological staging and treatment provided over a contemporary time period. The retrospective nature of this study and the use of multiple pathologists assessing TRG on an individual basis are potential limitations. However, the data was vigorously collected prospectively and the use of multiple pathologists reflects the usefulness of TRG in clinical practice and is pragmatic. A debate also remains as to what system to use to assess a local tumour response to neoadjuvant therapy<sup>[10,11,14,15,17-19]</sup>. The use of TRG is not without controversy as significant tumour regression has been reported in patients who underwent surgery alone, in up to 13.7% of cases. It has been suggested that this reflects tumour growth within abundant stroma and/or lymphocytic infiltration leading to partial tumour regression<sup>[21]</sup>. While the association of lymphocytic infiltration and stromal features with survival in cancer is not new



No. at risk	0	1	2	3	4	5	6
TRG 1-2	36	28	18	12	7	5	0
TRG 3-5 and LN downstaged	30	25	16	9	5	4	3
TRG 3-5 and LN not downstaged	70	35	16	7	5	1	1

Figure 3 Kaplan-Meier curves of patients undergoing multimodal therapy ( $n = 136$ ) grouped based on a combination of tumour regression grade and lymph node downstaging ( $P < 0.0001$ ). TRG: Tumour Regression Grade; LN: Lymph node.

their association with survival in OAC is yet to be fully understood and the clinical impact is unknown<sup>[32]</sup>.

Although a good pathological response of the primary tumour might be expected to represent a prognostic predictor after NAC, the low response rate observed following NAC remains problematic. In this study we observed a significant response rate of 26.5% ( $n = 36$ ) as assessed by TRG. However when lymph node downstaging is also considered this proportion increases to 48.5% ( $n = 66$ ). It can be hypothesised that patients who have a partial response to NAC reflected by downstaging of lymph nodes with modest or no response in the primary tumour (TRG 3-5) may be the most appropriate to be considered for trials of adjuvant treatment; as there is limited data from other disease sites to suggest only patients responding to neoadjuvant treatment benefit from further treatment<sup>[33]</sup>. This is relevant as the role of adjuvant therapy in esophageal cancer is controversial due to concerns over the additional benefit of post operative treatment over neoadjuvant alone<sup>[8]</sup> and toxicity<sup>[34]</sup>, and has resulted in the lack of adoption in the United Kingdom<sup>[1]</sup>. What is clear is that the group of patients with no significant downstaging and ypN<sub>1</sub> post neoadjuvant treatment have a particularly poor outlook. This group urgently requires identification at diagnosis and new trial treatments. This requires the ongoing studies of prognostic and predictive biomarkers from this cohort and others to yield meaningful and validated results.

One can now begin to consider an evolving algorithm for perioperative treatment of OAC that may involve induction chemotherapy followed by an early assessment of response and the curtailment of, or a change of, neoadjuvant therapy for non-responders. Further analysis of the primary tumour and lymph nodes after surgery would



direct patients with modest or no tumour response (TRG 3-5) to NAC, but with nodal downstaging, to adjuvant therapy. This kind of stratified therapy will be supported by ongoing studies of biomarkers and molecular imaging. The contribution of the tumour microenvironment is also likely to offer new targets for therapy and may be the place to look to explain the different responses to therapy observed between otherwise similar tumours.

In summary, this study has shown that a response to NAC in the primary tumour and in the lymph nodes is associated with improved outcomes after surgery for adenocarcinoma of the esophageal and gastro-esophageal. A previously unidentified group of patients who appear to have a poor tumoural response to NAC (TRG 3-5) do benefit from NAC with nodal downstaging and increased DFS.

We propose that methods to assess the pathological response to NAC are refined so that both the response in the primary tumour and the regional lymph nodes is used to guide selection of tailored post operative treatment strategies, identify biomarkers of response to chemotherapy, provide prognostic information and assess multimodal therapies.

## COMMENTS

### Background

Adenocarcinoma of the esophagus and esophageal adenocarcinoma (OAC) is a significant and increasing health problem in many countries; linked to rates of obesity, smoking, gastro-esophageal reflux disease and Barrett's oesophagus. At presentation, even in operable cases, tumours are often locally advanced (T3N1) with multi-institutional randomised studies of surgery alone giving 5 years survival rates in the order of 15%-24%. So as well as a focus on earlier detection and screening of at risk groups, clinical research has focused on adjuvant and specifically neo-adjuvant treatments prior to resection.

### Research frontiers

Neoadjuvant chemotherapy can be considered one standard of care, with a modest improvement in outcome over surgery alone; detailed in a recent meta-analysis as HR = 0.83 (95%CI: 0.71-0.95), or an absolute benefit of 5%-10% at 2 years. A key focus now is on identifying optimum neoadjuvant approaches (which chemotherapy regimens, chemoradiotherapy, small molecule inhibitors, biologic agents etc) and which patients should receive them e.g., patients with human epidermal growth factor receptor (HER)-2 over expressing tumours receiving a Trastuzumab containing regimen.

### Innovations and breakthroughs

To date prognostic information for OAC has been from standard clinicopathological data, and bar HER-2 expression predictive markers of response to treatment are lacking. The authors cannot predict at diagnosis who is going to gain from neoadjuvant treatment. Globally collaborative groups have been set up to generate large clinical datasets to link patient outcomes to molecular features: groups such as the oesophageal cancer clinical and molecular stratification study group in the United Kingdom, which are beginning to highlight important molecular determinants of OAC behaviour and identify attractive targets for therapy. The expectation is this will lead to valuable prognostic information and also identify who should, and should not proceed to a particular neoadjuvant strategy.

### Applications

The identification here that both T and N downstaging post neoadjuvant treatment need to be accounted for will help refine clinical datasets and provide prognostic information, as well as inform decisions concerning adjuvant treatment.

### Terminology

When reporting the anatomical extent of cancer after preoperative treatment has been given pathologists include the prefix "y" to the PTNM.

### Peer review

This study is an excellent clinical research as it confirms the association between regression grade and prognosis in a large and histologically homogeneous group of patients treated with platinum based triplet chemotherapy and

staged uniformly. It contains novel findings that are clinically relevant to physicians treating oesophageal cancer and assessment of both T and N responses to neoadjuvant therapy may be of relevance and interest to specialists treating other solid tumours.

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## Hepatitis B virus subgenotype A1 predominates in liver disease patients from Kerala, India

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### Abstract

**AIM:** To molecularly characterize hepatitis B virus (HBV) isolates from Kerala and to relate them to the clinical manifestation of infection.

**METHODS:** Sera and clinical data were collected from 91 patients diagnosed with chronic HBV infection and HBV-related hepatocellular carcinoma (HCC). HBV from 44 HCC, 22 cirrhotic and 25 chronic hepatitis patients were genotyped by sequencing of the complete S region or by restriction fragment length polymorphism assays. The basic core promoter/precore region was sequenced. The complete surface DNA sequences were assembled and aligned manually, and then compared with the sequences of HBV of genotypes (A-J) from GenBank. The evolutionary history was inferred using the Neighbor-Joining method and the evolutionary distances computed using the Kimura 2-parameter method. Bootstrapping was performed using 1000 replicates. The TaqMan BS-1 probe was used to quantify HBV DNA at a lower detection limit of approximately 20 IU/mL. Continuous variables were compared using an independent Student's *t* test. The  $\chi^2$  test or Fisher's exact test was used to compare categorical variables. The differences were considered statistically significant at  $P < 0.05$ .

**RESULTS:** Irrespective of disease status, the predominant genotype was A (72%); 95% belonging to subgenotype A1, followed by genotypes D (27%) and C (1%). HCC patients infected with subgenotype A1 were significantly younger than those infected with D. Mutation A1762T/G1764A was significantly associated with HCC in both genotypes A and D. Mutation G1862T was more frequent in subgenotype A1 ( $P < 0.0001$ ), and in combination with A1762T/G1764A, it was significantly associated with HBV from HCC patients. Mutation C1766T/T1768A was significantly associated with



genotype A ( $P = 0.05$ ) and HCC ( $P = 0.03$ ). The preS2 start codon M1T/I mutation was unique to genotype A strains (15.6%) from all disease groups and occurred at a higher frequency in isolates from HCC patients ( $P = 0.076$ ). A higher frequency of preS deletion mutants (33.3%) was observed in genotype A from HCC compared with non-HCC patients, but did not reach statistical significance. The preS2:F22L mutation was found in genotypes A and D.

**CONCLUSION:** Kerala is the first Indian state in which subgenotype A1 has been found to predominate in liver disease patients who developed HCC at a relatively young age.

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**Key words:** Hepatocellular carcinoma; Cirrhosis; Chronic hepatitis; Phylogenetic analysis; Genotype; India

**Core tip:** This study shows the predominance of subgenotype A1 in liver disease patients in Kerala, and its high prevalence in hepatocellular carcinoma (HCC) patients. Subgenotype A1 could be more hepatocarcinogenic and HCC could develop at an earlier age, regardless of host ethnicity. The S open reading frame of subgenotype A1 isolates from Kerala clustered separately within the "Asian" cluster and encoded distinct subgenotype A1 amino acids. A higher frequency of G1862T was detected compared with subgenotype A1 isolates from other geographical regions. This is the first time that preS deletion mutants have been described in Indian HCC patients.

Gopalakrishnan D, Keyter M, Shenoy KT, Leena KB, Thayumanavan L, Thomas V, Vinayakumar KR, Panackel C, Korah AT, Nair R, Kramvis A. Hepatitis B virus subgenotype A1 predominates in liver disease patients from Kerala, India. *World J Gastroenterol* 2013; 19(48): 9294-9306 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9294.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9294>

## INTRODUCTION

Hepatitis B virus (HBV) is the prototype member of the family *Hepadnaviridae*. HBV replicates by reverse transcription using a polymerase that lacks proof reading ability, and sequence heterogeneity is a feature of this virus. Phylogenetic analysis of HBV full-length genomes has led to the classification of HBV into nine genotypes (A-I), defined by an intergroup divergence in the complete HBV genome sequence of 7.5% or more. A tenth genotype J, which was found in a single individual, has been proposed<sup>[1]</sup>. Genotypes A, B, C, D, F and I are further classified into subgenotypes. Most genotypes, and some subgenotypes, display distinct geographical distri-

butions. Moreover, HBV genotypes and, in some cases, subgenotypes, have been shown to play an important role in the clinical consequences of the infection, as well as in the response to antiviral treatment.

HBV infection remains a significant global health problem, with an estimated two billion people infected and more than 240 million chronic carriers of the virus, leading to 600000 deaths from the clinical consequences of infection, including cirrhosis, liver failure and hepatocellular carcinoma (HCC). With a population of more than 1.2 billion people, India has the second largest global pool of chronic HBV infection and HBV is the major cause of liver disease in India<sup>[2]</sup>.

Most studies have estimated the hepatitis B surface antigen (HBsAg) carrier rate to be between 2% and 8%, placing India within the zone of intermediate endemicity. An HBsAg prevalence rate of 2.97% was found among the rural population<sup>[3]</sup>, and a meta-analysis has reported the mean prevalence in the general population of India as 3.3%<sup>[4]</sup>. However, these estimates have been questioned because, according to Phadke and Kale<sup>[5]</sup>, the often quoted estimate for India of 4.7% was obtained by incorrectly pooling results of a set of studies including unrepresentative high risk groups and also equating the single test HBsAg positivity rate with the carrier rate. By correcting for these errors, they estimated a carrier rate of 1.4%.

The known HBV genotype distribution in India is summarized in Figure 1. Overall, at approximately 65%, genotype D predominates, being the dominant genotype in Delhi in the north, Pune in the west and the Nicobar Islands in the south. Genotype A has been found in approximately 30%, with the highest frequency found in northern India. At approximately 5%, genotype C is found in the minority, with the highest frequency in eastern and southern India. The subgenotypes that have been described in India include A1, A2, C1, C2, D1, D2, D3, D5 and D9<sup>[6-9]</sup>.

Kerala is the most densely populated state of India, with a population of 33 million. HBsAg prevalence of 0.5% in the normal population has been reported in northern Kerala<sup>[10]</sup> and an HBsAg prevalence of 1.5% was detected among voluntary blood donors from Trivandrum, South Kerala<sup>[11]</sup>. There is a paucity of information on the prevalence of HBV genotypes and the respective subgenotypes in Kerala, as well as their association, if any, with different clinical manifestations following infection with HBV. We investigated the distribution of HBV genotypes/subgenotypes among patients with different clinical manifestations of HBV infection and characterized the viral isolates molecularly.

## MATERIALS AND METHODS

### Patients

The cross-sectional study was conducted from January 2005 to December 2009 during which sera and clinical data were collected from 91 patients diagnosed with chronic HBV infection and HBV-related HCC from



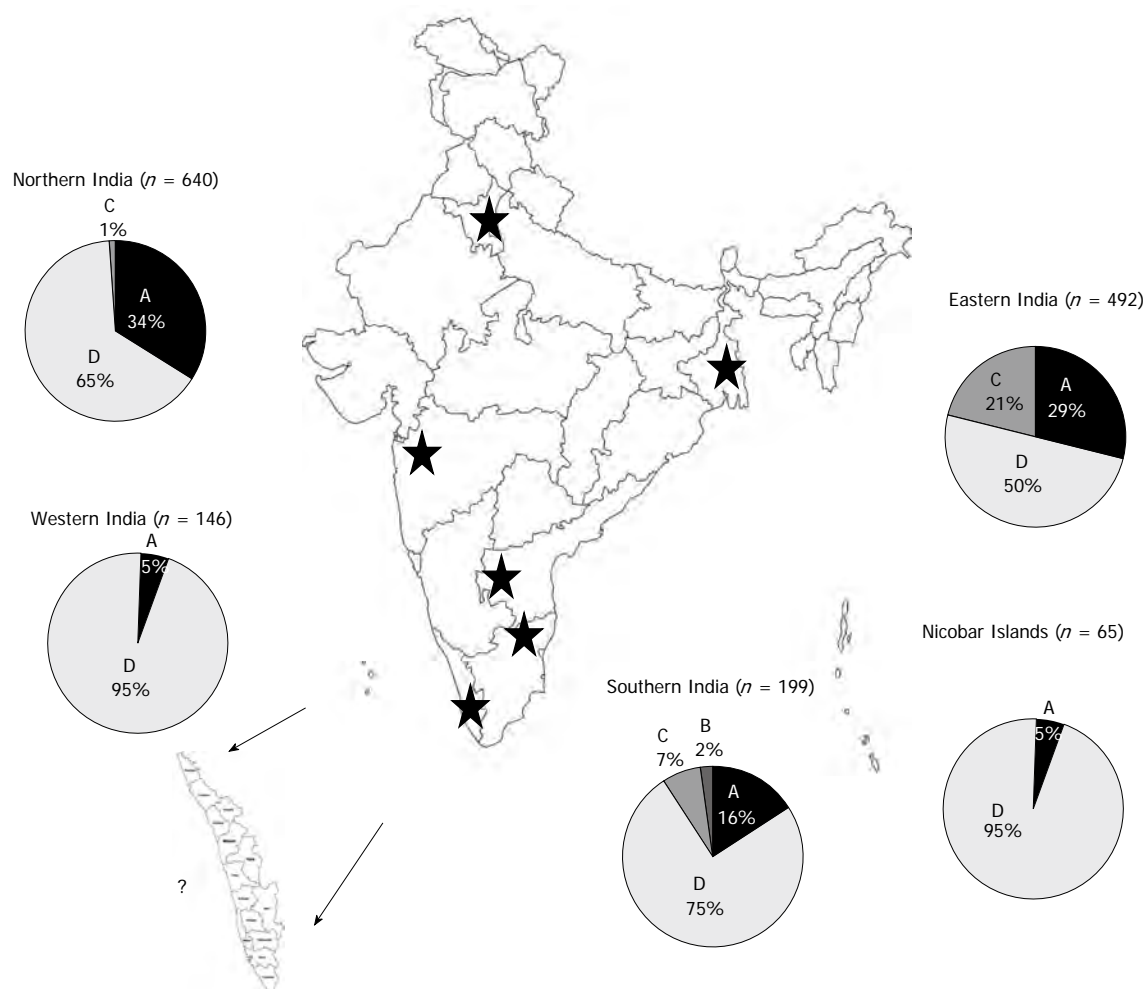


Figure 1 Prevalence of genotypes in different geographical areas of India compiled from previous reports. Northern<sup>[22,23,28,41,42]</sup>, Western<sup>[18,43]</sup>, Eastern<sup>[8,24,32,43-46]</sup>, Southern<sup>[6,47]</sup>, Nicobar Islands<sup>[48]</sup>. No hepatitis B virus genotyping data was available for Kerala before this study.

Medical College Trivandrum, Kerala, India. The serum samples were stored at  $-80^{\circ}\text{C}$  until use. A serum alanine transaminase (ALT) level of  $< 10$  times the upper limit of normal (ULN), a serum bilirubin level of less than 2.5 times the ULN and detectable HBsAg for  $\geq 6$  mo were used as inclusion criteria. The presence of the hepatitis B e antigen (HBeAg) was examined at the time of screening. All patients were negative for antibodies to hepatitis C virus, hepatitis D virus and human immunodeficiency virus. The study protocol conformed to the 1975 Declaration of Helsinki. The ethics committees of the Medical College Trivandrum, India and the University of the Witwatersrand, South Africa approved the study.

The diagnosis of HBV-related liver disease was based on clinical data, laboratory tests, liver biopsy and imaging studies. The patients were classified into three groups: group- I (HCC): the 44 patients with HCC were diagnosed by ultrasound scan and elevated serum  $\alpha$ -fetoprotein levels ( $\geq 400$  ng/mL) and the presence of a lesion of  $\geq 5$  cm; group- II (CR-Cirrhosis): 22 patients, with necro-inflammatory damage, fibrosis with nodule formation confirmed by liver biopsy, and with ultrasonographical evidence of portal hypertension; group-III

(CH-Chronic Hepatitis): 25 patients, with HBsAg positive status for  $\geq 6$  mo with normal or intermittently elevated ALT (1.5 times the ULN). Patients in this group were considered for liver biopsy on the basis of elevated ALT levels and HBeAg-status, and diagnosed with cirrhosis using histological activity index (HAI) and Fibrosis scores.

### Serological assays

All serum samples were screened for HBsAg and HBeAg using enzyme linked immunosorbent assay kits (DiaSorin S.P.A, Italy), according to the manufacturer's instructions. Laboratory evaluation included routine liver biochemistry (ALT and aspartate transaminase levels), total bilirubin, albumin, alkaline phosphatase, total protein and prothrombin time. Liver function tests were performed to find necro-inflammatory activity using a Hitachi 902 Fully Automated Chemistry Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). The ULN of ALT (40 IU/L) was used for diagnosis.

### Real-time polymerase chain reaction quantification of HBV DNA

Polymerase chain reaction (PCR) primers, HBV-Taql

and HBV-Taq2, covering a region of the S gene (321 to 401 from the *EcoRI* site) with a FAM/TAMRA labeled TaqMan BS-1 probe were used to quantify HBV DNA in an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, United States). The second WHO International Standard for HBV Nucleic Acid Amplification Techniques (product code 97/750 National Institute for Biological Standards and Control; Hertfordshire, United Kingdom), which has a final concentration of  $10^6$  IU/mL, was used as the internal standard. The lower detection limit of our assay was approximately 20 IU/mL. The conversion formula of IU = copies/4.7 was used<sup>[12]</sup>.

### PCR and restriction fragment length polymorphism assay for genotyping and molecular characterization

Total HBV DNA was extracted from serum using a QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The complete S open reading frame (ORF) was amplified using nested PCR.

Primers S1F 5'-CAATCGCCGCGTCGCAGAA-GATCTCAATC-3' (2410-2439 from the *EcoRI* site) and S1R 5'-TCCAGACCXGCTGCGAGCAAAACA-3' (1314-1291 from the *EcoRI* site) were used for the first round and S2F 5'-AATGTTAGTATTCCTTGGACT-CATAAGGTGGG-3' (2451-2482 from the *EcoRI* site) and S2R 5'-AGTTCGCGAGTATGGATCGGCAGAG-GA-3' (1280-1254 from the *EcoRI* site) were used for the second round PCR using previously reported reaction conditions<sup>[13]</sup>. The samples that did not amplify in the full S region were genotyped using restriction fragment length polymorphism (RFLP)<sup>[14]</sup>. Subgenotypes of A were also determined using a previously described RFLP assay, which uses the *StuI* recognition site, 5' AGG↓CCT3' at position 967-972 from the *EcoRI* site, found only in subgenotype A2 and genotype D, but not in subgenotype A1<sup>[15]</sup>. Thus, subgenotypes A1 and A2 could be differentiated. The basal core promoter (BCP)/Pre C region of HBV isolates was amplified using nested PCR.

Primers BCP1F 5'-GCATGGAGACCACCGT-GAAC-3' (1606-1625 from the *EcoRI* site) and BCP1R 5'-GGAAAGAAGTCCGAGGGCAA-3' (1974-1955 from the *EcoRI* site), were used for the first round and BCP2F 5'-CATAAGAGGACTCTTGGACT-3' (1653-1672 from the *EcoRI* site) and BCP2R 5'-GGCAAAAAACAGAG-TAACTC-3' (1959-1940 from the *EcoRI* site) were used for the second round, using previously reported reaction conditions.

### Sequencing

The amplicons were prepared for direct sequencing using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit and sequencing was performed with the ABI 3130XL Genetic analyzer (Applied Biosystems). The complete S ORF was analyzed as three overlapping fragments<sup>[13]</sup>.

### Phylogenetic analysis

The complete surface DNA sequences were assembled and aligned manually using MEGA 5 (<http://www.megasoftware.net/mega.php>). The sequences were compared with the sequences of HBV of genotypes (A-J) from GenBank. The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances computed using the Kimura 2-parameter method. Bootstrapping was performed using 1000 replicates to determine the support for the specific nodes. The accession numbers of HBV isolates sequenced in this study have been deposited in GenBank as KC752137-KC752206.

### Statistical analysis

Data were represented as mean  $\pm$  SD. Continuous variables were compared using an independent Student's *t* test. The  $\chi^2$  test or Fisher's exact test was used to compare categorical variables. Odds ratio was calculated to assess the risk of HCC. All *P* values were two sided, and the difference was considered statistically significant for *P* < 0.05. The analysis was performed using Statistical package for Social Sciences (SPSS 15) program (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Genotyping and phylogenetic analyses of HBV isolated from liver disease patients

Of the 91 HBsAg-positive sera, 86 were successfully genotyped using either RFLP or phylogenetic analysis of the S region (Table 1). Using the Lindh RFLP assay<sup>[14]</sup> for 36 HBV isolates, 30 belonged to genotype A and six to genotype D. Of the 30 genotype A isolates, 28 were subgenotype A1 and two were subgenotype A2, as determined by an alternative RFLP<sup>[15]</sup>.

Following phylogenetic analysis of the complete S ORF of 50 isolates, 32 belonged to genotype A (subgenotype A1:A2, 31:1) (Figure 2A), 17 to genotype D (subgenotypes D1:D2:D3, 4:12:1) (Figure 2B) and one to genotype C (subgenotype C1) (Figure 2A). The genotype A strains belonged to serotype *adw*2 (84.4%) and *ayw*1 (15.6%). The genotype D strains were of serotype *ayw*3 (58.8%), *ayw*2 (35.3%) and *adw*3 (5.9%). The single subgenotype C1 strain was *adr*.

The subgenotype A1 isolates split into an "African" and an "Asian" cluster<sup>[16]</sup> (Figure 2A). The 31 subgenotype A1 isolates from Kerala clustered within the Asian clade as a separate monophyletic clade and encoded the distinct subgenotype A1 amino acids, preS1:Q54, preS1:V74, preS1:A86, and preS1:V91 in the preS1 region and preS2:L32 in the preS2 region<sup>[17]</sup>. The majority of the isolates in the "Asian" cluster, including the Kerala isolates, had preS1:S5, preS1:S6, preS1:F25. The isolates in the African cluster displayed greater variation, with preS1:S5, preS1:S6; preS1:S5, preS1:A6 or preS1:5L, preS1:6P. There were, however, a number of amino acids in the preS1 and preS2 regions that differentiated the Kerala clade

**Table 1** Demographic, clinical and virological characteristics of hepatitis B surface antigen-positive patients with different disease profiles

Characteristic		Group I HCC (n = 44)	Group II CR (n = 22)	Group III CH (n = 25)	Total (n = 91)
Demographic and clinical data	Gender (M/F)	33/11	18/4	18/7	69/22
	Age (yr) (mean $\pm$ SD)	48.70 $\pm$ 10.94 <sup>a</sup>	40.68 $\pm$ 11.52	26.28 $\pm$ 11.10	40.87 $\pm$ 14.66
	ALT, IU/L	73.50 $\pm$ 11.65	70.50 $\pm$ 41.09	56.32 $\pm$ 30.01	67.45 $\pm$ 37.74
Virological characteristics	HBeAg positive <sup>1</sup>	7 (24.14)	4 (14.3)	9 (37.5)	20 (26.67)
	HBV DNA log <sub>10</sub> copies/mL <sup>1</sup>	4.79 $\pm$ 1.41 <sup>a</sup>	3.38 $\pm$ 1.69	3.27 $\pm$ 2.12	4.03 $\pm$ 1.83
	Number genotyped by direct sequencing/RFLP	21/19	9/12	19/6	86
	Genotype A	33 (82.5) <sup>a</sup>	14 (66.6)	15 (60)	62 (72.1)
	Subgenotype A1	30	14	15	59 (95)
	Subgenotype A2	3	-	-	3 (5)
	Genotype C	-	1 (4.8)	-	1 (1.2)
	Genotype D	7 (17.5)	6 (28.6)	10 (40)	23 (26.7)
	Subgenotype D1 <sup>2</sup>	-	1	3	
	Subgenotype D2	4	2	6	
	Subgenotype D3	1	-	-	

<sup>a</sup> $P < 0.05$  vs chronic hepatitis. <sup>1</sup>Depletion of serum allowed the viral loads to be determined for only 28, and hepatitis B e antigen for 29 HCC sera;

<sup>2</sup>Subgenotyping of genotype D was performed by direct sequencing. CH: Chronic hepatitis; HCC: Hepatocellular carcinoma; CR: Cirrhosis; SD: Standard deviation; ALT: Alanine aminotransferase; RFLP: Restriction fragment length polymorphism; HBeAg: Hepatitis B e antigen.

from other Asian strains. The majority of Kerala strains (22/31; 71%), had preS1:V48 in the preS1, whereas V/I/N/T was found in the other clades. In the preS2, 28/31 (90%), had preS2:T7, whereas the other Asian strains had either preS2:T7 or preS2:A7. In contrast to the other Asian strains that had preS2:T37, the Kerala strains had preS2:N37, as did the strains in the African clade. PreS2:P54 in the preS2 was found in 90% of the Kerala strains, whereas the other clades had a higher diversity of amino acids at this position (Figure 2). A cut off of 60% amino acid sequence identity was used to define the consensus sequence within the clades.

The Keralite genotype D isolates had the characteristic 33-nucleotide deletion in the preS1 region and had a relatively well-conserved polymerase overlapping preS1/preS2/S region when compared to subgenotype A1 sequences. The preS2 signature amino acids, preS2:I42, preS2:S43 and preS2:L54, were found in the Keralite strains belonging to subgenotype D1<sup>[7]</sup>; however, they differed by having preS2:39V instead of preS2:39A.

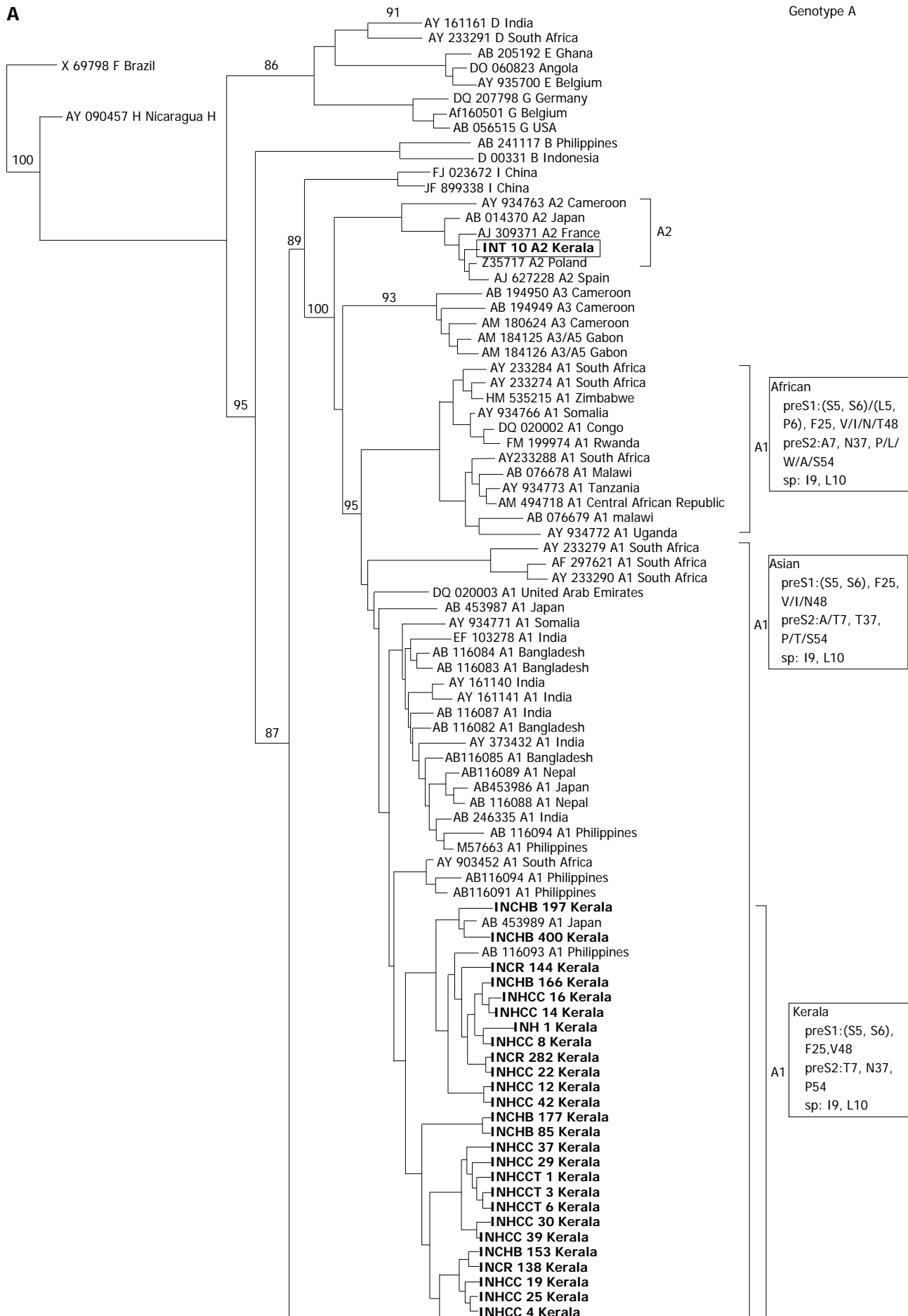
### Demographic, clinical and virological characteristics

In all three disease groups, the frequency of males was significantly higher (Table 1). Patients with HCC were significantly older than CH patients ( $P = 0.0001$ ). Twenty seven percent of the whole cohort was HBeAg-positive, with no significant difference in the frequency between the three disease groups. HBeAg-positive individuals were significantly younger than HBeAg-negative ( $32.1 \pm 17.9$  years vs  $39.6 \pm 11.1$  years,  $P = 0.032$ ) and had higher viral loads ( $5.4 \pm 1.8$  log<sub>10</sub> IU/mL vs  $3.6 \pm 1.6$  log<sub>10</sub> IU/mL,  $P = 0.016$ ). The ALT levels differed significantly between HBeAg-positive and negative patients ( $52.3$  IU/L vs  $37.4$  IU/L,  $P = 0.012$ , equal variances not assumed). HCC patients had higher viral load defined by HBV DNA level  $\geq 4.7 \times 10^4$  IU/mL compared with the non-HCC

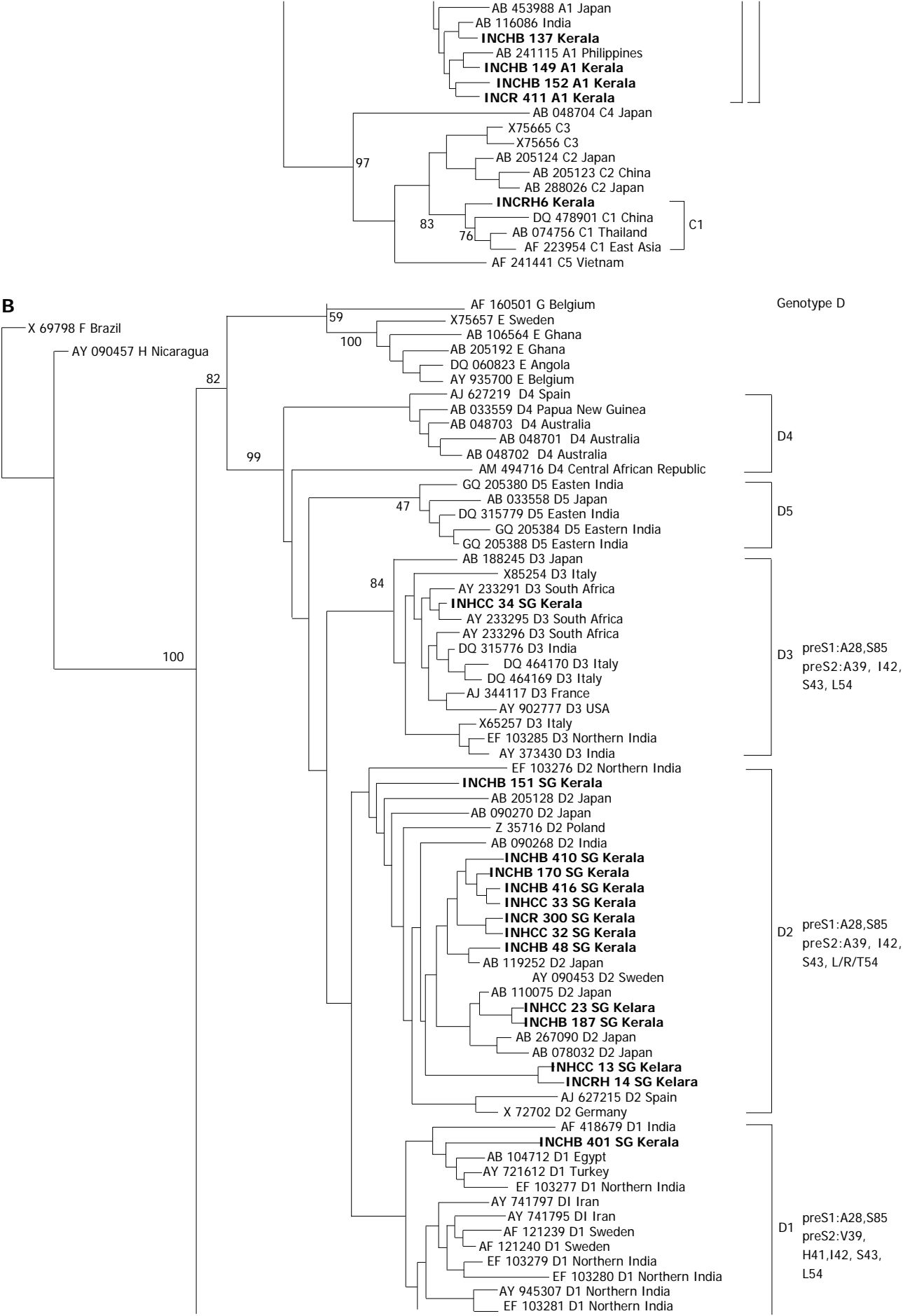
patients ( $18/29$  (62.1%) vs  $10/33$  (30.3%), respectively,  $P = 0.012$ , OR = 3.76, 95%CI: 1.16-12.52). The mean ALT values did not vary significantly between the three disease groups.

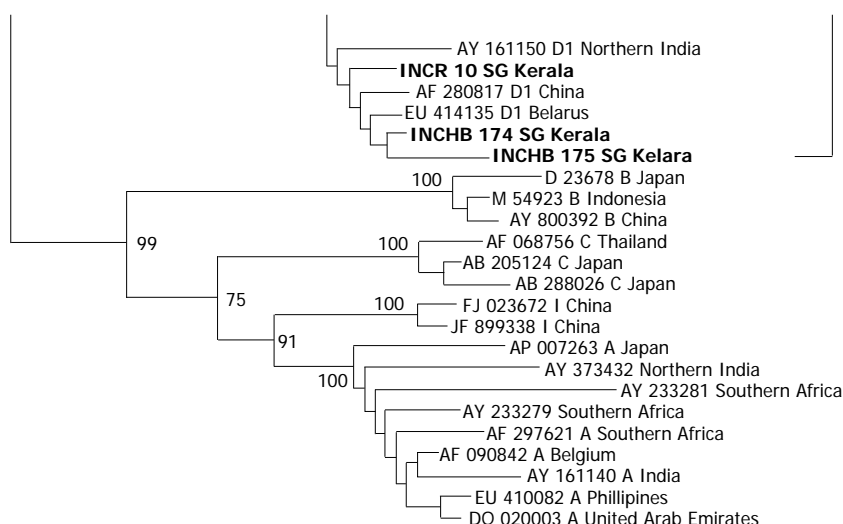
The majority of patients (72%) were infected with HBV genotype A, 27% with genotype D and one patient was infected with genotype C. The majority of genotype A strains (95%) belonged to subgenotype A1, and three to A2. Compared with the other disease groups, HCC patients were predominantly infected with subgenotype A1 ( $P < 0.05$ ). Subgenotypes D1, D2 and D3 were found, with D2 (70.6%) predominating followed by D1 (23.5%) and a single strain of D3. Age, HBV viral load, the frequency of HBeAg-positivity and ALT levels, did not differ between those patients infected with genotype A and D in all the three groups. However, HBeAg-negative HCC patients, infected with genotype A, were significantly younger ( $44.1 \pm 8.0$  years) than those infected with genotype D ( $53.0 \pm 8.8$  years) ( $P = 0.02$ ). There was no significant difference in the mean HAI ( $5.8 \pm 2.8$  vs  $4.6 \pm 3.1$ ) and fibrosis scores ( $1.0 \pm 1.8$  vs  $2.0 \pm 2.0$ ) between those with genotypes A and D. Genotype A was seen in 70% of the patients with HAI  $\geq 4$ .

Detection of mutations in the BCP/Pre C region was performed for 63 HBV isolates (Table 2). BCP and/or Pre C mutants were detected in 63% of the isolates, with different mutational patterns found in genotypes A and D (Figure 3). Mutation 1773T was characteristic of genotype A and 1773C of genotype D, with no significant difference in the frequency of the mutation in the disease groups. Mutation G1896A occurred only in genotype D, whereas C1766T and G1862T occurred more frequently in genotype A (Figure 3). The double mutation A1762T/G1764A was found in 26 (41.2%) isolates, with the single mutation, G1764A, occurring in 12 (19%) isolates. Both the single and double BCP mutations were significantly









**Figure 2** Phylogenetic relationships among complete preS1/pre S2/S sequences (nt 2854-835 numbering according to GenBank accession AY233274). A: Subgenotype A1 from hepatitis B virus (HBV) positive patients from Kerala (marked in bold) compared with sequences obtained from GenBank established using the neighbor joining method; B: Genotype D isolates from HBV positive patients from Kerala (marked in bold) compared with HBV isolates obtained from GenBank established using the neighbor joining method. Bootstrap statistical analysis was performed using 1000 replicates. Each sequence obtained from GenBank is designated by its accession number and its country of origin. The characteristic amino acids in the preS1 and polymerase spacer regions are indicated next to the sequences or relevant clades.

**Table 2** Multiple logistic regression analysis of basal core promoter/precore region in different disease groups

		HCC		Non HCC (CR/CH)		OR (95%CI)	P value
		Gen A (n = 20)	Gen D (n = 6)	Gen A (n = 26)	Gen D (n = 11)		
Basic core promoter/ pre core region	A1762T/G1764A + G1764A only	16 (80)	5 (83)	14 (54)	3 (27)	20.2 (6.3-65) <sup>a</sup>	0.008 <sup>a</sup>
	C1766T/T1768A	9 (45)	1 (5)	5 (25)	0	25 (7.3-86) <sup>a</sup>	0.03 <sup>a</sup>
						14.3 (1.7-119) <sup>d</sup>	0.05 <sup>d</sup>
	1773T (genotype A)	19 (95)	-	25	-	-	NS <sup>a</sup>
	1773C (genotype D)	-	4	-	7	-	NS <sup>a</sup>
	G1862T	18 (90)	2 (33)	21 (81)	1 (9)	30.33 (5.62-192.6) <sup>d</sup>	NS <sup>a</sup>
							0.0001 <sup>d</sup>
	G1896A	0	4 (67)	0	2 (18)	-	NS <sup>a</sup>
							0.0002 <sup>c</sup>
	A1762T/G1764A + G1862T	13 (65)	1 (2)	13 (50)	0	4.81 (0.6-39.4) <sup>b</sup>	NS <sup>a</sup>
Complete S region	Pre-S deletions	5 (33.3) <sup>e</sup>	0	3 (17.6)	1 (9)	1.89 (0.54-6.60) <sup>b</sup>	NS <sup>b</sup>
							NS <sup>d</sup>

<sup>a</sup>Comparison between hepatocellular carcinoma (HCC) and non-HCC, all genotypes; <sup>b</sup>Comparison between HCC and non-HCC, restricted to genotype A isolates; <sup>c</sup>Comparison between HCC and non-HCC, restricted to genotype D isolates; <sup>d</sup>Comparison between genotype A and D isolates; <sup>e</sup>Percentage out of 15 genotype A HCC isolates that were sequenced in the S region. CR: Cirrhosis; CH: Chronic hepatitis.

associated with HCC in both genotypes A and D ( $P = 0.03$ ). Mutation C1766T/T1768A was significantly associated with HCC and found predominantly in subgenotype A1 ( $P = 0.05$ ) (Table 2). Although G1862T was significantly associated with subgenotype A1, occurring in 85%, there was no significant difference between its presence in HCC and non-HCC patients (Table 2) and between isolates from HBeAg-positive and -negative patients (38.5% *vs* 61.5%, respectively;  $P = 0.08$ ). However, in combination with A1762T/G1764A, G1862T was significantly associated with HCC in patients infected with subgenotype A1 ( $P = 0.0004$ ). There was no correlation between the presence of BCP/Pre C mutations with either the age, gender, or the viral loads.

PreS deletion mutants, whose patterns are depicted in Table 3, were detected in nine isolates from five HCC and four CH, but in none of the CR patients. Overall, seven different types of preS mutations were detected (Table 3). The mean age of the patients, with and without preS deletions, did not differ significantly ( $35.4 \pm 11.5$  years *vs*  $37.0 \pm 15.8$  years,  $P = 0.78$ ), nor did the HBV DNA ( $3.8 \pm 2.0$  *vs*  $4.2 \pm 1.7$ ,  $P = 0.62$ ) and mean ALT levels ( $58.2 \pm 20.8$  *vs*  $60.0 \pm 28.2$ ,  $P = 0.88$ ). A higher frequency of preS deletion mutants was observed in HCC patients infected with genotype A, although this did not reach statistical significance (Table 2). The preS2 start codon M1T/I mutation, was unique to genotype A strains, occurring in 5/32 (15.6%) isolates from all disease groups

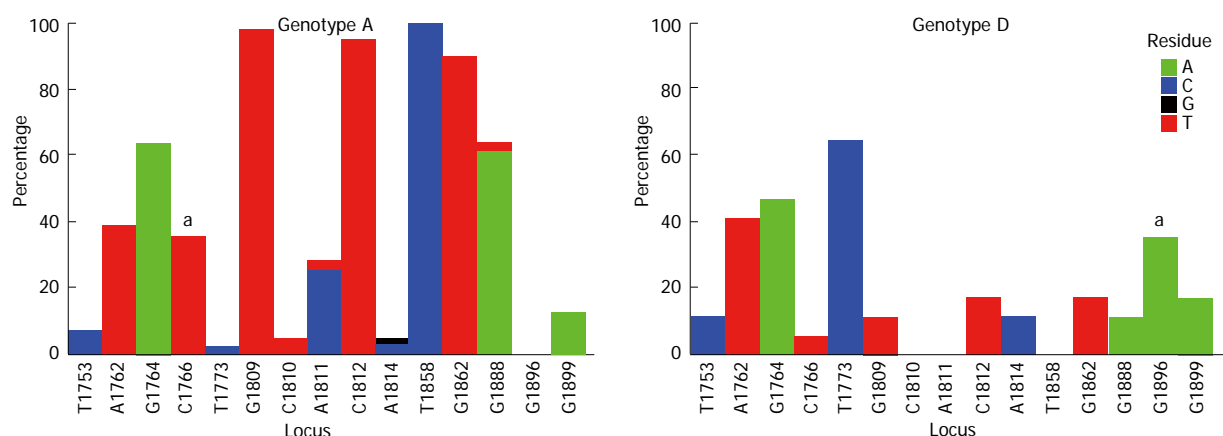


Figure 3 Comparison of the distribution of mutations in the basic core promoter/precore region (1742-1901 from the *EcoRI* site) in genotypes A (62 isolates) and D (23 isolates). Graphs showing the percentage of mutant residues relative to the reference motif found at the 15 loci of interest (1753, 1762, 1764, 1766, 1773, 1809-1812, 1814, 1858, 1862, 1888, 1896 and 1899). The study sequence files were submitted to the Mutation Reporter Tool<sup>[49]</sup> to produce the graphs. The reference motifs used were TAGCTGCACACGGGG (genotype A) and TAGCTGCACATGGGG (genotype D) for comparison. This is also shown by the letter preceding each locus on the X-axis. To facilitate direct comparisons between the graphs, conserved loci were not suppressed and the Y-axis was scaled to 100% by selecting the appropriate controls on the input page of the Mutation Reporter Tool. Nucleotides: A (green), C (dark blue), G (black), T (red). <sup>a</sup>Significantly associated with the respective genotype.

Table 3 Summary of the pre-S mutations prevalent among the three clinical groups and genotypes

Isolate	Age/sex	Clinical status	Subgenotype	PreS1	PreS2					Functions affected
				Start codon	Nucleotide from the		Amino acids from preS2			
					EcoRI start		start codon			
					Start codon	Deletion size	Position	Deletion size	Position	
CHB42	40/M	CH	A1	ATG	ACG	-	-	-	-	A
HCC12	60/M	HCC	A1	ATG	ACG	-	-	-	-	A
HCC25	50/M	HCC	A1	ATG	ACG	-	-	-	-	A
CHB137	16/F	CH	A1	Deletion	ATG	18	2854-2871	6	1-6	I
CHB170	29/F	CH	D2	ATG	ATG	21	35-55	7	16-22	T, B, P
CHB202	33/M	CH	A1	ATG	ATG	24	28-51	8	13-21	T, B, P
CHB413	35/M	CH	A1	ATG	ATG	6	49-54	2	21-22	B, P
HCC29	50/F	HCC	A1	ATG	ATA	33	22-54	11	11-22	A, T, B, P
HCC30	32/M	HCC	A1	ATG	ATG	24	30-53	8	13-21	T, B, P
HCC37	30/F	HCC	A1	ATG	ATG	33	24-56	11	11-22	T, B, P
HCC39	55/M	HCC	A1	ATG	ATG	33	24-56	11	11-22	T, B, P
HCCT3	38/M	HCC	A1	ATG	ATA	54	1-54	18	4-22	A, T, B, P, M

A: PreS2 initiation codon abolished; M1T, M1I; I: PreS1 initiation start codon abolished; M: Morphogenesis domain ps1:103-119 and ps2: 1-4; T: T cell epitope ps1: 109-119/ps2: 1-13; B: B cell epitope, amino acids 14-26; P: Putative neutralizing anti-preS2 antibody, amino acids 1-26; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B.

and occurred at a higher frequency in isolates from HCC patients ( $P = 0.076$ ). The preS2: F22L/I mutation was detected in 13 isolates (genotype A-9/32, 28%; genotype D4/17, 23%). The F22 mutation was significantly associated with HCC (10/13, 77%) compared with CR (2/13, 15%) and CH (1/13, 8%), respectively ( $P = 0.0065$ ). This significance remained when comparing genotype D isolates only, but not genotype A isolates alone.

## DISCUSSION

The present study demonstrated that genotype A was the most prevalent HBV genotype infecting liver disease patients in Kerala. This prevalence differed from other geographical regions of India, where genotype D predominates or occurs at an equal prevalence with geno-

type A (Figure 1). Ninety five percent of the genotype A isolates belonged to subgenotype A1, which has also been found to be the predominant subgenotype of A in India<sup>[18]</sup>.

The Keralite subgenotype A1 strains clustered with the Asian subgenotype strains but differed from them in some molecular characteristics in the preS2 region, which they shared with African strains. A minority of genotype A strains belonged to subgenotype A2, which was previously described to be restricted to the peripheral blood lymphocytes of eastern Indian blood donors<sup>[19]</sup>. Subgenotype A1 of HBV was the first subgenotype to be recognized and is the dominant genotype A strain in Africa, with unique molecular characteristics that differentiate it from A2, the genotype A strain prevailing outside Africa. Subgenotype A1 has its origin in Africa and its

global dispersal coincides with historical events, including the slave trade and colonization<sup>[20]</sup>. Calicut and Cochin on the west coast of Kerala were major sea ports frequented by both the Dutch East India Company and Portuguese colonists<sup>[21]</sup>.

The patients infected with genotype A and D did not differ from each other in terms of age, HBV viral loads, the frequency of HBeAg-positivity and ALT levels (Table 1). However, compared to cirrhotic and chronic hepatitis patients, a significantly higher proportion of HCC patients were infected with subgenotype A1, and HBeAg-negative HCC patients infected with subgenotype A1 were significantly younger than HCC patients infected with genotype D. Although the number of HCC patients in the present study is relatively low, the results concur with a South African study that showed that patients infected with subgenotype A1 had a 4.5 fold increased risk of developing HCC compared with those infected with non-A genotypes and they developed the cancer 6.5 years earlier<sup>[15]</sup>, thus intimating a higher hepatocarcinogenic potential of subgenotype A1, regardless of host ethnicity. However, studies with a larger number of HCC patients would be required to confirm this. These findings differ from a New Delhi study, which found a comparable distribution of genotype A and D between disease groups and that genotype D, and not genotype A, was associated with HCC and were of younger age<sup>[22]</sup>. Other studies from New Delhi<sup>[23]</sup> and Western India<sup>[24]</sup> found no association between genotype A, D and disease progression. The subgenotypes of A were not differentiated in any of these three studies.

Irrespective of the genotype, the frequency of the *A1762T/G1764A* and *1764A* mutations was significantly higher in HCC patients compared with non-HCC patients. The majority of HCC patients were infected with subgenotype A1. Similarly, the BCP double mutation occurred at a higher frequency in HCC patients compared with asymptomatic carriers in southern Africa, where subgenotype A1 predominates<sup>[25]</sup>. This was not the case in Western India where genotype D is prevalent<sup>[26]</sup>. Although the BCP mutants have been reported to contribute to the HBeAg-negative phenotype by downregulating precore mRNA transcription<sup>[27]</sup>, in the present study, and in agreement with others<sup>[28]</sup>, there was no correlation between the presence of the *A1762T/G1764A* mutation and HBeAg-negativity.

The double mutation C1766T/T1768A was significantly associated with HCC and subgenotype A1 (Table 2). The T1768A mutation results in F132Y in HBx and may play a synergetic role with K130M and V131I, introduced by *A1762T/G1764A*, leading to carcinogenesis<sup>[29]</sup>. Moreover, mutation C1766T/T1768A has been reported as an independent predictor of cirrhosis in HBeAg-negative patients and is associated with higher viral replication by increasing the encapsidation of pg RNA<sup>[30]</sup>.

Mutation G1862T was found in 85% of subgenotype A1 isolates (Table 2). Previously, G1862T was detected in 79% of global subgenotype A1 isolates, but in none

of the subgenotype A2 isolates, and has been shown to be a characteristic of subgenotype A1<sup>[31]</sup>. This high frequency of G1862T in the Keralite strains is much higher than that reported in either Eastern Indian and Southern African studies (60%<sup>[32]</sup> and 25%<sup>[33]</sup>, respectively). In the present study, the combination of *A1762T/G1764A* and G1862T was significantly associated with HCC in patients infected with subgenotype A1. A previous study showed that G1862T was significantly associated with HBeAg-negativity in South African HCC patients<sup>[34]</sup>, but not in asymptomatic carriers<sup>[33]</sup>. Moreover, mutation *A1762T/G1764A* is found frequently in South African HCC patients, but not in asymptomatic carriers<sup>[25]</sup>, and the majority of South African HCC patients are infected with subgenotype A1<sup>[15]</sup>.

The present study showed a significant association of the preS2:F22L mutation with the development of HCC, particularly in genotype D. Recent studies identified the F22L mutation in the preS2 region as a risk factor for HCC among patients infected with genotype C<sup>[35]</sup>, and showed significant association of this mutation with liver cirrhosis in Eastern India<sup>[36]</sup>. Our study supports the possibility that F22L may be associated with severe liver disease progression.

The preS deletion and initiation codon mutations were prevalent in strains isolated from all clinical groups (Table 3). This is the first study to describe the preS mutants from Indian HCC patients infected with subgenotype A1. A strong correlation between preS mutants and the development of HCC has been shown in patients infected with genotypes B or C<sup>[37,38]</sup>. In studies carried out in isolates belonging to genotypes B and C, mutated envelope proteins were shown to accumulate within the hepatocyte endoplasmic reticulum (ER) and result in a characteristic histopathological hallmark of HCC, known as ground glass hepatocytes<sup>[39]</sup>. HBV induced ER stress has been shown to dysregulate several cell cycle regulatory pathways, which may contribute to hepatocarcinogenesis<sup>[40]</sup>.

In conclusion, genotypes A and D were isolated from liver disease patients in Kerala, Southern India, with subgenotype A1 predominating. The relatively high prevalence of subgenotype A1 in HCC patients supports previous studies in Africa, which showed an association of subgenotype A1 with HCC and its development at a younger age<sup>[15]</sup>. This association appears not to depend on host ethnicity. The combination of BCP and/or preC mutations, as well as the described preS mutations, could lead to the accumulation of replicative intermediates and viral proteins, contributing to viral integration and cellular stress or damage. These combined characteristics could induce severe liver disease, including HCC, and should be explored further.

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## COMMENTS

## Background

Genotype D, followed by genotype A, has been reported to predominate in India. Hepatitis B virus (HBV) isolates with the A1762T/G1764A variations are prevalent in hepatocellular carcinoma (HCC) patients. In addition, variation G1862T has been reported to be a characteristic of subgenotype A1. Subgenotype A1 has been reported to be more hepatocarcinogenic in southern Africans, who develop HCC at a younger age than those infected with other genotypes.

## Research frontiers

The hepatocarcinogenic potential of subgenotype A1 of HBV has been linked to disease progression in regions outside India. Subgenotype A1 has its origin in Africa and its global dispersal coincides with historical events, including the slave trade and colonization.

## Innovations and breakthroughs

This is the first study to report the predominance of subgenotype A1 in liver disease patients in India and its high prevalence in HCC patients. The relatively high prevalence of subgenotype A1 in HCC patients supports previous studies in Africa that showed an association of subgenotype A1 with HCC and its development at a younger age. The S open reading frame of subgenotype A1 isolates from Kerala clustered within the Asian clade as a separate clade and encoded distinct subgenotype A1 amino acids. The subgenotype A1 isolates from Kerala had a higher frequency of G1862T compared to subgenotype A1 isolates from other geographical regions. This is the first time that preS deletion mutants have been described in Indian HCC patients. Pre-S2: F22L was found in genotypes A and D.

## Applications

The prevalence of different mutations in the various genotypes of HBV may serve as biomarkers for disease risk and development of HCC. The differences in the geographical distribution of genotypes and subgenotypes of HBV may require different treatment algorithms. Knowledge of HBV genotypes and/or mutations may facilitate personalized treatment.

## Terminology

A genotype is generally defined as the genetic constitution of an organism. In the case of viruses, the term genotype applies to the forms into which the genomic sequence has stabilized after a prolonged period of time and that are replication competent. The genotypes of HBV are defined by an intergroup divergence of more than 7.5%-8% in the complete genome sequence and by more than 4% at the level of the S gene. The term subgenotype is used to identify subgroups of HBV genotypes with an intergroup nucleotide difference between 4% and 8% across the complete genome.

## Peer review

The study contributes to the understanding of the relationship between HBV variability and clinical outcomes in different populations worldwide. The relationship between the preS deletion and HCC in this study is a critical and hot point.

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## Anti-miRNA-221 sensitizes human colorectal carcinoma cells to radiation by upregulating PTEN

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### Abstract

**AIM:** To investigate the regulative effect of miRNA (miR)-221 on colorectal carcinoma (CRC) cell radiosensitivity and the underlying mechanisms.

**METHODS:** A human CRC-derived cell line was cultured conventionally and exposed to different doses of X-rays (0, 2, 4, 6 and 8 Gy). The total RNA and protein of the cells were extracted 24 h after irradiation, and the alteration of miR-221 and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene mRNA expression was detected by real-time reverse transcriptase polymerase chain reaction (PCR). The protein alteration of PTEN in the cells was detected by Western blotting. Caco2 cells were pretreated with or without anti-PTEN-siRNA prior to the addition of pre-miR-221 or anti-miR-221 using Lipofectamine 2000. Colony formation assay and flow cytometry analysis were used to measure the surviving cell fraction and the sensitizing enhancement ratio after irradiation. Ad-

ditionally, PTEN 3'-untranslated region fragment was PCR amplified and inserted into a luciferase reporter plasmid. The luciferase reporter plasmid construct was then transfected into CRC cells together with pre-miR-221 or anti-miR-221, and the luciferase activity in the transfected cells was detected.

**RESULTS:** The X-ray radiation dose had a significant effect on the expression of miR-221 and PTEN protein in human Caco2 cells in a dose-dependent manner. The miR-221 expression level improved gradually with the increase in irradiation dose, while the PTEN protein expression level reduced gradually. miR-221 expression was significantly reduced in the anti-miR-221 group compared with the pre-miR-221 and negative control groups ( $P < 0.01$ ). Anti-miR-221 upregulated expression of PTEN protein and enhanced the radiosensitivity of Caco2 cells ( $P < 0.01$ ). Moreover, the inhibitory effect was dramatically abolished by pretreatment with anti-PTEN-siRNA, suggesting that the enhancement of radiosensitivity was indeed mediated by PTEN. A significant increase of luciferase activity was detected in CRC cells that were cotransfected with the luciferase reporter plasmid construct and anti-miR-221 ( $P < 0.01$ ).

**CONCLUSION:** Anti-miR-221 can enhance the radiosensitivity of CRC cells by upregulating PTEN.

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**Key words:** Colorectal carcinoma; miR-221; Phosphatase and tensin homolog deleted on chromosome 10; Radiosensitivity

**Core tip:** Previous studies have shown that miRNA (miR)-221 expression is elevated in radioresistant colorectal carcinoma (CRC) cells; however, it is unknown whether and how miR-221 controls cellular response to irradiation. We demonstrated that knock-down of miR-221 upregulated phosphatase and tensin



homolog deleted on chromosome 10 (PTEN) expression, and PTEN was identified as a direct target of miR-221 in CRC. Upregulated PTEN expression suppressed AKT activity and increased radiation-induced cell death, enhancing radiosensitivity in CRC cells. This study provides evidence for antioncogenic activity of anti-miR-221 in the irradiation of CRC and may be a useful biomarker or therapeutic target in CRC.

Xue Q, Sun K, Deng HJ, Lei ST, Dong JQ, Li GX. Anti-miRNA-221 sensitizes human colorectal carcinoma cells to radiation by upregulating PTEN. *World J Gastroenterol* 2013; 19(48): 9307-9317 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9307.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9307>

## INTRODUCTION

Colorectal carcinoma (CRC) is one of the most frequent cancers and a common cause of cancer-related death worldwide, with an increasing incidence expected in the next few decades<sup>[1]</sup>. The overall incidence of CRC is 5% in the general population and the 5-year survival rate ranges from 40% to 60%<sup>[2]</sup>. The national comprehensive cancer network guidelines on CRC treatment include radiotherapy as standard for patients with a high risk of recurrence (<http://www.nccn.org/index.asp>). However, the radiotherapeutic efficiency is often limited by the occurrence of radioresistance, reflected as a diminished susceptibility of the irradiated cells to undergo apoptosis. As a result, this therapeutic strategy cannot substantially improve the survival rate<sup>[3]</sup>. With the understanding of the molecular biology of CRC, it has been recognized that development of radioresistance is related to changes of tumor environment and the dysregulation of certain genes, including some genes involved in a variety of cell signaling pathways as well as oncogenes and tumor suppressor genes<sup>[4]</sup>.

miRNAs are a new class of small noncoding RNAs that regulate the expression of target genes through translational repression or mRNA cleavage/decay<sup>[5]</sup>. Genome-wide studies have demonstrated that miRNA genes are frequently located at cancer-associated genomic regions, indicating the potential roles of miRNAs in tumorigenesis<sup>[6]</sup>. miRNAs play an important role in the multistep processes of carcinogenesis, either by oncogenic or tumor suppressor function in CRC<sup>[7]</sup>. However, only a few studies have determined the roles of miRNAs in radiation response in CRC<sup>[8]</sup>. miRNA (miR)-221, encoded in tandem from a gene cluster located on chromosome X, is a recently discovered miRNA and is involved in tumor development by regulating cell proliferation cycle<sup>[9]</sup>. In our previous study, we have demonstrated that miR-221 promotes CRC occurrence and progression, which makes it a potential antitumor candidate for treatment and prevention of CRC<sup>[10]</sup>. However, the

miR-221 response for CRC to survive radiation-induced injury remains largely unknown.

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene, located at 10q23.3, encodes a central domain with homology to the catalytic region of protein tyrosine phosphatases<sup>[11]</sup>. This gene is an important regulator of protein phosphatases and 3'-phosphoinositol phosphatases. PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3), the second messenger produced by phosphoinositide 3-kinase, to regulate negatively the activity of the serine/threonine protein kinase, AKT<sup>[12]</sup>. PTEN is inactivated in some malignant tumors, resulting in AKT hyperactivation, thereby promoting cell proliferation, inhibition of apoptosis, and enhanced cell invasion and radioresistance<sup>[13]</sup>. miRNAs, specifically miR-221, have been established as regulators of PTEN expression<sup>[14]</sup>. However, how miR-221 affects PTEN in CRC radiation has not been elucidated. Therefore, in this study, we observed the effect of miR-221 on the radiosensitivity of CRC cells and its underlying mechanisms.

## MATERIALS AND METHODS

### Cell culture and transfection

Human CRC-derived cell lines, including HT-29, Lovo, SW-480, Caco2, and control human umbilical vein endothelial cells (HUVECs), provided by Shanghai Institutes For Biological Science, CAS, were resuscitated routinely, resuspended with RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, United States), 100 kU/mL penicillin G and 100 g/L streptomycin, and then planted in a 25-cm<sup>2</sup> culture bottle and incubated in a 5% CO<sub>2</sub> humidified atmosphere at 37 °C. The media were changed every 3 d and the cells were trypsinized using trypsin/edetic acid when they reached 80%-90% confluence. Cells aged at passages 4-8 were used for the experiments. The day before transfection, cells were seeded in antibiotic-free medium. Cells ( $1 \times 10^4$ /well) were seeded in a 96-well plate, incubated for 24 h to allow them attach to the bottom of the well, and then transfected with 50 nmol/L negative control, pre-miR-221 or anti-miR-221 oligonucleotides, respectively (Shanghai GenePharma, Shanghai, China). Transfection of miRNAs was carried out using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen, Carlsbad, CA, United States). The above experiment was repeated at least three times.

### Cell proliferation analysis of transfected cells by MTT assay

The status of cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl trozolum bromide (MTT; Amresco, Solon, OH, United States) assay. In short, exponentially growing CRC cells were adjusted to  $1.5 \times 10^4$  cells/mL with RPMI-1640, planted in 96-well plates (Corning, Corning, NY, United States) at 200  $\mu$ L/well, and incubated for 12 h. After transfection with 50 nmol/L pre-miR-221 or anti-miR-221 and incubation for

48 h (five duplicate wells for each sample), 20  $\mu$ L/well MTT (5 g/L) was added to each well. The medium was removed after 4 h incubation and 100  $\mu$ L/well dimethylsulfoxide was added to dissolve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunity implement (Bio-Rad 2550, Hercules, CA, United States) at 490 nm. Cellular proliferation inhibition rate (CPIR) was calculated using the following formula: CPIR = (1 - average *A* value of experimental group/average *A* value of control group)  $\times$  100%. The above experiment was repeated at least three times.

### miRNA target prediction

The analysis of miR-221-predicted targets was performed using the algorithms TargetScan (<http://targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>) and MiRanda (<http://www.microrna.org/microrna/home.do>).

### Luciferase activity assay

The human 3'-untranslated region (UTR) of the PTEN gene was amplified by PCR using the primers 5'-CGATTC-TAGAAATCATGTTCTGGTGG-3' for PTEN-3'-UTR-Forward and 5'-GCATTCTAGAATTCTGCA-CAGTAAGCATA-3' for PTEN-3'-UTR-Reverse and cloned into the *Xba*I site of the pGL3-control vector (Promega, Madison, WI, United States), downstream of the luciferase gene, to generate the vector pGL3-PTEN. For luciferase assay, the CRC cells were cultured in 24-well plates and transfected with 500 ng of either pGL3-PTEN or pGL3-control vector and 50 pmol pre-miR-221, anti-miR-221 or negative control. Transfection was performed using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. At 24 h after transfection, firefly luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega, Madison, WI, United States). The above experiment was repeated at least three times.

### Radiation exposure

Irradiation was performed at room temperature in a linear accelerator (Varian 600; Palo Alto, CA, United States) at a dose rate of 3.2 Gy/min. Monolayer cells were plated into six-well plates, placed 100 cm from the source, and exposed to the specified dose (0, 2, 4, 6 and 8 Gy) of X-rays<sup>[14]</sup>.

### Detection of miR-221 and PTEN mRNA expression by real-time reverse transcriptase polymerase chain reaction

Total RNA was extracted with routine Trizol reagent (Invitrogen). The precipitation was dissolved in diethylpyrocarbonate-treated water. Nucleic acid protein analyzer (Beckman Coulter, Fullerton, CA, United States) was used to determine RNA concentration. The purity and integrity of RNA were identified by two aspects:  $A_{260\text{nm}}/A_{280\text{nm}} \geq 1.8$ , and a band ratio of 28S to 18S RNA  $\geq 1.5$  in formaldehyde denaturing gel electrophoresis. miR-221 and PTEN mRNA was quantified as described previously<sup>[10]</sup>. The comparative  $2^{-\Delta\Delta CT}$  method was used for relative

quantification and statistical analysis. The above experiment was repeated at least three times.

### Detection of target protein expression by Western blotting

The cells were rinsed twice with cold phosphate buffered solution (PBS) buffer, and were then lysed in an ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, Sussex, United Kingdom). The samples were cleared by centrifugation at 13000 *g* for 10 min. Fifty micrograms of protein from the tissue was subjected to SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes (Immobilon, Bedford, MA). After blocking in 20 mmol/L Tris-HCl, pH 7.6 (containing 150 mmol/L NaCl, 0.1% Tween-20, and 5% nonfat dry milk), membranes were incubated with primary antibodies against target protein or  $\beta$ -actin (used as a sample loading control) overnight at 4 °C and then incubated with horseradish-peroxidase-conjugated secondary antibody. The blot was developed using the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, United States) according to the manufacturer's instructions and the protein imprinting band was obtained. The above experiment was repeated at least three times.

### Colony formation assay

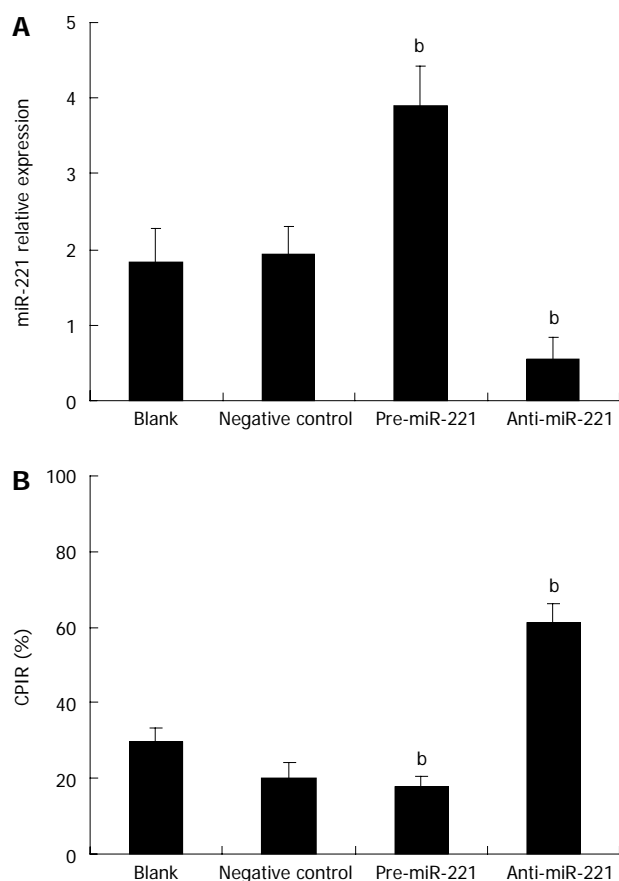
At 24 h after irradiation, all cells were trypsinized and counted. Corresponding numbers of cells were seeded into 10-cm dishes containing RPMI-1640 supplemented with 10% FBS, in triplicate, incubated for 14 d to allow colony growth, and colonies were stained with crystal violet. Colonies containing  $\geq 50$  cells were counted. The plating efficiency was calculated by dividing the average number of colonies per dish by the number of cells plated. Survival fractions were calculated by normalization to the plating efficiency of appropriate control groups. The above experiment was repeated at least three times.

### Flow cytometry assay

The effects of miR-221 and irradiation on CRC cell death were examined by flow cytometry. Pretreated CRC cells were harvested and washed twice with PBS, fixed with 70% ethanol at -20 °C for 30 min, and stored at 4 °C overnight, then washed with PBS again, treated with 100 mL 100 mg/L RNase at 37 °C for 30 min, and stained with 100 mL 50 mg/L propidium iodide at 4 °C for 30 min in the dark. The multiplication cycle and apoptotic rate were assayed using flow cytometry, and the data were analyzed using CellQuest software. The percentages of necrotic and apoptotic cells were measured by calculating the ratio of the number of corresponding cells to the number of total cells. For each sample, 10000 cells were measured.

### Statistical analysis

All data in the experiment are presented as mean  $\pm$  SD.



**Figure 1** Modulation of miR-221 expression and cell proliferation in Caco2 cells. A: Expression of miR-221 was detected by real-time RT-PCR. Expression of U6 snRNA was used as internal control; B: The status of cell proliferation was determined by MTT assay. CPIR in the presence of pre-miR-221 or anti-miR-221 was compared with those of controls.  $n = 6$ , mean  $\pm$  SD; <sup>b</sup> $P < 0.01$  vs control group.

Comparisons between groups were analyzed with one-way ANOVA and Student-Newman-Keuls  $Q$  test using SPSS version 15.0 (SPSS, Chicago, IL, United States).  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Modulation of miR-221 expression in CRC cell lines

To study the expression pattern of miR-221 in CRC cells, we performed real-time RT-PCR to detect miR-221 expression in four CRC-derived cell lines. The expression values of miR-221 in HT-29, Lovo, SW-480 and Caco2 were  $4.094 \pm 0.208$ ,  $1.122 \pm 0.138$ ,  $3.927 \pm 0.232$  and  $1.831 \pm 0.149$ , respectively. A significant overexpression of miR-221 was observed in all four CRC cell lines relative to HUVEC ( $0.223 \pm 0.047$ ,  $P < 0.01$ ). The Caco2 cell line was chosen for both pre-miR-221 and anti-miR-221 transfection in the successive experiment because it exhibits, among the four cell lines tested, an intermediate miR-221 expression level.

To determine the biological impact of miR-221 in the CRC-derived cell line, Caco2 cells were transfected with pre-miR-221 or anti-miR-221 to increase or reduce

miR-221 level, respectively. Real-time RT-PCR analysis revealed that introduction of pre-miR-221 caused a significant increase of miR-221 value; conversely, anti-miR-221 caused a significant decrease of miR-221 value (Figure 1A,  $P < 0.01$ ). These strategies were then used as the basis of the remaining experiments.

We further tested whether the cell proliferation potential of the transfected CRC cells was modified and the status of cell proliferation was determined by MTT assay. We observed a significant increase in proliferation after transfection of pre-miR-221. In contrast, anti-miR-221 significantly decreased cell proliferation (Figure 1B,  $P < 0.01$ ). These data indicate that cell proliferation can be significantly enhanced by increase of miR-221 expression, a result which strongly supports the potential oncogenic activity of miR-221 in CRC.

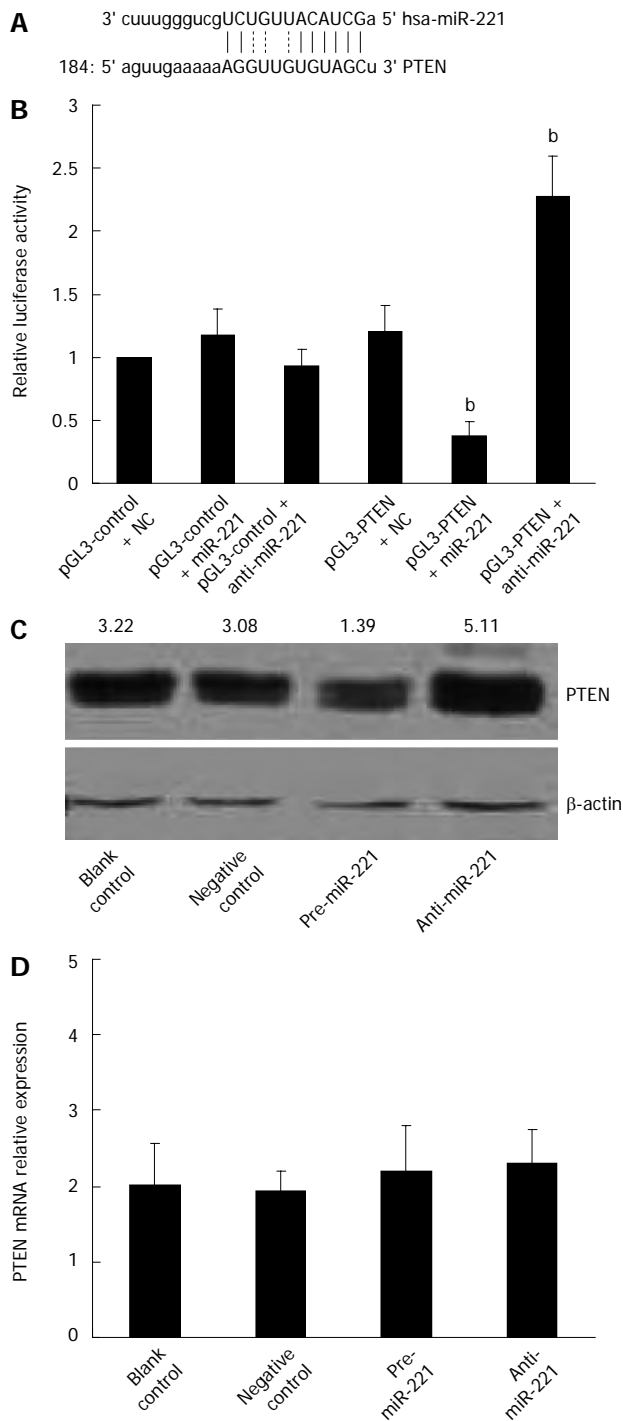
### PTEN is a target of miR-221 in CRC

Most miRs are thought to control gene expression by base-pairing with the miR-recognizing elements found in their messenger target. We utilized all three currently available major prediction programs, TargetScan, Miranda and PicTar, to analyze the potential interactions between miR-221 and PTEN. All these algorithms reveal a potential miR-221 target site in the PTEN mRNA 3'-UTR region (Figure 2A). To demonstrate the direct interaction between miR-221 and PTEN mRNA, we cloned PTEN-3'-UTR segment, which includes a potential target site for miR-221, downstream of the pGL3 luciferase reporter gene, to generate the pGL3-PTEN vector. This vector was cotransfected into Caco2 cells together with pre-miR-221 or anti-miR-221. Luciferase activity in Caco2 cells cotransfected with pGL3-PTEN vector and miR-221 was decreased markedly compared with negative controls. On the contrary, luciferase activity in Caco2 cells transfected with anti-miR-221 was increased significantly compared with negative controls (Figure 2B). These results support the bioinformatic prediction indicating the 3'-UTR of PTEN mRNA as a target for miR-221.

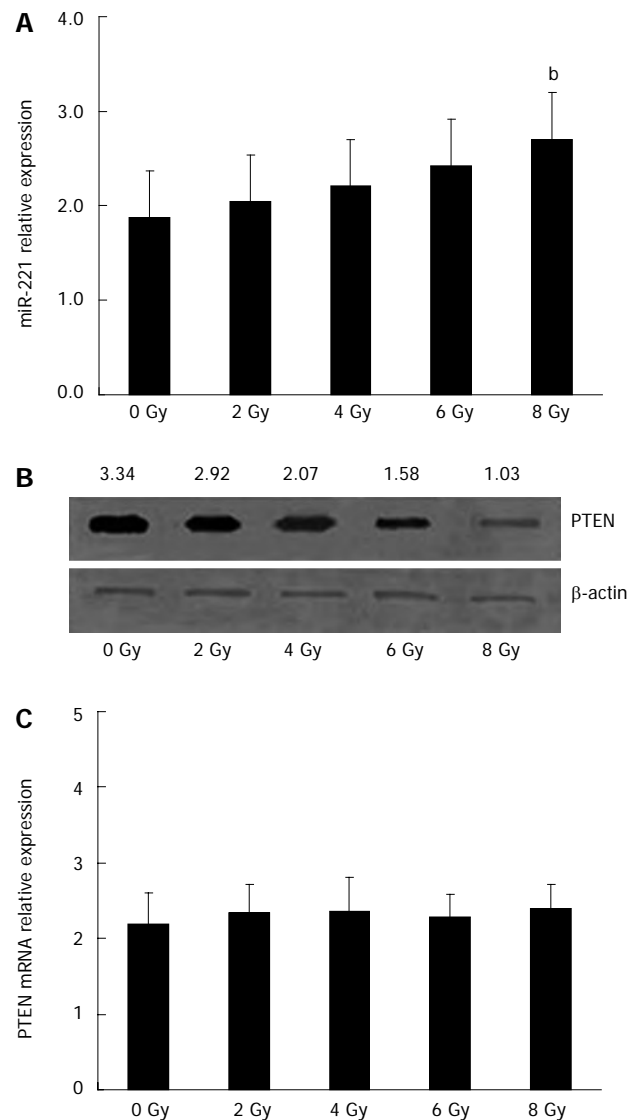
To check whether miR-221 actually affects PTEN expression in CRC cells, we analyzed the consequence of the ectopic expression of miR-221. We transfected the pre-miR-221 or anti-miR-221 into Caco2 cells, and we searched for changes in PTEN protein levels by Western blotting. Introduction of miR-221 caused a significant increase of miR-221 value and decreased PTEN protein levels. Conversely, anti-miR-221 caused a significant decrease of miR-221 value and increased PTEN protein amounts (Figure 2C). No significant changes in the PTEN mRNA levels were observed in the cells either transfected with miR-221 or anti-miR-221 (Figure 2D). This result strongly validates a post-transcriptional regulation of PTEN protein by miR-221, and also excludes its role in PTEN mRNA degradation.

### miR-221 modulates Caco2 cell radiosensitivity

To determine whether miR-221 and PTEN were involved in the cellular response to radiotherapy in CRC,



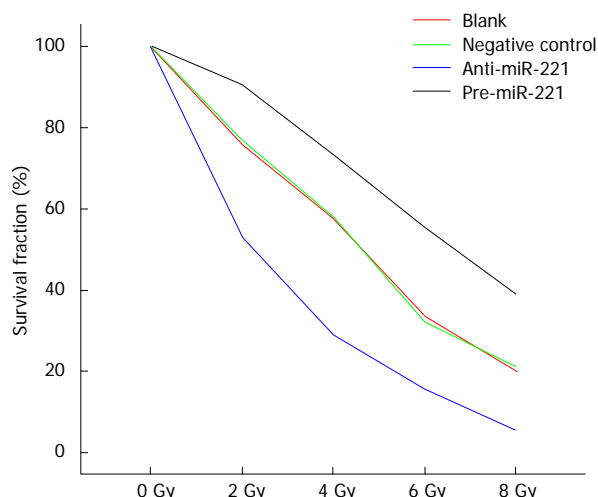
**Figure 2** miR-221 regulated expression of phosphatase and tensin homolog deleted on chromosome 10 in colorectal carcinoma-derived cells. **A:** Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) 3'-UTR site potentially targeted by miR-221 as predicted by Miranda; **B:** Luciferase activity assay showing direct interaction between miR-221 and PTEN 3'-UTR site. Firefly luciferase reporter activity in the presence of both pGL3-PTEN vector and pre-miR-221 or anti-miR-221 was compared with that of the controls. Luciferase activity in Caco2 cells cotransfected with pGL3-PTEN vector and pre-miR-221 was decreased markedly compared with the negative control. Luciferase activity in Caco2 cells transfected with anti-miR-221 was increased significantly compared with the negative control, <sup>b</sup> $P < 0.01$  vs control group; **C:** Western blotting showing PTEN protein expression in Caco2 cells transfected with pre-miR-221 or anti-miR-221.  $\beta$ -actin was used as a housekeeping gene to normalize PTEN protein expression. The relative PTEN protein levels normalized against  $\beta$ -actin are shown at the top of each panel; **D:** Real-time RT-PCR analysis showing PTEN mRNA expression in Caco2 cells transfected with pre-miR-221 or anti-miR-221. All the results are representative of three independent experiments.



**Figure 3** Effects of X-ray dose on miR-221 and phosphatase and tensin homolog deleted on chromosome 10 expression in colorectal carcinoma-derived cells. **A:** Expression of miR-221 was detected by real-time RT-PCR. The miR-221 expression level improved in a dose-dependent manner with the increase in radiation dose, <sup>b</sup> $P < 0.01$  vs control group; **B:** Expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein was detected by Western blotting,  $\beta$ -actin was used as a housekeeping gene to normalize PTEN protein expression. The relative PTEN protein levels normalized against  $\beta$ -actin are shown at the top of each panel. The expression of PTEN protein was decreased in a dose-dependent manner with the increase in radiation dose; **C:** Expression of PTEN mRNA was detected by real-time RT-PCR. No significant changes in the PTEN mRNA levels were observed in the cells exposed to irradiation. The results are representative of three independent experiments.

Caco2 cells were exposed to different doses of X-rays to observe the regular pattern of miR-221 and PTEN. The radiation dose had a significant effect on the expression of miR-221 and PTEN protein in human Caco2 cells in a dose-dependent manner. The miR-221 expression level improved gradually with the increase in radiation dose, while the PTEN protein expression level reduced gradually (Figure 3A and B). However, no significant changes in the PTEN mRNA levels were observed in the cells exposed to irradiation (Figure 3C). All these results gave us a first hint that the expression of miR-221 might be





**Figure 4** miR-221 modulates colorectal carcinoma cell radiosensitivity. Caco2 cells transfected with negative control, pre-miR-221 or anti-miR-221 oligonucleotides were exposed to 0-8 Gy radiation and incubated for 14 d prior to fixation, staining and assessment of colony formation. The colony formation assays were performed in triplicate.

one of the mechanisms acting to regulate radiosensitivity in CRC cells negatively.

To determine whether miR-221 affected Caco2 cell radiosensitivity, cells were transfected with pre-miR-221 or anti-miR-221 and colony formation was assessed following 0-8 Gy radiation. Transfection of Caco2 cells with pre-miR-221 significantly increased survival following 0-8 Gy radiation compared to blank and negative controls. Conversely, transfection of Caco2 cells with anti-miR-221 significantly decreased survival following radiation exposure (Figure 4). The  $D_0$  value, the radiation dose required to reduce the level of cell survival from 100% to 37%, which is considered a measure of the intrinsic radiosensitivity of the cell, was calculated following genetic manipulation of miR-221. The quasi-threshold dose ( $D_q$ ) value represents the sublethal damage repair capacity of the cells, which is also a sensitive indicator for the evaluation of cell radiosensitivity. Blank control cells, cells transfected with negative control or pre-miR-221 or anti-miR-221 oligonucleotides exhibited  $D_0$  values of 1.681, 1.666, 2.208 and 1.068 Gy, respectively. The sensitization enhancement ratio (SER), calculated by determining the ratio of the  $D_0$  of the control group *vs* treated cells, was 1.009, 0.761 and 1.574 for negative control-, pre-miR-221-, or anti-miR-221-treated cells, respectively (Table 1), indicating a radiosensitization potential for targeting miR-221.

To study the mechanism of miR-221 knockdown-induced radiosensitization, we measured irradiation-induced cell death in cells transfected with negative control or anti-miR-221 by flow cytometry. We found that in unirradiated cells transfected with anti-miR-221, there was an increase in necrosis and apoptosis compared to that in the controls. This is consistent with our previous observations that miR-221 knockdown leads to inhibition of cell growth<sup>[10]</sup>. More interestingly, in irradiated cells, anti-miR-221 transfection enhanced cell death (Figure

**Table 1** Impact of miR-221 expression on Caco2 cell radiosensitivity

Group	$D_0$	$D_q$	SF <sub>4</sub>	SER
Blank control + irradiation	1.681	5.630	0.5966	
Negative control + irradiation	1.666	5.813	0.5858	1.009
Pre-miR-221 + irradiation	2.208	7.828	0.7453	0.761
Anti-miR-221 + irradiation	1.068	4.655	0.2984	1.574

Caco2 cells were transfected with negative control, pre-miR-221 or anti-miR-221 oligonucleotides.  $D_0$  and  $D_q$  were determined by standardized software, and the sensitization enhancement ratio (SER) was calculated by determining the ratio of the  $D_0$  of the control group *vs* treated cells. SF<sub>4</sub>: Surviving fraction at 4 Gy.

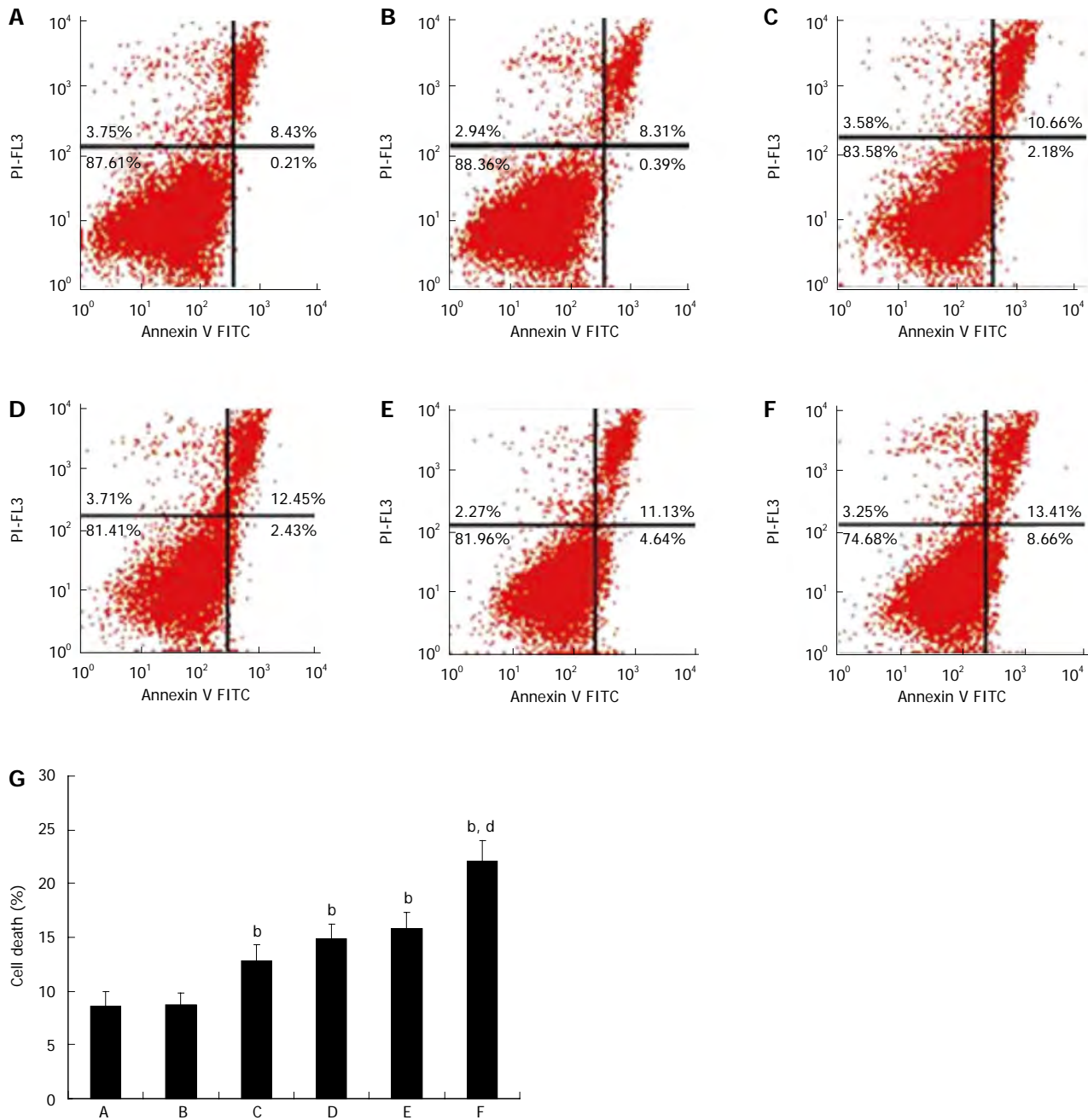
5), demonstrating a synergistic effect of miR-221 knock-down with irradiation. Collectively, these results provide strong evidence that miR-221 regulates the radiosensitivity of Caco2 cells.

### Enhancement of anti-miR-221 on CRC cell radiosensitivity is mediated by PTEN

PTEN is a tumor suppressor protein, thus, we hypothesized that anti-miR-221 might sensitize Caco2 cells to radiotherapy by upregulating PTEN protein expression. To study further downstream pathways of miR-221, we conducted Western blotting to look at phosphorylation of AKT, a downstream target of PTEN. In Caco2 cells transfected with anti-miR-221, there was a significant increase in PTEN expression accompanied by downregulation of AKT phosphorylation (Figure 6A).

Additionally, if anti-miR-221 enhancement of CRC cell radiosensitivity was indeed mediated by PTEN, we would expect that the PTEN-specific and irreversible antagonist, anti-PTEN-siRNA, would abolish this effect. To test this hypothesis, we measured the cell radiosensitivity variations induced by pre-miR-221 or anti-miR-221 in CRC cells previously transfected with anti-PTEN-siRNA. The aim of this experiment was to study if and how the PTEN-depleted cellular environment responds to pre-miR-221 or anti-miR-221 addition and irradiation. Caco2 cells were pretreated with or without anti-PTEN-siRNA (80 nmol/L) for 24 h prior to the addition of pre-miR-221 (50 nmol/L) or anti-miR-221 (50 nmol/L) and the status of cell radiosensitivity was determined by colony formation assay following different doses of X-ray exposure. The data showed that a reduction of PTEN dosage by means different from miR-221 overexpression led to analogous outcomes: when we transfected Caco2 cells with anti-PTEN-siRNA, which was able to reduce both PTEN mRNA and protein by about 80% (Figure 6B and C), we observed a sharp increase in cell survival following radiation as compared with the negative controls (Figure 6D). Thus, reducing PTEN levels in CRC cells, either by miR-221 overexpression or by anti-PTEN-siRNA transduction, is sufficient to induce a comparable cell survival increase.

When pre-miR-221 was transfected into Caco2 cells previously treated with anti-PTEN-siRNA, we observed



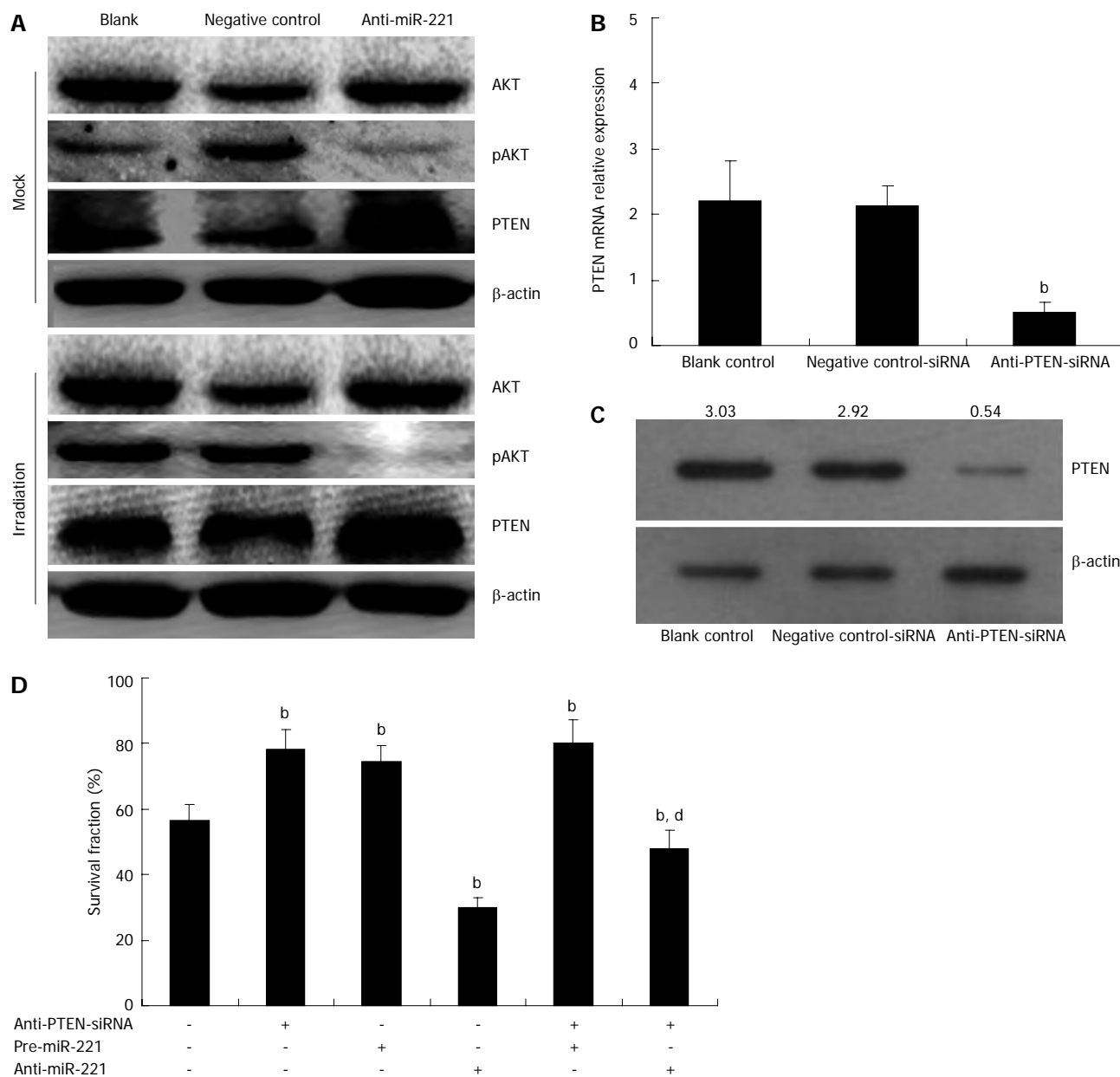
**Figure 5** Effects of anti-miR-221 on irradiation-induced death in Caco2 cells. The percentages of necrotic and apoptotic cells were measured by calculating the ratio of the number of corresponding cells to the number of total cells. A: Blank control; B: Caco2 cells transfected with negative control; C: Caco2 cells transfected with anti-miR-221; D: Caco2 cells under irradiation; E: Caco2 cells transfected with negative control under irradiation; F: Caco2 cells transfected with anti-miR-221 under irradiation. The right upper quadrant (FITC/PI) shown as necrotic cells. The right lower quadrant (FITC/PI) shown as apoptotic cells; G: The status of cell death was determined by flow cytometry. Groups A-F are described as above.  $n = 3$ , mean  $\pm$  SD; <sup>b</sup> $P < 0.01$  vs blank or negative control group. <sup>d</sup> $P < 0.01$  vs sole anti-miR-221 or irradiation group.

that anti-PTEN-siRNA and miR-221 seemed to cooperate to increase the survival rate (Figure 6D). However, when anti-miR-221 was transfected into Caco2 cells previously treated with anti-PTEN-siRNA, we observed that the suppression of cell survival by anti-miR-221 was partially abrogated by anti-PTEN-siRNA (Figure 6D, Table 2). These results indicated that the inhibitory effect of anti-miR-221 on CRC cell survival following irradiation was partially, but not completely, mediated by PTEN, suggesting that anti-miR-221 could also activate some PTEN-in-

dependent signaling pathways to repress CRC cell growth in addition to the up-regulation of PTEN.

## DISCUSSION

Radiotherapy is one of the most important treatment methods for CRC. However, only one-third of CRC patients are highly responsive to radiation. The other patients are relatively resistant to radiation, and they tend to progress even after high-dose treatment<sup>[15-17]</sup>. Factors



**Figure 6** Ectopic expression of miR-221 affects the radiosensitivity of colorectal carcinoma cells by targeting phosphatase and tensin homolog deleted on chromosome 10. **A:** miR-221 regulates the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/AKT pathway. Caco2 cells transfected with negative control or anti-miR-221 were mock treated or irradiated (4 Gy). Total cell lysates were obtained for Western blotting using the indicated antibodies. Caco2 cells were pretreated with or without anti-PTEN-siRNA (80 nmol/L) for 24 h prior to the addition of pre-miR-221 (50 nmol/L) or anti-miR-221 (50 nmol/L); **B, C:** Real-time RT-PCR and Western blotting showing PTEN mRNA and protein reduced markedly after transfection with anti-PTEN-siRNA ( $^cP < 0.01$  vs blank control and negative control group); **D:** The status of cell radiosensitivity was determined by colony formation assay. The suppression of Caco2 cell survival fraction at 4 Gy by anti-miR-221 was partially, but not completely, abrogated by anti-PTEN-siRNA (SF<sub>4</sub> from 29.77% to 47.88%).  $n = 3$ , mean  $\pm$  SD;  $^bP < 0.01$  vs negative control group;  $^dP < 0.01$  vs sole anti-miR-221 group.

leading to CRC radioresistance include location, size, and microenvironment such as inadequate vascular supply<sup>[18,19]</sup>. More importantly, cellular and genetic factors that are related to radiation responses may explain radiation-resistant cellular phenotypes<sup>[20,21]</sup>.

Activation of oncogenes and inactivation of tumor suppressor genes lead to aberrant activity of signal transduction pathways, and radioresistance can be a result of abnormal functioning of these signaling pathways<sup>[22-24]</sup>. PTEN functions as a tumor suppressor gene, specifically by negatively regulating the AKT/PKB signaling

pathway<sup>[25]</sup>. Previous studies have shown that PTEN is a dual-specificity phosphatase possessing both lipid and protein phosphatase activities. Activated PTEN affects a dephosphorylation of PIP3, generates PIP2, and decreases the phosphorylation level of AKT, which result in cell growth arrest and apoptosis<sup>[26]</sup>. Genetic inactivation of PTEN is a hallmark of many cancers, including CRC, and reduced expression occurs in many other tumor types. Deficiency of PTEN in the intestine has been reported to induce precancerous polyps, *via* the induction of formation and fission of crypts, structures located

**Table 2** Impact of PTEN on miR-221-mediated Caco2 cell radiosensitivity

Group	$D_0$	$D_q$	SF <sub>4</sub>	SER
Blank control + irradiation	1.677	5.590	0.5667	
Anti-PTEN-siRNA + irradiation	2.327	7.788	0.7569	0.718
Anti-miR-221 + irradiation	1.082	4.702	0.2977	1.550
Anti-miR-221 and anti-PTEN-siRNA + irradiation	1.511	5.010	0.4788	1.110

Caco2 cells were pretreated with anti-PTEN-siRNA for 24 h prior to the addition of anti-miR-221.  $D_0$  and  $D_q$  were determined by standardized software, and the sensitization enhancement ratio (SER) was calculated by determining the ratio of the  $D_0$  of the control group *vs* treated cells. SF<sub>4</sub>: Surviving fraction at 4 Gy.

at the base of the intestine containing a rapidly dividing pool of intestinal stem cells<sup>[27,28]</sup>. Moreover, restoring PTEN expression in PTEN-deficient tumor cells has been shown to enhance radiosensitivity; however, little is known regarding the impact of miRNAs on PTEN expression in CRC<sup>[29,30]</sup>.

Zhang *et al.*<sup>[31]</sup> studied the miR-221 expression in a gastric cancer-derived cell line and demonstrated that PTEN was the target of miR-221. miR-221 is a newly discovered miRNA which is upregulated in multiple malignant tumors such as hepatocellular carcinoma, bladder cancer, and pancreatic cancer, and facilitates tumors entering S phase from G<sub>0</sub>/G<sub>1</sub> phase by inhibiting the expression of cyclin dependent kinase inhibitor (CDKI)<sup>[32]</sup>. miR-221 therefore represents an attractive candidate for selective treatment with miR-221-specific inhibitor. miR-221 expression is abnormally increased in CRC and can promote CRC development, which has been confirmed by our previous studies<sup>[10]</sup>. However, the mechanism by which miR-221 modulates the malignant phenotype, including radioresistance, within CRC remains unknown. Here, we observed miR-221 upregulation in several human CRC-derived lines compared with HUVEC, corroborating the findings of our previous studies<sup>[10]</sup>. In this study, we predicted that PTEN would be a target gene of the miR-221 by computer-aided algorithm. Moreover, we found binding sites for human miR-221 in the PTEN 3'-UTR by using luciferase activity assay, suggesting that miR-221 might affect PTEN expression. Indeed, we demonstrated that introduction of miR-221 caused a significant increase of miR-221 value and decreased PTEN protein levels. Conversely, anti-miR-221 caused a significant decrease in miR-221 value and increased PTEN protein. Based upon these findings, we hypothesized PTEN as a target of miR-221 in CRC to regulate cell radiosensitivity.

As PTEN is a target of miR-221, and has been described previously as an important regulator of radiation sensitivity, these results suggest that increasing PTEN expression by silencing miR-221 could enhance the radiosensitivity of CRC cells. In this study, transfection of Caco2 cells with pre-miR-221 significantly increased survival following X-ray exposure compared to blank and negative controls. Conversely, transfection of Caco2

cells with anti-miR-221 significantly decreased survival following irradiation. Indeed, we proved that the Caco2 cells were sensitized to radiation by knockdown of miR-221; however, whether PTEN was the sole or main target for miR-221 regulation of radiosensitivity remains unknown. Thus, by using the PTEN-specific antagonist, anti-PTEN-siRNA, we demonstrated that the regulatory effect of anti-miR-221 on CRC cell radiosensitivity was partially, but not completely, mediated by PTEN, suggesting that miR-221 could regulate other PTEN-independent signaling pathways to enhance CRC radiosensitivity. It has been previously shown that CDKN1B/p27 and CDKN1C/p57 are also the target of miR-221 and consistently, CDKN1B/p27 and CDKN1C/p57 expressions inversely correlate in most cancers with miR-221 over-expression, which suggests that the inhibitory effect of anti-miR-221 on CRC cell radiosensitivity is only partially abrogated by anti-PTEN-siRNA<sup>[33]</sup>. We think that our results, which identify PTEN as a target for miR-221 in the context of CRC cell lines, fit well within a dynamic view of the miRNA-mediated regulation of gene expression: it is well known and widely predicted that the relationship between miRNAs and target mRNAs is not a “one to one” connection, because the same mRNA can be regulated by more than one miRNA, and that the choice of how many and which miRNAs target one 3'-UTR is strongly determined by the specific cellular environment<sup>[34-36]</sup>. An miRNA that regulates targets playing opposite roles in the control of cell proliferation may act as a tumor suppressor in some cancers and as an oncogene in others, depending on which targets are driving tumorigenesis in that specific cellular milieu.

In summary, we demonstrated that miR-221 could regulate CRC cell radiosensitivity by targeting PTEN. Our data suggest that upregulation of PTEN expression by transfection of anti-miR-221 has important biological effects on the radiosensitivity of CRC cells. These results identify anti-miR-221 as a potential therapeutic approach for CRC *via* upregulation of PTEN. However, it is noteworthy that the results in this study are based on only one cultured CRC cell line that might not necessarily comprehensively reflect other lines and the *in vivo* situation. Therefore, further experiments are required to elucidate the antitumor mechanisms of anti-miR-221 in *in vivo* systems.

## COMMENTS

### Background

miRNAs regulate gene expression by mainly binding to the 3'-untranslated region (UTR) of target mRNAs, leading to mRNA degradation or translation inhibition. miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate.

### Research frontiers

Colorectal carcinoma (CRC) is one of the most dangerous malignancies in China. Previous studies have shown that miR-221 expression is elevated in radioresistant CRC cell lines; however, it is not known whether and how miR-221 controls the cellular response to irradiation. In this study, the authors investigated the alterations of miR-221 and phosphatase and tensin homolog



deleted on chromosome 10 (PTEN) gene expression in CRC cells after X-ray irradiation, and the mechanisms underlying the enhancement of radiosensitivity to irradiation in CRC cells transfected with anti-miR-221.

### Innovations and breakthroughs

Some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. This study showed that knocking down miR-221 by antisense oligonucleotides upregulated PTEN expression and PTEN was identified as a direct target of miR-221 in CRC. Moreover, up-regulated PTEN expression suppressed AKT activity and increased radiation-induced cell death, resulting in enhancement of radiosensitivity in CRC cells.

### Applications

This study indicated that anti-miR-221 enhanced the radiosensitivity of CRC cells by upregulating PTEN, and miR-221 might be a novel potential strategy for CRC treatment.

### Peer review

Anti-miR-221 could enhance the radiosensitivity of CRC cells by upregulating PTEN. This study provides evidence for the antioncogenic activity of anti-miR-221 in the irradiation of CRC and this may be a useful biomarker or therapeutic target in CRC.

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## Effectiveness of a hydroxynaphthoquinone fraction from *Arnebia euchroma* in rats with experimental colitis

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also determined inflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  level by ELISA, Western blotting and immunohistochemistry to explore the potential mechanisms of HM.

**RESULTS:** After intracolonic instillation of TNBS, animals developed colitis associated with soft stool, diarrhea and marked colonic destruction. Administration of HM significantly attenuated clinical and histopathologic severity of TNBS-induced colitis in a dose-dependent manner. It abrogated body weight loss, diarrhea and inflammation, decreased macroscopic damage score, and improved histological signs, with a significant reduction of inflammatory infiltration, ulcer size and the severity of goblet cell depletion (all  $P < 0.05$  vs TNBS alone group). HM could reduce MPO activity. In addition, it also decreased serum TNF- $\alpha$  level and down-regulated TNF- $\alpha$  expression in colonic tissue. This reduction was statistically significant when the dose of HM was 10 mg/kg ( $P < 0.05$  vs TNBS alone group), and the effect was comparable to that of mesalazine and showed no apparent adverse effect. The underlying mechanism may be associated with TNF- $\alpha$  inhibition.

**CONCLUSION:** These findings suggest that HM possesses favourable therapeutic action in TNBS-induced colitis, which provides direct pharmacological evidence for its clinical application.

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**Key words:** *Arnebia euchroma* (Royle) Johnst; Hydroxynaphthoquinones; Inflammatory bowel disease; 2,4,6-trinitrobenzene sulfonic acid-induced colitis; Tumor necrosis factor

**Core tip:** Current therapies for inflammatory bowel disease are limited by lack of effectiveness, drug refractoriness or severe adverse effects. Therefore, there is

### Abstract

**AIM:** To evaluate the potential effectiveness of hydroxynaphthoquinone mixture (HM) in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.

**METHODS:** Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. Disease progression was monitored daily by observation of clinical signs and body weight change. At the end of the experiment, macroscopic and histopathologic lesions of rats were scored, and myeloperoxidase (MPO) activity was determined. We

an urgent need for more effective and safe therapeutic approaches. Due to their favourable effect and less side effects, looking for novel agents from herbal and natural products has been a research focus for a long time. Previous studies demonstrate that hydroxynaphthoquinones exert therapeutic action on chronic inflammatory disease. In this study, hydroxynaphthoquinones showed beneficial effect on 2,4,6-trinitrobenzene sulfonic acid-induced colitis *via* tumor necrosis factor- $\alpha$  inhibition, and the effect was comparable to that of mesalazine. This provides pharmacological evidence for its clinical application.

Fan HY, Zhang ZL, Liu K, Yang MY, Lv WH, Che X, Xu H, Song WW. Effectiveness of a hydroxynaphthoquinone fraction from *Arnebia euchroma* in rats with experimental colitis. *World J Gastroenterol* 2013; 19(48): 9318-9327 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9318.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9318>

## INTRODUCTION

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing inflammatory intestinal disorder<sup>[1]</sup>. The incidence and prevalence of IBD are now increasing worldwide. The highest incidences have been reported in North American and Northern Europe. In recent years, the incidence rates appear to be increasing in developing countries in Europe and Asia with westernisation of lifestyle and industrialization, including China, South Korea and India<sup>[2]</sup>. The exact pathogenesis remains elusive, but thus far, IBD is thought to be the results of interaction between genetic alterations and environment factors that induce an aberrant mucosal immune response, in which inflammatory cytokines play a critical role in the induction of colonic tissue damage<sup>[3,4]</sup>. Available therapies for IBD include conventional anti-inflammatory agents (such as 5-aminosalicylates and corticosteroids), immune modulators and biological therapy. Biological therapy aims at antagonizing pro-inflammatory molecules. Thus, inflammatory cytokines are the most logical targets for IBD treatment. Among various cytokines, tumor necrosis factor (TNF)- $\alpha$  is the cytokine that has been widely studied. Currently, the use of TNF- $\alpha$  blockers is the only licensed biological therapy for IBD and several TNF- $\alpha$  blockers (infliximab, adalimumab and certolizumab) have been applied in clinical practice. Although these available agents have shown clinical benefits to some degree, they are not entirely effective and have multiple adverse effects. Furthermore, IBD management requires long-term treatment that often leads to drug refractoriness or intolerance<sup>[3]</sup>. Patients who are unresponsive to the current therapy still suffer from this common disease. Therefore, it is necessary to develop novel therapeutic approaches.

Zicao, the dried root of *Arnebia euchroma* (Royle) Johnston, is a traditional Chinese herbal medicine. It has been

used in China for thousands of years for the treatment of various diseases<sup>[5,6]</sup>. Naphthoquinones (also termed as hydroxynaphthoquinones in Pharmacopoeia of China) have been identified as the main representative active ingredients of *Zicao*. Hydroxynaphthoquinones mainly consist of alkannin and shikonin as well as their derivatives. Modern pharmacological studies have demonstrated that hydroxynaphthoquinones possess multiple biological activities such as anti-inflammation, wound healing, antibacterial and antifungal<sup>[5-9]</sup>. In particular, shikonin exerts significant anti-arthritis and immunomodulatory effects. It could inhibit the expression and transcriptional activation of TNF- $\alpha$  and reduce the production of inflammatory mediators<sup>[10-12]</sup>. Furthermore, the ointment containing alkannin derivatives has been applied successfully to the treatment of traumatic ulcers and acute anal fissures<sup>[6]</sup>. Based on these characteristics, we hypothesized that hydroxynaphthoquinones may prevent and even cure IBD.

In previous research, our group isolated a mixture of hydroxynaphthoquinone alkannin derivatives and validated its protective effect against experimental arthritis and its analgesic effect, as well as its modulatory effect on the serum TNF- $\alpha$  level<sup>[13,14]</sup>. Chemical analysis identified seven constituents: alkannin, acetylalkannin,  $\beta$ -acetoxyisovalerylalkannin, deoxyalkannin,  $\beta$ , $\beta'$ -dimethylacrylalkannin,  $\alpha$ -methylbutyrylalkannin, and isovalerylalkannin<sup>[14]</sup>. The objective of this study was to investigate the potential therapeutic action of this hydroxynaphthoquinone mixture (HM) in the murine model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley (SD) rats (weight, 200-220 g) were purchased from the Animal Department of the College of Medicine, Beijing University [certificate No. SCXK (Jing) 2006-0008]. All animals were allowed to acclimate for at least 1 wk at a temperature of  $24 \pm 1^\circ\text{C}$  and humidity of  $55\% \pm 5\%$ . All rats were housed in cages with food and tap water *ad libitum*. The experiment procedures were approved by Office of Experimental Animal Management Committee of Shandong Province, China.

### Materials and reagents

HM was provided by Shandong Target Drug Research Co. Ltd. Mesalazine slow-release granule was the product of Ethypharm Industries (France). TNBS was supplied by Sigma Chemical Co. (United States). Polyclonal anti-TNF- $\alpha$  antibody was purchased from Santa Cruz Biotechnology (CA, United States). Anti- $\beta$ -actin antibody was obtained from Beyotime Institute of Biotechnology (Jiangsu Province, China). Rabbit anti-goat IgG was purchased from Boster Biotechnology (Wuhan, Hubei Province, China). Myeloperoxidase (MPO) detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TNF- $\alpha$  ELISA Kit was the



product of R and D System (United States), which was obtained from Shanghai Chuanxiang Biotechnology Co. Ltd (Shanghai, China).

### Induction of colitis and study design

After a 24 h fasting, rats were anaesthetized with pentobarbital, and then TNBS (80 mg/kg, dissolved in 50% ethanol) was instilled into the colon<sup>[15]</sup>. Control rats received saline instead. Animals were orally administered with mesalazine 100 mg/kg and HM (2.5, 5, 10 mg/kg) 24 h after TNBS instillation, daily for 7 d. Body weight and diarrhea of rats were recorded daily. On day 8, rats were anaesthetized with pentobarbital. Blood was collected from the abdominal aorta of rats. The serum was prepared by centrifuging the blood at 3000 g for 15 min for TNF- $\alpha$  assay. The colon was removed and placed on an ice-cold plate, cleared of fat and mesentery, and blotted on filter paper. Then, the colon was longitudinally opened, washed gently with ice-cold saline and blotted dry with filter paper. Finally, colon tissue was weighted. Its length was measured and the extent of macroscopic damage was evaluated. Afterwards, the colon was divided into several segments. One segment of the colon (approximately 2 cm) was used for histological examination. The rest of tissue segments were snap-frozen in liquid nitrogen and stored at -80 °C for MPO activity measurement and Western blotting analysis.

### Macroscopic damage evaluation

Macroscopic colonic damage was assessed using a magnifying glass by an independent observer and was scored. The scale for macroscopic damage ranged from 0-10 and was based on the appearance of ulceration, thickening of the bowel wall, sites of ulceration and sites of inflammation<sup>[16]</sup>.

### Histopathological assessment

The tissue was fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H and E). Colonic damage and inflammation were scored blindly according to the criteria described previously<sup>[17]</sup>.

### Determination of myeloperoxidase activity

The assessment of MPO activity is a well established biochemical assay for quantifying intestinal inflammation<sup>[18]</sup>. The colonic samples (100 mg) were thawed and homogenized on ice in PBS buffer containing hexadecyl trimethyl ammonium bromide to prepare a 5% homogenate. The subsequent assay was performed according to the manufacturers' instructions. The absorbance was read at 460 nm using visible spectrophotometer. MPO activity was determined using the *O*-dianisidine method and the final results were expressed as units per gram of wet tissue.

### TNF- $\alpha$ assay

Serum was obtained from all groups of rats on the final

experimental day and then stored at -20 °C until analysis. The supernatant was used to measure the level of TNF- $\alpha$  with a rat TNF- $\alpha$  ELISA kit. The procedure was performed according to the manufacturer's instructions.

### Immunohistochemical analysis

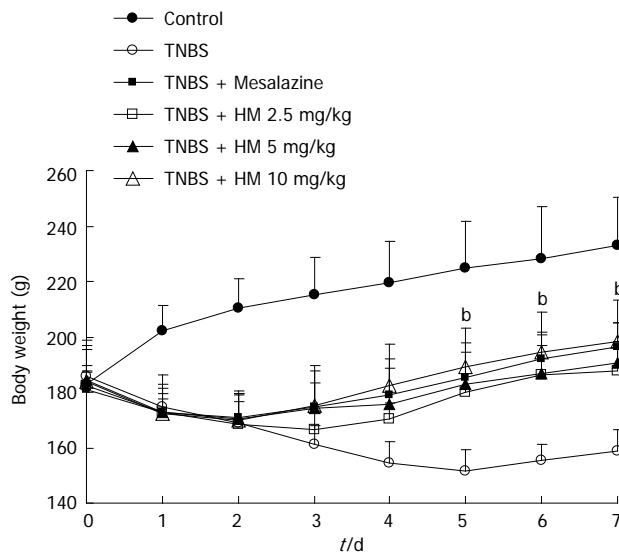
Paraffin-embedded colonic tissue sections (5  $\mu$ mol/L) were fixed in 4% paraformaldehyde, deparaffinized and dehydrated through graded ethanol. The sections were washed three times with PBS for 5 min each, blotted drying, and then treated with 3% hydrogen peroxide for 30 min at room temperature to block the endogenous peroxidase activity. Afterwards, the sections were immersed in antigen retrieval solution (citrate buffer, pH 6.0) for 10 min. This was followed by rinsing with PBS. After blocking with normal goat serum for 30 min at 37 °C, sections were co-incubated with a primary anti-TNF- $\alpha$  antibody (1:150 dilution in PBS) overnight and then with a peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature. Thereafter, the sections were incubated with 3,3-diaminobenzidine (DAB) reagents for 10 min, counterstained with hematoxylin, dehydrated and mounted for microscopy analysis. The intensity of immunoreactivity was examined with a pathological image analyzer and IMAGE-PRO PLUS analyzing program. The results were represented as mean integrated optical density (A) value for each sample.

### Western blotting analysis

Frozen colonic tissues (60 mg) were thawed and later mechanically homogenized on ice. Homogenates were centrifuged at 10000 g for 20 min and total proteins were collected from the supernatant. The protein concentration was measured with a BCA protein assay kit. The protein was boiled in SDS sample loading buffer (Tris-HCl pH 6.8; 10% SDS; 0.5% bromophenol blue; 50% glycerine; 5%  $\beta$ -mercaptoethanol) for 5 min. Then equal amounts of protein (120  $\mu$ g) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis for 60 min, the protein was transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% skim milk and saturated in Tris buffered saline with 1% Tween 20 for 1 h at room temperature. Subsequently, the membrane was incubated with the primary anti-TNF- $\alpha$  antibody (diluted 1:100 in TBST) overnight at 4 °C. After that, the membrane was washed three times for 5 min each and probed with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:10000 in TBST). Protein was detected using an enhanced chemiluminescence (ECL) detection kit (Beyotime Institute of Biotechnology), and bands were visualized by exposure to photographic film. Densitometric analysis of protein bands was performed using Quantity One v4.4.0.36 analyzer software (BIO-RAD).

### Statistical analysis

Statistical analysis was performed using SPSS software 11.5 for windows. All results are expressed as mean  $\pm$



**Figure 1** Treatment with hydroxynaphthoquinone mixture ameliorates body weight loss of 2,4,6-trinitrobenzene sulfonic acid-induced rats. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. Disease progression was monitored by observation of clinical signs and body weight change. Data are represented as mean  $\pm$  SD of 8 animals of each group. <sup>b</sup> $P < 0.01$  vs TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

SD. Comparisons between groups of nonparametric data were made with the Kruskal-Wallis test followed by the Mann-Whitney *U* test. For evaluation of the body weight and TNF- $\alpha$  level, one-way ANOVA followed by Tukey's *post hoc* test was used.  $P < 0.05$  was considered significant.

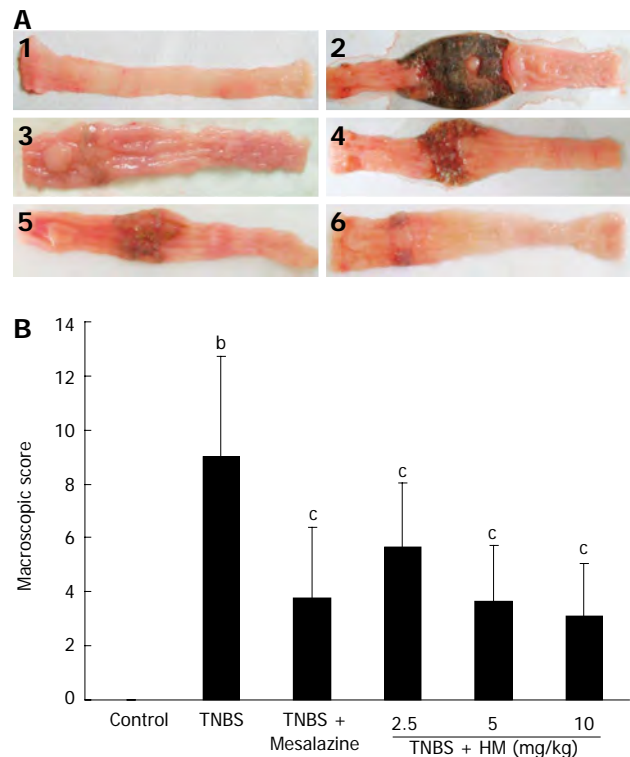
## RESULTS

### HM improves clinical symptoms in rats with TNBS-induced colitis

After intracolonic instillation of TNBS, animals developed colitis associated with soft stool and diarrhea. All TNBS-treated rats had profound weight loss compared with the weight gain seen in controls (all  $P < 0.01$ ). Rats treated with HM and mesalazine gradually recovered the lost body weight beginning on day 3, accompanied by improving symptoms (Figure 1).

### HM reduces colonic macroscopic damage in rats with TNBS-induced colitis

Control animals showed no colonic damage and the colonic damage score was zero. Rats in the TNBS group displayed hyperemia, thickening of the bowel, necrosis, inflammation, and a large area of ulceration. Moreover, moderate to severe adhesion of the colon to the surrounding organs was also observed. The macroscopic colon damage score was significantly increased. The severity of colonic destruction was markedly ameliorated after oral administration of HM and mesalazine, with reduced area of inflammation and ulcer (Figure 2).



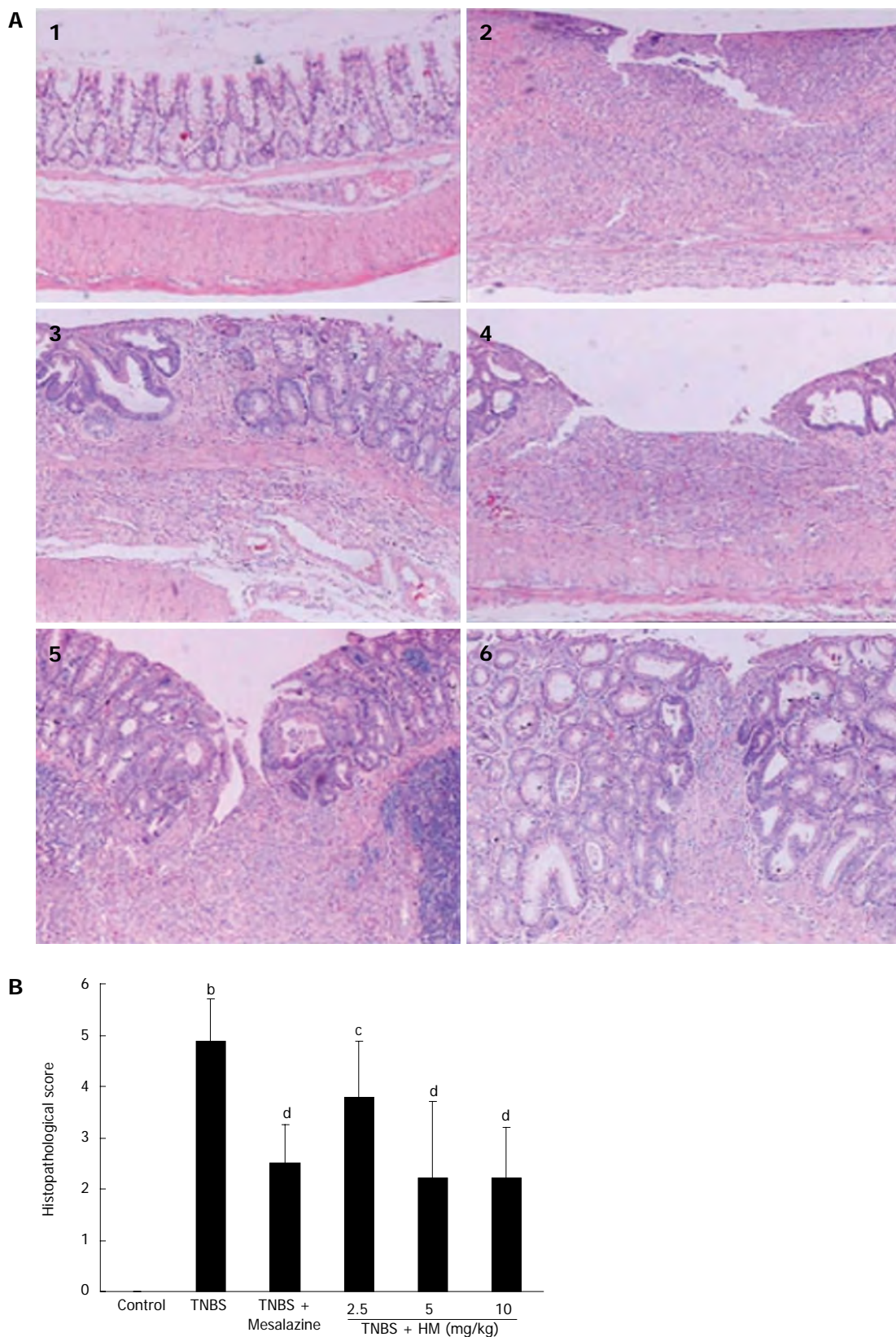
**Figure 2** Hydroxynaphthoquinone mixture reduces colonic macroscopic damage in rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. A: Intestinal macroscopic changes; B: Macroscopic pathological scores. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. 1: Control; 2: Rat treated with TNBS alone; 3: Rat treated with TNBS and mesalazine; 4-6: Rats treated with TNBS and HM (2.5, 5, 10 mg/kg). Data are represented as mean  $\pm$  SD of 8 animals of each group. <sup>b</sup> $P < 0.01$  vs control; <sup>c</sup> $P < 0.05$  vs TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

### HM prevents TNBS-induced histopathological change

There was no histopathological change in the colons of control rats. Histologic evaluation of the colon of TNBS-treated rats showed transmural inflammation involving all layers of the bowel. The inflammatory process was associated with patchy ulceration, epithelial cell loss, pronounced depletion of goblet cells, distortion of the tubular glands, numerous inflammatory cell infiltrations, and dilated crypts. When rats were administered with HM, these histologic signs were much improved, with significant reduction of inflammatory infiltration and ulcer size. The transmural involvement of the lesions was reduced, and the goblet cell depletion was less severe (Figure 3).

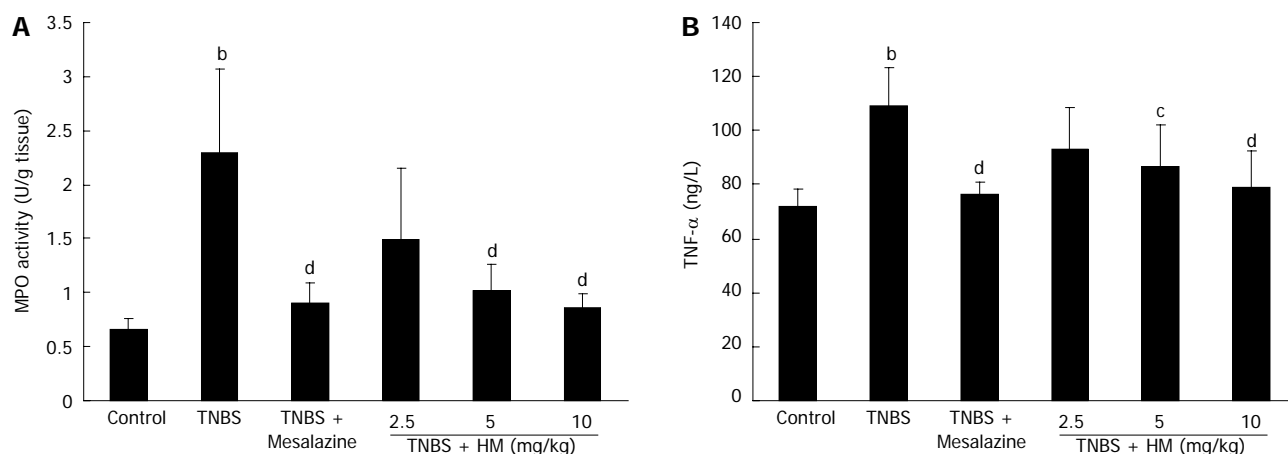
### HM reduces MPO activity in rats with TNBS-induced colitis

Myeloperoxidase in the intestine is a well-known enzyme that is directly correlated with the degree of neutrophil infiltration<sup>[18]</sup>. As shown in Figure 4A, MPO activity was markedly increased in colonic tissue following TNBS instillation compared to controls ( $P < 0.01$ ,  $2.29 \pm 0.78$  U/g *vs*  $0.65 \pm 0.11$  U/g tissue). After treatment with HM,



**Figure 3** Hydroxynaphthoquinone mixture prevents 2,4,6-trinitrobenzene sulfonic acid-induced histopathological changes. **A:** Intestinal histopathological features; **B:** Pathological scores of representative colonic samples of each group. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. Histopathological analysis was performed in HE-stained sections of colons. 1: Control; 2: Rat treated with TNBS alone; 3: Rat treated with TNBS and mesalazine; 4-6: Rats treated with TNBS and HM (2.5, 5, 10 mg/kg). Data are represented as mean  $\pm$  SD of 5 animals of each group. <sup>b</sup> $P < 0.01$  vs control; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs TNBS alone. HM: Hydroxynaphthoquinone mixture (original magnification,  $\times 100$ ); TNBS: 2,4,6-trinitrobenzene sulfonic acid.





**Figure 4** Hydroxynaphthoquinone mixture decreases myeloperoxidase activity (A) and serum tumor necrosis factor- $\alpha$  level (B) in rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. TNF- $\alpha$  level was determined by ELISA. Data are represented as mean  $\pm$  SD of 8 animals of each group. <sup>b</sup> $P < 0.01$  vs control; <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.01$  vs TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid; MPO: Myeloperoxidase; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

MPO activity were reduced to be  $1.49 \pm 0.66$ ,  $1.02 \pm 0.24$  and  $0.86 \pm 0.13$  U/g tissue for HM 2.5, 5 and 10 mg/kg, respectively. The effect was significant at doses of 5 and 10 mg/kg. This indicated that treatment with HM attenuated the degree of inflammation response. The similar efficacy for mesalazine was also observed.

#### HM decreases serum TNF- $\alpha$ level in rats with TNBS-induced colitis

As depicted in Figure 4B, serum TNF- $\alpha$  level was obviously higher in TNBS-treated rats compared with that in controls. In contrast, pretreatment with HM and mesalazine prevented the increase in TNF- $\alpha$  level. The mean serum TNF- $\alpha$  levels were determined to be  $71.90 \pm 6.33$  ng/L for the control group,  $109.05 \pm 14.30$  ng/L for the TNBS group,  $76.16 \pm 4.64$  ng/L for the mesalazine group,  $93.00 \pm 15.24$  ng/L for the HM 2.5 mg/kg group,  $86.96 \pm 15.26$  ng/L for the HM 5 mg/kg group and  $78.72 \pm 13.94$  ng/L for the HM 10 mg/kg group.

#### HM attenuates immunostaining for TNF- $\alpha$ in rats with TNBS-induced colitis

Representative samples of each group were immunohistochemically stained for the expression of TNF- $\alpha$ . As shown in Figure 5A1, colonic section obtained from the control group showed negative staining. TNBS-treated rats showed strongly positive staining for TNF- $\alpha$ . Positively stained cells appeared in the mucosal and submucosal inflammatory cells (Figure 5A2 and B). Administration of HM attenuated the degree of TNF- $\alpha$  staining in the colon tissue. Significantly less positive cells were observed in tissues from HM 5 mg/kg- and 10 mg/kg-treated rats than in those from TNBS-treated rats (Figure 5A4, A5 and Figure 5B).

#### HM decreases TNF- $\alpha$ expression in colonic tissue of rats with TNBS-induced colitis

As shown in Figure 6, higher TNF- $\alpha$  expression was ob-

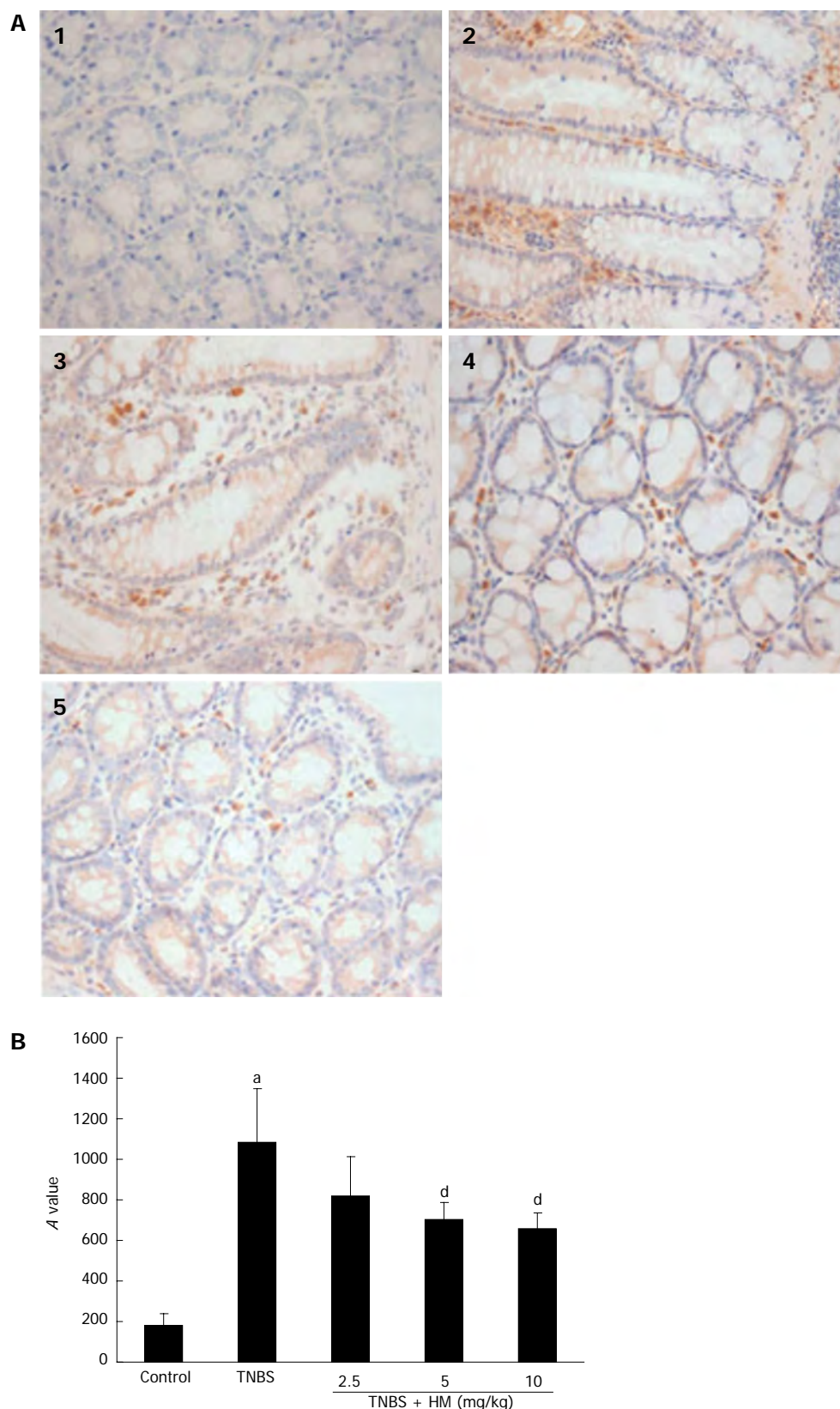
served after TNBS instillation as compared with controls. HM treatment reduced the expression of TNF- $\alpha$  in a dose-dependent manner.

## DISCUSSION

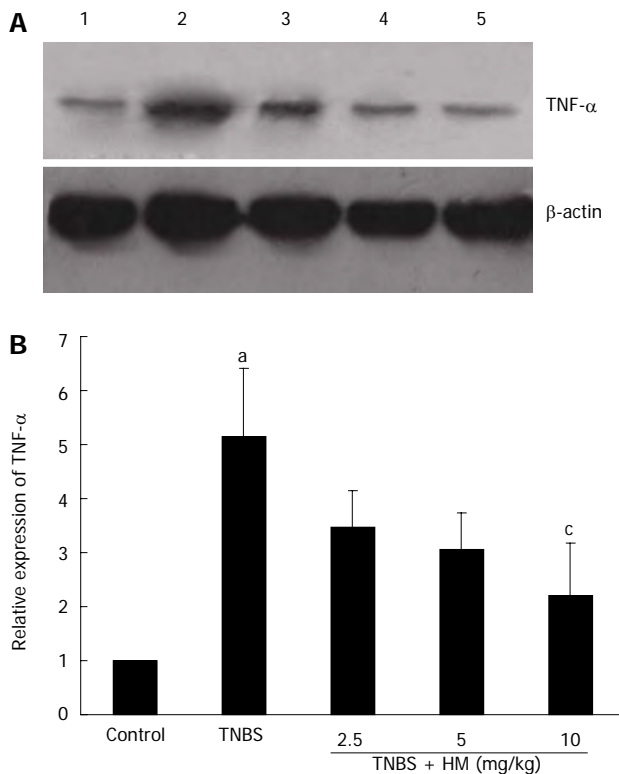
Many plant-derived extracts or chemicals have pharmacological effects and clinical benefits, which provide a great potential in improving the symptoms of IBD. The published literature has revealed that several natural products exhibit encouraging anti-IBD activity by inhibition of cytokine production, such as flavonoids or polyphenolic compounds<sup>[19]</sup>. TNBS-induced colitis is a Th1 cell-mediated inflammatory disease, associated with excessive secretion of cytokines as a consequence of exaggerated macrophage and neutrophil infiltration and activation, giving rise to transmurally inflamed intestinal mucosa, which displays clinical, biochemical, and pathological similarities to human CD. It is a commonly used experimental model system to test potential therapeutic agents<sup>[20]</sup>. Thus, the present study was performed to investigate the potential effect of HM on CD using TNBS-induced colitis.

The results obtained from the study demonstrate for the first time the efficacy of HM in intestinal inflammation, confirming the hypothesis mentioned in the introduction section that hydroxynaphthoquinones possess the ability to attenuate symptoms of IBD. Administration of HM at the onset of the disease ameliorated the clinical severity of the wasting disease, abrogating body weight loss, diarrhea, inflammation and the area of ulceration. This beneficial effect was further evidenced by histological evaluation, with a marked reduction in the extent and severity of inflamed tissue damage and infiltration of inflammatory cells. The beneficial effect was also established by a decrease in MPO activity. As mentioned above, MPO is a marker of neutrophil infiltration. It has been reported that MPO activity is increased in several





**Figure 5** Immunostaining for tumor necrosis factor- $\alpha$ . A: Immunostaining sections; B: Integrated optical density (A) values of representative colonic samples of each group. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) 24 h after TNBS instillation. Colons were excised and stained with anti-TNF- $\alpha$  antibody. 1: Control; 2: Rat treated with TNBS alone; 3-5: Rats treated with TNBS plus HM (2.5, 5, 10 mg/kg). Data are represented as mean  $\pm$  SD of 4 animals of each group. <sup>a</sup> $P$  < 0.05 vs control; <sup>d</sup> $P$  < 0.01 vs TNBS alone. HM: Hydroxynaphthoquinone mixture (original magnification,  $\times$  400); TNBS: 2,4,6-trinitrobenzene sulfonic acid; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .



**Figure 6** Hydroxynaphthoquinone mixture decreases tumor necrosis factor- $\alpha$  expression in colonic tissue of rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. A: Tumor necrosis factor (TNF)- $\alpha$  protein bands; B: Integrated optical density (A) values of protein bands. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) 24 h after TNBS instillation. Protein extracts were obtained from colons and TNF- $\alpha$  expression level was detected by Western blotting analysis. Lane 1: Controls; lane 2: Rats treated with TNBS alone; lane 3-5: Rats treated with TNBS plus HM (2.5, 5, 10 mg/kg). Data are represented as mean  $\pm$  SD of 4 animals of each group. <sup>a</sup> $P < 0.05$  vs control; <sup>b</sup> $P < 0.05$  vs TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

experimental colitis models, including TNBS-induced colitis<sup>[18]</sup>, and it is widely used to quantify intestinal inflammation and assess the degree of inflammation. Thus, a reduction of MPO activity can be interpreted as a manifestation of the anti-inflammatory effect of a given compound<sup>[18,21,22]</sup>. Inhibition of MPO activity by HM was consistent with the results observed in the histological examination, in which the level of inflammatory cell infiltration in colonic tissues was lower in HM-treated animals than in TNBS-treated rats.

Mesalazine was used as a reference in this study. Several reports suggest that mesalazine acts locally in the colon. After intragastric administration of mesalazine, it is absorbed from intestinal lumen and concentrates in the mucosa. The effectiveness of the drug directly depends on its mucosal concentration<sup>[23,24]</sup>. In the present experiment, oral administration with mesalazine 100 mg/kg resulted in a significant improvement in intestinal lesions, which was consistent with previous findings<sup>[24,25]</sup>. The effect of HM 10 mg/kg was comparable to that of mesalazine.

The anti-inflammatory effect and protection against tissue injury exerted by HM was further confirmed by the

down-regulation of TNF- $\alpha$  in serum and colonic tissue. TNF- $\alpha$  is an important inflammatory mediator and plays a key role in the colonic damage. Among the various cytokines involved in the pathogenesis of TNBS colitis, TNF- $\alpha$  appears to be a key regulator since it has been reported that TNBS-induced colitis could not be induced in TNF- $\alpha$ -deficient mice and is far more severe in mice that over-express this inflammatory cytokine<sup>[26]</sup>. Different agents interfering with TNF- $\alpha$  signaling have been successful in the treatment of subsets of CD patients, and display favourable therapeutic effect, particularly TNF- $\alpha$  blockers<sup>[27]</sup>. Moreover, the majority of previous preclinical studies and current therapeutic approaches<sup>[27-29]</sup> have confirmed the idea that therapy that can address an essential element of a final common pathologic pathway participating in IBD could potentially treat this disease<sup>[30]</sup>. Therefore, we assessed the impact of HM on TNF- $\alpha$  signaling and explored the underlying mechanism. The results obtained from the present study showed that HM significantly lowered TNF- $\alpha$  level in serum and reduced TNF- $\alpha$  expression in colonic tissue. This indicates that the preventive effect of HM against TNBS-induced colonic injury is directly or indirectly related to its TNF- $\alpha$  inhibition. However, given that many other signaling pathways and transcription factors are involved in the regulation of TNF- $\alpha$  activity, additional research is needed to examine the exact mechanisms of action of HM.

In summary, the present study demonstrates that treatment with HM attenuates the clinical symptoms of TNBS-induced colitis, resulting in significant histological improvement, reduced MPO activity and TNF- $\alpha$  level in serum, and down-regulation of TNF- $\alpha$  expression in colonic tissue. The underlying mechanism of action of HM may be associated with TNF- $\alpha$  inhibition. These results provide supporting evidence for the clinical application of HM.

## COMMENTS

### Background

The incidence and prevalence of inflammatory bowel disease (IBD) are increasing at a disturbing rate worldwide, and now it has become a global disease. Current therapies for IBD are limited by lack of effectiveness, drug refractoriness or severe adverse effects. Therefore, there is an urgent need for more effective and safe therapeutic approaches.

### Research frontiers

Looking for novel agents from herbal or natural products has been a research focus for a long time. Many plant-derived extracts or chemicals have pharmacological effects and clinical benefits. The published literature has revealed that several natural products exhibit encouraging anti-IBD activity by inhibition of cytokine production, such as flavonoids or polyphenolic compounds. Hydroxynaphthoquinones have been identified as the main active ingredients of *Zicao*, a traditional Chinese herbal medicine, and possess potent anti-inflammatory, wound healing and antibacterial activities. Yet no studies have evaluated the therapeutic effect of hydroxynaphthoquinones on treating inflammatory intestinal diseases so far. In this study, the authors isolate a hydroxynaphthoquinone mixture (HM) from *Zicao*, which mainly contains seven alkannin derivatives, and demonstrate that HM showed beneficial effect on TNBS-induced colitis.

### Innovations and breakthroughs

This is the first study to report that hydroxynaphthoquinones exert therapeutic

effect on mucosal inflammation by modulating tumor necrosis factor- $\alpha$ , and the effect of hydroxynaphthoquinones is comparable to that of mesalazine, a classical anti-IBD drug.

### Applications

Elucidation of the effect and mechanism of action of hydroxynaphthoquinone mixture (HM) in the treatment of inflammatory intestinal disease provides pharmacological evidence for its further development as a candidate for treating IBD, and for its clinical application.

### Peer review

This study describes the beneficial effects of HM from *Arnebia euchroma* in an experimental model of colitis in rats. It revealed that HM significantly attenuated the severity of mucosal lesions. The authors have made a complex and interesting study.

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## Latent hepatitis B is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C

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### Abstract

**AIM:** To study the potential association between hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC), cirrhosis and latent hepatitis B (LHB) infection, defined as the absence of detectable serum hepatitis B surface antigen (HBsAg) and the presence of hepatitis B core antibody (HBcAb).

**METHODS:** This retrospective analysis is comprised of 185 cirrhotic patients with HCC who were hepatitis C virus antibody (HCV Ab) (+) and HBsAg(-) at Wayne State University between 1999 and 2008. From these, 108 patients had HCV polymerase chain reaction confirmation of viremia while the remaining (77) were considered to have CHC on the basis of a positive HCV Ab and the absence of any other cause of liver disease. Controls were drawn from our institutional database from the same time period and consisted of 356 HBsAg(-) age, race and gender matched patients with HCV RNA-confirmed CHC and without evidence of HCC. A subgroup of controls included 118 matched patients with liver cirrhosis.  $\chi^2$  test and *t* test were used for data analysis.

**RESULTS:** Seventy-seven percent of patients in all 3 groups were African Americans. Patients with HCC

had a significantly higher body mass index ( $P = 0.03$ ), a higher rate of co-infection with human immunodeficiency virus (HIV) ( $P = 0.05$ ) and a higher prevalence of alcohol abuse ( $P = 0.03$ ) than the controls. More patients with HCC had LHB than controls (78% vs 39%,  $P = 0.01$ ). Sixty three percent of patients with HCC were both hepatitis B surface antigen (HBsAb)(-) and HBcAb(+) compared to 23% of controls ( $P < 0.01$ ). When compared to cirrhotic controls, the frequency of HBcAb(+) remained higher in patients with HCC (78% vs 45%,  $P = 0.02$ ). Patients with HCC were more likely to be both HBsAb(-) and HBcAb(+) than the cirrhotic controls (63% vs 28%,  $P = 0.01$ ). Although not statistically significant, 100% of CHC and HIV co-infected patients with HCC ( $n = 11$ ) were HBcAb(+) when compared to controls (44%;  $n = 9$ ).

**CONCLUSION:** These data suggest that LHB occurs at a significantly increased frequency in patients with CHC and HCC than in patients with CHC without HCC.

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**Key words:** Hepatocellular carcinoma; Chronic hepatitis C; Latent hepatitis B; Hepatitis C virus

**Core tip:** Latent hepatitis B (LHB) has recently received significant attention among researchers and clinicians managing chronic liver disease. It is defined as a combination of hepatitis B surface antigen negative and hepatitis B core antibody positive. The potential association of LHB with hepatocellular carcinoma among patients with chronic hepatitis C infection has been studied and reported in this manuscript.

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## INTRODUCTION

Emerging data suggest that the mortality rate in cirrhotic patients with hepatocellular carcinoma (HCC) is rising whereas the mortality rate from other complications of cirrhosis is either stable or declining<sup>[1]</sup>. In the United States, chronic hepatitis C (CHC) accounts for the majority of cases of HCC. Among patients with CHC, factors such as older age, male gender, severity of liver disease, metabolic syndrome and poor response to interferon therapy are established risk factors for hepatocarcinogenesis<sup>[1]</sup>. “Latent hepatitis B (LHB)”, defined as the presence of detectable hepatitis B core antibody (HBcAb) with undetectable hepatitis B surface antigen (HBsAg)(-) in serum and usually with detectable HBV DNA in hepatocytes, has not been studied as a risk factor for HCC in the United States<sup>[2]</sup>. Patients with previous exposure to hepatitis B virus but with no evidence of chronic infection are HBsAg(-) and HBcAb(+). This finding alone is now considered as unrecognized LHB<sup>[3]</sup>. In a large study, the majority of patients with LHB had detectable hepatitis B DNA (HBV DNA) in serum as well as in liver tissue<sup>[4]</sup>. Various other studies have also confirmed the same findings<sup>[5,6]</sup>. This led to the identification of a unique group of patients who are HBcAb(+) and at risk for latent hepatitis B.

Early studies from the 1990s suggested that patients with HCC in the absence of chronic hepatitis B and C had detectable covalent closed circular hepatitis B DNA (ccc DNA) in liver parenchyma although they were HBsAg(-) in serum. These patients were considered to have “occult hepatitis B”<sup>[7]</sup>. A single prospective study by Squadrito *et al.*<sup>[8]</sup> revealed that among HBsAg(-) patients with CHC, patients with occult hepatitis B with ccc DNA in liver biopsy specimens were at a higher risk for the development of HCC. With the availability of highly sensitive real-time polymerase chain reaction (PCR) assays for the measurement of HBV DNA, tissue analysis for HBV DNA is largely unnecessary to make a diagnosis of latent hepatitis B<sup>[2]</sup>. Patients with cirrhosis from alcoholic and non-alcoholic fatty liver disease are also at a significantly higher risk for developing HCC when associated with LHB particularly in those who were HBcAb(+) but HBsAg(-)<sup>[9]</sup>. Injection drug users, patients on hemodialysis, patients with CHC and human immunodeficiency virus (HIV)-infected patients are at increased risk for LHB<sup>[10]</sup>. In patients with CHC, occult hepatitis B seems to be associated with rapid progression of liver disease<sup>[11]</sup>. Studies from areas with high prevalence of chronic hepatitis B have associated occult hepatitis B with HCC among patients with CHC<sup>[12,13]</sup>. This association is much stronger among CHC patients who are non-responders to currently available therapy<sup>[14]</sup>. Another study reported that although occult hepatitis B may not have a significant impact on response of CHC to interferon, it does increase

the risk for HCC among non-responders but not among responders<sup>[15]</sup>.

A large multicenter Japanese study concluded that CHC patients with LHB are at a significantly higher risk for developing HCC<sup>[16]</sup>. In the same study, interferon was less effective in preventing HCC in patients with LHB when compared to those without evidence of previous HBV exposure. This association was independent of the presence of HBV DNA in serum and therefore, LHB is clinically and prognostically more relevant than serum DNA status.

The above referenced studies establishing LHB as a risk factor for development of HCC are from countries with high endemicity for chronic hepatitis B infection. We studied this potential association among predominantly African American patients with CHC and cirrhosis in an area with low endemicity for chronic hepatitis B.

## MATERIALS AND METHODS

This retrospective study, included patients with CHC who were diagnosed with HCC between January 1999 and December 2008 at the Detroit Medical Center, Detroit, Michigan. The primary sites were the Wayne State University Gastroenterology clinic and the Department of Pathology at Harper University Hospital in Detroit. Patients with a diagnosis of HCC who were > 18 years old, hepatitis C Ab(+) and HBsAg(-) were included in our study. The diagnosis of HCC was made either by histopathology or by non-invasive criteria *i.e.*, alpha fetoprotein (AFP) > 200 ng/mL and a mass lesion in the liver with radiological features typical for HCC observed on two or more imaging modalities (European Association for the Study of the Liver, EASL criteria)<sup>[17]</sup>. The study group consisted of both inpatients and outpatients although the majority were outpatients.

A control comparison group consisted of patients with CHC who were HBsAg(-) without evidence of HCC. These patients were drawn from our institutional database of CHC patients. They were age, race and gender matched to the cases and were from the same time period. Patients with HIV and CHC coinfection were included in the study and were part of a subset analysis. The study was approved by the Institutional Review Board at Wayne State University and the Detroit Medical Center.

A total of 185 patients fulfilled the selection criteria and were evaluated for inclusion in the study. Of these, 108 had serum RNA confirmation of CHC by PCR, while CHC viremia was presumed in the remaining 77. A total of 356 matched (1:2) non-HCC controls with CHC were selected from our database. All controls had PCR confirmation of CHC, were HBsAg(-) and were selected from the same time period. Non HCC controls included 118 matched patients with cirrhosis diagnosed by histopathology or by clinical criteria. Since the majority of patients with CHC who develop HCC have underlying advanced liver disease<sup>[10]</sup>, a selected sub-group of matched

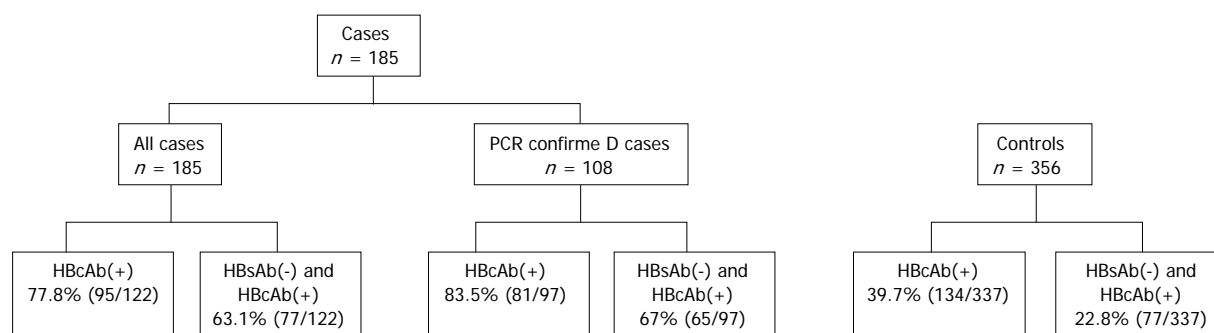


Figure 1 Hepatitis B serology in cases and controls. HBcAb: Hepatitis B core antibody; HBsAb: Hepatitis B surface antigen; PCR: Polymerase chain reaction.

**Table 1** Baseline characteristics of hepatocellular carcinoma cases

	HCV Ab(+), HCV RNA(+) (n = 108)	HCV Ab(+) (n = 77)	P value
Age (yr), mean $\pm$ SD	60.88 $\pm$ 8.6	59.42 $\pm$ 7.4	0.88
Males	73.10%	67.53%	0.32
African American	75.90%	72.70%	0.22
	n = 70	n = 52	0.20
BMI (kg/m <sup>2</sup> )	29.56 $\pm$ 6.11	27.88 $\pm$ 5.2	
HIV coinfection	10.20%	7.60%	0.16
Heavy alcohol use	37.03%	48.05%	0.04

HCV Ab: Hepatitis C virus antibody; HCV DNA: Hepatitis C DNA; BMI: Body mass index; HIV: Human immunodeficiency virus.

controls with cirrhosis and CHC was utilized in the analysis (Figure 1). In addition to demographic data, alcohol intake was assessed by chart review. Patients were classified into three categories based on alcohol consumption. Those who consumed 1 to 2 servings of liquor or wine a week or  $\leq 6$  beers (12 oz) a week were categorized as “mild drinkers”. Patients with alcohol consumption that exceeded this amount were considered “heavy drinkers”. The third group was designated as “non drinkers”. Lab values closest to the date of the diagnosis of HCC were collected. In controls, lab values at the time of their initial evaluation were recorded.

### Statistical analysis

Data was analyzed using SPSS version 12.  $\chi^2$  test was used to analyze nominal data while *t* test was used to compare means among groups. Univariate analysis was then performed after controlling for covariates in the final analysis. Among cases, baseline characteristics were compared in patients with PCR confirmation of CHC and in those without PCR confirmation (Table 1). Since these groups were identical in baseline characteristics, they were combined for subsequent analysis. Additionally, subset analysis of African-American patients, patients with PCR confirmation of CHC, and cirrhotic patients was performed.

## RESULTS

The mean age of patients with HCC was 60 years, and

71% were male (Table 2). More than seventy five percent of patients in each group were African-American. HCC was diagnosed by biopsy in 129 patients and by non-invasive (EASL) criteria in the remainder. Patients with HCC had a significantly higher body mass index (BMI), AFP, aspartate aminotransferase, alanine aminotransferase and a more prolonged prothrombin time (PT), but they had a lower albumin and platelet count. HIV-HCV coinfection was seen more commonly in patients with HCC (8.1%) than in controls (2.5%,  $P = 0.05$ ). While mild alcohol consumption was not different in both groups, patients with HCC were more likely to be heavy drinkers (42% *vs* 27%; Table 2). Furthermore, HCV patients without HCC were more likely to be non-drinkers (32%) compared to patients with HCC (11%,  $P < 0.01$ ; Table 2).

HBcAb was positive in 78% of patients with HCC but in only 40% of controls ( $P = 0.01$ ). When hepatitis B surface antibody (HBsAb) status was determined, 63% of HCC cases were both HBsAb(-) and HBcAb(+) as compared to only 23% of controls ( $P < 0.01$ ). When analysis was restricted to patients with cirrhosis, the prevalence of HBcAb was higher in cirrhotic controls at 42%, and the combination of HBsAb(-) and HBcAb(+) was also more prevalent when compared to total controls (27.6% *vs* 63.1%,  $P < 0.01$ ). Despite this difference in prevalence of HBsAb and HBcAb among control groups, overall prevalence remained significantly higher in patients with HCC (63.1% *vs* 22.8%). Although statistical significance was not achieved, 100% of HIV-HCV coinfecting patients with HCC (44.4%) were HBcAb(+) when compared to  $< 50\%$  among coinfecting controls.

Univariate analysis predicting HCC showed that HBcAb(+) status had an odds ratio of 1.9 (95%CI: 1.28-3.04,  $P = 0.02$ ) where as a combination of HBsAb(-) and HBcAb(+) had a higher odds ratio of 3.24 (95%CI: 2.28-4.62,  $P < 0.01$ ). In this analysis, BMI, albumin, PT, alcohol consumption and HIV coinfection were identified covariates. When regression analysis was performed after controlling for these covariates, these odds ratios were 1.84 (95%CI: 1.22-3.08,  $P = 0.01$ ) and 2.98 (95%CI: 2.12-5.08,  $P < 0.01$ ) respectively. Analysis of cirrhotic patients when controlled for covariates showed that HBcAb(+) had an odds ratio of 1.66 and a combination of HBsAb(-) and HBcAb(+) was at 2.10 (95%CI: 2.12-4.04,  $P < 0.01$ ).

**Table 2** Comparison of variables in cases and controls

	CHC with HCC ( <i>n</i> = 185)	CHC without HCC ( <i>n</i> = 356)	<i>P</i> value
Age (yr), mean ± SD	60.3 ± 9.71	59.72 ± 9.2	
Males	70.80%	71.10%	
Race AA	74.60%	78.70%	
CAU	21.60%	18.50%	
Other	3.80%	2.80%	
BMI (kg/m <sup>2</sup> )	<i>n</i> = 122	<i>n</i> = 320	
	28.8 ± 6.01	27.26 ± 5.9	
Albumin	<i>n</i> = 178	<i>n</i> = 289	
	2.67 ± 0.7	3.88 ± 0.6	< 0.01
PT (s)	<i>n</i> = 171	<i>n</i> = 242	
	16.69 ± 8.6	11.57 ± 2.5	< 0.01
AFP (ng/mL)	<i>n</i> = 163	<i>n</i> = 284	
	99035.1 ± 263605.5	21.12 ± 90.4	
Platelets	<i>n</i> = 172	<i>n</i> = 337	
	176.5 ± 127	202.98 ± 83.4	0.04
ALT (IU/L)	<i>n</i> = 166	<i>n</i> = 345	
	263.8 ± 518.2	78.19 ± 58.3	< 0.01
AST (IU/L)	<i>n</i> = 160	<i>n</i> = 324	
	283.8 ± 63.5	75.2 ± 55.9	< 0.01
HIV co-infection	8.10%	2.50%	0.05
Alcohol			
Mild	29.7%	27.50%	NS
Heavy	41.60%	27%	0.03
Non drinkers	10.80%	31.70%	< 0.01

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HIV: Human immunodeficiency virus; BMI: Body mass index; PT: Prothrombin time; AFP: Alpha feto-protein; NS: Not significant.

Subset analysis of African-American patients with cirrhosis when controlled for covariates resulted in an odds ratio of 2.08 (95%CI: 1.42-3.60, *P* < 0.01) when HBcAb was positive and 2.58 (95%CI: 1.82-4.44, *P* < 0.01) when HBsAb was negative in addition to a positive HBcAb.

## DISCUSSION

In this study, HCC and advanced liver disease, shown by higher transaminases, lower albumin and platelet count and prolonged prothrombin time were associated with increased frequency of HBcAb(+) among patients with CHC. Previous studies have also noted an increased association of HBcAb(+) with advanced liver disease and HCC<sup>[1,11,12,14,16,18]</sup>. However, these data, as well as a prospective study<sup>[16]</sup>, originated from regions of relatively high prevalence for both chronic hepatitis B and HCC. Our study presents findings from an area of relatively low endemicity in a population comprised predominantly of urban African-Americans, yet the prevalence of HBcAb(+) was even higher in our study (74%) than previously reported<sup>[11,14]</sup>. Multivariate analysis restricted to this group of patients revealed a much stronger association of LHB with HCC (Table 3). Our study included a total of 418 African-American patients and is by far the largest analysis studying this association in a select group.

Certain potential limitations to our study need further discussion. A majority of patients with HCC (66%) were diagnosed by histopathology. This is largely due to inclusion of patients before the EASL non-invasive criteria

**Table 3** Univariate analysis predicting the following

	OR (95%CI)	<i>P</i> value
HCC in patients with CHC		
HBcAb(+)	1.90 (1.28-3.04)	0.02
HBsAb(-) and HBcAb(+)	3.24 (2.28-4.62)	< 0.01
HCC in cirrhotic patients with CHC		
HBcAb(+)	1.54 (1.18-2.54)	0.02
HBsAb(-) and HBcAb(+)	2.14 (1.68-3.82)	0.01
HCC in patients with CHC when controlled for covariates <sup>1</sup>		
HBcAb(+)	1.84 (1.22-3.08)	0.01
HBsAb(-) and HBcAb(+)	2.98 (2.12-5.08)	< 0.01
HCC in cirrhotic patients with CHC when controlled for covariates <sup>1</sup>		
HBcAb(+)	1.66 (1.22-3.24)	0.01
HBsAb(-) and HBcAb(+)	2.10 (1.72-4.04)	< 0.01
HCC in cirrhotic African American patients with CHC when controlled for covariates <sup>1</sup>		
HBcAb(+)	2.08 (1.42-3.6)	< 0.01
HBsAb(-) and HBcAb(+)	2.58 (1.82-4.44)	< 0.01

<sup>1</sup>Body mass index, albumin, prothrombin time, human immunodeficiency virus co-infection and alcohol consumption. HCC: Hepatocellular carcinoma; CHC: Chronic hepatitis C; HBcAb: Hepatitis B surface antibody; HBsAb: Hepatitis B core antibody.

for diagnosis of HCC were proposed<sup>[17]</sup>. One could argue that lack of RNA confirmation of CHC viremia in 40% of our cases detracts from our conclusions. However, patients with or without RNA confirmation of HCV viremia had similar baseline characteristics and no other etiology for their liver disease, and were therefore appropriately grouped together. Alcohol consumption is a well established risk factor for HCC among patients with chronic liver disease. In the present study, details pertaining to alcohol consumption were not available in some patients despite extensive review of both inpatient and outpatient medical records. Every effort was made to classify patients based on alcohol consumption when information was available. Controls, who were drawn from a prospectively maintained institutional database were more likely to have reliable information regarding alcohol intake. Nevertheless, in concurrence with previous literature, our results suggest that BMI and alcohol consumption play an important role in the progression to HCC in cirrhotics with CHC.

Although the majority of patients with HCC were drawn from an outpatient setting, some were hospitalized and likely sicker. Controls were selected from a predominantly outpatient database. This may account for the significantly higher AFP and transaminases in the CHC patients with HCC. Although PCR measurement of serum HBV DNA assessment was not accomplished in our patients, we do not consider this as a significant limitation. In previous studies, HBcAb had a stronger association with HCC than serum HBV DNA among patients with CHC<sup>[5,6]</sup>. Latent hepatitis B, defined as a previous exposure to hepatitis B [HBcAb(+) and HBsAg(-)] is a clinically more relevant tool for predicting risk for HCC than is the assessment of HBV DNA in serum or liver tissue.

Another limitation to the study is that the frequency



of smoking, injection drug use and diabetes was not analyzed. Patients with HIV-HCV co-infection have rapid progression of liver disease and development of HCC. Liver disease is the leading cause of mortality in these patients<sup>[19-21]</sup>. Of 20 patients with HIV-HCV coinfection, 11 patients had HCC and all 11 were HBcAb(+) compared to 40% among coinfecting controls. Although this difference did not achieve statistical significance, due to low numbers, this raises the intriguing observation that HIV-HCV coinfecting patients with HCC may have an increased frequency of LHB.

Our study is consistent with previous studies from areas with high prevalence of hepatitis B that suggests that LHB increases the risk for HCC. Furthermore, patients sero-negative for HBsAb are at even higher risk, which may suggest longer time since acquisition of HBV. Our data also suggest that African-Americans have a greater risk of HCC when associated with LHB. There is additional data from our institution (preliminary) to support the observation that patients with CHC and LHB are more likely to have advanced liver disease and respond poorly to Interferon-based therapies. The current study takes this concept further by associating LHB with HCC.

In conclusion, LHB occurs at a significantly increased frequency in patients with CHC and HCC than in patients with CHC without HCC. This association is even stronger in African Americans. It is important to recognize this risk during surveillance for HCC in these patients.

## COMMENTS

### Background

Patients with chronic hepatitis C (CHC) and cirrhosis have an increased risk of developing hepatocellular carcinoma (HCC). Several risk factors for this progression have so far been identified. The authors studied the potential association between HCC in patients with CHC, cirrhosis and latent hepatitis B (LHB) infection, defined as the absence of detectable serum hepatitis B surface antigen (HBsAg) and the presence of hepatitis B core antibody (HBcAb).

### Research frontiers

LHB has recently received significant attention among researchers and clinicians managing chronic liver disease. It is defined as a combination of HBsAg(-) and HBcAb(+). The potential association of LHB with HCC among patients with chronic CHC has been studied and reported in this manuscript.

### Innovations and breakthroughs

Interestingly, subset analysis among human immunodeficiency virus-CHC co-infected patients showed a 100% association of HCC with LHB suggesting much higher association in this group. This identifies a unique group of CHC patients at much higher risk for development of HCC.

### Peer review

Risk factors for HCC in patients with chronic hepatitis C are only partially understood. Here the authors showed that there is a clear association between latent hepatitis B infection and HCC development in an area with low endemicity for chronic hepatitis B.

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## Inhibitor of differentiation proteins do not influence prognosis of biliary tract cancer

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### Abstract

**AIM:** To investigate the expression and clinical relevance of inhibitor of differentiation (ID) proteins in biliary tract cancer.

**METHODS:** ID protein expression was analyzed in 129 samples from patients with advanced biliary tract cancer (BTC) (45 extrahepatic, 50 intrahepatic, and 34

gallbladder cancers), compared to normal controls and correlated with clinical and pathological parameters.

**RESULTS:** ID1-3 proteins are frequently overexpressed in all BTC subtypes analyzed. No correlation between increased ID protein expression and tumor grading, tumor subtype or treatment response was detected. Survival was influenced primary tumor localization (extrahepatic *vs* intrahepatic and gall bladder cancer, OS 1.5 years *vs* 0.9 years *vs* 0.7 years,  $P = 0.002$ ), by stage at diagnosis (OS 2.7 years in stage I *vs* 0.6 years in stage IV,  $P < 0.001$ ), resection status and response to systemic chemotherapy. In a multivariate model, ID protein expression did not correlate with clinical prognosis. Nevertheless, there was a trend of shorter OS in patients with loss of cytoplasmic ID4 protein expression ( $P = 0.076$ ).

**CONCLUSION:** ID protein expression is frequently deregulated in BTC but does not influence clinical prognosis. Their usefulness as prognostic biomarkers in BTC is very limited.

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**Key words:** Biliary tract cancer; Cholangiocarcinoma; Inhibitor of differentiation; Prognostic factors

**Core tip:** Cholangiocarcinoma present as heterogeneous tumors with generally poor prognosis. Molecular changes that drive tumor development are poorly understood, and no valid prognostic markers other than stage and performance status have been identified. Here we analyzed the protein expression of the four inhibitor of differentiation (ID)-proteins by immunohistochemistry in 129 patients with advanced biliary tract cancer, which showed a deregulated ID protein expression in cancer cells and this protein expression partly correlated with the overall survival of patients. Therefore the ID-proteins maybe useful prognostic markers.

Harder J, Müller MJ, Fuchs M, Gump V, Schmitt-Graeff A, Fischer R, Frank M, Opitz O, Hasskarl J. Inhibitor of differentiation proteins do not influence prognosis of biliary tract cancer. *World J Gastroenterol* 2013; 19(48): 9334-9342 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9334.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9334>

## INTRODUCTION

Cholangiocarcinomas/biliary tract cancers (BTC) form a heterogeneous group of tumors consisting of intrahepatic mass forming type biliary tract cancer (IHC), perihilar Klatskin tumors, extrahepatic BTC (EHC), and gallbladder cancer (GBC)<sup>[1,2]</sup>. More than 50% of tumors are diagnosed at an advanced stage. Prognosis is dismal with a mean overall survival of 7 to 8 mo. Although expression of oncogenes such as *K-ras*, *c-myc*, *c-neu*, *c-met*, and *hcl-2*, or inactivation of tumor suppressor genes like p53 in BTC has been described<sup>[3,4]</sup>, the pathogenesis and molecular biology of this tumor entity is poorly understood. A recent analysis of liver-fluke associated biliary tract cancer on eight tumors and matched normal tissue identified mutations in three signaling pathways, namely histone modification, G protein activation, and genomic instability<sup>[5]</sup>. Validation of 15 of the identified 187 mutated genes in another 46 cases of BTC confirmed the role of p53, K-ras, and SMAD-4, and identified additional mutations in genes not prior associated to BTC<sup>[5]</sup>.

The inhibitor of DNA-binding (ID) proteins, ID1-4, are members of the larger family of basic Helix-Loop-Helix (bHLH) transcription factors, which share a basic domain necessary for DNA-binding<sup>[6]</sup>. ID proteins lack this DNA-binding domain and inhibit transcription of target genes such as p21<sup>CIP1/WAF1</sup>, p16<sup>INK4B</sup>, and pRb by forming DNA-binding incompetent heterodimers with other bHLH factors. Various cellular processes are regulated by individual ID-proteins: Inhibition of cellular differentiation by interference with differentiation-specific bHLH and non-bHLH transcription factors<sup>[7]</sup>, extension of cellular life span<sup>[8-10]</sup>, regulation of angiogenesis<sup>[11]</sup> and maintenance of embryonic and adult stem cells<sup>[12]</sup>, and chromosomal instability<sup>[13,14]</sup>. ID expression is deregulated in many tumors including pancreatic cancer<sup>[6]</sup> a malignancy somehow related to BTC. In some cases ID-expression is associated with poor clinical prognosis<sup>[15]</sup>. Until now, no data on ID protein expression in biliary tract cancer is available, although methylation of the ID4 promoter has been reported in some instances<sup>[16]</sup>.

To investigate the role of ID proteins in BTC we analyzed the expression of ID proteins 1-4 in tumor specimen from 129 patients with advanced BTC and in 9 normal controls by immunohistochemistry (IHC).

## MATERIALS AND METHODS

### Archival tumor samples and controls

The institutional database of the University Medical Cen-

ter Freiburg, Germany, was retrospectively searched for patients presenting with advanced BTC between 1996 and 2007. Archival hematoxylin and eosin (HE) stained slides from routinely processed paraffin-embedded samples collected at time of the initial diagnosis were reviewed to verify the diagnosis and to choose representative blocks for further evaluation. Control tissue samples included normal liver with normal biliary structures obtained from 9 male autopsy cases that had died without any evidence of liver or cardiac disease. Samples from tonsils, lymph nodes and colon mucosa were used as positive controls. The study was in accordance with the ethical standards set by the institutional ethics committee and was conducted according to the declaration of Helsinki.

### Staging

Staging was performed according to UICC/AJCC recommendations.

### Immunohistochemistry

Fresh serial sections were cut at 2 µm from the original diagnostic paraffin-embedded tissue blocks, mounted on Superfrost Plus slides, dried overnight at 37 °C, deparaffinized in xylene and rehydrated through a graded series of alcohol solutions. Antigen retrieval was performed in Target Retrieval Solution (ID1, ID2, ID4: pH6; ID3: pH8; Dako, Glostrup, Denmark) at 95 °C for 60 min. Immunostaining was performed using a semi-automatically autostainer (Dako). The sections were incubated for 60 min at room temperature with primary polyclonal rabbit antibodies against relevant ID proteins (Santa Cruz Biotechnology, Santa Cruz, CA, United States) at different dilutions (ID1, C-20: sc-488 at 1:150; ID2: C-20: sc-489 at 1:1000; ID3, C-20: sc-490 at 1:100; ID4: H-70: sc-13047 at 1:100). After washing with PBS, the samples were incubated with biotinylated goat anti-polyvalent antibodies for 15 min and subsequently by streptavidin alkaline phosphatase for 15 min according to the labeled streptavidin-biotin (LSAB) method. The ID protein binding sections was visualized by K 5005, Fast-Red-Chromogen (Dako) detection for 10 min. Nuclei were counterstained with Mayer's hemalaun solution. Vascular smooth muscle cells served as internal positive controls for the ID proteins. As a negative control, the primary antibody was omitted, with all other experimental conditions kept constant. The staining specificity was further confirmed by blocking the antibody binding to the antigen with the corresponding blocking peptides for ID1, ID2 and ID3 (Santa Cruz). No blocking peptide was commercially available for ID4.

### Scoring of the immunohistochemical staining

Two blinded observers independently evaluated all cases. For cases with discordant scoring results, a consensus was obtained by reevaluating the slides. When the tumor samples showed a heterogeneous staining pattern, the "hot spots" showing the highest staining intensity were selected for further quantification. Cytoplasmic and nuclear immunolabeling were evaluated. Cytoplasmic ex-



**Table 1** Patient characteristics, survival and best response to treatment *n* (%)

Age (yr)	Median (62.2)		Range (32-84)	
Sex	Male	Female		
	67 (51.9)	62 (48.1)		
BTC subtype	EHC	IHC	GBC	Total
	45 (34.9)	50 (38.8)	34 (26.4)	129
Stage	I	II	III	IV
	10 (7.8)	20 (15.5)	40 (31.0)	65 (50.4)
Grade	G1	G2	G3	
	7 (5.4)	81 (62.8)	41 (31.8)	
Treatment	BSC	Cx	Sx	Total
	56 (43.4)	64 (49.6)	9 (7.0)	129
Response to Cx	PR	SD	PD	
	11 (17.2)	27 (42.2)	26 (40.6)	
OS	BSC	Cx	Sx	All
(mo)	15.1	17.7	41.8	18.3

BSC: Best supportive care; Sx: Surgery; Cx: Chemotherapy; PR: Partial remission; SD: Stable disease; PD: Progressive disease; EHC: Extrahepatic BTC; IHC: Intrahepatic BTC; GBC: Gallbladder cancer.

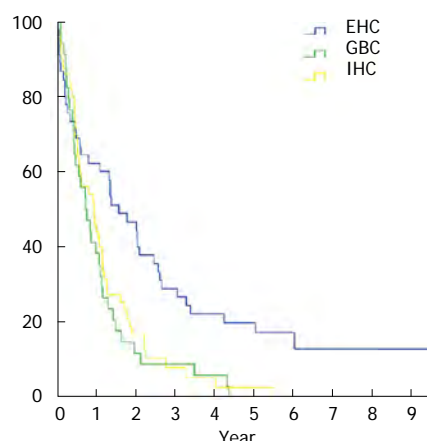
pression was scored according to the staining intensity (0, negative; 1, low; 2, moderate to 3, strong). Nuclear staining was scored according to the percentage of positive nuclei. At least 100 nuclei were assessed in the hot spots at high magnification ( $\times 400$ ). The percentage of positive cells was rated as follows: 0: 0%-10%, 1: 11%-50%, 2: 51%-80%, and 3: 81%-100% of tumor cell nuclei positive.

### Statistical analysis

Statistical analysis was performed using SPSS 15.0 statistical software (SPSS, Inc, Chicago, Illinois). Both the analysis of the association of ID1-4 expression levels in BTC with the overall survival (OS) and the correlation of tumor subtypes with OS were performed by the chi-square test with  $P < 0.05$  considered as statistically significant. Overall survival was defined as the interval from date of diagnosis (histopathology) until death from any cause. Additionally to the analysis with the whole study cohort ( $n = 129$ ), the patients were divided into the two following subgroups: Patients treated by chemotherapy ( $n = 64$ ) and patients not treated by chemotherapy ( $n = 56$ ). Survival estimates were calculated using the Kaplan-Meier method. To test for independent relevance of the candidate prognostic factors a multivariate Cox proportional hazards regression model was fit for each of the ID-proteins. All baseline characteristics plus cytoplasmic and nuclear expression of the respective ID-protein were included in the model. Tumor stage and grading were dichotomized (1/2 *vs* 3/4).

## RESULTS

We identified 129 cases of advanced BTC (unresectable at time of presentation in our institution) that had been treated at our institution between June 1998 and June 2005. Thirty-five percent ( $n = 45$ ) were of extrahepatic origin (EHC), 39% ( $n = 50$ ) were of intrahepatic origin



**Figure 1** Survival of patients with biliary tract cancer by primary tumor localization. Kaplan-Meier estimates of overall survival in patients with extrahepatic biliary tract cancer (BTC) ( $n = 45$ , blue line), intrahepatic BTC ( $n = 50$ , yellow line), and gall bladder cancer ( $n = 34$ , green line).

(IHC), and 26% ( $n = 34$ ) were gallbladder cancers (GBC). Median age at diagnosis was 62.2 years (range 32-84 years) with even gender distribution (48% female, 52% male). The majority of patients had developed metastatic disease; most tumors were G2 tumors (63%). Patients were treated with best supportive care ( $n = 56$ ) and various chemotherapeutic regimens ( $n = 64$ ). Nine patients had become secondary resectable (R0). Chemotherapy regimens were 5-FU or gemcitabine based, often in combination with cisplatin or oxaliplatin. Response to treatment was monitored by CT, MRI or ultrasound according to the standard WHO criteria (WHO, 1979). Eleven patients (17%) showed a partial remission, 27 (42%) had stable disease, and 26 patients (41%) had progressive disease despite chemotherapy. Follow-up data were available from all 129 patients with a median follow-up of 16.5 mo. The median overall survival for all patients was 18.3 mo (Table 1).

To identify prognostic subgroups standard clinical prognostic variables were correlated with clinical outcome. As expected from clinical experience, site of the primary tumor clearly influenced prognosis. Patients with EHC had a significantly longer OS compared to patients with IHC and GBC (Median OS 15.1 years *vs* 0.9 years *vs* 0.7 years,  $P = 0.002$ ) (Figure 1). Overall survival from time of diagnosis was also influenced by stage at diagnosis (Stage I : 2.7 years *vs* stage II : 2.0 years *vs* stage III 0.9 years *vs* stage IV 0.6 years,  $P < 0.001$ ). Resection status also influenced survival, which was longer in patients with completely resected (R0) tumors compared to incompletely resected (R1/R2) and not surgically treated tumors (OS 2.0 years *vs* 1.3 years *vs* 0.6 years,  $P < 0.001$ ). Likewise, OS was better in patients whose tumors responded to chemotherapy (PR 2.0 years *vs* SD 1.3 years *vs* PD 0.6 years,  $P = 0.003$ ). To exclude an influence of curative resection ( $n = 9$ ) on the results, a sensitivity analysis excluding these cases was performed that confirmed the results ( $P = 0.004$ ). Because of the small sample size no additional subgroup analyses were performed.

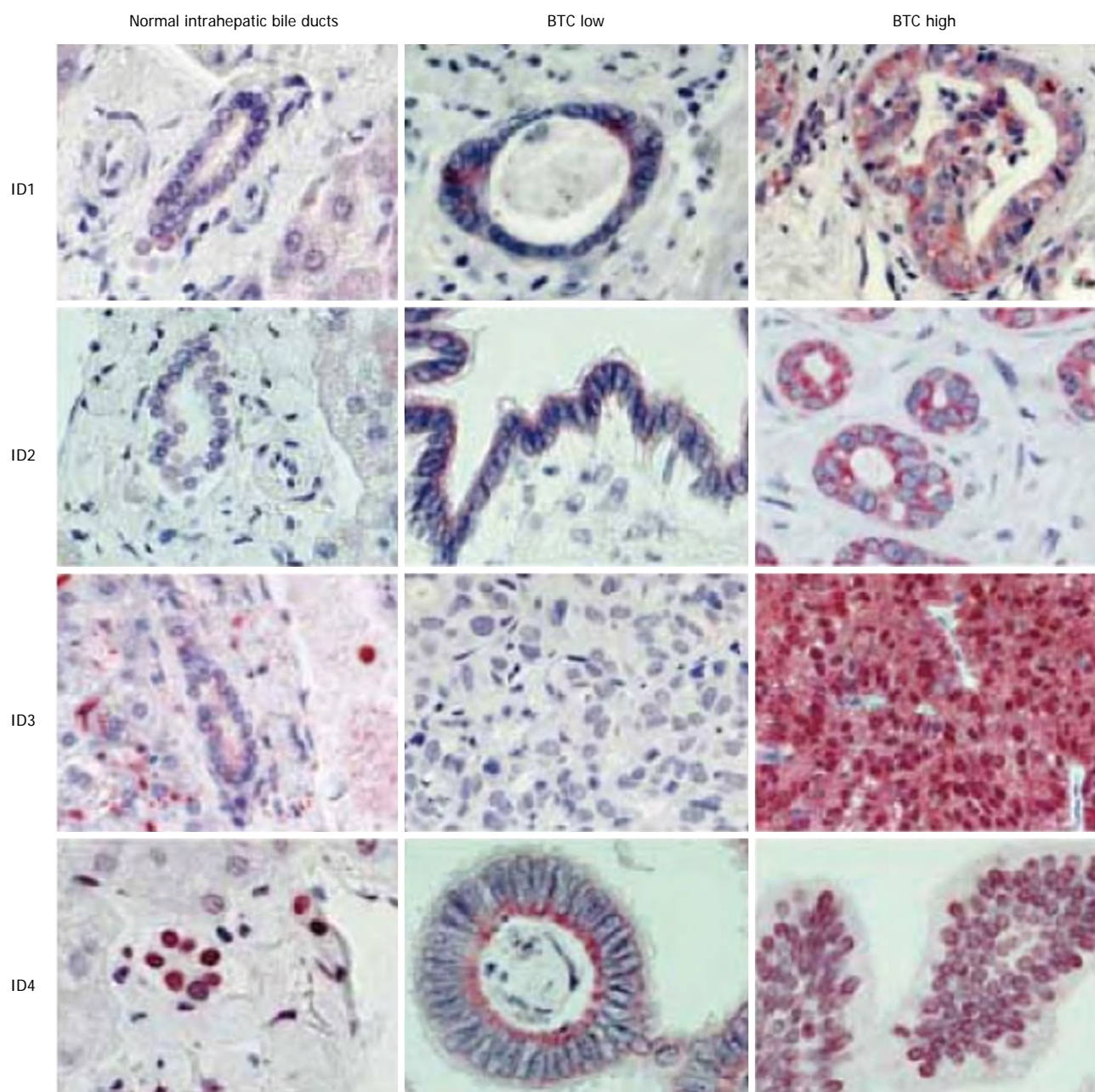


Figure 2 Inhibitor of differentiation protein expression in normal intrahepatic bile ducts and biliary tract cancer. Representative photographs. Depicted are representative stains of normal intrahepatic bile ducts, low and high expressing biliary tract cancer cases for each inhibitor of differentiation (ID) protein.

### *ID proteins are frequently expressed in BTC*

To determine the role of the ID proteins in BTC, we analyzed ID protein expression and subcellular localization of ID proteins in archival samples from all 129 patients collected at initial diagnosis. Normal bile ducts expressed only low amounts of ID proteins with the exception of ID4, where strong nuclear expression of ID4 was detected in all cases analyzed. In contrast to normal controls, high levels of ID1, ID2 and ID3 were detected in the majority of BTC cases (Figure 2). Table 2 summarizes the results.

Detailed analysis of expression levels and subcellular localization of the ID proteins showed clear differences between normal bile ducts and BTC. While expression

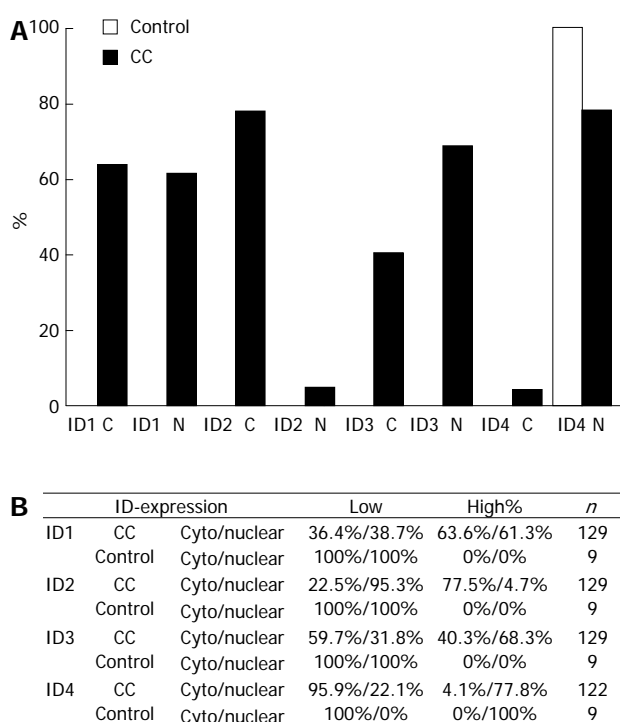
of ID1, ID2, and ID3 was undetectable or low in normal tissue, similarly high cytoplasmic and nuclear ID1 expression was detected in 63.6%, and 61.3% of BTC cases (Figure 3). Likewise, high ID2 expression was detected, which was mainly cytoplasmic (77.5% cytoplasmic *vs* 4.7% nuclear). ID3 was highly expressed in the cytoplasm (40.3%) and even more pronounced in the nucleus (68.3%). In contrast, all normal controls analyzed expressed high levels of nuclear ID4, but only low levels of cytoplasmic ID4. In BTC, this predominant nuclear ID4 staining was reduced to 77.8%.

To investigate the clinical relevance of the above findings, ID protein expression was correlated with overall survival (OS), tumor grade, tumor stage, prior

**Table 2** Inhibitor of differentiation protein expression and tumor characteristics *n* (%)

	Total ( <i>n</i> )	IHC	EHC	GBC	Stage I	Stage II	Stage III	Stage IV	G1	G2	G3
ID1 neg	17	6 (5)	6 (5)	5 (4)	1 (1)	2 (2)	4 (3)	10 (8)	1 (1)	9 (7)	7 (5)
ID1 pos	112	44 (34)	39 (30)	29 (22)	9 (7)	18 (14)	30 (23)	55 (43)	6 (5)	72 (56)	34 (26)
ID2 neg	29	13 (10)	9 (7)	7 (5)	1 (1)	3 (2)	9 (7)	16 (12)	2 (2)	19 (15)	8 (6)
ID2 pos	100	37 (29)	36 (28)	27 (21)	9 (7)	17 (13)	25 (19)	49 (38)	5 (4)	62 (48)	33 (26)
ID3 neg	25	9 (7)	9 (7)	7 (5)	1 (1)	7 (5)	3 (2)	14 (11)	2 (2)	15 (12)	8 (6)
ID3 pos	104	41 (32)	36 (28)	27 (21)	9 (7)	13 (10)	31 (24)	51 (40)	5 (4)	66 (51)	33 (36)
ID4 neg	26	11 (9)	9 (7)	6 (5)	2 (2)	7 (6)	9 (7)	8 (7)	1 (1)	15 (12)	10 (8)
ID4 pos	96	35 (29)	35 (29)	26 (21)	8 (7)	13 (11)	23 (19)	52 (43)	6 (5)	61 (50)	29 (24)

EHC: Extrahepatic biliary tract cancer (BTC); IHC: Intrahepatic BTC; GBC: Gallbladder cancer; G: Grade.



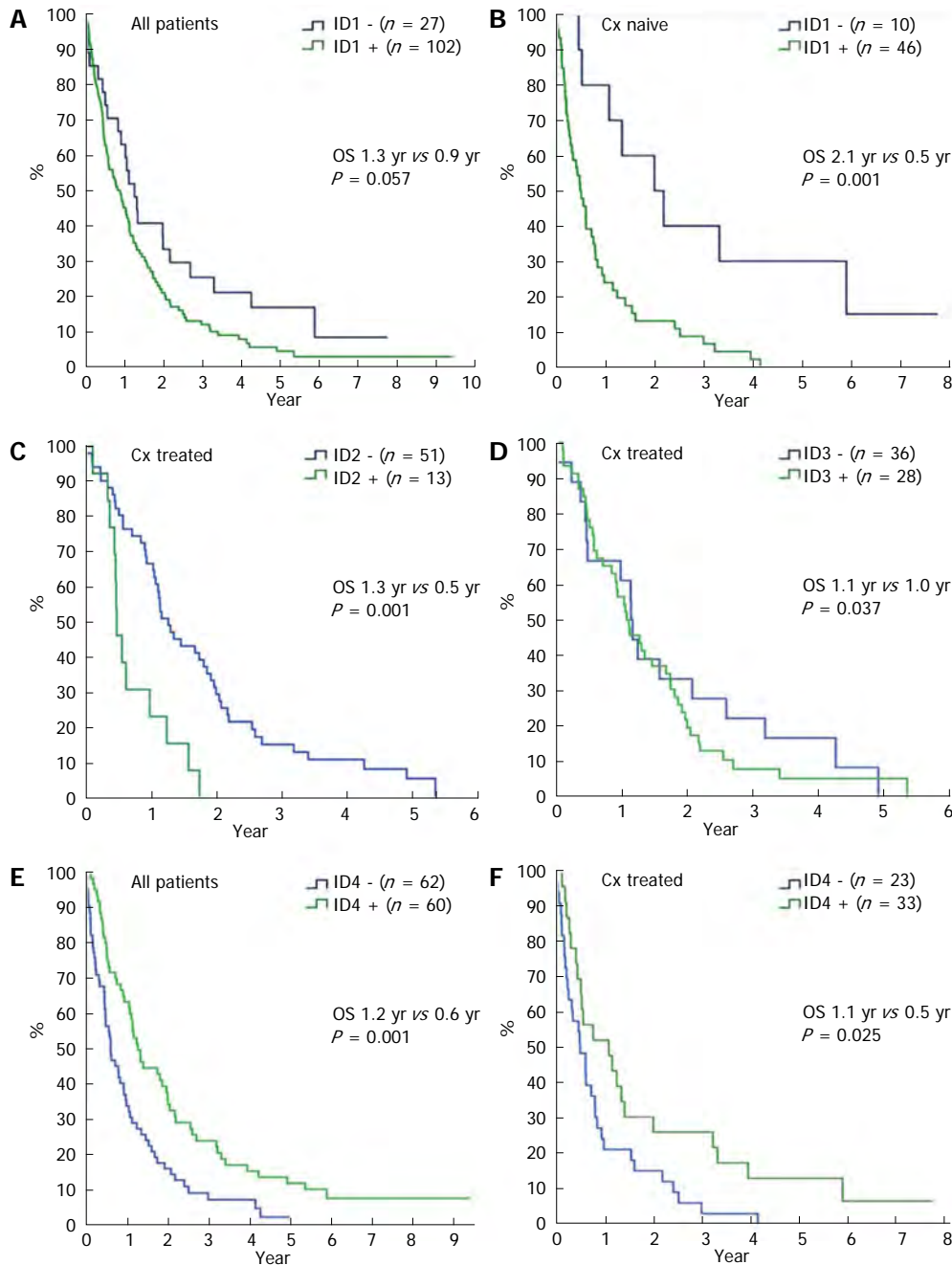
**Figure 3** Proportion of inhibitor of differentiation protein expression in biliary tract cancer and normal intrahepatic bile ducts. A: Percentage of biliary tract cancer (BTC) with high expression of the respective inhibitor of differentiation (ID) protein in BTC (black bars) and normal intrahepatic bile ducts (control; grey bar); B: Summary of ID protein expression. Overall ID protein expression ID protein expression was scored in low (0+1) and high (2+3) and assessed for nuclear and cytoplasmic (cyto) staining pattern. Cytoplasmic staining intensity was scored 0 (negative) to 3 (strong), nuclear expression was scored based on the percentage of positive nuclei (0: 0%-10%; 1: 11%-50%; 2: 51%-80%; and 3: 81%-100%). For ID4, only 122 samples could be analyzed. C: Cytoplasmic expression; N: Nuclear expression.

chemotherapy, and response to chemotherapy. Correlation of ID expression with OS was calculated for the whole study cohort ( $n = 129$ ;  $n = 122$  for ID4) and for the subgroups of patients treated with chemotherapy ( $n = 64$ ) and patients not treated by chemotherapy ( $n = 56$ ). While neither cytoplasmic nor nuclear ID1 expression was correlated with OS, a clear trend for shorter OS was observed for nuclear negative ( $n = 27$ ) *vs* nuclear positive ( $n = 102$ ) cases (0.9 years *vs* 1.2 years,  $P = 0.058$ ) (Figure 4A). Further subgroup analyses identified a strong correlation of ID1 expression and overall survival in che-

motherapy naïve patients ( $n = 56$ ). Here, patients without nuclear ( $n = 10$ ) ID1 expression had an OS of 2.1 years compared to 0.5 years in patients with nuclear ( $n = 46$ ) ID1 expression ( $P = 0.001$ ) (Figure 4B). ID2 did not have a prognostic value for the overall study population ( $P = 0.79$  for cytoplasmic expression,  $P = 0.28$  for nuclear expression). Nevertheless, in the subgroup of patients who had received chemotherapy ( $n = 64$ ) patients without nuclear ID2 expression ( $n = 51$ ) had a significantly better prognosis than patients with nuclear ID2 expression ( $n = 13$ ), with OS of 1.3 years and 0.5 years, respectively ( $P = 0.001$ ) (Figure 4C). As for ID1 and ID2, ID3 expression in the overall study population did not correlate with OS ( $P = 0.28$  for cytoplasmic, and  $P = 0.44$  for nuclear ID3 expression). The subgroup of patients who had received chemotherapy ( $n = 64$ ) patients without cytoplasmic ID3 expression ( $n = 36$ ) had a slightly better prognosis than patients with cytoplasmic ID3 expression ( $n = 28$ ), with OS of 1.0 years and 1.1 years, respectively ( $P = 0.037$ ) (Figure 4D). Using the same subgroup analyses as for the other IDs, no relevant subgroup was identified. Quite strikingly, while nuclear ID4 expression did not correlate with OS ( $P = 0.27$ ), cytoplasmic ID4 expression seemed to correlate with prognosis (Figure 4E). Overall survival in patients with cytoplasmic ID4 expression ( $n = 60$ ) was 1.2 years compared with 0.6 years in patients without cytoplasmic ID4 expression ( $n = 62$ ,  $P = 0.001$ ) in univariate analysis. This was reproduced in the subgroup of patients who had not received prior chemotherapy ( $n = 56$ ). Here, OS was 1.1 years *vs* 0.5 years in patients with cytoplasmic ( $n = 23$ ) *vs* no cytoplasmic ( $n = 33$ ) ID4 expression ( $P = 0.025$ ) (Figure 4F). Analyses of ID1-4 expression and other clinical-pathological variables failed to show any significant correlation (data not shown). Specifically, ID expression levels were similar in EHC, IHC, and GBC.

To test the relevance of the above findings a multivariate Cox proportional hazards regression model was used. Multivariate testing confirmed the importance of tumor localization, surgical treatment, and response to chemotherapy as factors influencing survival (Table 3). Patients with extrahepatic BTC have the best prognosis (HR = 0.32, 95%CI: 0.18-0.6,  $P < 0.0005$ ), as have patients who had been treated surgically (HR = 0.3, 95%CI: 0.17-0.55,  $P < 0.0001$ ). Patients responding to chemotherapy with





**Figure 4** Kaplan-Meier survival estimates for overall survival from time of diagnosis for patients with biliary tract cancer expressing ID1-ID4 with or without systemic chemotherapy. A: Survival in patients ( $n = 129$ ) with (green line) and without nuclear ID1 expression (blue line); B: Survival in patients who have not received systemic chemotherapy ( $n = 56$ ) with (green line) or without nuclear ID1 expression (blue line); C: Survival in patients who have received systemic chemotherapy ( $n = 64$ ) with (green line) or without nuclear ID2 expression (blue line); D: Survival in patients who have not received systemic chemotherapy ( $n = 64$ ) with (green line) or without cytoplasmic ID3 expression (blue line); E: Survival in patients ( $n = 122$ ) with (green line) and without cytoplasmic ID4 expression (blue line); F: Survival in patients who have not received systemic chemotherapy ( $n = 56$ ) with (green line) or without cytoplasmic ID4 expression (blue line). Cx: Chemotherapy.

a partial remission (PR) or disease stabilization (SD) likewise seem to have a better clinical prognosis (HR = 0.43, 95%CI: 0.20-0.91,  $P = 0.267$ ; and HR 0.4, 95%CI: 0.22-0.71,  $P = 0.002$ , respectively). The effects of the ID proteins observed in univariate testing did not reach statistical significance in this multivariate model. Only tumors with loss of cytoplasmic ID4 expression showed a trend for shorter survival (HR = 0.68, 95%CI: 0.45-1.04,  $P = 0.0736$ ).

## DISCUSSION

Our analysis of 129 cases of biliary tract cancer confirmed the importance of primary tumor localization, tumor stage, resection status and response to treatment as strong predictors for patients' prognosis. This difference in survival was independent of other confounding factors such as stage, resection, or response to chemotherapy. We believe that our findings are valid, as also patients who



**Table 3** Multivariate analysis of clinical characteristics and inhibitor of differentiation protein expression

	ID1			ID2			ID3			ID4		
	HR	95%CI	P value	HR	95%CI	P value	HR	95%CI	P value	HR	95%CI	P value
Gender (male)	1.17	0.76-1.80	0.4717	1.07	0.71-1.64	0.7363	1.03	0.68-1.56	0.8877	1.29	0.82-2.02	0.267
IHC	0.57	0.33-0.97	0.0399	0.66	0.39-1.11	0.1193	0.72	0.42-1.23	0.2312	0.75	0.44-1.28	0.295
EHC	0.32	0.18-0.60	0.0003	0.36	0.20-0.64	0.0005	0.36	0.20-0.63	0.0003	0.32	0.18-0.57	0.0001
Stage III-IV	1.51	0.80-2.83	0.2037	1.5	0.80-2.80	0.2051	1.72	0.91-3.25	0.0928	1.24	0.65-2.35	0.5129
Grade 3	1.57	0.99-2.50	0.0532	1.58	1.01-2.47	0.0471	1.43	0.90-2.27	0.1326	1.53	0.96-2.45	0.0732
Surgery	0.3	0.17-0.55	< 0.0001	0.35	0.20-0.61	0.0003	0.33	0.18-0.61	0.0004	0.31	0.17-0.55	< 0.0001
Age	0.99	0.97-1.01	0.2327	0.99	0.97-1.01	0.3835	0.99	0.97-1.01	0.3118	0.99	0.97-1.02	0.6452
Response to Cx PD	0.81	0.44-1.47	0.4836	0.83	0.45-1.53	0.5442	0.65	0.35-1.20	0.1669	0.7	0.37-1.31	0.261
Response to Cx SD	0.4	0.22-0.71	0.002	0.4	0.22-0.72	0.0021	0.33	0.19-0.60	0.0002	0.37	0.20-0.67	0.0012
Response to Cx PR	0.43	0.20-0.91	0.0267	0.48	0.23-0.98	0.0426	0.46	0.22-0.94	0.0329	0.5	0.25-1.01	0.0531
Nuclear low	0.93	0.46-1.88	0.8447	1.41	0.80-2.50	0.2333	1.32	0.62-2.79	0.4721	0.94	0.38-2.33	0.8979
Nuclear high	1.2	0.65-2.20	0.5563	1.51	0.19-11.84	0.696	0.97	0.47-2.00	0.9275	0.63	0.27-1.47	0.2813
Cytoplasmic low	0.81	0.34-1.91	0.6276	0.59	0.07-4.80	0.6195	1.0	0.60-1.68	0.9948	0.68	0.45-1.04	0.0736
Cytoplasmic high	0.81	0.34-1.90	0.6261	0.64	0.08-5.04	0.6703	0.71	0.33-1.54	0.3866	0.31	0.03-2.85	0.3028

$n = 129$ , number of events = 119,  $r^2 = 0.386$  for ID1-3,  $r^2 = 0.392$  for ID4; EHC: Extrahepatic biliary tract cancer (BTC); IHC: Intrahepatic BTC; Cx: Chemotherapy; PR: Partial remission; SD: Stable disease; PD: Progressive disease; ID: Inhibitor of differentiation.

had not been curatively resected had a significantly longer OS ( $P = 0.004$ ). Hence, information of primary BTC localization should be prospectively evaluated and used for stratification in clinical trials in advanced BTC to verify its prognostic relevance. It is hard to speculate on factors influencing this different prognosis. One report could hint to a role of the multidrug resistance proteins (MRP) showing lower levels of MRP3 in EHC compared to gallbladder carcinomas<sup>[17]</sup>. Alternatively, differential gene expression as of matrix metalloproteinases and growth factors might contribute to the different biological behavior of EHC<sup>[3,18]</sup>.

While data on ID protein expression in BTC was scarce, more comprehensive data is available for ID expression in hepatocellular carcinoma (HCC). In HCC, ID1 is frequently expressed and higher ID1 expression was reported to correlate with decreased p16<sup>INK4A</sup> expression<sup>[19]</sup>. ID protein expression decreased with loss of differentiation, suggesting a role of ID proteins in early carcinogenesis<sup>[20]</sup>. Contrasting these results are data that imply a role of ID1 in progression, metastasis, and tumor vessel formation<sup>[19,21-23]</sup>. Immunohistochemical analysis of liver tissue from 112 patients with liver cirrhosis showed elevated ID1 expression in 38% ( $n = 42$ ) of patients. These patients were at higher risk of developing HCC<sup>[23]</sup>. In an analysis of 80 matched pair biopsies of HCC and normal liver, and cirrhotic and chronic hepatitis samples no ID1-protein expression was observed in normal liver, whereas moderate to strong ID1 expression was detected in 50% of HCCs. In 60 matched pairs of primary tumor and metastasis, expression in the metastases was higher (90%) than in the primary tumors (42%), correlating with increased VEGF expression<sup>[22]</sup>. In a nude mouse model ID1 induced VEGF by stabilizing HIF alpha, and antisense-inhibition of ID1 resulted in decreased tumor growth due to decreased VEGF expression and decreased tumor vascularization<sup>[22]</sup>.

Analysis of ID protein expression in our cohort of cholangiocarcinomas showed expression of all four

ID proteins. ID4 was the only ID protein expressed in normal bile ducts. Neither overall ID protein expression levels nor subcellular localization correlated with clinical prognosis. While loss of ID4 protein expression was frequently detected and patients with ID4 negative tumors had a trend for shorter OS this needs to be confirmed in a larger sample. The role of ID4 on tumor development is still not fully understood. Recent studies revealed that the ID4 gene can be silenced through promoter hypermethylation in various tumors, including BTC<sup>[16,24-30]</sup>, suggesting a tumor suppressive role. On the other hand ID4 was identified as an upstream regulator of BRCA1 in breast and ovarian cancer<sup>[31]</sup>. Also supporting an oncogenic role of ID4 are reports describing activating translocations of ID4 in some patients with bladder cancer and a subset of patients with acute lymphoblastic leukemia<sup>[32-34]</sup>. Our data suggest a tumor suppressive role of ID4 in BTC, as patients without or with low cytoplasmic levels of ID4 had shorter overall survival. While hypothesis generating, this must be confirmed and validated in a larger patient population. In summary, we have shown that ID protein expression is deregulated in biliary tract cancer but that their use as prognostic biomarkers is very limited.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Cholangiocarcinoma present as heterogeneous tumors with generally poor prognosis. Molecular changes that drive tumor development are poorly understood, and no valid prognostic markers other than stage and performance status have been identified.

### Innovations and breakthroughs

To investigate the role of inhibitor of differentiation (ID) proteins in biliary tract cancer (BTC) we analyzed the expression of ID proteins 1-4 in tumor specimen

from 129 patients with advanced BTC and in 9 normal controls by immunohistochemistry.

### Applications

Authors have shown that ID protein expression is deregulated in biliary tract cancer but that their use as prognostic biomarkers is very limited.

### Peer review

The authors described the expressions of ID1-4 proteins in specimens of cholangiocarcinoma and compared to the normal control. An interesting paper looking for new histological parameters in cholangiocarcinoma.

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## Smoothelin, a new marker to determine the origin of liver fibrogenic cells

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### Abstract

**AIM:** To explore this hypothesis that smooth muscle cells may be capable of acquiring a myofibroblastic phenotype, we have studied the expression of smoothelin in fibrotic conditions.

**METHODS:** Normal liver tissue ( $n = 3$ ) was obtained from macroscopically normal parts of hepatectomy, taken at a distance from hemangiomas. Pathological specimens included post-burn cutaneous hypertrophic scars ( $n = 3$ ), fibrotic liver tissue ( $n = 5$ ), cirrhotic tissue (viral and alcoholic hepatitis) ( $n = 5$ ), and hepatocellular carcinomas ( $n = 5$ ). Tissue samples were fixed

in 10% formalin and embedded in paraffin for immunohistochemistry or were immediately frozen in liquid nitrogen-cooled isopentane for confocal microscopy analysis. Sections were stained with antibodies against smoothelin, which is expressed exclusively by smooth muscle cells, and  $\alpha$ -smooth muscle actin, which is expressed by both smooth muscle cells and myofibroblasts.

**RESULTS:** In hypertrophic scars,  $\alpha$ -smooth muscle actin was detected in vascular smooth muscle cells and in numerous myofibroblasts present in and around nodules, whereas smoothelin was exclusively expressed in vascular smooth muscle cells. In the normal liver, vascular smooth muscle cells were the only cells that express  $\alpha$ -smooth muscle actin and smoothelin. In fibrotic areas of the liver, myofibroblasts expressing  $\alpha$ -smooth muscle actin were detected. Myofibroblasts co-expressing  $\alpha$ -smooth muscle actin and smoothelin were observed, and their number was slightly increased in parallel with the degree of fibrosis (absent in liver with mild or moderate fibrosis; 5% to 10% positive in liver showing severe fibrosis). In cirrhotic septa, numerous myofibroblasts co-expressed  $\alpha$ -smooth muscle actin and smoothelin (more than 50%). In hepatocellular carcinomas, the same pattern of expression for  $\alpha$ -smooth muscle actin and smoothelin was observed in the stroma reaction surrounding the tumor and around tumoral cell plates. In all pathological liver samples,  $\alpha$ -smooth muscle actin and smoothelin were co-expressed in vascular smooth muscle cells.

**CONCLUSION:** During development of advanced liver fibrosis, a subpopulation of myofibroblasts expressing smoothelin may be derived from vascular smooth muscle cells, illustrating the different cellular origins of myofibroblasts.

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**Key words:** Smooth muscle cells; Myofibroblasts;  $\alpha$ -smooth muscle actin; Smoothelin; Fibrosis/cirrhosis; Hepatocellular carcinoma

**Core tip:** In fibrotic conditions, it has been suggested that smooth muscle cells can acquire a myofibroblastic phenotype. To explore this hypothesis, we studied the expression of smoothelin, a specific marker of end-stage differentiation of smooth muscle cells, in cutaneous and hepatic fibrotic conditions, using immunohistochemistry and confocal microscopy. We showed that during advanced liver fibrosis, a subpopulation of  $\alpha$ -smooth muscle actin-expressing myofibroblasts also express smoothelin and thus may be derived from vascular smooth muscle cells. This finding, which illustrates the different potential cellular origins of myofibroblasts involved in liver fibrogenesis, may represent an interesting tool to distinguish advanced stages of cirrhosis.

Lepreux S, Guyot C, Billet F, Combe C, Balabaud C, Bioulac-Sage P, Desmoulière A. Smoothelin, a new marker to determine the origin of liver fibrogenic cells. *World J Gastroenterol* 2013; 19(48): 9343-9350 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9343.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9343>

## INTRODUCTION

Smoothelin, a constituent of the smooth muscle cell cytoskeleton, has been described as a marker of end-stage differentiation of smooth muscle cells because it has been found only in contractile smooth muscle cells<sup>[1-3]</sup>. Smoothelin has two major isoforms in adults, which are expressed in a tissue specific manner: a 59 kDa isoform, smoothelin-A, which is expressed in visceral and urogenital tissues, such as the digestive tract, bladder, and prostate; and a 110 kDa isoform, smoothelin-B, which is expressed in blood vessel walls<sup>[4]</sup>. Transient synthesis of a third smoothelin isoform has been detected in embryonic striated muscle cells in chicken<sup>[5]</sup>. In cultured smooth muscle cells, smoothelin colocalizes with  $\alpha$ -smooth muscle actin stress fibers<sup>[3]</sup> and smoothelin can bind to  $\alpha$ -smooth muscle actin, which suggested a direct role of smoothelin in contraction<sup>[6]</sup>. In contrast, cells with smooth muscle cell-like features, such as myofibroblasts, do not express smoothelin<sup>[3]</sup>. Myofibroblasts are contractile cells that express  $\alpha$ -smooth muscle actin<sup>[7]</sup>. These cells are involved in tissue repair processes and, particularly, in extracellular matrix deposition and remodeling<sup>[8]</sup>. In normal connective tissues, myofibroblasts are rare<sup>[9]</sup>. After tissue injury, myofibroblasts appear, and it is commonly accepted that most are derived from locally recruited connective tissue fibroblasts<sup>[10]</sup>. In the liver, the most important cells involved in fibrogenesis are hepatic stellate cells and portal fibroblasts, which are able to differentiate into myofibroblasts and are responsible for matrix deposition<sup>[11]</sup>. However, the involvement of different cell

types with various origins, such as smooth muscle cells, circulating cells or bone marrow-derived cells has also been suggested in the establishment of liver fibrosis/cirrhosis<sup>[12-16]</sup>. However, the degree to which this process contributes to fibrosis remains a matter of intense debate and is likely to be context-dependent. To determine the possible contribution of smooth muscle cells to the appearance of myofibroblasts during fibrotic processes, we studied the expression of smoothelin in different diseases that show myofibroblast involvement; *i.e.*, post-burn cutaneous hypertrophic scars, liver fibrosis and cirrhosis, and hepatocellular carcinomas.

## MATERIALS AND METHODS

### Human liver samples and tissue processing

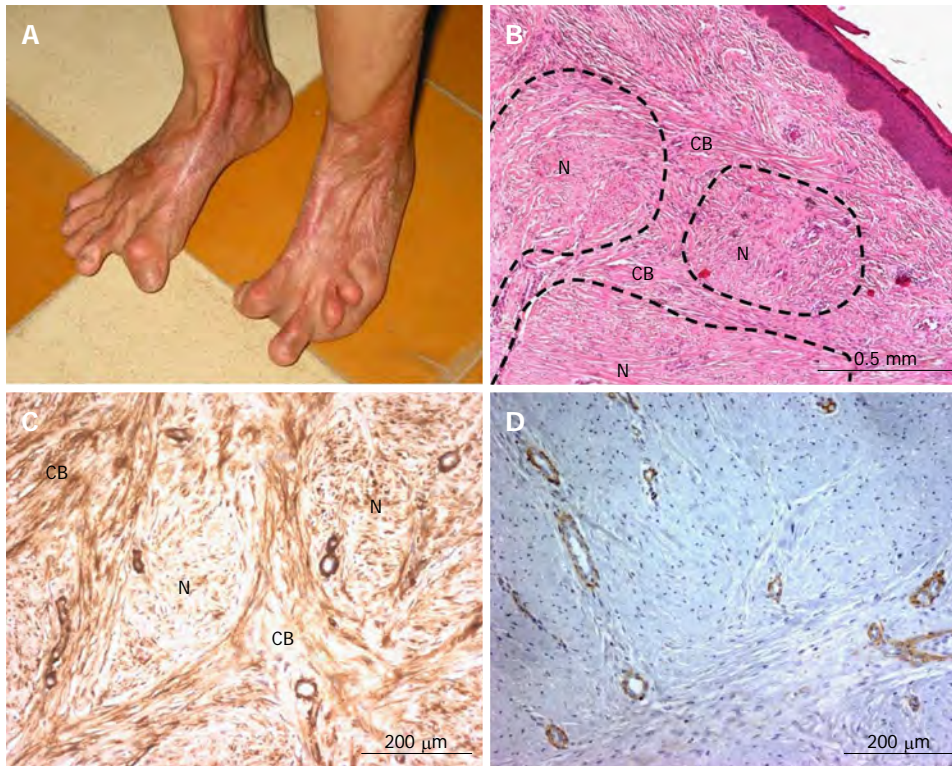
Human tissue samples used in this study were selected from the files of the tissue bank of the Department of Pathology (CHU Bordeaux, Pellegrin Hospital, Bordeaux, France). Normal liver tissues ( $n = 3$ ) were obtained from macroscopically normal parts of livers after hepatectomy, taken at a distance from hemangiomas. Pathological specimens included post-burn cutaneous hypertrophic scars ( $n = 3$ ); fibrotic liver tissue ( $n = 5$ ), ranging from F1 to F3 stage according to the Metavir score<sup>[17]</sup>; cirrhotic tissues (viral and alcoholic hepatitis) ( $n = 5$ ); and hepatocellular carcinomas ( $n = 5$ ). Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and processed for diagnostic purposes and immunohistochemistry, or were immediately frozen in liquid nitrogen-cooled isopentane for confocal microscopy analysis. The procedures were carried out in accordance with the European Guidelines for the use of human tissues.

### Immunohistochemistry and confocal microscopy

Mouse monoclonal antibodies against smoothelin (immunoglobulin G, IgG1 clone R4A, which reacts with smoothelin A and B, MUBio Products, Maastricht, The Netherlands), and  $\alpha$ -smooth muscle actin (IgG2a clone 1A4, Dako SA, Trappes, France) were used. These two antibodies have been extensively used and their specificity has been clearly documented<sup>[1,18]</sup>. Immunohistochemistry was essentially performed as previously described<sup>[19]</sup>. Briefly, after incubation with the first antibody, the epitopes were detected using the Vectastain<sup>®</sup> ABC system (Vector Laboratories, Peterborough, United Kingdom) or the Envision<sup>™</sup> system (DakoCytomation, Trappes, France), with diaminobenzidine as the color substrate. Slides were then counterstained with hematoxylin.

For double immunofluorescence, cryostat sections were first incubated with the antibodies against smoothelin and  $\alpha$ -smooth muscle actin, then with a TRITC-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL, United States) and an Alexa Fluor<sup>®</sup> 488 goat anti-mouse IgG2a (Molecular Probes, Eugene, OR, United States). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

The specificity of staining was confirmed by incubation



**Figure 1**  $\alpha$ -smooth muscle actin and smoothelin expression in a cutaneous hypertrophic scar. A: Following a burn injury, a retractive hypertrophic scar was observed (from Vincent Casoli, Plastic Surgery and Burns Unit, University Hospital of Bordeaux, France); B: Hematoxylin and eosin staining shows typical hypertrophic scar architecture with nodules (N) surrounded by cell bundles (CB); C:  $\alpha$ -smooth muscle actin is expressed in vascular smooth muscle cells and myofibroblasts both in and around the nodules; D: Smoothelin is only expressed by vascular tunica media smooth muscle cells.

tion in non-immune serum and in the absence of the primary antibody. For immunohistochemistry, sections were examined with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany). Images were acquired with an AxioCam camera (Carl Zeiss Vision, Hallbergmoos, Germany) by means of the AxioVision image processing and analysis system (Carl Zeiss Vision). For double immunofluorescence, sections were analyzed with a confocal microscopy (LSM Meta 510, Carl Zeiss Microscopy).

For quantitative evaluation of staining, cell counting was performed in the septa of fibrotic and cirrhotic livers, and in the stroma reaction of hepatocellular carcinoma. Vessels were not included in this evaluation. For each field, the ratio of the number of smoothelin-positive and  $\alpha$ -smooth muscle actin-positive cells over the number of cells only expressing  $\alpha$ -smooth muscle actin was calculated. The analysis was performed on an average of 10 fields/zone using the  $\times 40$  objective. Only positive cells containing a nucleus were counted.

## RESULTS

### $\alpha$ -smooth muscle actin and smoothelin expression in hypertrophic scars

In many situations, hypertrophic scars develop significant contractile activity (because of the presence of myofibroblasts) (Figure 1A), and are usually organized in a nodular pattern (Figure 1B). By immunohistochemistry,  $\alpha$ -smooth

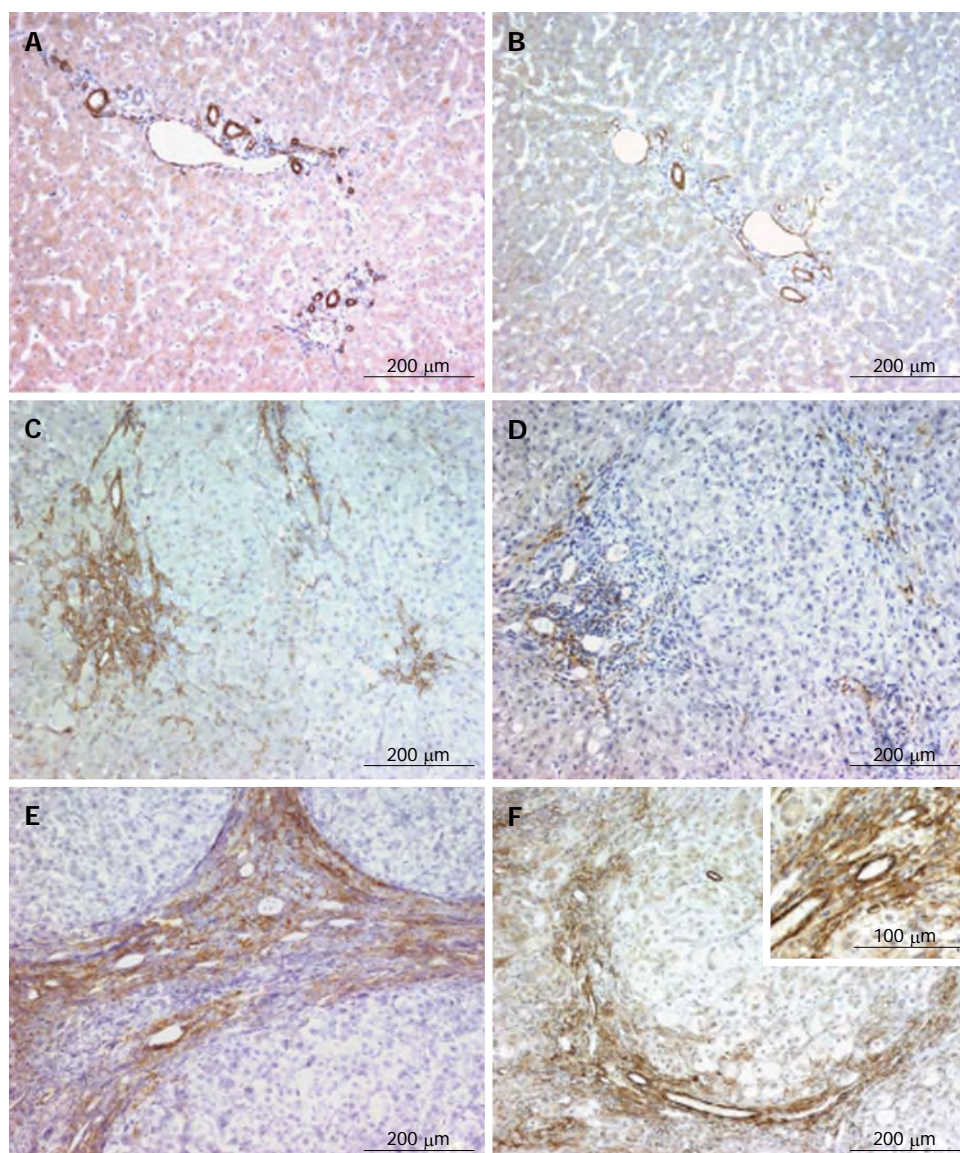
muscle actin was detected in blood vessel walls and in numerous myofibroblasts present in and around the nodules (Figure 1C), whereas smoothelin was exclusively shown in the vessel walls (Figure 1D). Our data showed that myofibroblasts present in hypertrophic scars do not express smoothelin.

### $\alpha$ -smooth muscle actin and smoothelin expression in normal and pathological livers

In the normal liver,  $\alpha$ -smooth muscle actin was exclusively expressed by the smooth muscle cells within the tunica media of portal arteries and veins, and of the centrilobular veins (Figure 2A). The expression of smoothelin in the normal liver was also detected in the smooth muscle cells of these vessels (Figure 2B). Smoothelin and  $\alpha$ -smooth muscle actin showed a similar distribution.

In livers with mild or moderate fibrosis (F1-F2), myofibroblasts present in fibrotic areas clearly expressed  $\alpha$ -smooth muscle actin, but did not express smoothelin (data not shown). In livers showing severe fibrosis (F3), strong staining for  $\alpha$ -smooth muscle actin was detected in myofibroblasts present in fibrotic areas (Figure 2C). Weak expression of smoothelin was present in a few myofibroblasts (Figure 2D). In cirrhotic livers (F4), myofibroblasts present in cirrhotic septa expressed high amounts of  $\alpha$ -smooth muscle actin (Figure 2E). Numerous myofibroblasts also expressed smoothelin (Figure 2F). In all stages of liver fibrosis,  $\alpha$ -smooth muscle actin





**Figure 2**  $\alpha$ -smooth muscle actin and smoothelin expression in normal, fibrotic and cirrhotic livers. A and B: In the normal liver,  $\alpha$ -smooth muscle actin (A) and smoothelin (B) show similar expression in tunica media smooth muscle cells of the portal arteries and veins and in the centrilobular veins; C and D: In severe fibrosis, myofibroblasts express high amounts of  $\alpha$ -smooth muscle actin (C), while smoothelin is only expressed at low levels (D) ( $\alpha$ -smooth muscle actin and smoothelin are coexpressed in vascular smooth muscle cells); E and F: In the cirrhotic liver, myofibroblasts express  $\alpha$ -smooth muscle actin in fibrotic septae (E) and numerous myofibroblasts also express smoothelin (F); however, smoothelin expression is lower compared with  $\alpha$ -smooth muscle actin expression (insert).  $\alpha$ -smooth muscle actin and smoothelin are co-expressed in vascular tunica media smooth muscle cells (F).

and smoothelin were co-expressed in vascular smooth muscle cells (Figure 2C-F).

The co-localization of  $\alpha$ -smooth muscle actin and of smoothelin in the same cells was confirmed by double immunofluorescence (Figure 3). All cells expressing smoothelin also expressed  $\alpha$ -smooth muscle actin, including vascular smooth muscle cells and myofibroblasts. Moreover, a quantitative evaluation revealed that in livers showing severe fibrosis (F3), 5% to 10% of myofibroblasts co-expressed  $\alpha$ -smooth muscle actin and smoothelin. In cirrhotic septa, more than 50% of myofibroblasts co-expressed  $\alpha$ -smooth muscle actin and smoothelin.

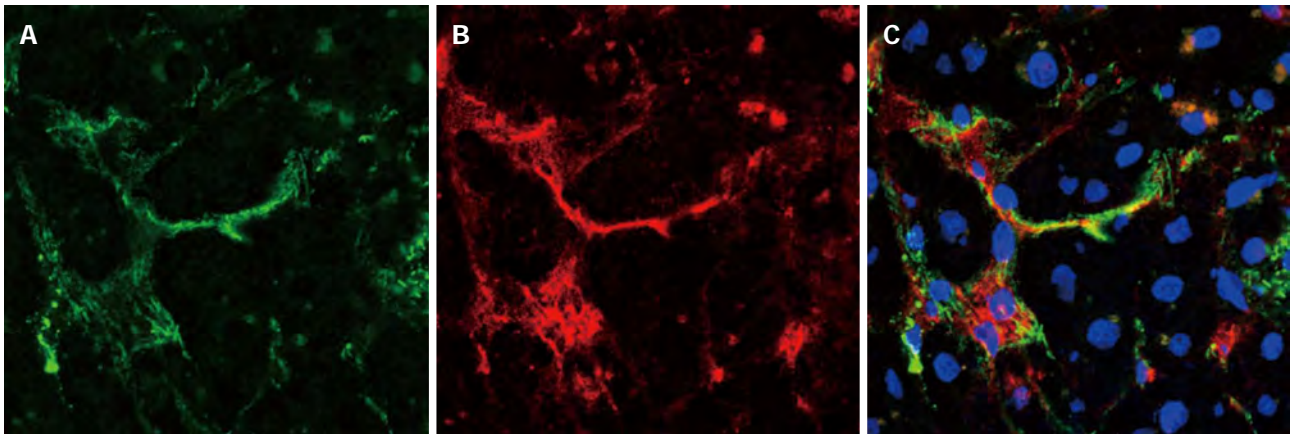
In hepatocellular carcinomas, we observed the same expression pattern of  $\alpha$ -smooth muscle actin and smoothelin in the stroma reaction surrounding the tumor (Figure

4A and B). These two proteins were also expressed by fusiform pericyte-like cells between endothelial cells of the sinusoidal capillaries and tumoral cell plates (Figure 4C and D).

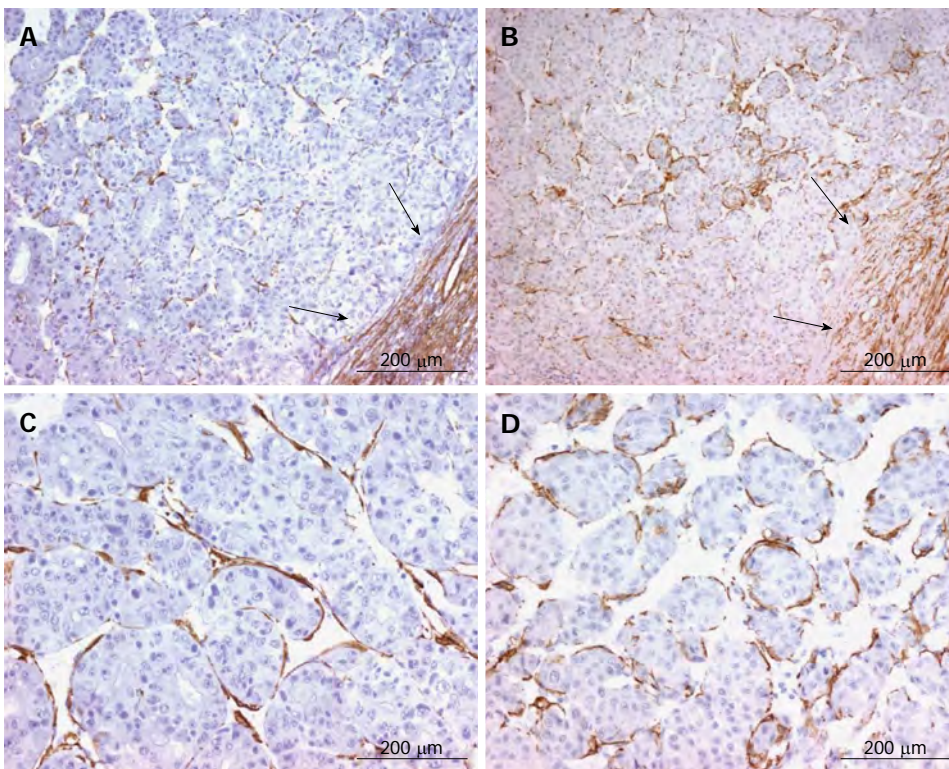
## DISCUSSION

In normal conditions, several cell types that have contractile properties express  $\alpha$ -smooth muscle actin, such as smooth muscle cells and, to a lesser extent, pericytes. Smooth muscle cells display a large variation in phenotype among, and even within, tissues and organs. Different patterns of marker expression reflect, in part, the heterogeneity of smooth muscle cell subpopulations and their phenotypic modulation<sup>[20]</sup>. Smoothelin, a recently





**Figure 3** Cellular co-localization of  $\alpha$ -smooth muscle actin and smoothelin in a fibrotic liver. A:  $\alpha$ -smooth muscle actin expression (green); B: Smoothelin expression (red); C: Merged (nuclei are stained with DAPI). Smoothelin-expressing cells also express  $\alpha$ -smooth muscle actin.



**Figure 4**  $\alpha$ -Smooth muscle actin and smoothelin expression in hepatocellular carcinoma. A and B:  $\alpha$ -smooth muscle actin (A) and smoothelin (B) are expressed similarly in the stroma reaction surrounding the tumor (arrows) and around tumoral hepatocytes; C and D:  $\alpha$ -smooth muscle actin (C) and smoothelin (D) are expressed by pericyte-like cells underlying capillaries between tumoral hepatocytes.

described smooth muscle cell marker, illustrates these phenotypic features. Firstly, smoothelin expression varies, with isoform A being expressed in visceral smooth muscle cells, and isoform B being expressed in vascular smooth muscle cells<sup>[4]</sup>. Secondly, smoothelin is expressed only by fully differentiated smooth muscle cells and not by proliferative or non-contractile smooth muscle cells<sup>[1]</sup>. Thirdly, visceral smoothelin expression is different according to the location of the smooth muscle cells within the organ. For example, in the digestive tract and urinary tract, most of the smooth muscle cells of the muscularis propria express smoothelin, but the smooth muscle cells

of the muscularis mucosae do not<sup>[21,22]</sup>. During fibrotic processes, it is assumed that it is mainly fibroblasts that are recruited and acquire a myofibroblastic phenotype, *i.e.*, a smooth muscle cell-like phenotype. However, it has been suggested that smooth muscle cells can also contribute to the appearance of myofibroblasts. Similarly to smooth muscle cells, fully differentiated myofibroblasts express  $\alpha$ -smooth muscle actin and are contractile cells; in addition, myofibroblasts are responsible for extracellular matrix deposition. Like myofibroblasts observed with-in bladder carcinoma<sup>[21]</sup>, blood vessel adventitia<sup>[23]</sup> or the airway wall of patients with asthma<sup>[24]</sup>, our study shows



that myofibroblasts in cutaneous hypertrophic scars do not express smoothelin. In the skin, myofibroblasts are mostly derived from locally recruited connective tissue fibroblasts, which, prior to injury, do not express  $\alpha$ -smooth muscle actin or smoothelin<sup>[8]</sup>. These fibroblasts, when transformed into myofibroblasts, express  $\alpha$ -smooth muscle actin but not smoothelin. Additionally, it has been shown that, in hypertrophic scars, a population of fibrogenic circulating cells, called fibrocytes, is recruited and participates in scar formation<sup>[25]</sup>. Here, we showed that, in hypertrophic scars, myofibroblasts do not express smoothelin, which suggests that these myofibroblasts probably do not derive from local smooth muscle cells. However, our study showed that, in fibrotic liver diseases, myofibroblasts can express smoothelin. Double immunofluorescence and confocal analysis confirmed the co-expression of  $\alpha$ -smooth muscle actin and smoothelin in the same cells. This expression is stronger in parallel with the progression and the chronicity of the fibrotic lesion: i.e., there are more smoothelin-expressing myofibroblasts within advanced stages of fibrosis or cirrhosis than in the first stages of fibrosis. Smoothelin expression may thus appear gradually during fibrosis development; therefore, smoothelin could represent a good marker for evaluating the prognosis of liver fibrosis. In the liver, numerous cell types can be recruited and acquire a myofibroblastic phenotype; generally these are fibroblasts within the portal tract and hepatic stellate cells in the space of Disse<sup>[11]</sup>. As shown here, these cells do not express smoothelin. However, myofibroblasts can also originate from the smooth muscle cells present in portal vessels and centrilobular veins<sup>[11]</sup>, which, as described here, do express smoothelin. In chronic hepatic schistosomiasis, it has been suggested that smooth muscle cells from the artery and vein tunica media are involved in the portal fibrogenesis that occurs in this pathology<sup>[12]</sup>. In the experimental model of porcine serum-induced fibrosis in rats, mesenchymal cells, located around the centrilobular vein, called second layer cells, participate in the formation of fibrotic septa<sup>[26]</sup>. The expression of smoothelin within smooth muscle cells can also vary. In early atherosclerotic lesions, smoothelin expression decreases in the vicinity of the affected area<sup>[27,28]</sup>; however, when the plaques become “quiescent”, smoothelin expression can be detected again<sup>[2]</sup>. Finally, we cannot exclude the possibility that the expression of smoothelin within septal myofibroblastic cells is a residual smooth muscle feature, underlining the possible smooth muscle cell origin of a subpopulation of myofibroblasts. Lastly, the tumoral stroma is the connective tissue surrounding tumoral cells<sup>[29]</sup>. No smoothelin-expressing stromal cells were described in published studies of bladder carcinoma<sup>[21,30]</sup>. In hepatocellular carcinoma, we show that numerous  $\alpha$ -smooth muscle actin expressing myofibroblasts in the stroma reaction also express smoothelin, underlining the possible smooth muscle origin of these myofibroblasts. Surprisingly, we also showed that, in hepatocellular carcinomas, cells located between endothelial cells and tumoral hepatocytes express  $\alpha$ -smooth muscle actin and smoothelin. Pericytes and hepatic stellate cells,

also termed liver-specific pericytes<sup>[31,32]</sup>, can express  $\alpha$ -smooth muscle actin, but not smoothelin<sup>[2]</sup>. This pattern of smoothelin expression, which was not found in the fibrotic liver, illustrates the complete reorganization of the capillarized liver sinusoids during tumor development, with the appearance of specific cells, and may represent an additional feature for diagnosing hepatocellular carcinomas. In summary, we hypothesize that during liver fibrosis, a subpopulation of myofibroblasts express smoothelin. These myofibroblasts may be derived from smooth muscle cells coming from vascular walls, which are recruited to participate in the fibrotic process. These cells expressing smoothelin are recruited in advanced stages of cirrhosis, when a significant vascular reorganization occurs, including portovenous and arteriovenous shunting; therefore, smoothelin could be a useful marker to distinguish advanced stages of cirrhosis. In addition, in hepatocellular carcinomas, smoothelin expression shows a very specific pattern. These data again illustrate the different cellular origins of the so-called myofibroblastic cells involved in liver fibrogenesis.

## COMMENTS

### Background

Myofibroblasts are fibrogenic and contractile cells involved in remodeling and healing processes. In most organs, myofibroblasts are rare in normal physiological conditions. After tissue injury, myofibroblasts appear, which are derived from local stromal cells, such as resident fibroblasts or organ specific stromal cells, such as hepatic stellate cells in the liver.

### Research frontiers

To explore the origin of myofibroblasts, the authors studied the expression of smoothelin, a constituent of the smooth muscle cell cytoskeleton, in normal and fibrotic livers, in hepatocellular carcinoma and in a cutaneous hypertrophic scar. Immunohistochemistry was used to identify smoothelin-expressing cells.

### Innovations and breakthroughs

This is the first study exploring smoothelin expression in the normal liver, fibrotic liver and in hepatocellular carcinoma. The results show an increase in expression that correlated with the degree of fibrosis, suggesting the progressive involvement of resident smooth muscle cells in myofibroblast recruitment. In hepatocellular carcinoma, smoothelin is expressed by pericyte-like cells between the endothelial cells of the capillaries and the tumoral cell plates.

### Applications

This preliminary study underlines that tracing the origin of myofibroblasts may be useful to evaluate the degree of fibrosis in the liver and the level of reorganization in hepatocellular carcinomas.

### Terminology

Smoothelin is a constituent of the smooth muscle cell cytoskeleton. It has been described as a marker of end-stage differentiation of smooth muscle cells because its expression has been found only in contractile smooth muscle cells.

### Peer review

This is a good descriptive study in which the authors explore the expression pattern of a smooth muscle marker, smoothelin, within normal, fibrotic and tumoral livers, as well as in a cutaneous hypertrophic scar. The results are interesting and suggest that smoothelin expression increases with the degree of fibrosis, allowing the evaluation of the transition between fibrosis and cirrhosis. Moreover, in hepatocellular carcinoma, smoothelin is expressed by some stromal cells present beneath endothelial cells of the capillary surrounding the tumoral cell plates and this distinctive feature could be useful for hepatocellular carcinoma diagnosis.

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## Epidemiology and clinical features of cystic hydatidosis in Western Sicily: A ten-year review

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### Abstract

**AIM:** To assess retrospectively the epidemiological and clinical aspects of cystic echinococcosis (CE) and to evaluate follow-up and response to treatment in patients affected by CE.

**METHODS:** From January 2000 to December 2010, all patients affected by CE at the Infectious Diseases Units of the University of Catania and of Basilotta Hospital in Nicosia-Enna, were enrolled as participants in the study. Epidemiological, clinical and laboratory data were collected for each patient. Diagnosis of CE was performed using clinical imaging and laboratory parameters. Response to treatment was categorized as follows: "cure" as the disappearance or complete calcification of cyst/s; "improvement" as a reduction in the diameter and/or

number of existing cysts; and "impairment" as an increase in the diameter and/or number of existing cyst/s and the onset of relapses (*i.e.*, the onset of new cyst/s and an increase in the diameter of previously existing cyst/s and/or complications. Immunoglobulin E (IgE) titers and eosinophil percentages were evaluated at diagnosis, at six months after the initiation of treatment and again in the case of relapse. Hyper-eosinophilia was defined as an eosinophil percentage of  $\geq 6\%$ .

**RESULTS:** Thirty-two patients were diagnosed with CE in our Unit during the research period, with a male-female ratio of 2:1. At the time of diagnosis, 40% of patients presented a single CE cyst. Sixty percent showed multi-organ involvement. The liver-lung localization ratio was 2:1. Patients below the age of 50 at diagnosis were more likely to have multiple cysts (73.7% *vs* 35.5%,  $P < 0.05$ ). Regarding treatment, 30 patients were treated medically and 16 surgically. Fourteen patients were treated both medically and surgically. Relapses were seen to be less frequent in patients treated with albendazole before and after surgery. Complete cure or an improvement was achieved in 23 patients. Impairment was observed in one patient. Two patients showed no improvement. Relapses were more frequent in those patients treated before 2005. At diagnosis, 71% of patients were positive for specific CE IgE, and 56.3% showed an eosinophil percentage of  $\geq 6\%$ . Patients who were diagnosed with hyper-eosinophilia developed complications more frequently than the other patients, but did not suffer relapses.

**CONCLUSION:** On the basis of our results, we propose cystic echinococcosis screening for family members of patients, appropriate pre- and post-surgery treatment and the assessment of anti-echinococcus IgE titer or eosinophil percentage as a therapy response marker in settings with limited resources.

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**Key words:** Cystic echinococcosis; Hydatid disease; Cestode infections; Epidemiology; Diagnosis

**Core tip:** On the basis of the data presented, we suggest the use of specific immunoglobulin E detection and eosinophil percentage counts as therapeutic response markers, particularly in settings with limited resources. We also recommend: (1) routine screening for cystic echinococcosis in relatives (and/or close associates) of patients to facilitate the diagnosis of asymptomatic infection; (2) extension of the follow-up period after surgical and/or medical treatment for the early diagnosis of relapses; and (3) appropriate pre- and post-surgery therapy.

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## INTRODUCTION

Human cystic echinococcosis (CE), or hydatid disease, is a parasitic zoonosis caused by the larval stages of the cestode *Echinococcus granulosus*. The definitive hosts of this parasite are usually members of the canid family, such as dogs, which develop the adult worm in the gut following ingestion of the larvae that are present in the tissues of the intermediate host. Following the ingestion of eggs that are expelled in the feces of the definitive host, larval cysts then go on to develop in the visceral tissue of the intermediate hosts (typically sheep and goats and occasionally, humans), particularly in the liver and lungs.

CE is found worldwide, especially where livestock breeding and farming are widespread and in areas where human, definitive and natural intermediate hosts are found in close proximity. In the human host, a hydatid cyst can lead to life-threatening complications, such as cyst rupture, with possible anaphylactic shock, the spread of new cysts, and bacterial infection. In Italy, Sicily is one of the most endemic areas for CE because of the high levels of farming and livestock breeding, with an average annual incidence of 3.2/100000 inhabitants<sup>[1]</sup>. CE is often underdiagnosed because it is frequently a silent condition that develops over several years and whose symptoms are only apparent when compression of internal organs occurs.

The aim of this study was to assess retrospectively the epidemiological and clinical characteristics of CE, and evaluate follow-up and response to treatment in patients affected by this disease.

## MATERIALS AND METHODS

From January 2000 to December 2010 all CE patients

admitted to the Infectious Diseases Units of the University of Catania and of Basilotta Hospital in Nicosia-Enna were enrolled as participants in the study. Epidemiological, clinical and laboratory data were collected from the clinical records of each patient. Diagnosis of CE was made using clinical, imaging and laboratory parameters.

Epidemiological data included sex, age, race, job, place of residence, possible contact with stray dogs or hunting dogs, countryside activities, hunting and presence of other people in the family affected by CE. Patients were classified as being “exposed” or “not exposed” to CE on the basis of occupation and recreational activities potentially at risk of acquiring CE. Chest X-ray and abdominal ultrasound provided the parameters for an initial diagnosis of CE. None of the patients enrolled in this study were assessed using high-sensitivity tests, such as specific anti-*Echinococcus* immunoglobulin E (IgE)-ELISA ImmunoCAP or on the basis of micro/macrosopic morphology alone. Serum alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (gamma-GT) and alkaline phosphatase (ALP) values were also collected.

Where appropriate, treatment consisted of albendazole at a dosage of 10-15 mg per kg per day, administered in two separate doses, each individual course of treatment lasting up to 3 mo. Ultrasound and X-ray assessed the response to treatment after 1 year. A complete “cure” was defined as the disappearance or the complete calcification of the cyst/s, thereby rendering them non-viable. “Improvement” was defined as a reduction in the diameter and/or number of cysts and/or partial calcification, and “impairment” as an increase in the diameter and/or number of cyst/s and the onset of relapses and/or complications. A “relapse” was defined as the onset of new cyst/s (regardless of location) and/or an increase in diameter of previously existing cyst/s.

Response to treatment was recorded together with details of any adverse events, the severity of such events and any further complications. Correlations between side effects and albendazole treatment were assessed using the Naranjo algorithm<sup>[2]</sup>. Side effect severity was measured using the FDA drug reaction severity scale<sup>[3]</sup>.

As the average time before relapse was observed to be  $30 \pm 6.4$  mo, for the purposes of calculating the relapse rate, the decision was made to include only those patients diagnosed before 2008. IgE titers were evaluated at diagnosis, at six months after beginning treatment and again in the case of relapse. Eosinophil percentages were evaluated at diagnosis and six mo after starting treatment. Hyper-eosinophilia was defined as an eosinophil percentage of  $\geq 6\%$ .

### Ethics statement

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. The Ethics Committee of the University of Catania approved the study. All patients provided written informed consent for their data to be analyzed.

### Statistical analysis

Anti-*echinococcus* IgE was performed using ELISA Immu-

**Table 1** Main characteristics of the 32 patients affected by cystic echinococcosis *n* (%)

Characteristics	Value
Age (yr)	
Median (range)	46 ± 16.5
Sex	
Male	21 (65.7)
Female	11 (34.3)
Risk factors	
Exposed	14 (43.7)
Not exposed	18 (56.3)
Comorbidities	
Affected	17 (53.1)
Not affected	15 (46.9)
Diagnosis	
Abdominal ultrasound	16 (50)
Chest X-ray	9 (28.1)
Unknown	7 (21.9)
Hydatid cyst	
Single	13 (40)
Multiple	18 (60)
Treatment	
Medical	30 (93.5)
Surgical	16 (50)
Medical/surgical	14 (46.8)

no-CAP (Phadia AB, Uppsala). Inferential statistical analysis was conducted using the  $\chi^2$  test with Yates' correction, Fishers' exact test, Mann-Whitney's *U* test and Student's *t*-test.

## RESULTS

### Gender and age

Thirty-two patients were diagnosed with CE in the course of this study, of whom 21 were male and 11 were female, with a male-female ratio of 2:1. Their median age was 46 ± 16.5 years.

### Risk factors

Fourteen patients, (43.7%) were classified as having been exposed to CE. In 12 of these cases (85.7%), exposure was a result of occupation (dog or cattle breeders, farmers, butchers, veterinarians), and in two cases (14.2%) exposure occurred during recreational activities (hunting). A strong correlation was observed between exposure to risk factors and the presence of other subjects affected by CE in the patients' extended families (Figure 1A). Comorbidities were observed in 17 (53.1%) patients, hypertension in nine, diabetes in four, chronic pulmonary diseases in two, hepatitis B in one and hepatitis C in one.

### Diagnosis and localization

CE was detected in 16 patients (50%) by abdominal ultrasound, and in nine (28.1%) by chest X-ray, though in some cases diagnostic examinations were not carried out directly in the two units involved in the study. When diagnosed, 13 patients (40%) were observed as having a single hydatid cyst either in the liver (nine patients) or in the lung (four patients). Nineteen patients (60%) presented

multiple cysts with multi-organ involvement. Multiple cysts were observed more frequently (73.7% *vs* 35.5%, *P* < 0.05) in patients below 50 years of age at the time of diagnosis (Figure 1B). When diagnosed, 24 patients (75%) were symptomatic, the most frequent symptom being a dry cough. The liver-lung cyst localization ratio was 1.8:1 and although pulmonary cysts tended to be more frequently symptomatic than hepatic cysts, this difference was not significant (Figure 1C).

### Medical and surgical treatment

Thirty patients were treated medically, receiving an average of two cycles of albendazole (6 mo treatment; interquartile range of two to four cycles). Sixteen patients were treated surgically, while 14 patients were treated both medically and surgically. Table 1 summarizes the main characteristics of the 32 patients affected by CE. Of the 30 medically treated patients, 10 developed severe adverse events: leukopenia was observed in four patients, pancytopenia in one patient, and an increase in ALT levels in five patients (for three of whom it resulted in the discontinuation of treatment). Gamma-GT and ALP levels were monitored during albendazole treatment and were found to be normal. The onset of adverse events was not related to ALT levels observed before treatment with albendazole. Of the 30 patients treated with albendazole alone or in combination with surgery, 23 had either a complete cure of CE (disappearance or calcification of cysts) or an improvement (reduction in either the number or size of cysts). An increase in cyst diameter (impairment) was observed in one patient. Four patients could not be evaluated for response to treatment because the cysts were surgically removed immediately after therapy. Two patients showed no improvement.

### Relapses

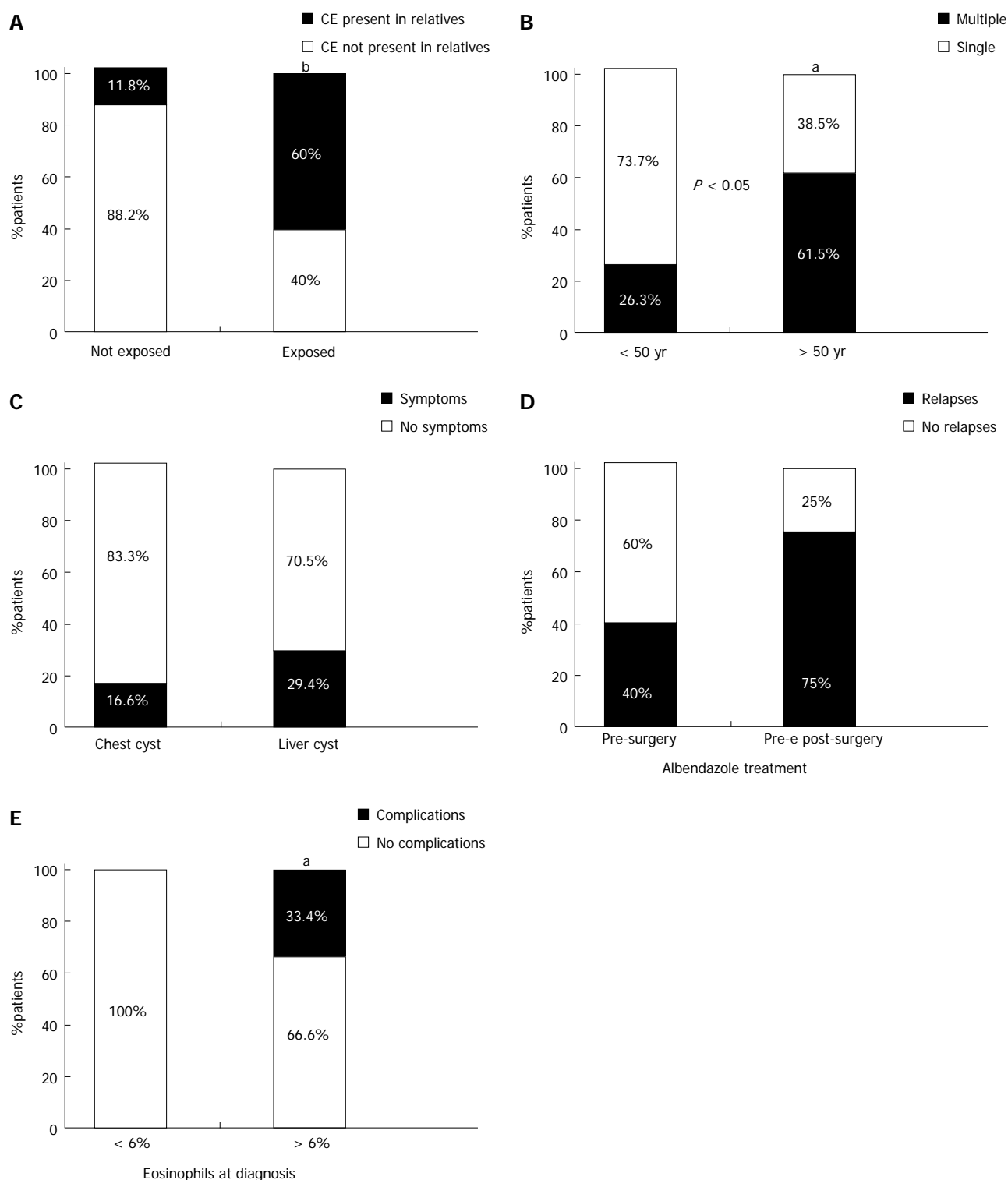
Of the 21 patients diagnosed before 2005, five patients (23.8%) relapsed. Relapses tended to be more frequent in patients that were treated with albendazole before surgical treatment alone than in those treated both before and after surgery, although this difference was not statistically significant (Figure 1D).

### Complications

Complications were observed in six patients (18.8%), these being cyst rupture, mostly traumatic, in five cases and bacterial infection in one case.

### Specific IgE and eosinophil cell count

At diagnosis, 71% of patients were positive for specific CE IgE. The median value of anti-echinococcus IgE titer was 16 kU<sub>A</sub>/L (25<sup>th</sup> percentile = 2; 75<sup>th</sup> percentile = 24.3; min = 0; max = 34). Six months after beginning treatment with albendazole, 60% of patients were positive for specific IgE. A significant reduction in anti-echinococcus IgE titer values was also observed (IgE titer median = 1.2; 25<sup>th</sup> percentile = 0.75; 75<sup>th</sup> percentile = 9.5;



**Figure 1 Correlation between different groups.** A: Correlation between exposure of patients to risk factors and presence of relatives affected by cystic echinococcosis (CE); B: Correlation between age at diagnosis and presence of single or multiple CE; C: Correlation between cysts location and presence of symptomatic CE; D: Correlation between treatment modality and development of relapses; E: Correlation between rate of eosinophils at diagnosis and development of complications. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.

min = 0; max = 369, *P* < 0.05). In cases of recurrence (five patients), the median value of anti-echinococcus IgE titer was 22 kU<sub>A</sub>/L (25<sup>th</sup> percentile = 4.93; 75<sup>th</sup> percentile = 38.5; min = 1.24; max = 42).

An eosinophil percentage of  $\geq 6\%$  was observed in 18 patients (56.3%) at diagnosis. After 6 mo of albendazole

treatment, a significant reduction in eosinophil count was observed, with only three patients (12%) showing more than 6%. The median percentage value at diagnosis was 5% (25<sup>th</sup> percentile = 3; 75<sup>th</sup> percentile = 9; min = 0; max = 35.2), while 6 mo after beginning treatment with albendazole this has fallen to 2% (25<sup>th</sup> percentile = 1; 75<sup>th</sup> percentile

= 3; min = 0.75; max = 12), with a highly significant difference in titres registered at these two times ( $P < 0.001$ ).

Patients with hyper-eosinophilia at diagnosis developed complications, but not relapses, more frequently than other patients (33.4% *vs* 0%,  $P < 0.05$ ) (Figure 1E). Patients with positive specific IgE results at diagnosis had a higher median eosinophil count than those with negative specific IgE.

## DISCUSSION

As hydatid cysts usually develop slowly, CE is often an under-diagnosed (and consequently under-treated) disease. It is often difficult to assess its prevalence in a given area. The aim of this study was to assess the epidemiological and clinical characteristics of CE and to evaluate responses to therapy and follow-up by analyzing patterns in patients affected by the disease admitted to two Infectious Diseases Departments from 2000 to 2010.

### Gender and age

During the ten-year observation period, 32 patients were diagnosed with CE, with a male/female ratio of 1.8/1. This ratio is comparable with that reported in other endemic areas and perhaps reflects the more frequent occupational exposure of males to the risk of infection<sup>[4-7]</sup>. The slow development of hydatid cysts, the nature of the host's immune response, and the structure of the most typically affected tissues, may all contribute to late diagnosis<sup>[6,7]</sup>.

### Risk factors

Of the total number of patients, 43% were classified as having been exposed to CE through specific risk factors. This is probably caused by the sharing of occupational and/or recreational risk factors among patients and their relatives. It is, therefore, crucial to evaluate patient exposure in family contexts and suggest CE screening for relatives and co-workers.

### Diagnosis and localization

Abdominal ultrasound was used as the primary diagnostic tool for 50% of patients. Ultrasound is widely considered one of the best hepatic diagnostic tools for CE, as it allows diagnosis even before the evidence of an increase in specific IgE antibodies<sup>[8,9]</sup>. In addition, abdominal ultrasound is fundamental for the staging of hepatic CE purposes and for establishing appropriate treatment options<sup>[10,11]</sup>. At the time of diagnosis, 60% of patients had multiple cysts. Those below the age of 50 were more likely to have multiple cysts ( $P < 0.05$ ) than those diagnosed later. This probably reflected the fact that multiple cysts tend to become more symptomatic than single cysts.

In other studies, the majority of CE patients diagnosed during non-hospital based screening programs in endemic areas did not present any symptoms of CE<sup>[12]</sup>. By contrast, our patients presented CE-related symptoms in 75% of cases. This may reflect the higher probability that an already symptomatic patient is more likely to be

referred to a hospital for investigation and/or treatment. In addition, a higher percentage of symptomatic patients was observed among patients affected by pulmonary-CE compared with hepatic-CE. This may be because hepatic hydatid cysts can grow for many years before symptom onset, while the symptoms of pulmonary CE tend to be more evident. Screening programs in endemic areas show a prevalence of hepatic-CE that is higher than pulmonary-CE, with a liver:chest localization ratio of 6-12:1<sup>[12]</sup>. In the patients of this study, the ratio was around 2.5:1, thus confirming the greater probability of finding cases of pulmonary-CE rather than hepatic CE in a hospital setting<sup>[12]</sup>.

### Medical and surgical treatment

The patients in this study were given albendazole in 93.8% of cases, with an average treatment duration of 6 mo and a maximum of 120 mo in one patient affected by non-surgical multi-organ CE. Studies have shown that Albendazole is more effective than mebendazole in the treatment of CE<sup>[13]</sup>. Some studies suggest treating CE patients with albendazole for no less than 3 mo, but no longer than 6 mo, longer treatment being offered only to patients with non-abdominal and non-pulmonary CE<sup>[14,15]</sup>.

Response to medical treatment was evaluated by means of ultrasound and X-ray, performed 1 year after beginning of therapy with albendazole. A complete cure (disappearance or calcification of cysts) or improvement (reduction in number or size of cysts) was achieved in 76% of patients treated with albendazole alone or in combination with surgery. This confirmed the results from other studies conducted on around 2000 CE patients treated with benzimidazoles, which showed a response to therapy of 50%-70% after a 12-mo follow-up<sup>[16-18]</sup>. Albendazole-related adverse events are usually self-limiting and rarely severe. However, in CE, factors such as the longer period of treatment needed, the frequently older age of patients and drug-cyst interaction, may lead to higher occurrence and severity<sup>[17-20]</sup>. One third of the patients in the current study developed moderate to severe adverse events. In three of the five hypertransaminasemia cases, this led to the discontinuation of treatment with this drug. Similarly, in a study carried out by Gil-Grande *et al.*<sup>[15]</sup>, 10%-20% of CE patients were seen to develop hypertransaminasemia, which was always reversible after drug discontinuation or during the pauses between albendazole cycles<sup>[13-19]</sup>.

The surgical treatment of CE can potentially lead to a complete cure of the disease and is indicated in the following cases: for the removal of large cysts with multiple daughter vesicles; for cysts exerting pressure on adjacent vital organs; in the case of cysts communicating with the biliary tree (as an alternative to percutaneous treatments or PTs); or where there are single superficial liver cysts and infected cysts (when PTs are not possible). In non-surgical cases, alternative techniques such as Puncture Aspiration Injection Respiration (PAIR) can be considered<sup>[6]</sup>. In our study, 50% of the patients were surgically treated, but only half of these cases were given albendazole both pre- and



post-surgery. The WHO recommendations stipulate that albendazole should be given at least 4 d before surgery and for 3 mo following surgery<sup>[20]</sup>. Other studies suggest that a 3 mo albendazole cycle before surgery is more effective in reducing cyst vitality, rather than treatment for only 4 d or even one mo<sup>[15]</sup>. Appropriate post-surgical albendazole therapy has also been observed to prevent CE relapses because of surgical dissemination<sup>[13-24]</sup>.

### Relapses

Among the patients of this study, 60% of those treated with albendazole before surgery alone suffered a relapse, compared to only 25% of those treated both before and after surgery. This suggests that appropriate medical therapy offered to the patient both pre- and post-surgery is necessary.

The literature reports a relapse incidence in the treatment of CE of between 0% and 30%, with an average time for the onset of relapse symptoms of 3-4 years<sup>[21]</sup>. The patients in this study developed symptomatic relapses, hepatic in 80% of cases and pulmonary in 20% of cases, in an average time of 20.7 mo after the cessation of treatment or following surgery, with a relapse rate of 23.8%.

### Complications

Six of the patients in this study (18.8%) developed complications, these being bacterial super-infection of a cyst in one case and cyst rupture (mostly traumatic and easily managed using a surgical approach) in five cases. The most frequent complication reported in CE endemic areas is cyst rupture into the biliary tree (5%-17%), followed by super-infection (5.1%)<sup>[25,26]</sup>.

### Specific IgE and eosinophil cell count

ELISA for specific anti-echinococcus IgG and IgE titer detection is frequently used in CE serological diagnosis and screening, as it requires a short preparation time, has a relatively limited cost, and shows a sensitivity and specificity of 95%<sup>[27-30]</sup>. In our study, at the time of diagnosis, 71% of patients were positive for specific CE IgE, with a median titer of 16 kUA/ which dropped significantly to 1.2 kUA/ (with 60% of positive specific IgE patients) 6 mo after beginning treatment with albendazole. This confirmed that monitoring IgE titer variation over time is likely to be more helpful in evaluating CE activity than considering single absolute values<sup>[31]</sup>. However, it should be noted that fluctuations in specific IgE titer that are unrelated to the clinical stage of the disease are possible. Extremely prolonged positivity (*e.g.*, 3-7 years) is possible even after surgical cyst treatment<sup>[32-34]</sup>. Persistence of high specific IgE titer beyond 3-7 years usually indicates the onset of a relapse. This appeared to be confirmed by the patients of this study that developed relapses: their median specific IgE value was 22 kUA/ at the time of relapse diagnosis<sup>[32-34]</sup>. On this basis, it is possible to claim that in CE-affected patients, specific IgE titer detection can be considered as a useful tool in post-therapeutic follow-up to predict cure and eventual relapse.

In the literature, CE diagnosis and follow-up eosinophil cell counts have usually been considered of limited value because it is significantly high in no more than half of CE affected patients, as confirmed in our own study<sup>[4]</sup>. In this study, although 56.3% of patients presented a percentage value of eosinophils  $\geq 6\%$  (defined as hyper-eosinophilia) at diagnosis, six months after beginning treatment with albendazole, only 12% of patients continued to display hyper-eosinophilia. At diagnosis, the median percentage value of eosinophils was 5%; however, 6 mo after beginning treatment with albendazole, this value had dropped to 2%. This significant difference in titers registered at the two different times ( $P < 0.001$ ) may be a result of the positive response to therapy obtained in the majority of patients. Finally, we observed that patients with positive specific IgE at diagnosis had a higher median eosinophil percentage than those with negative specific IgE ( $P < 0.05$ ). This suggested that, in settings with limited resources, or where specific IgE detection is not feasible, eosinophil percentage value at diagnosis could be used as an effective tool for post-therapeutic follow-up, to assess the response to therapy and as an aid in patient prognosis.

In conclusion, on the basis of our data, three main proposals can be made. Firstly, we advocate the routine screening for CE in relatives (and/or close associates) of patients classified as being exposed to CE in settings where this is feasible. We also recommend the use of anti-echinococcus IgE titer as a response marker of albendazole therapy (in low resource settings this could be replaced by eosinophil percentage value). Our results also highlight the need for an extended and well managed period of follow-up after surgical and/or medical therapy, as indicated by the average time for the onset of relapse, which in our patients was about 2 years. Finally, while the surgical approach remains the first-line treatment in most CE cases, appropriate medical therapy before and after surgery should also be considered a useful tool to reduce the incidence of relapse<sup>[35]</sup>.

## COMMENTS

### Background

Endemic in sheep raising areas, cystic echinococcosis (CE) is a neglected disease. This neglect is also reflected in the clinical management of CE, which has evolved over the last few decades with little or no comparative evaluation of the efficacy, effectiveness, rate of adverse events and relapse rates. In addition, CE is often under-diagnosed as it is frequently undetected, developing over the course of several years.

### Research frontiers

On the basis of their data, some proposal can be made: (1) routine screening for CE in relatives (and/or close associates) of patients exposed, according to occupation and recreational activities, who are potentially at risk for acquiring CE, to speed up CE diagnosis; (2) extended and manage the period of follow-up after surgical and/or medical therapy to avoid late CE relapse diagnosis; and (3) administer appropriate medical therapy before and after surgery to reduce the incidence of relapse.

### Innovations and breakthroughs

These findings suggest that anti-echinococcus immunoglobulin E (IgE) titer may be successfully replaced by eosinophil percentage count in low resource settings as a marker of response to therapy.

## Applications

Cystic echinococcosis is a helminthic zoonosis that can affect humans, most frequently involving the liver or the lungs. Symptoms typically show themselves only when the compression of internal organs occurs. In humans, the natural history of CE can lead to life-threatening complications, such as cyst rupture (with possible anaphylactic shock), the spread of new cysts, and bacterial infection.

## Terminology

Cystic echinococcosis is a helminthic zoonosis that can affect human beings, most frequently involving the liver or the lungs, showing symptoms only when the compression of internal organs occurs. In humans, the natural history of CE can lead to life-threatening complications, such as cyst rupture, with possible anaphylactic shock, the spread of new cysts, and bacterial infection.

## Peer review

Cystic echinococcosis is a helminthic zoonosis that can affect humans, most frequently involving the liver or the lungs. Symptoms typically show themselves only when the compression of internal organs occurs. In humans, the natural history of CE can lead to life-threatening complications, such as cyst rupture (with possible anaphylactic shock), the spread of new cysts, and bacterial infection.

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## Risk of cancer, with special reference to extra-intestinal malignancies, in patients with inflammatory bowel disease

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### Abstract

**AIM:** To determine the incidence and characteristics of intestinal and extra-intestinal cancers among patients with inflammatory bowel disease in a Spanish hospital and to compare them with those of the local population.

**METHODS:** This was a prospective, observational, 7-year follow-up, cohort study. Cumulative incidence, incidence rates based on person-years of follow-up and relative risk were calculated for patients with inflammatory bowel disease and compared with the background population. The incidence of cancer was determined using a hospital-based data registry from Hospital Universitario de Fuenlabrada. Demographic data and details about time from diagnosis of inflammatory bowel disease to occurrence of cancer, disease extent, inflammatory bowel disease treatment, cancer therapy and cancer evolution were also collected in the inflammatory bowel disease cohort.

**RESULTS:** Eighteen of 590 patients with inflammatory bowel disease developed cancer [cumulative incidence = 3% (95%CI: 1.58-4.52) vs 2% (95%CI: 1.99-2.11) in the background population; RR = 1.5; 95%CI: 0.97-2.29]. The cancer incidence among inflammatory bowel disease patients was 0.53% (95%CI: 0.32-0.84) per patient-year of follow-up. Patients with inflammatory bowel disease had a significantly increased relative risk of urothelial carcinoma (RR = 5.23, 95%CI: 1.95-13.87), appendiceal mucinous cystadenoma (RR = 36.6, 95%CI: 7.92-138.4), neuroendocrine carcinoma (RR = 13.1, 95%CI: 1.82-29.7) and rectal carcinoid (RR = 8.94, 95%CI: 1.18-59.7). Colorectal cancer cases were not found.

**CONCLUSION:** The overall risk of cancer did not significantly increase in our inflammatory bowel disease patients. However, there was an increased risk of urinary bladder cancer and, with less statistical power, an increased risk of appendiceal mucinous cystadenoma and of neuroendocrine tumors. Colorectal cancer risk was low in our series.

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**Key words:** Extra-intestinal cancer; Inflammatory bowel disease; Cancer risk; Background population; Urothelial carcinoma; Appendiceal mucinous cystadenoma; Neuroendocrine carcinoma; Rectal carcinoid

**Core tip:** Several studies have reported increased rates of colorectal cancer in patients with inflammatory bowel diseases but limited data are available regarding incidence of extraintestinal malignancies in these patients. The present study demonstrates a higher risk of urinary bladder cancer, mucinous cystadenoma of the appendix and of neuroendocrine tumors, and a low colorectal cancer risk, in patients with inflammatory bowel dis-



ease in our environment. We raised the question of whether current cancer screening strategies need to be reviewed and adapted to the characteristics of each patient.

Algaba A, Guerra I, Castaño Á, de la Poza G, Castellano VM, López M, Bermejo F. Risk of cancer, with special reference to extra-intestinal malignancies, in patients with inflammatory bowel disease. *World J Gastroenterol* 2013; 19(48): 9359-9365 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9359.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9359>

## INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic condition that involves several portions of the gastrointestinal tract and includes periods of activity of variable severity. This pathology can be associated with involvement of other organs in 35% of patients with IBD, with rheumatologic, ophthalmologic and dermatologic disorders being the most common extra-intestinal manifestations<sup>[1,2]</sup>.

To date, a number of studies have reported increased rates of colorectal cancer (CRC) in patients with IBD<sup>[3-8]</sup>. The reported risk varies widely between studies due to the different methodologies used. The current trend published in the most recent studies suggests a lower rate of CRC than previously described<sup>[9]</sup>. The location and duration of IBD<sup>[6-12]</sup>, as well as previous family history of CRC<sup>[13,14]</sup> and primary sclerosing cholangitis<sup>[15,16]</sup>, have been described as determinant factors associated with CRC. Additionally, the severity of chronic inflammation of the colon is another important risk factor for this kind of cancer in patients with both ulcerative colitis (UC) and Crohn's disease (CD)<sup>[17,18]</sup>. IBD-specific and non-IBD specific medications have also been associated with an increased or decreased risk of CRC<sup>[19,20]</sup>.

On the other hand, limited and disparate data are available for incidences of extra-intestinal malignancy in these patients<sup>[21-24]</sup>. In addition, several of these studies are not population based, or have retrospective designs. Other studies only contain information on cumulative cancer risk or represent select populations.

We present a prospective, cohort study designed to determine the incidence and characteristics of intestinal and extra-intestinal cancers among patients with IBD in our environment and to compare these incidences with those of the local population.

## MATERIALS AND METHODS

### Patients and design

This was a prospective, observational, 7-year follow-up, cohort study. We identified all cases of cancer observed between January 2005 to the end of December 2011 in a cohort of patients diagnosed with IBD in our hospital ( $n = 590$ ). Diagnosis of IBD was confirmed by routine clinical, radiological, endoscopic and histological cri-

teria<sup>[25]</sup>. The incidence and characteristics of intestinal and extra-intestinal cancers were obtained in the IBD group and were compared to those of the background population ( $n = 222219$ ). The incidence of cancer in our population was determined using a hospital-based data registry from Hospital Universitario de Fuenlabrada. This registry contains all cases of cancer diagnosed and/or treated in a 7-year follow-up period in the patients of our area. In each case, cancer was confirmed by a pathologist who classified the lesions according to the International Classification of Diseases for Oncology (ICD-O-3 histology codes).

Demographic data and details of time from diagnosis of IBD to occurrence of cancer, disease extent according to the Montreal Classification<sup>[26]</sup>, IBD treatment, cancer therapy and cancer evolution were also collected in the IBD cohort. The study was approved by the Research Ethics Committee at Hospital Universitario de Fuenlabrada.

### Statistical analysis

The descriptive analysis of quantitative variables calculated the mean and standard deviation or median and interquartile range (IQR) depending on whether or not the data were normally distributed. Qualitative variables were expressed as percentages with 95%CI. Cumulative incidences for each cancer were calculated for patients with IBD and compared with the background local population. The incidence rates based on person-years of follow-up and relative risk (RR) with 95%CI were also analysed. Analysis was performed assuming that the IBD cohort had the same risk of developing malignancies as the general population.

## RESULTS

Eighteen of 590 patients with IBD were diagnosed with cancer between 2005 and 2011 in our hospital. The clinical and demographic characteristics of all patients with IBD are shown in Table 1. The cumulative incidence of cancer was 3% (95%CI: 1.58-4.52) *vs* 2% (95%CI: 1.99-2.11) in the local population; RR = 1.5; 95%CI: 0.97-2.29. The mean age in IBD patients with a diagnosis of cancer was  $49.9 \pm 11.9$  years; 61.1% were males. In the cohort of patients with IBD, 9 had CD (50%) and 9 UC (50%). The clinical characteristics of these patients are shown in Table 2. By type of IBD, the cumulative incidence was 2.8% (95%CI: 0.86-4.84) for CD *vs* 3.5% (95%CI: 1.06-5.92) for UC patients. At the time of cancer diagnosis, 33% of patients were being treated with thiopurines [median duration of treatment was 6 mo (IQR 2.4-27)], and one patient (6%) was on anti-tumour necrosis factor-alpha therapy (39 mo of treatment with adalimumab). The median time from IBD diagnosis to cancer development was 54 mo (IQR 21-111). The cancer incidence among IBD patients was 0.53% (95%CI: 0.32-0.84) per patient-year of follow-up.

Ten different kinds of cancers were identified. The

**Table 1 Clinical features of the total studied inflammatory bowel disease patients *n* (%)**

Clinical features	Value
Age (yr, mean $\pm$ SD)	43.4 $\pm$ 13.8
Gender	
Female	305 (51.70)
Male	285 (48.3)
Type of IBD	
CD	313 (53.05)
UC	256 (43.39)
IBDU	21 (3.56)
Disease extension (UC) <sup>1</sup>	
Proctitis	61 (23.83)
Left-sided colitis	124 (48.44)
Pancolitis	71 (27.82)
Age at diagnosis (CD) <sup>1</sup>	
A1 < 17	24 (7.67)
A2 17-40	208 (66.45)
A3 > 40	81 (25.88)
Disease location (CD) <sup>1</sup>	
L1 ileal	112 (35.78)
L2 colic	76 (24.28)
L3 ileocolic	111 (35.46)
L4 upper gastrointestinal tract	6 (1.92)
L1 + L4	5 (1.60)
L3 + L4	3 (0.96)
Behaviour (CD) <sup>1</sup>	
B1 non-stricturing non-penetrating	179 (57.19)
B2 stricturing	30 (9.58)
B3 penetrating	41 (13.10)
B1 + perianal disease	51 (16.29)
B2 + perianal disease	1 (0.32)
B3 + perianal disease	11 (3.52)
Immunosuppressive or biological treatment	
Thiopurines	259 (43.90)
Anti-TNF- $\alpha$ drugs	84 (14.23)

<sup>1</sup>In accordance with the Montreal classification. IBD: Inflammatory bowel disease; IBDU: Inflammatory bowel disease type unclassified; CD: Crohn's disease; UC: Ulcerative colitis.

specific types of cancer observed in the cohort of IBD patients are shown in Table 3. Compared with the local population, patients with IBD had a significantly increased RR of urothelial carcinoma, mucinous cystadenoma of the appendix, neuroendocrine carcinoma and rectal carcinoid (Table 4). The RR of breast, skin, stomach, pancreas, lung and liver cancers were not significantly different with respect to the background local population (Table 4). CRC diagnoses were not found, and only two patients had biopsies with low grade dysplasia despite dysplasia screening by colonoscopy being performed following standard recommendations<sup>[27]</sup>.

All patients with a diagnosis of urinary bladder cancer were men: 2 UC and 2 CD, 1 smoker and 3 former smokers. All patients with breast cancer had a previous family history (none of the remaining patients had family history for other types of tumours).

Regarding the evolution of cancer, 16 of the 18 patients diagnosed with cancer (88.9%) needed oncological surgery and 27.7% (*n* = 7) were treated with chemotherapy or radiation therapy. Treatment was maintained in 50% of patients on thiopurines at the time of their cancer diagnosis. In the remaining patients, immunosup-

**Table 2 Clinical features of the cohort of patients with inflammatory bowel disease and cancer diagnosis *n* (%)**

Features	Value
Disease extension (UC) <sup>1</sup>	
Proctitis	1 (11.1)
Left-sided colitis	4 (44.45)
Pancolitis	4 (44.45)
Age at diagnosis (CD) <sup>1</sup>	
A2 17-40	8 (88.89)
A3 > 40	1 (11.11)
Disease location (CD) <sup>1</sup>	
L1 ileal	4 (44.45)
L2 colic	2 (22.22)
L3 ileocolic	2 (22.22)
L3 + L4 upper gastrointestinal tract	1 (11.11)
Behaviour (CD) <sup>1</sup>	
B1 non-stricturing non-penetrating	7 (77.78)
B2 stricturing	1 (11.11)
B1 + perianal disease	1 (11.11)
Treatment at time of cancer diagnosis	
Aminosalicylates	10 (55.56)
Thiopurines	6 (33.34)
Anti-TNF- $\alpha$ drugs	1 (5.55)
Nonspecific inflammatory bowel disease treatment	1 (5.55)
Immunosuppressive or biological treatment previous cancer diagnosis	
Thiopurines	1 (5.55)
Anti-TNF- $\alpha$ drugs	1 (5.55)

<sup>1</sup>In accordance with the Montreal classification. CD: Crohn's disease; UC: Ulcerative colitis; TNF- $\alpha$ : Tumor necrosis factor  $\alpha$ .

**Table 3 Specific cancers diagnosed in the cohort of patients with inflammatory bowel disease between 2005 and 2011**

Location	<i>n</i>	Histological type
Urinary bladder	4	Urothelial carcinoma
Breast	3	Carcinoma
Skin	1	Melanoma
	2	Basal cell carcinoma
Appendix	2	Mucinous cystadenoma
Stomach	1	Adenocarcinoma
Pancreas	1	Adenocarcinoma
Lung	1	Adenocarcinoma
Liver	1	Hepatocellular carcinoma
Small intestine	1	Neuroendocrine carcinoma
Rectum	1	Carcinoid

pressive therapy was withdrawn and in only one of them was thiopurine therapy reintroduced three years later in agreement with the oncologist's recommendations. An association between thiopurine treatment and malignancy risk was not found. Adalimumab treatment was also withdrawn after the cancer diagnosis. Patients were followed up for an average of  $3.5 \pm 2.3$  years from tumour diagnosis. During this period, two patients died (11.1%) due to cancer (patients with lung and liver cancer diagnoses) and 3 (16.7%) had tumour recurrence.

## DISCUSSION

The present cohort study of patients with IBD revealed

**Table 4** Cumulative incidences in both the inflammatory bowel disease cohort and the non-inflammatory bowel disease cohort and relative risk for different types of cancer

Cancer site	Cumulative incidence in IBD cohort	Cumulative incidence in local population	RR	95%CI
Urinary bladder	0.68	0.13	5.23	1.95-13.87 <sup>a</sup>
Breast	0.51	0.26	1.95	0.63-5.87
Melanoma	0.17	0.06	2.56	0.34-17.63
Basal cell carcinoma	0.33	1.26	0.26	0.06-1.00
Appendix	0.33	0.009	36.6	7.92-138.4 <sup>a</sup>
Stomach	0.17	0.06	2.83	0.41-20.9
Pancreas	0.17	0.028	6.07	0.84-40.4
Lung	0.17	0.067	2.54	0.34-17.6
Liver	0.17	0.037	4.59	0.64-32.5
Small intestine	0.17	0.013	13.1	1.82-29.7 <sup>a</sup>
Rectum	0.17	0.019	8.94	1.18-59.7 <sup>a</sup>

<sup>a</sup> $P < 0.05$ . IBD: Inflammatory bowel disease.

that the overall risk of cancer did not significantly increase in our IBD patients compared to the background population. However, the study found patients with IBD to have an increased risk of developing urothelial carcinoma and, with less statistical power, an increased risk of mucinous cystadenoma and neuroendocrine tumours.

The results obtained in our population regarding overall risk of cancer and risk of urinary bladder cancer are in accordance with those reported previously by Pedersen *et al.*<sup>[23]</sup>. The association between CD and urinary bladder cancer described in other populations by other authors<sup>[23,28]</sup> could be related to the high prevalence of smokers. Pedersen *et al.*<sup>[23]</sup> explain in their meta-analysis that tobacco smoking could have a causal role in the development of a number of cancers including urinary bladder cancer. They did not find this association in UC patients. In our study, out of the two patients diagnosed with urothelial carcinoma with UC and two patients with CD, only one patient was a smoker at time of cancer diagnosis, while the remaining three patients were former tobacco users. Although different factors may be involved in the development of bladder cancer, the results obtained in several studies, including our work, indicate that tobacco could be a key factor and it would be recommended to encourage CD patients to quit smoking. A recent publication finds that the risk of bladder cancer in former smokers remains elevated more than 32 years after quitting, even among those with moderate smoking histories<sup>[29]</sup>. Nonsteroidal anti-inflammatory drugs seem to have a chemo-preventive role in urothelial carcinoma of the bladder in subjects who have quit for long periods<sup>[30]</sup>. Dietary factors may also affect the risk of these carcinomas<sup>[31-33]</sup>. Ros *et al.*<sup>[32]</sup> suggest that high consumption of certain types of vegetables and fruits may reduce the risk of aggressive or non-aggressive urothelial cell carcinoma of the bladder.

On the other hand, the increased risk of appendiceal mucinous cystadenoma and neuroendocrine tumours observed in the present study were not described before in

the meta-analysis carried out by Pedersen *et al.*<sup>[23]</sup>.

Mucocele of the appendix is a rare group of lesions that includes four histological types: retention cyst, mucosal hyperplasia, cystadenoma and cystadenocarcinoma. In our study, IBD patients were at an increased risk of mucinous cystadenoma of the appendix. Although a causal relationship between IBD and this type of mucocele cancer is still being elucidated, some authors have suggested that obstruction of the appendiceal orifice might play a role in the development of appendiceal mucocele. This obstruction could be due to inflammation by IBD or associated with colorectal neoplasm<sup>[34-36]</sup>.

Several publications suggest that appendectomy appears to reduce the extent and recurrence of UC and is associated with a less severe course of this pathology<sup>[37,38]</sup>. These findings could support the hypothesis that an appendectomy related to mucinous cystadenoma diagnosis may have a secondary beneficial effect on the severity and the course of UC. In contrast, data regarding CD patients are controversial<sup>[39,40]</sup>. In our series, the two patients with mucocele of the appendix had CD and ileocolic involvement.

The present study also identified one case of neuroendocrine carcinoma of the small intestine and one carcinoid of the rectum. Carcinoids and, in particular, neuroendocrine neoplasms other than carcinoids are uncommon tumours and are infrequently described in UC and CD. Some authors suggest that a permanent inflammation at the level of the colon could increase the number of neuroendocrine cells and help the development of this kind of neoplasm<sup>[41]</sup>. Greenstein *et al.*<sup>[42]</sup> included eleven patients with IBD-associated carcinoid tumours (11 in the appendix, 2 in the ileum). They found that all carcinoids were diagnosed incidentally after surgery for IBD. These findings are consistent with our results, in which the patient was diagnosed with carcinoid of the rectum during a colectomy. Sigel *et al.*<sup>[43]</sup> evaluated 14 cases of neuroendocrine neoplasms. All of the tumours arose in areas with IBD involvement. Tumour sites were the rectum (6 cases), appendix (4 cases), small bowel (2 cases) and sigmoid colon (2 cases). Conversely, in our case, the patient with neuroendocrine carcinoma of the small intestine had UC (extensive colitis) without ileum involvement.

Concerning the IBD-specific medications, it has been reported that patients with IBD who receive thiopurines are at increased risk of non-melanoma skin cancer or lymphoproliferative disorders<sup>[44,45]</sup>. However, data about other malignancies are less clear. Moreover, studies in patients taking azathioprine for a long time are scarce. In the present study, a possible effect of thiopurines on the risk of extra-colonic cancer was evaluated but a clear association between variables was not found; this probably could be due to the small size of our series and the short period of treatment.

A higher RR of CRC has been described in patients with IBD<sup>[5,7,46]</sup>. However, in the present study, CRC was not found despite dysplasia screening by colonoscopy. These data are consistent with current publications

that show a lower incidence of CRC than previously reported<sup>[9,47-49]</sup>. Some authors have found that only IBD patients with a longer disease duration, extensive disease and an IBD diagnosis at a young age have a significantly higher risk of CRC. IBD patients without these characteristics would have a similar risk for colorectal cancer as the general population<sup>[9,47,48,50]</sup>. IBD-specific and non-IBD specific medications have also been associated with an increased or decreased risk of CRC<sup>[19,20,51]</sup>. The anti-inflammatory action of IBD drugs such as 5-ASA seems to have a protective effect on the occurrence of CRC<sup>[52,53]</sup>. Jess *et al.*<sup>[48]</sup> suggest that changes in IBD-specific treatment may reduce the risk of CRC among UC patients. Currently, it has also been suggested that agents that control chronic inflammation, such as thiopurines and tumour necrosis factor- $\alpha$  antagonists, could have a protective role against the development of CRC<sup>[48,50,54,55]</sup>.

Study design could also have an influence on the CRC risk obtained. Patients with IBD from population-based cohorts have a lower risk for CRC than reference centre cohorts<sup>[47]</sup>. Regarding this fact, we present a cohort study that included all patients with IBD followed in our hospital. We believe that this cohort of patients is a good representative for the entire IBD population in our area. Our hospital is a reference centre with a specific unit for patients with IBD. There are no other hospitals or reference centres in our area. In our opinion, only an insignificant number of patients with IBD are treated by general practitioners at primary care centres. In addition, the incidence of IBD is not lower than that expected for our population: 590 cases in a population of 222219 people. The present manuscript has the value of its prospective design and its well-defined population using a data registry that contains all cases of cancer diagnosed and/or treated in a 7-year follow-up period. In this regard, the results obtained in this survey probably will serve as a base for future studies aimed at elucidating the real risk of extra-intestinal malignancies and the utility of current cancer screening strategies used in these patients.

However, the observations obtained in the present study should be considered with caution due to the potential limitations of this study, which included a small number of cases over a limited period of time.

In conclusion, our prospective study revealed that patients with IBD in our area have a similar overall risk of cancer as the local population. However, they are at a higher risk of developing specific types of extra-intestinal cancers such as bladder, appendiceal cystadenoma or neuroendocrine tumours. Smoking and specific IBD characteristics could be risk factors associated with the development of cancer, so it is recommended that patients be encouraged to quit smoking. On the other hand, the present study did not show a higher risk of CRC, in line with recent publications that suggest a lower incidence of CRC than previously reported. Further evaluations are required to know if the current CRC screening strategies need to be reviewed and adapted to patients according to the characteristics of their IBD.

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## COMMENTS

### Background

To date, many studies have analyzed the rates of colorectal cancer in patients with inflammatory bowel disease. The reported risk varies widely between studies due to the different methodologies used. However, limited and disparate data are available for incidences of extra-intestinal malignancy in these patients.

### Research frontiers

Inflammatory bowel disease is a chronic condition that involves several portions of the gastrointestinal tract but also can be associated with involvement of other organs. Accordingly with the special characteristics of this pathology, the risk of development of different kind of cancers in these patients could be different from the general population.

### Innovations and breakthroughs

The present study demonstrates a higher risk of urinary bladder cancer, appendiceal mucinous cystadenoma and of neuroendocrine tumors and a low colorectal cancer risk in patients with inflammatory bowel disease in a Spanish hospital setting. These results could suggest the revision and adaptation of current cancer screening strategies according to characteristics of each patient.

### Applications

The results obtained in this survey could serve as the basis of future studies aimed at elucidating the real risk of extra-intestinal malignancies and the utility of current cancer screening strategies used in these patients.

### Terminology

Appendiceal mucinous cystadenoma is a rare tumour of the appendix characterized by a cystic dilatation of the appendiceal lumen with stasis of mucus inside it.

### Peer review

The authors present a prospective, cohort study designed to determine the incidence and characteristics of intestinal and extra-intestinal cancers among patients with inflammatory bowel disease and to compare these incidences with those of the local population.

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## Transforming growth factor- $\beta$ and toll-like receptor-4 polymorphisms are not associated with fibrosis in haemochromatosis

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### Abstract

**AIM:** To investigate the role of genetic polymorphisms in the progression of hepatic fibrosis in hereditary haemochromatosis.

**METHODS:** A cohort of 245 well-characterised C282Y homozygous patients with haemochromatosis was studied, with all subjects having liver biopsy data and DNA available for testing. This study assessed the association of eight single nucleotide polymorphisms (SNPs) in a total of six genes including toll-like receptor 4 (TLR4), transforming growth factor-beta (TGF- $\beta$ ), oxoguanine DNA glycosylase, monocyte chemoattractant protein 1, chemokine C-C motif receptor 2 and interleukin-10 with liver disease severity. Genotyping was performed using high resolution melt analysis and sequencing. The results were analysed in relation to the stage of hepatic fibrosis in multivariate analysis incorporating other cofactors including alcohol consumption and hepatic iron concentration.

**RESULTS:** There were significant associations between the cofactors of male gender ( $P = 0.0001$ ), increasing age ( $P = 0.006$ ), alcohol consumption ( $P = 0.0001$ ), steatosis ( $P = 0.03$ ), hepatic iron concentration ( $P < 0.0001$ ) and the presence of hepatic fibrosis. Of the candidate gene polymorphisms studied, none showed a significant association with hepatic fibrosis in univariate or multivariate analysis incorporating cofactors. We also specifically studied patients with hepatic iron loading above threshold levels for cirrhosis and compared the genetic polymorphisms between those with no fibrosis vs cirrhosis however there was no significant effect from any of the candidate genes studied. Importantly, in this large, well characterised cohort of patients there was no association between SNPs for TGF- $\beta$  or TLR4 and the presence of fibrosis, cirrhosis or increasing fibrosis stage in multivariate analysis.

**CONCLUSION:** In our large, well characterised group



of haemochromatosis subjects we did not demonstrate any relationship between candidate gene polymorphisms and hepatic fibrosis or cirrhosis.

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**Key words:** Haemochromatosis; Genetic polymorphism; Liver fibrosis; Toll-like receptor 4; Interleukin 10; Monocyte chemoattractant protein 1; Chemokine (C-C motif) ligand 2; Transforming growth factor beta; 8-oxoguanine DNA glycosylase

**Core tip:** This study does not support the previously proposed role of mutations in both toll-like receptor 4, transforming growth factor-beta in the progression of hepatic fibrosis associated with hereditary haemochromatosis.

Wood MJ, Powell LW, Dixon JL, Subramaniam VN, Ramm GA. Transforming growth factor- $\beta$  and toll-like receptor-4 polymorphisms are not associated with fibrosis in haemochromatosis. *World J Gastroenterol* 2013; 19(48): 9366-9376 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9366.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9366>

## INTRODUCTION

It is generally believed that genetic factors may influence the progression of hepatic fibrosis in chronic liver disease of differing aetiologies. Many case-control studies have been performed in an attempt to elucidate these genetic influences, however, results have been inconsistent. Possible explanations for this include relatively small sample sizes and the difficulties in controlling for factors such as the duration of hepatic insult (*e.g.*, in chronic hepatitis C virus infection and disease co-morbidities (*e.g.*, alcohol)<sup>[1]</sup>. In hereditary haemochromatosis iron accumulation begins in early adulthood in males and despite similarity in the age of onset, there is a highly variable disease progression both in iron loading and in hepatic fibrosis progression<sup>[2-6]</sup>. It is likely that genetic factors play a role in influencing both iron accumulation and the development of cirrhosis<sup>[7]</sup>. The aim of this study was to explore potential genetic polymorphisms involved in hepatic disease progression in haemochromatosis with particular attention to candidate molecules associated with the processes of hepatic fibrogenesis. This study was conducted using a well-characterised cohort of patients with HFE-associated hereditary haemochromatosis with known fibrosis stage and quantitative hepatic iron loading.

Candidate genes for analysis were chosen based either on their existing association between gene mutations and fibrogenesis in other disease aetiologies, or their demonstrated role in hepatic stellate cell biology and hepatic injury/fibrosis. Candidate genes included:

(1) molecules associated with hepatic inflammation including monocyte chemoattractant protein 1 (MCP-1), the MCP-1 receptor, chemokine C-C motif receptor 2 and interleukin-10 (IL10); and (2) mediators of hepatic injury/inflammation/fibrosis including transforming growth factor-beta (TGF- $\beta$ ), toll-like receptor 4 (TLR4) and human 8-oxoguanine DNA glycosylase (hOGG1).

MCP-1, also known as chemokine (C-C motif) ligand 2, is a cytokine belonging to the CC chemokine family which acts as a potent inducer of monocyte, macrophage and hepatic stellate cell migration<sup>[8-11]</sup> and is involved in the early stages of hepatic inflammation and fibrogenesis<sup>[9]</sup>. Several clinical studies have shown an association between single nucleotide polymorphisms (SNPs) in the *MCP-1* gene and fibrosis in various organs including liver, kidney, and skin<sup>[12-14]</sup> although others have shown conflicting results. The MCP-1 receptor, CCR2, mediates much of chemokine response of MCP-1<sup>[15]</sup>. While the precise role of CCR2 in human liver disease is relatively unknown, studies investigating CCR2 variants in alcoholic liver disease or liver carcinoma have produced varying results<sup>[16,17]</sup>. IL10 acts as an anti-inflammatory cytokine<sup>[18]</sup> in different forms of human chronic liver disease, regulating inflammatory and fibrogenic responses (reviewed in<sup>[19]</sup>). In haemochromatosis, hepatic IL10 mRNA expression is decreased<sup>[20]</sup>.

TGF- $\beta$  has been described as a “master switch” in hepatic fibrosis due to its central role in the activation of hepatic stellate cells and the production of fibrillar collagen *via* Smad<sup>[21]</sup>. SNPs in the *TGF- $\beta$*  gene have previously been studied in a relatively small cohort of subjects with haemochromatosis where results suggested that the presence of the proline substitution (C) may accelerate hepatic fibrosis<sup>[22]</sup>. TLRs are a group of receptors involved in both the recognition of pathogens and in mediating non-infectious and ischaemic causes of liver injury<sup>[23,24]</sup>. TLR4 has been shown to be associated with signalling leading to hepatic fibrosis, with hepatic stellate cells being the main direct mediator promoting fibrogenesis *via* TGF- $\beta$  signalling<sup>[25]</sup>. Studies designed to assess the role of *TLR4* polymorphisms in the susceptibility to inflammatory or infectious diseases have provided conflicting results<sup>[26,27]</sup>. In haemochromatosis, however, one study has shown that a *TLR4* polymorphism was associated with clinical disease without any notable effect on iron loading<sup>[28]</sup>, although again this was conducted in a relatively small cohort of patients. hOGG1 is an enzyme responsible for repairing the 8-oxo-7,8-dihydroguanine 8 lesion of DNA subjected to oxidative stress. Although oxidative stress is one of the common mechanisms for hepatocyte injury and an inflammatory cascade, particularly in iron loading, to our knowledge no studies have considered the role of *OGG1* gene polymorphisms in the progression of liver damage.

Few studies have assessed the contribution of polymorphisms in genes associated with hepatic injury and fibrosis in the phenotypic disease expression in haemochromatosis. Those that have were limited by both



cohort size and the lack of well characterised patients with liver biopsy-proven fibrosis staging and quantitative hepatic iron loading. This study represents one of the largest cohorts of patients with haemochromatosis to be assessed for the role of SNPs associated with hepatic disease expression.

## MATERIALS AND METHODS

### Ethics statement

All subjects in this study provided written informed consent and the study was approved by the Human Research Ethics Committees of the Royal Brisbane and Women's Hospital (RBWH) and the Queensland Institute of Medical Research (QIMR), Brisbane, Australia. Written informed consent was witnessed and documented in each patient hospital file, a procedure approved by both ethics committees.

### Study subjects

The subjects in this study were derived from the Haemochromatosis Database at the QIMR. This is a cohort recruited over approximately 30 years from clinical review at the RBWH. The database lists more than 2400 patients of whom 722 are C282Y homozygous. The database includes clinical and laboratory data, with DNA available from a subset of these patients.

Inclusion criteria for selection for this study were: (1) genetic testing confirming C282Y homozygosity; (2) patients had previously undergone a liver biopsy for clinical indications and information was available with respect to iron loading and fibrosis stage; and (3) patients had previously provided blood for the extraction of DNA from peripheral white cells or were available to do so prospectively.

Patients were excluded from this study if aged less than 16 years at the time of liver biopsy as iron loading at this age may indicate the presence of other mutations in iron homeostatic genes. Patients with viral hepatitis were excluded. Excessive alcohol consumption was not an exclusion criterion however this information was included in the data collection.

Control subjects were obtained from the QIMR DNA bank and represented a selection of healthy subjects who had previously provided peripheral blood for the extraction of DNA. Those with European ethnicity were preferentially utilised for this study in order to provide a genetically comparable group for the study subjects. Allele frequencies were also compared to subjects in the International HapMap project selected from United States residents with Northern and Western European ancestry<sup>[29]</sup>.

### Rationale for candidate gene analysis

While this cohort of haemochromatosis patients is one of the largest and most well characterised studied to date, investigation using genome wide association in a cohort of this size was not considered viable, thus we

used a candidate gene approach.

**MCP-1 and CCR2:** Human MCP-1 production is regulated in part by a region 1.8 to 2.7 kb upstream of the transcriptional start site and a polymorphism at position -2518 (G/A rs1024611) affects the transcriptional activity<sup>[30]</sup>. Individuals with a G allele at this site (G/A or G/G) produce more MCP-1 from monocytes in response to stimulation with interleukin-1 $\beta$ <sup>[30]</sup>. A variant in the CCR2 gene Val64Ile (A/G rs1799864) has been shown to be associated with a delay in progression in human immunodeficiency virus<sup>[31]</sup> and other studies have shown a possible role for this polymorphism in inflammatory conditions such as sarcoidosis and atherosclerosis<sup>[32,33]</sup>. Thus, the MCP1-2518 and CCR2-190 SNPs were chosen for study.

**IL10:** The promoter region of the *IL10* gene has several polymorphisms at positions -1082 (G/A: rs1800896), -819 (C/T: rs1800871) and -592(C/A: rs1800872) with only three haplotypes found in Caucasian populations: GCC, ACC and ATA<sup>[34]</sup>. Heritability factors are thought to account for some of the variability in IL10 production although environmental influences are also important<sup>[35,36]</sup>. The role of specific SNPs in differential IL10 production is controversial<sup>[37-40]</sup>. The GCC promoter haplotype may have greater transcriptional activity compared to the ATA and ACC haplotypes<sup>[35,41]</sup> and this would be consistent with other studies showing decreased IL10 production in those with the -1082A genotype<sup>[34,39,42]</sup>. Most studies considering the relationship between *IL10* promoter polymorphisms and liver fibrosis in HCV infected patients have failed to show statistically meaningful effects although many of these have suffered from small sample sizes (reviewed in<sup>[43]</sup>). Therefore, although it seems attractive to consider IL10 as a mediator of hepatic inflammation and fibrosis, promoter polymorphisms in this gene have not been unambiguously linked with either cytokine production or hepatic fibrosis. For this study, IL10-1082 and IL10-592 SNPs were selected for investigation.

**TGF- $\beta$ :** A single nucleotide polymorphism in the *TGF- $\beta$*  gene at position -915, codon 25(G/C: rs1800471) results in an amino acid substitution of proline for arginine. Leukocytes from those homozygous for the arginine (G) molecule at this site appear to produce more TGF- $\beta$  in response to stimuli suggesting this is the "high producing" genotype<sup>[44]</sup>. This report has been challenged by other groups who have suggested that there may be important differences between total TGF- $\beta$  secretion and bioavailable forms<sup>[45,46]</sup>. The TGF $\beta$ -915 SNP was used in this evaluation of disease expression susceptibility in haemochromatosis.

**TLR4:** Two common missense mutations in the *TLR4* gene have been suggested to have functional significance with defective signalling resulting. Aspartic acid substi-

**Table 1** Gene specific primers for polymerase chain reaction amplification and dissociation analysis

Gene	Forward primer	Reverse primer
MCP1-2518	TTTCTTGACAGAGCAGAAGTGGGAG	TTGCTGGCTGAGTGTTCACATAGG
CCR2 190	ATACCAACGAGAGCGGTGAAGAAG	AAAGCAGATCAGAGATGGCCAGG
IL10-592	AAAGGAGCCTGGAACACATCCTGT	AAAGTTCCAAGCAGCCCTTCCAT
IL10-1082	TCCAAGACAACACTACTAAGGCTTC	GCTGGATAGGAGGTCCCTTACTTT
TGF- $\beta$	CTACCGCTGCTGTGGCTACTGGT	TCACCAGTCCATGTCGATAGTCT
TLR4-299	CCGATTAGCATACTTAGACTACTACCTC	CCTTCAATAGTCACACTCACCAGG
TLR4-399	GCTTGAGTTTCAAAGGTTGCTGTCTC	GCCCAAGAAGTTTGAACATCATGGTAA
hOGG1	ACCCTCTACAGGTGCTGTTCAGT	CCTTGGAAACCCTTCTGCGCTTT

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF- $\beta$ : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

**Table 2** Polymerase chain reaction profiles for candidate gene high resolution melt analysis

Gene	Annealing temperature	Extension temperature	Melt analysis range
MCP-1	58 °C	72 °C	74 to 84 °C
CCR-2	58 °C	72 °C	74 to 84 °C
IL10-592	57 °C	72 °C	75 to 85 °C
IL10-1082	59 °C	72 °C	70 to 81 °C
TGF- $\beta$	65 °C (20 s)	72 °C (20 s)	79 to 89 °C
TLR4 299	57 °C	68 °C	66 to 78 °C
TLR4 399	58 °C	70 °C	69 to 81 °C
hOGG1	57 °C	68 °C	78 to 88 °C

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF- $\beta$ : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

tuted for glycine at amino acid position 299 (Asp299Gly) (A/G: rs4986790) and 399 (Thr399Ile) (C/T: rs4986791) are said to induce hypo-responsiveness to lipopolysaccharide although this has not been supported by all investigations<sup>[47-49]</sup>. The CC variant of the Thr399Ile polymorphism was one SNP included in a panel of tests demonstrating discrimination of patients with advanced fibrosis in chronic hepatitis C<sup>[50]</sup>. Functional studies have shown that the polymorphisms in TLR4 which confer protection from hepatic fibrosis are associated with a reduced threshold for HSC apoptosis and attenuation of fibrogenic responses stimulated by MCP-1, BAMBI and IL6<sup>[51]</sup>. Both TLR4-299 and TLR4-399 SNPs were assessed in the present study.

**OGG1:** It can be shown on paraffin sections of diseased liver that there is increased staining of 8-oxodG consistent with oxidative DNA damage to hepatocytes and this does not appear to be specific to any particular type of liver disease<sup>[52]</sup>. A polymorphism exists in the human OGG1 gene leading to Ser326Cys conversion and affecting the function of this glycosylase due to changes in localization and phosphorylation (C/G: rs1052133)<sup>[53]</sup>. There is some epidemiological evidence to suggest that certain tumours may have an increased prevalence in those with the variant of OGG1<sup>[54]</sup>. Functional studies

have shown that individuals with the GG genotype in Ser326Cys have a reduced capacity for repair of DNA damage compared to wild types or heterozygous subjects<sup>[55]</sup>. Thus OGG1-326 was included for evaluation in this study.

### SNP mutation analysis, real-time PCR and sequencing

Patients provided blood samples, often at the time of therapeutic venesection to allow DNA extraction from buffy coats using a high salt extraction method. DNA was utilised in all PCR experiments at a concentration of 25 ng/ $\mu$ L. Primers were designed using Genbank to obtain genetic sequences adjacent to the area of interest and using specific software (Primer Quest) to optimise the temperature difference for denaturing and minimise secondary structures (Table 1). Specificity was tested using specific software (Blast). Primers were purchased from Sigma-Aldrich Pty Ltd (NSW, Australia).

High resolution melt (HRM) analysis (Corbett Life Science Rotor-Gene<sup>TM</sup> 6000 HRM) was used to evaluate nucleotide sequences based on the dissociation profile of the fragment of DNA containing the polymorphism when amplification of the template occurs in the presence of specific dye. Sensimix HRM<sup>TM</sup> (Quantace, London) was used in PCR reactions with the following volumes for 1 reaction to give a total reaction volume of 25  $\mu$ L. Each gene was amplified and analysed under optimised conditions (Table 2).

From initial HRM analysis, subjects with variable dissociation characteristics were selected for sequencing in order to confirm the presence of the suspected gene polymorphism. Once identified, a sample from these subjects was used in each experiment to provide a positive control. Any sample that could not be genotyped with HRM analysis was subjected to sequencing. Each patient sample was tested in duplicate. All experiments contained negative control samples (H<sub>2</sub>O) to confirm the absence of contamination by PCR product. All patient and control sample identities were number coded for inclusion in experiments.

Sequencing was performed with ABI BigDye Version 3.1 according to specific instructions and using primers designed to incorporate the area of interest.

**Table 3** Patient characteristics grouped according to the presence or absence of hepatic fibrosis (univariate analysis) *n* (%)

Parameter	No fibrosis ( <i>n</i> = 136)	Fibrosis present ( <i>n</i> = 109)	<i>P</i> value
Male gender	72 (53)	89 (82)	0.0001
Age at biopsy (yr) (mean ± SD)	39.7 (14.9)	44.3 (12.4)	0.006
Alcohol (g/d) (median: range)	5 (0-120)	20 (0-200)	0.0001
Steatosis present <sup>1</sup>	36 (37)	42 (53)	0.03
Serum ferritin (µg/L) (median: range)	531 (33-3000)	2134 (155-6000)	0.0001
HIC (µmol/g dw) (median: range)	124 (20-537)	218 (43-847)	< 0.0001
Iron grade			
0	1 (1)	0	
1	5 (4)	0	
2	27 (20)	7 (6)	
3	63 (46)	25 (23)	
4	40 (29)	77 (71)	< 0.0001

<sup>1</sup>Steatosis data available for 176 subjects (72% of total cohort).

### Statistical analysis

Normally distributed data were summarized by mean and standard deviation and differences tested by Student's *t* test. Non-parametric data were summarized by median and range and tested by Mann-Whitney or Kruskal-Wallis test. Categorical variables were summarized by frequencies and tested by Pearson  $\chi^2$  or Fisher's Exact test. Ordinal multivariate logistic regression analysis was performed using increasing hepatic fibrosis grade as the outcome variable incorporating predictor variables including age, gender, alcohol consumption and the genetic polymorphism in question. Results are presented as OR with 95%CI. *P* values of < 0.05 or less were considered significant. Polymorphisms were grouped in both possible ways for testing but reporting has been limited to that commonly described in the literature. Grouping for the outcome variable of hepatic fibrosis was done in several ways including "no fibrosis (F0) *vs* any hepatic fibrosis (F1-4)", "minimal fibrosis (F0-2) *vs* advanced fibrosis (F3-4)" and as an ordered logistic regression analysis with increasing hepatic fibrosis grade as the outcome variable. In order to consider those patients with the greatest difference in clinical outcome the analysis was repeated incorporating only those patients with heaviest iron loading (iron grade 3 and 4) and comparing those with no fibrosis (F0) against those with advanced fibrosis (F3/F4). Stata/IC software (version 10.1; StataCorp LP, College Station, TX) was used for statistical analysis. Deviation from Hardy-Weinberg equilibrium was tested for each gene using an on-line calculation tool (<http://www.oege.org/software/hwe-mr-calc.shtml>).

## RESULTS

Of the 245 C282Y homozygous patients included in this study, 161 (66%) were male. The majority of biopsies performed in these patients (82%) were done so prior

**Table 4** Allele frequency in candidate genes assessed in patient and control groups by high resolution melt analysis: Compared to published data for Caucasian ethnicity populations

Gene	Patient group	Control group	Published results <sup>1</sup>
<i>MCP1</i>	A 0.729	A 0.736	A 0.695
	G 0.271	G 0.264	G 0.305
<i>CCR2</i>	G 0.892	G 0.976	G 0.892
	A 0.108	A 0.024	A 0.108
<i>TGFβ</i>	G 0.918	G 0.892	G 0.887
	C 0.082	C 0.108	C 0.113
<i>hOGG1</i>	C 0.695	C 0.694	C 0.776
	G 0.305	G 0.306	G 0.224
<i>IL10-1082</i>	G 0.539	G 0.500	G 0.531
	A 0.461	A 0.500	A 0.469
<i>IL10-592</i>	C 0.794	C 0.794	C 0.792
	A 0.206	A 0.206	A 0.208
<i>TLR4 299</i>	A 0.934	A 0.930	A 0.967
	G 0.066	G 0.070	G 0.033
<i>TLR4 399</i>	C 0.934	C 0.931	C 0.955
	T 0.066	T 0.069	T 0.045

<sup>1</sup>NCBI dbSNP (HapMap CEU-Utah residents with Northern and Western European ancestry from the CEPH collection). *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

to 1996 when the *HFE* gene was cloned and therefore many patients are likely to have been biopsied for diagnostic purposes. Fibrosis stages were as follows; F0: 136 (56%), F1: 26 (11%), F2: 23 (9%), F3: 13 (5%), F4: 47 (19%). The demographic, laboratory and histological characteristics of the patients with and without hepatic fibrosis are summarized in Table 3. Of those with the grade of steatosis reported, 56% had no steatosis present and 12% had grade 2 or 3 steatosis. Male gender, age, alcohol consumption, steatosis and iron indices from serum and liver sections all showed significant associations with the presence of hepatic fibrosis, as has been previously demonstrated<sup>[56,57]</sup>.

Allele frequencies for the genes of interest were assessed in patient and control populations and these results compared to published data for Caucasian groups (Table 4). There was a significant difference in allele frequencies for the *CCR2* gene polymorphism when comparing the haemochromatosis and control populations (*P* = 0.001). This appeared to be due to an unexpectedly low number of heterozygous and A allele homozygous control subjects. When the patient population was compared to data published from the International HapMap Project there was no significant difference (*P* = 0.985). For all other genes there were no significant differences in allele frequencies between patient and control populations.

No patient or control subject was identified to have the uncommon homozygous polymorphism (Pro/Pro or C/C) in the *TGF-β* gene but as this is present in low frequency in the population this is not an unexpected finding. For the analyses of this polymorphism, Arg/Arg (G allele homozygosity) was compared to Arg/Pro (G/C).

**Table 5** Genetic polymorphisms in Hereditary Haemochromatosis patients grouped according the presence or absence of advanced fibrosis and subjected to univariate analysis *n* (%)

Gene	No/minimal fibrosis (F0-2)	Advanced fibrosis (F3-4)	<i>P</i> value
<i>MCP1</i>			
AA	99 (53.5)	28 (46.7)	0.546
AG	76 (41.1)	27 (45.0)	
GG	10 (5.4)	5 (8.3)	
<i>CCR2</i>			
GG	142 (78.0)	50 (84.8)	0.401
AG	37 (20.3)	9 (15.3)	
AA	3 (1.7)	0 (0)	
<i>TGFβ</i>			
GG	154 (83.2)	51 (85)	0.749
GC	31 (16.8)	9 (15)	
<i>hOGG1</i>			
CC	99 (53.8)	26 (43.3)	0.352
CG	64 (34.8)	25 (41.7)	
GG	21 (11.4)	9 (15)	
<i>IL10-1082</i>			
GG	50 (27.1)	17 (28.3)	0.806
GA	97 (52.4)	33 (55.0)	
AA	38 (20.5)	10 (16.7)	
<i>IL10-592</i>			
CC	120 (64.9)	37 (61.7)	0.173
AC	58 (31.3)	17 (28.3)	
AA	7 (3.8)	6 (10.0)	
<i>TLR4 299</i>			
AA	159 (86.4)	54 (91.5)	0.305
AG	24 (13.0)	4 (6.8)	
GG	1 (0.6)	1 (1.7)	
<i>TLR4 399</i>			
CC	161 (87.0)	53 (89.8)	0.502
CT	23 (12.4)	5 (8.5)	
TT	1 (0.5)	1 (1.7)	

*MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

All genes showed no deviation from Hardy-Weinberg equilibrium in the haemochromatosis patient population except the *OGG1* polymorphism ( $P = 0.03$ ). This may relate to selective pressure in this group. The control population showed no deviation from Hardy-Weinberg equilibrium.

No significant associations were present between the polymorphisms of any of the candidate genes and fibrosis stage when the patients were grouped into those with minimal or no fibrosis (F0-2) and compared with those having severe fibrosis (F3-4) (Table 5). Each candidate gene was assessed in ordered logistic regression analysis incorporating increasing fibrosis stage as outcome before and after adjustment for age, gender, iron loading and alcohol consumption and in each analysis, there was no statistically significant effect from the SNP of interest on fibrosis stage (Table 6). Analyses were repeated incorporating data on steatosis grade however this produced no statistically significant effect. Iron loading, alcohol consumption, male gender and age remained important in multivariate analyses as previously reported<sup>[2,4-6,58-62]</sup>.

**Table 6** Multivariate ordered logistic regression analysis determining role of genetic polymorphisms in increasing hepatic fibrosis stage

Gene	OR	95%CI	<i>P</i> value	Adjusted <i>P</i> value <sup>1</sup>
<i>MCP1</i>	1.11	0.69-1.80	0.660	0.681
<i>CCR2</i>	0.71	0.36-1.32	0.284	0.432
<i>TGFβ</i>	1.03	0.54-1.95	0.936	0.740
<i>hOGG1</i>	0.94	0.44-1.98	0.864	0.588
<i>IL10-1082</i>	1.10	0.65-1.88	0.720	0.897
<i>IL10-592</i>	0.82	0.50-1.35	0.441	0.639
<i>TLR4 299</i>	0.73	0.35-1.53	0.403	0.745
<i>TLR4 399</i>	0.87	0.42-1.81	0.706	0.990

<sup>1</sup>Adjusted for age at biopsy; gender; iron grade and alcohol consumption. *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

After considering only those patients with heaviest grades of iron loading (iron grade 3 and 4) there was no significant association with hepatic fibrosis (F0 vs F3/F4) and any of the genetic polymorphisms studied when assessed in univariate or multivariate analysis (after adjustment for age, gender and alcohol consumption) (Table 7). A threshold hepatic iron concentration for cirrhosis of 236 μmol/g dry weight has previously been identified in this haemochromatotic patient cohort<sup>[59]</sup>; this cut off was used to isolate those with the greatest risk of hepatic fibrosis and subjects with no fibrosis (F0) were again compared to those with advanced disease (F3/F4) (Table 8). Age and alcohol consumption were important in disease progression in this group but no difference was seen for any of the genetic polymorphisms studied. Gender was not a significant risk factor in this cohort with very heavy iron stores.

## DISCUSSION

Studies investigating the clinical penetrance of haemochromatosis have consistently identified the severity of iron loading, male gender and alcohol consumption as being crucial factors in determining the risk of liver fibrosis (reviewed in<sup>[7]</sup>). It is clear that steatosis accelerates the hepatic injury<sup>[57]</sup> and that diabetes is a risk factor for advanced fibrosis<sup>[56]</sup>. Despite these known risk factors, family studies have suggested a clustering of phenotypes that may indicate a role for genetic disease modifiers quite separate to those influencing iron loading. Relatively few studies have explored this area in haemochromatosis subjects, particularly when compared to a large body of literature that exists with respect to viral hepatitis.

Many candidate gene studies have suffered from methodological flaws which increase the risk of misleading results. Perhaps the most common scenario is a small subject group which allows the finding of a false positive result due to chance. Although haemochromatosis is relatively common in terms of genetic diseases, its expression remains uncommon in the general popula-



**Table 7 Genetic polymorphism frequencies in patients with heavy iron loading (Grade 3 and 4) grouped according to no fibrosis (F0) vs advanced fibrosis (F3/F4)**

Gene	No fibrosis (F0) (n = 103)		Advanced fibrosis (F3/4) (n = 60)		P value	Adjusted P value <sup>1</sup>
MCP1	AA 53/103	AG/GG	AA 28/60	AG/GG 32/60	0.555	0.433
	51.50%	50/103 48.5%	46.70%	53.3%		
CCR2	GG 81/100	AG/AA	GG 50/59	AG/AA 9/59	0.549	0.277
	81%	19/100 19%	84.70%	15.3%		
TGF $\beta$	GG 86/103	CG/CC 17/103	GG 51/60	CG/CC 9/60	0.800	0.968
	83.50%	16.5%	85%	15%		
hOGG1	CC 54/102	CG/GG 48/102	CC 26/60	CG/GG 34/60	0.238	0.790
	52.90%	47.1%	43.30%	56.7%		
IL10-1082	AA/AG 75/103	GG 28/103	AA/AG 43/60	GG 17/60	0.874	0.998
	72.8%	27.20%	71.80%	28.30%		
IL10-592	AA/AC 34/103	CC 66/103	AA/AC 23/60	CC 37/60	0.492	0.659
	33.0%	67.0%	38.3%	61.7%		
TLR4 299	AA 87/102	AG/GG 15/102	AA 54/59	AG/GG 5/59	0.248	0.848
	85.3%	14.7%	91.5%	8.5%		
TLR4 399	CC 89/103	CT/TT 14/103	CC 53/59	CT/TT 6/59	0.524	0.985
	86.4%	13.6%	89.8%	10.2%		

<sup>1</sup>Adjusted for age:gender:alcohol consumption. MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF- $\beta$ : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

**Table 8 Logistic regression analysis performed in subjects with HIC > 236  $\mu$ mol/g dw and comparing outcome of F0 vs F3/4 (univariate analysis) (n = 47)**

Factor	OR	95%CI	P value
Age	1.07	1.00-1.15	0.034
Alcohol	1.03	1.01-1.05	0.011
Female gender	0.27	0.05-1.55	0.140
MCP1	1.20	0.37-3.87	0.76
CCR2	0.74	0.13-4.12	0.73
TGF $\beta$	0.58	0.09-3.52	0.553
hOGG1	1.50	0.47-4.77	0.492
IL10-1082	1.89	0.48-7.44	0.363
IL10-592	0.98	0.30-3.21	0.980
TLR4 299	1.17	0.77-7.79	0.868
TLR4 399	1.24	0.19-8.19	0.824

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: interleukin-10; TGF- $\beta$ : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

tion. Added to this is the fact that many patients have no indication, or wish to undergo a liver biopsy, it is clear that establishing a sizable cohort of subjects for study is difficult. Our group of 245 C282Y homozygous patients not only had liver biopsy data available but had also provided blood samples for extraction and storage of DNA. This is likely to represent one of the largest groups in the international literature and represents a recruitment period spanning decades, including the pre-HFE era when liver biopsies were used for diagnosis of the disease. This allowed inclusion of patients with early and late stage disease thus avoiding a recruitment bias. In order to allow conclusions regarding the role of genetic polymorphisms, data are also needed about other factors. We have included information from this group relating to iron grade, age, alcohol and gender and used this information to perform multivariate analyses con-

sidering gene-environment interactions.

We selected biologically plausible genes for analysis of functionally significant polymorphisms based on known mechanisms of hepatic injury and liver fibrosis and considering the results of previous studies. This approach has been used to describe the role of SNPs in many polygenic diseases although it does risk returning a null result. Genome wide association studies have since become an alternative approach which removes any need for a mechanistic approach to gene selection however these studies require very large patient cohorts in order to describe associations with a small effect on disease.

We did not find a role for the single nucleotide polymorphisms studied in genes coding MCP-1, TGF- $\beta$ , IL10, OGG1, TLR4 or CCR2 when considered in univariate analysis with fibrosis stage or when incorporated in multivariate analysis including gender, iron loading, alcohol consumption and age. Genotyping was performed using high resolution melt analysis with confirmation of grouping controls with sequencing. Data were considered in several different analyses with the outcome variables of liver fibrosis grouped as being either present or absent, minimal or advanced and finally as an ordered logistic regression approach (F0-F4). Likewise, the predictor variables (genetic polymorphisms) were considered in all possible combinations but none proved to have a statistically significant association with the outcome variable. We particularly examined those patients where there is greatest variability in liver fibrosis despite significant iron loading. We used the previously published HIC threshold for cirrhosis in this cohort, *i.e.*, HIC > 236  $\mu$ mol/g dry weight<sup>[59]</sup>, and compared the groups with no fibrosis (F0) against those with advanced fibrosis (F3/F4) who had this significant level of hepatic iron deposition. We hypothesized that these patients are most likely to have other factors accounting for disease progression; however, it was still evident that there was no significant

effect seen in any of the genes tested. In this subgroup analysis of patients with significant iron loading, it is apparent that alcohol consumption and age remain the important determinants of liver disease and this is consistent with previous studies. Although our overall study size is large, it must be noted that this subgroup with very heavy iron loading is much smaller and it is possible that this accounts for the lack of association.

Our results are not consistent with previous studies which have been performed in smaller cohorts. A European study reported that the TLR4 Asp299Gly gene polymorphism modulated phenotypic expression of haemochromatosis and described the effect on both liver histology and on an amalgamated clinical expression including liver disease, arthropathy, joint disease, cardiomyopathy and endocrine disease<sup>[28]</sup>. The grouping of such diverse types of clinical expression is unusual when considering candidate gene testing and one may wonder whether this is biologically plausible. This study included 99 patients but of these, only 52 had histology available and 29 of these had liver iron quantification. Although allele frequencies were similar to our cohort, we did not replicate the SNP association with liver disease in a group almost five times larger and suspect that the European results may represent a type 1 error.

Similarly, a previous investigation into the role of TGF- $\beta$  mutations in 149 biopsied haemochromatosis patients concluded that those with the proline substitution at codon 25 were more likely to be grouped into an outcome variable of cirrhosis (F4) *vs* all other stages (F0-3). This grouping could be considered somewhat arbitrary and it would be interesting to know the genotype frequencies across other fibrosis stages. A recent meta-analysis considering the role of TGF- $\beta$  polymorphisms in liver disease (mainly viral hepatitis) concluded a lack of effect upon fibrosis progression which is in keeping with our results<sup>[63]</sup>.

There are substantial difficulties in studying disorders with polygenic and environmental interactions. Future research directions may involve non-targeted analysis of either whole genomes or exomes but this is likely to require greater numbers of subjects who have been well characterised in terms of liver disease and co-factors. Collaborations between research institutions would allow more patients to be involved in such studies but newer non-invasive tests of liver fibrosis such as transient elastography will allow assessment for liver disease in almost all patients and capture a much greater proportion of C282Y homozygous subjects in such studies.

In conclusion, the role of chemokines, chemokine receptors, oxidative stress and inflammatory mediators in fibrogenesis in haemochromatosis is established; however, the influence of genetic polymorphisms in the molecules studied here is less clear. In contrast to other published associations, in our large, well-characterised group of C282Y homozygous subjects we did not demonstrate any relationship between *MCP1*, *CCR2*, *TGF $\beta$* , *IL10*, *OGG1* and *TLR4* single nucleotide polymorphisms and

hepatic fibrosis or cirrhosis. Future studies utilising techniques such as exome sequencing may provide a better approach to identify genetic polymorphisms associated with hepatic fibrosis progression in haemochromatosis.

## COMMENTS

### Background

Hereditary haemochromatosis can lead to liver fibrosis and cirrhosis however not all patients with iron loading develop this complication. It is thought that genetic polymorphisms influence this process however previously reported studies may have had methodological flaws.

### Research frontiers

Factors associated with an increased risk of hepatic fibrogenesis have been the subject of investigation and many of the clinical cofactors are now established. Genetic factors have proven more difficult to determine although international collaborations investigating this area are ongoing. This is one of the largest cohorts of C282Y homozygous patients studied in this field.

### Innovations and breakthroughs

The cohort studied in this paper is large and carefully characterised which allows us to accurately test the relationship between genetic polymorphisms, cofactors for liver injury and fibrosis stage. Authors have tested polymorphisms in molecules related to inflammation and hepatic fibrogenesis and found no significant relationship which is in contrast to previous papers. Their work suggests that further well designed studies are needed to determine what genetic factors influence fibrosis in iron loading.

### Applications

By understanding the molecular differences between patients who develop progressive liver disease and those who don't, they may eventually be able to develop therapeutic targets to modify disease development. Patients could also expect more personalised prognostic information and this may allow better informed treatment decisions.

### Terminology

Hereditary haemochromatosis due to homozygosity in the C282Y substitution in *HFE* is a genetic disorder seen in those of Northern European ancestry. It is one of the most common genetic disorders in this population and can lead to heavy iron loading in the liver and liver scarring (cirrhosis). The molecules studied in this paper are thought to be involved in mediating inflammation, signalling to fibrosis-producing cells or repair of oxidative stress within the liver.

### Peer review

The manuscript reports the lack of association between hepatic fibrosis risk and polymorphisms in the genes encoding toll-like receptor 4, transforming growth factor-beta, in a relatively large cohort of hemochromatosis patients. These results are not consistent with previous findings, which were obtained with error prone smaller cohorts of patients. The methodology employed here is appropriate and the paper is well written. The discussion puts the negative findings into context. The conclusions will be of interest to researchers and clinicians in the field of gastroenterology.

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## Efficacy and safety of tenofovir disoproxil fumarate in pregnancy for the prevention of vertical transmission of HBV infection

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= 24). All infants received 200 IU of hepatitis B immune globulin (HBIG) within 24 h postpartum and 20 µg of recombinant HBV vaccine at 4, 8, and 24 wk. Perinatal transmission rate was determined by hepatitis B surface antigen and HBV DNA results in infants at week 28.

**RESULTS:** At week 28, none of the infants of TDF-treated mothers had immunoprophylaxis failure, whereas 2 (8.3 %) of the infants of control mothers had immunoprophylaxis failure ( $P = 0.022$ ). There were no differences between the groups in terms of adverse events in mothers or congenital deformities, gestational age, height, or weight in infants. At postpartum week 28, significantly more TDF-treated mothers had levels of HBV DNA < 250 copies/mL and normalized alanine aminotransferase compared with controls (62% vs none,  $P < 0.001$ ; 82% vs 61%,  $P = 0.012$ , respectively).

**CONCLUSION:** TDF therapy during the second or third trimester reduced perinatal transmission rates of HBV and no adverse events were observed in mothers or infants.

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**Key words:** Hepatitis B; Tenofovir; Reverse transcriptase inhibitors; Vertical transmission; Chronic

**Core tip:** Tenofovir disoproxil fumarate use during late pregnancy reduced hepatitis B virus transmission in highly viremic hepatitis B e antigen positive mothers.

### Abstract

**AIM:** To evaluate the effects of tenofovir disoproxil fumarate (TDF) use during late pregnancy to reduce hepatitis B virus (HBV) transmission in highly viremic mothers.

**METHODS:** This retrospective study included 45 pregnant patients with hepatitis B e antigen (+) chronic hepatitis B and HBV DNA levels >  $10^7$  copies/mL who received TDF 300 mg/d from week 18 to 27 of gestation ( $n = 21$ ). Untreated pregnant patients served as controls ( $n$

Celen MK, Mert D, Ay M, Dal T, Kaya S, Yildirim N, Gulsun S, Barcin T, Kalkanli S, Dal MS, Ayaz C. Efficacy and safety of tenofovir disoproxil fumarate in pregnancy for the prevention of vertical transmission of HBV infection. *World J Gastroenterol* 2013; 19(48): 9377-9382 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

Hepatitis B virus (HBV) infection is a important medical problem affecting approximately 2 billion people globally<sup>[1]</sup>. The vertical transmission of HBV from hepatitis B surface antigen (HBsAg)-positive mothers to their infants at birth or in early infancy has a significant role in the endemicity of HBV infection and causes an increased risk of chronic hepatitis B (CHB)<sup>[2]</sup>. The prevention of perinatal or vertical transmission is crucial in the control of hepatitis B endemicity. Without immunoprophylaxis > 90% of infants, born to mothers with hepatitis B e antigen (HBeAg), become chronically infected with HBV. In recent years, active and passive immunoprophylaxis of newborns and universal vaccination programs have reduced the transmission rates of HBV<sup>[1-4]</sup>. It was reported that passive or active immunization within 12 h of birth may lead to the prevention of perinatal transmission of HBV<sup>[5]</sup>. However, some studies showed that HBV immunoprophylaxis fails in 10%-15% of infants<sup>[6,7]</sup>, mainly as a result of vertical infection<sup>[8-11]</sup>. A high level of maternal viremia is a significant factor in prophylaxis failure. A positive correlation between high maternal serum HBV DNA levels and an increased risk for vaccination breakthrough was found in these studies<sup>[8-11]</sup>. These data have introduced the idea of antiviral therapy in pregnant women with a high level of maternal viremia and high maternal serum HBV DNA levels.

Among the oral anti-HBV agents approved by United States Food and Drug Administration (FDA), Tenofovir disoproxil fumarate (TDF) is an effective agent due to its potency and resistance profile<sup>[13-15]</sup>. TDF is a nucleotide analog which inhibits reverse transcriptase and blocks HBV replication in liver cells<sup>[2,16]</sup>. In over 600 human immunodeficiency virus (HIV) mono-infected and HIV/HBV co-infected mothers, it was reported that TDF had a favorable efficacy and safety profile<sup>[16-18]</sup>. However, to our knowledge, there are limited data available in the literature on the safety and efficacy of TDF therapy during pregnancy in highly viremic mothers with chronic hepatitis B and its impact on the perinatal transmission of HBV.

In the current study, we evaluated the efficacy and safety of TDF use during late pregnancy to reduce HBV transmission in highly viremic HBeAg positive mothers.

## MATERIALS AND METHODS

### Patients

This was a retrospective study conducted in six hospitals in South-east Anatolia, Turkey. A total of 45 pregnant women, who were diagnosed with HBeAg-positive chronic hepatitis B before 12 wk of gestation between February 2010 and January 2012, were included in this study. Twenty-one patients were treated with TDF 300

mg orally once a day (Viread; Gilead Sciences, CA, United States) from week 18 to 27 of gestation ( $n = 21$ ) and served as the treated-group. Twenty-four untreated pregnant women with active hepatitis B infection served as the control group. The treated patients received TDF until the fourth week after delivery.

Eligibility criteria for inclusion in this study were: (1) pregnant women; (2) positive for serum HBsAg and HBeAg for a period of at least 6 mo; (3) HBV DNA levels  $\geq 7 \log_{10}$  copies/mL before initiation of TDF; (4) treatment-naïve patients; (5) patients without lamivudine resistance; and (6) patients without gestational diabetes, vaginitis, arrhythmia, anemia or proteinuria.

Forty-five pregnant women met all inclusion criteria and were included in the study. Mothers with HIV co-infection, pregnancy complications, or an abnormal sonographic examination were excluded from TDF therapy. Baseline demographic data and virological characteristics (age, race, HBeAg, and history of prior HBV therapy) of the pregnant women were recorded. Blood and urine beta-HCG were tested in all patients).

HBsAg, HBeAg, anti-HBe, HBV DNA, alanine aminotransferase (ALT), aspartate aminotransferase levels, and creatinine level were measured at intervals of 12 wk. Both the mothers and infants were evaluated at periodic intervals during the intrauterine period.

All newborns were evaluated for congenital malformations, hypothyroidism, and phenylketonuria at birth. Infant Apgar score, anthropometry, birth defects, history of immunoprophylaxis, mode of delivery and complications were evaluated and recorded.

HBV DNA was quantified using the Roche COBAS Amplicor HBV monitor assay which has a low limit of detection (LLD) of 500 copies/mL (Roche Molecular Diagnostics, Branchburg, NJ, United States). This assay was later replaced by the Roche COBAS TaqMan HBV Test with a LLD of 50 copies/mL (Roche Molecular Diagnostics). HBV serological markers were detected by enzyme-linked immunosorbent assay kits (Abbott Labs, North Chicago, IL, United States) on an ARCHITECT 2000 full automatic chemiluminescence immunoassay instrument (Abbott Labs, North Chicago, IL, United States) according to the manufacturer's instructions. Hearing screening was tested by Echo Screen (Madsen, Germering, Germany). Heel blood was taken from the infants after 72 h of breastfeeding and then dried blood-spot specimens on filter paper were sent to the laboratory for congenital phenylketonuria and hypothyroidism screening.

According to national and international treatment guidelines, all infants received 200 IU of hepatitis B immune globulin (HBIG, HyperHEP B solvent/detergent treated; Talecris Biotherapeutic, NC, United States) within 24 h postpartum and 20  $\mu$ g of recombinant HBV (Recombivax HB; Merck Sharp and Dohme, NJ, United States) vaccine (4, 8, and 24 wk). Infants were evaluated in terms of serum HbsAg and HBV DNA levels at postpartum weeks 4-28. Vertical transmission was evaluated by HBsAg testing of infant peripheral blood at 4-28 wk of age.

**Table 1** Maternal characteristics of the control group and tenofovir disoproxil fumarate-treated group

Maternal characteristics	Control group ( <i>n</i> = 24)	Treated group ( <i>n</i> = 21)
Mean age (yr)	26.9 ± 2.9	28.2 ± 4.1
HBV DNA (IU/mL)	8.31 log	8.28 log
ALT levels (U/L)	52 (19-77)	56 (22-71)
Serum creatinine levels (mg/dL)	0.81 (0.6-1.0)	0.79 (0.6-0.98)
Compensated cirrhosis	0 (0%)	2 (10%)

HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

**Table 2** Maternal outcomes in the control group and tenofovir disoproxil fumarate-treated group *n* (%)

Maternal outcomes	Control group ( <i>n</i> = 24)	Treated group ( <i>n</i> = 21)
HBV DNA < 50 IU/mL	0 (0)	13 (62)
Normalized ALT (U/L)	15 (61)	17 (82)
Elevated creatinine kinase (> 165 mg/dL)	0 (0)	1 (4.7)
Spontaneous abortion	1 (4)	0 (0)
Gestational diabetes	0 (0)	1 (4.7)
Vaginitis	0 (0)	1 (4.7)
Arrhythmia	0 (0)	1 (4.7)
Anemia	0 (0)	1 (4.7)
Proteinuria	1 (4.2)	2 (10)

HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

### Ethics

All participants gave their written informed consent and did not receive any compensation for taking part in this study. The study conformed to the standards set by the latest revision of the Declaration of Helsinki and was approved by the Ethical Committee.

### Statistical analysis

Statistical analysis was performed using Stata software version 10 (Computer Resource Center, Chicago, IL, United States). Measurement data were expressed as mean ± SD and compared with analysis of variance. Fisher's exact test was used for comparison of transmission rate. *P* < 0.05 was considered statistically significant.

## RESULTS

### Maternal characteristics

HBV DNA levels were > 2000000 IU/mL ( $10^7$  copies/mL) in all patients (treated-group and control group). The median maternal age was  $27.7 \pm 3.7$  years. Serum creatinine levels were within the normal ranges in all patients. Two patients in the treated-group had compensated cirrhosis (10%) (Table 1).

### Maternal outcomes

All the mothers in the treated-group continued to receive therapy during the study period. In the treated-group, all pregnant women delivered, however, one patient in the

control group had a spontaneous abortion at week 9.

In the treated-group, gestational diabetes was found in one patient, vaginitis in one patient, arrhythmia in one patient, and anemia in one patient. Three patients (14.3%) had proteinuria in the treated-group. Elevated creatinine kinase (CK) was detected in one (4.7%) patient in the treated-group at week 6, and reached the highest level (341 mg/dL) at week 8. At this time, the patient had no complaints or muscle function loss. Muscle function tests in this patient were normal, and she was diagnosed with asymptomatic CK elevation.

One patient (4.7%) in the treated-group had elevated levels of ALT (258 U/L) at week 7, however, ALT levels were normal at week 11 of treatment.

At postpartum week 28, significantly more TDF-treated mothers had levels of HBV DNA < 50 IU/mL (250 copies/mL) and normalized ALT compared with controls (62% *vs* none, *P* < 0.001; 82% *vs* 61%, *P* = 0.012, respectively). There were no differences in adverse effects in mothers between the groups (Table 2). The treated mothers had no hepatic flares until the fourth week after delivery.

### Infant characteristics and outcomes

At the 20<sup>th</sup> gestational week evaluation, no serious complications were observed in the infants of the treated-group, although 3 (14.3%) infants had growth retardation as shown on ultrasound screening. However, these infants did not show growth retardation at week 24<sup>th</sup> following ultrasound monitoring.

Birth weight was < 2500 g in two (4.7%) newborn. Hypothyroidism, phenylketonuria and congenital hearing loss were not observed in any of the newborn. At 28 wk, none of the infants whose mothers received TDF had immunoprophylaxis failure, whereas 2 (8.3%) of the infants of control mothers had immunoprophylaxis failure (HBsAg positivity was detected) (*P* = 0.022). Anti-HBs levels were < 100 mIU/mL in one (4.7%) of the vaccinated neonates of treated mothers, while levels were > 100 mIU/mL in the remaining (95%) vaccinated neonates of treated patients. No differences in infant congenital deformities, gestational age, height, or weight between the groups were observed (Table 3).

## DISCUSSION

In this retrospective study, we report the efficacy and safety of TDF in the prevention of vertical transmission (VT) in pregnant women with high viremia HBV infection<sup>[15]</sup>. Immunoprophylaxis with immediate HBIG and HBV vaccine after delivery effectively prevented VT in these cases. Failure of immunoprophylaxis is generally caused by high maternal viral load<sup>[9,11,15]</sup>. It has been clearly demonstrated that there is a correlation between intrauterine serum HBV DNA levels and perinatal transmission of HBV in pregnant women<sup>[15]</sup>. HBV DNA level was found to be an independent risk factor for failure of immunoprophylaxis in HBsAg-positive mothers with high HBV DNA levels



**Table 3** Outcome of infants born to control mothers and tenofovir disoproxil fumarate-treated mothers *n* (%)

Infant characteristics and outcomes	Infants of control group mothers ( <i>n</i> = 23)	Infants of treated group mothers ( <i>n</i> = 21)
Birth weight < 2500 g	1 (4.3)	1 (4.7)
Immunoprophylaxis failure	2 (8.3)	0 (0)
Anti-hepatitis B surface levels > 100 mIU/mL	19 (82)	20 (95)
Hypothyroidism	0 (0)	0 (0)
Phenylketonuria	0 (0)	0 (0)
Congenital hearing loss	0 (0)	0 (0)

( $\geq 6 \log_{10}$  copies/mL)<sup>[19]</sup>. For these reasons we included mothers with high levels of DNA in the present study.

There are many studies on the use of TDF to prevent VT in HIV mono-infected and HIV/HBV coinfecting mothers in the literature<sup>[17,18]</sup>. However, there are limited data on the use of TDF in pregnancy and in the prevention of VT in HBV mono-infected mothers. According to previous studies, early post-natal immunoprophylaxis with antiviral therapy in mothers in the third trimester was safe, well-tolerated, and effectively prevented VT of HBV<sup>[2,20]</sup>. Among five FDA approved oral anti-HBV agents, TDF and entecavir are the most effective agents due to their resistance profile and potency<sup>[13,15]</sup>. TDF and telbivudine are classified as category B (no evidence of risk to humans: either animal findings indicate risk, but human findings do not; or, if no adequate human studies have been conducted, animal findings are negative) for use in pregnancy, whereas lamivudine, entecavir, and adefovir are category C (risk cannot be ruled out: human studies are lacking, and animal studies are either positive for fetal risk, or are lacking). However, potential benefits may justify the potential risk in the FDA drug category for pregnancy<sup>[15]</sup>.

In a randomized, double-blind, placebo-controlled study with lamivudine, it was found that lamivudine therapy during late pregnancy can reduce HBV perinatal transmission in highly viremic mothers. This study demonstrated that infants in the lamivudine + vaccine + HBIG group had a significant decrease in the incidence of HBsAg seropositivity (10/56, 18% *vs* 23/59, 39%,  $P = 0.014$ ) and in detectable HBV DNA (11/56, 20% *vs* 27/59, 46%,  $P = 0.003$ ) compared to infants who received placebo + vaccine + HBIG. The results of this study suggested that lamivudine reduced HBV transmission from highly viremic mothers to their infants following immunization<sup>[20]</sup>. In another prospective, open-label controlled study evaluating the efficacy and safety of telbivudine use during late pregnancy, a striking decline in HBV DNA levels was seen from treatment onset to week 4, and remained at a low level from week 12. According to this study, 33% of the telbivudine-treated mothers and none of the untreated controls had DNA < 500 copies/mL at delivery and seven months after delivery, and the incidence of perinatal transmission was lower in the infants of telbivudine-treated mothers than in the controls (0% *vs* 8%;  $P = 0.002$ )<sup>[2]</sup>. In a case series by Pan *et al*<sup>[15]</sup>, TDF therapy in the third trimester was evaluated in eleven Asian women with HBV. In their un-

controlled study, a significant reduction in serum HBV-DNA was achieved at delivery compared with baseline, and all infants were HBsAg negative 28-36 wk after birth<sup>[15]</sup>. In our controlled study, at postpartum week 28 significantly more TDF-treated mothers had low levels of HBV DNA and none of the infants of 21 treated mothers had immunoprophylaxis failure. In light of these results, we suggest that TDF use in the third trimester is safe and effectively prevents VT of HBV from high viremic HBeAg-positive mothers.

When the potential benefit of an antiviral-agent is evaluated, adverse effects of that antiviral-agent should also be taken into consideration. These adverse effects include teratogenicity, long-term effects on bone development in the infant, post-treatment ALT flares, and HBV-resistant mutations. Studies have indicated that TDF can cause renal events in HIV patients and patients with preexisting renal disease. However, nephrotoxicity was not observed during a three-year period of TDF use in chronic HBV patients with preserved baseline renal function<sup>[21,22]</sup>. According to analyzed neonatal safety data from the Antiretroviral Pregnancy Registry (APR), the birth defect prevalence of earliest exposure commencing in the first trimester was 3.1% for lamivudine and 2.4% for TDF; earliest exposure commencing in the second or third trimester was 2.7% for lamivudine and 2.0% for TDF<sup>[23]</sup>. A meta-analysis of lamivudine in late pregnancy reported that no significant increase in adverse effects or complications in pregnancy was observed<sup>[24]</sup>. In the large-scale controlled study by Han *et al*<sup>[2]</sup>, no serious adverse events were noted in the telbivudine-treated mothers or their infants. In a Chinese study conducted in eight pregnant HBV women receiving TDF, HBV flares, an increase in creatinine and birth defects were not observed, and all newborn parameters were appropriate for gestational age<sup>[25]</sup>. Several clinical studies and the APR have stated that anti-viral agents for hepatitis are safe in pregnancy during the second/third trimester<sup>[15,23]</sup>. The relationship between TDF and fetal growth, particularly bone development is a matter of concern. Studies of pregnant monkeys showed that the use of TDF can cause reduced fetal growth and a reduction in fetal bone porosity within two months of starting maternal therapy<sup>[26]</sup>. TDF use in HIV-infected children has been reported to result in decreases in bone mineral density<sup>[27,28]</sup>. However, long-term safety data in infants perinatally exposed to TDF demonstrated no abnormal bone metabolism or growth impairment in these children<sup>[29,30]</sup>. In the study by Pan *et al*<sup>[15]</sup>, serum creatinine

levels were stable and within the normal range during TDF treatment in all mothers, and they did not encounter any adverse pregnancy outcomes and/or birth defects. Similarly, in the current study we did not observe any differences in adverse events in mothers or infant congenital deformities, gestational age, height, or weight between the groups.

In conclusion, this controlled study revealed that the use of TDF in highly viremic chronic hepatitis B mothers during the second or third trimester of pregnancy reduced the rate of perinatal transmission. Tenofovir disoproxil fumarate produced no adverse events in infants or mothers by 28 wk and is a safe and effective agent in pregnant women with high viremia.

## COMMENTS

### Background

The vertical transmission of hepatitis B virus (HBV) from hepatitis B surface antigen-positive mothers to their infants at birth or in early infancy has a significant role in the endemicity of HBV infection and causes an increased risk of chronic hepatitis B (CHB).

### Research frontiers

Among the oral anti-HBV agents approved by the Food and Drug Administration, tenofovir disoproxil fumarate (TDF) is an effective agent due to its potency and resistance profile. However, there are limited data available in the literature on the safety and efficacy of TDF therapy during pregnancy in highly viremic mothers with CHB and on its impact on the perinatal transmission of HBV. In this study, the authors demonstrated that TDF therapy during the second or third trimester in CHB mothers reduced perinatal transmission rates with no adverse events in mothers and their infants.

### Innovations and breakthroughs

This report highlighted the importance of TDF therapy in highly viremic CHB mothers during the second or third trimester to reduce perinatal transmission rates with no adverse events in infants or mothers. This is an important study which shows that TDF can be used in highly viremic mothers. Furthermore, this study suggests that TDF may be used in highly viremic CHB mothers.

### Applications

TDF therapy may represent a future strategy for CHB mothers during the second or third trimester.

### Terminology

TDF is a nucleotide analog which inhibits reverse transcriptase and blocks HBV replication in liver cells. TDF is a safe and effective agent which can be used during pregnancy in highly viremic mothers with CHB and does not increase perinatal transmission of HBV.

### Peer review

The authors studied the influence of TDF use on perinatal transmission of HBV infection. This is an interesting report.

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## Study of risk factors for gastric cancer by populational databases analysis

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### Abstract

**AIM:** To study the association between the incidence of gastric cancer and populational exposure to risk/protective factors through an analysis of international databases.

**METHODS:** Open-access global databases concerning the incidence of gastric cancer and its risk/protective factors were identified through an extensive search on the Web. As its distribution was neither normal nor symmetric, the cancer incidence of each country was categorized according to ranges of percentile distribution. The association of each risk/protective factor with exposure was measured between the extreme ranges of the incidence of gastric cancer (under the 25<sup>th</sup> percentile and above the 75<sup>th</sup> percentile) by the use of the Mann-Whitney test, considering a significance level of 0.05.

**RESULTS:** A variable amount of data omission was observed among all of the factors under study. A weak or nonexistent correlation between the incidence of gastric cancer and the study variables was shown by a visual analysis of scatterplot dispersion. In contrast,

an analysis of categorized incidence revealed that the countries with the highest human development index (HDI) values had the highest rates of obesity in males and the highest consumption of alcohol, tobacco, fruits, vegetables and meat, which were associated with higher incidences of gastric cancer. There was no significant difference for the risk factors of obesity in females and fish consumption.

**CONCLUSION:** Higher HDI values, coupled with a higher prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables and meat, are associated with a higher incidence of gastric cancer based on an analysis of populational global data.

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**Key words:** Gastric cancer; Risk factors; Epidemiologic factors; Environment; Public health

**Core tip:** An ecological study on gastric cancer based on public databases proved to be feasible and promising, and this method can be used to monitor the behavior of the disease globally. The results of this study indicated a higher level of development, coupled with the highest prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables and meat, among the countries with the highest incidences of gastric cancer. In contrast, a high consumption of vegetables was associated with a lower disease incidence in other countries.

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## INTRODUCTION

Although its incidence has been declining in many countries, gastric cancer is still the second leading cause of death from malignancy worldwide, accounting for 700349 deaths in 2002<sup>[1]</sup>.

The identification of risk factors associated with malignancies is of great importance because this knowledge can facilitate not only the development of policies aimed at the prevention of cancer occurrence but also the vigilance of at-risk groups regarding the early identification of new cases. Such identification is usually performed through observational studies, and especially case-control and cohort studies, which involve high-cost processes from a financial perspective and a long time to completion. However, those types of studies, although numerous, are usually conducted on limited population sizes, social profiles and geographic locations, with a consequent limitation of the generalizability of the results.

The systematic recording of the health data, social characteristics and habits of living populations and the consequent creation and maintenance of public databases have been made possible by the development of techniques for ecological study. These methods, by definition, allow a shift of the focus of analysis from the individual to the population. Ecological studies allow inferences about the effect of risk conditions on rates of diseases in populations. Despite limitations in measuring individual aspects and in the analysis of the cumulative effects of factors, such studies have great value due to their simplicity and low cost of analysis, may contribute to the development of health policies and may guide research efforts specific to large populations.

Given the notable differences in the frequency and distribution of gastric cancer in the various countries of the world and the operational difficulties of running traditional epidemiological studies on global demographic trends and risk factors, we performed a study on risk factors' association with gastric cancer through populational database analysis.

## MATERIALS AND METHODS

### Databases

The age-standardized incidences of stomach cancer in 184 countries were obtained from the public database maintained by the GLOBOCAN Project of the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO), considering the most recent data available (from 2008).

### GLOBOCAN project

Over the past 30 years, the IARC has published regular estimates of the incidence of and mortality from major types of cancer in several regions of the world at the national level, focusing on 184 countries, using new sources of data and improved methods of estimation. The results of the data collection in 2008 can be summarized as fol-

lows: (1) National incidence data - systematically collected by IARC member countries (62 countries); (2) Data from one or multiple local records - presented by the weighted average (75 countries); (3) Data derived from known frequencies of all types of cancer - adjusted for the known relative frequency of each type (13 countries); and (4) No data collected - presents data from neighboring countries in the same geographic region (34 countries).

Estimates are presented separately for each sex and divided into 10 age groups. These values are based on the most recent data available at the IARC and on publicly available information on the Internet, including data from the cancer registries of populational databases. These databases can cover an entire national population but more often cover smaller, subnational areas, and in developing countries, only large cities.

Furthermore, the degree of delay was taken into account by computing predictions. Although historical trends may not continue in the future, predictions based on linear trend patterns have been empirically shown to be reasonably accurate, particularly in the short term.

When historical data and a sufficient number of registered cases were available, the incidence rates were projected for 2008. Otherwise, the incidence rates of the most recent period were applied.

Regional models were used when data on the incidence in specific countries or locations were absent or when the data were considered to be of insufficient quality. In the absence of data, the values of neighboring countries in the same region were used.

### Risk and protective factors

For the selection of risk and protective factors described in the medical literature, an extensive review was conducted through the search engines MEDLINE and PubMed, using the following keywords and limiters: gastric cancer, risk factors, etiology, epidemiology and diet factors. After characterizing these factors, we performed a search of specific databases to determine populational exposure to the factors using the Google search engine, with emphasis on references from government agencies and nongovernmental organizations linked to each marker. The year of reference for each factor was 2008 or the closest available year.

### *Helicobacter pylori*

We analyzed data from studies published between 1990 and 2012 in PubMed and MEDLINE using the following key words and limiters: *Helicobacter pylori* (*H. pylori*), *Helicobacter*, *Helicobacter pylori*, incidence, prevalence and epidemiology. We performed this search because there is no global database containing the values of the incidence and prevalence of colonization by the agent. The values collected showed a lack of precision and a large omission of data, as we could identify references to only 54 of the 183 studied countries. Thus, we decided to exclude this risk factor from further analysis, despite its clinical relevance.

**Table 1** List of countries categorized by extreme incidences

< P25	Incidence	> P75	Incidence
Botswana	0.2	Chinese Taipei	15.6
Namibia	0.7	Poland	15.6
Malawi	0.8	Singapore	15.8
Lesotho	0.9	Austria	15.9
Sudan	1.0	Bosnia and Herzegovina	16.2
Swaziland	1.0	Azerbaijan	16.4
Tanzania	1.0	Bhutan	16.6
Central African Republic	1.1	Uruguay	16.7
Chad	1.1	Guatemala	17.0
Eritrea	1.1	Honduras	17.0
Gambia	1.1	Slovakia	17.2
Cameroon	1.2	Vietnam	17.3
Comoros	1.2	Spain	17.5
Equatorial Guinea	1.2	Peru	18.1
Gabon	1.2	Georgia	18.6
Niger	1.2	Germany	18.6
Nigeria	1.2	Kyrgyzstan	18.6
Republic of Congo	1.3	Romania	18.7
Djibouti	1.3	Moldavia	18.8
Gaza Strip	1.3	Jamaica	19.3
Maldives	1.3	Hungary	20.8
Mozambique	1.5	Costa Rica	20.9
Syria	1.5	Kazakhstan	21.4
United Arab Emirates	1.5	Armenia	21.8
Saudi Arabia	1.7	Slovenia	22.3
Egypt	1.8	Chile	22.4
Togo	1.8	Ecuador	22.4
Benin	1.9	Croatia	22.6
Burkina Faso	1.9	Mongolia	22.8
Ethiopia	1.9	Macedonia	22.9
Zambia	1.9	Bulgaria	23.5
Kuwait	2.0	Montenegro	23.6
Qatar	2.0	Italy	26.0
Sri Lanka	2.0	Ukraine	26.2
Iraq	2.1	Latvia	26.8
Yemen	2.1	Albania	26.9
Angola	2.2	Portugal	27.1
Sierra Leone	2.2	Lithuania	27.6
Republic of South Africa	2.2	Estonia	27.9
Ivory Coast	2.4	Russia	28.7
Laos	2.4	China	34.5
Oman	2.4	Belarus	36.4
Somalia	2.4	South Korea	56.3
Solomon Islands	2.5	Japan	80.2
Ghana	2.6		
Libya	2.6		
Vanuatu	2.6		

### Tobacco

Data were collected from the Global Health Observatory Data Repository of the WHO. The reference variable used was the percentage of the population using any tobacco product (age-standardized rate).

### Alcohol

Data were collected from the World Health Statistics database of the Global Health Observatory Data Repository of the WHO. The reference variable used was liters of pure alcohol/person/year.

### Obesity

Data were collected from the World Health Statistics

database of the Global Health Observatory Data Repository of the WHO. The reference variable used was the percentage of adults over 20 years that present a body mass index (BMI) > 30 kg/m<sup>2</sup>.

### Consumption of fruits, vegetables, legumes, meat and fish

Data were collected using the FAOSTAT tool of the Food and Agriculture Organization (FAO) database of the United Nations. The reference variable used was consumption in g/person/d.

### Salt consumption

We could not find databases regarding average *per capita* salt intake or consumption in different countries, so this factor was excluded from our analysis.

### Human development index

Data on the human development index (HDI) were collected from the database of Human Development Reports of the United Nations.

### Statistical analysis

The bivariate relationship of characteristics has been primarily studied by plotting data on the incidence of gastric cancer and various numerical indicators associated with risk in scatter plots. As the incidence of gastric cancer in 183 countries did not have a normal (Kolmogorov-Smirnov  $P < 0.001$ ) or symmetric (histogram analysis) distribution, the distribution of incidences was categorized into percentile ranges (10, 25, 50, 75 and 90). A new graphical analysis of the association between the incidence of cancer (categorized) and measures of exposure to each risk factor was performed using box plots.

The Mann-Whitney test was then used to test for differences in measures of exposure to each risk factor between the extreme ranges of incidence (under the 25<sup>th</sup> percentile and above the 75<sup>th</sup> percentile) (Table 1).

A database of collected data was created using the software MS Excel, and data analysis was performed using SPSS v. 13.0, considering a significance level of 0.05.

As the research was performed using open-access public databases, it was unnecessary to obtain express authorization from the maintainers of such data. The data's sources are properly cited along with the disclosure of the results of this study, as recommended by the sources.

We could not identify any conflicts of interest or ethical conflicts in the implementation of the present study, so submission for analysis by the Committee of Ethics in Research was not necessary, according to its own rules.

## RESULTS

In the cross-analysis of variables from different databases, which is the basis of this study, it was expected that values for each risk factor under study could not be found for all countries. Thus, the availability of data ranged from 58 countries for the prevalence of *H. pylori*

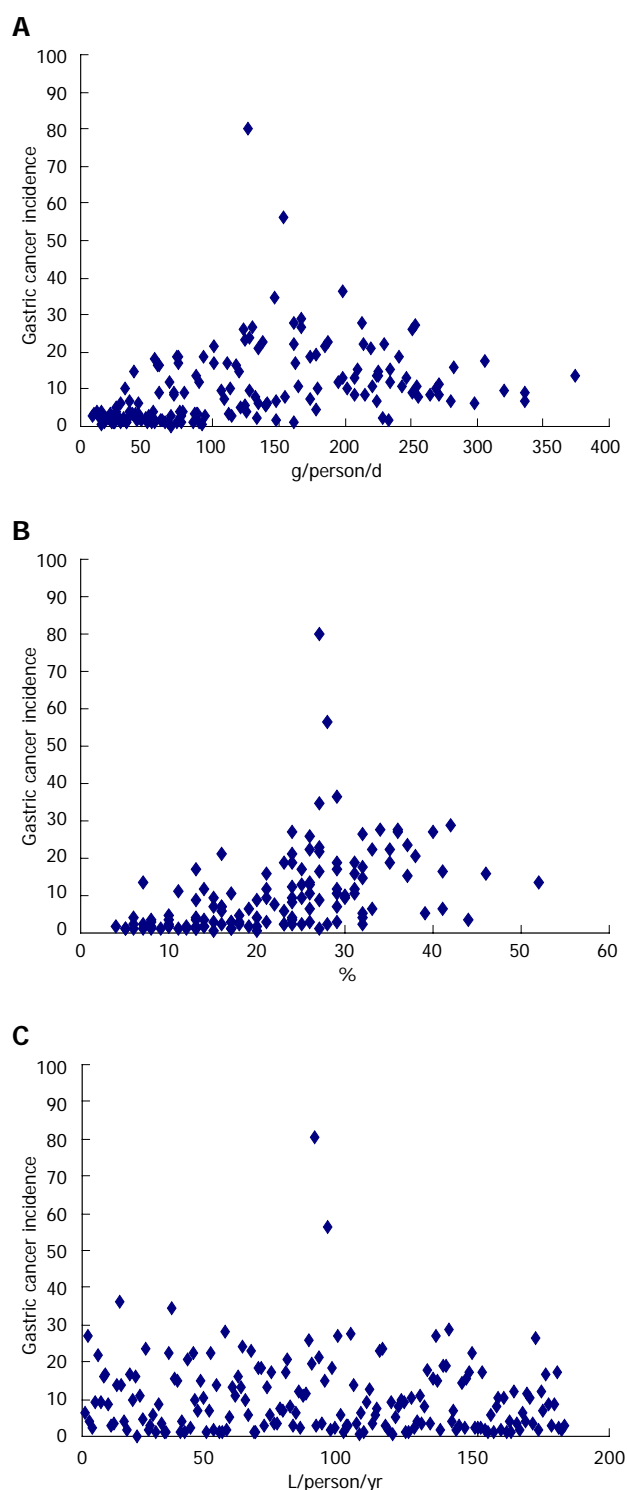


Figure 1 Several of the scatter plots. A: Meat; B: Tobacco; C: Alcohol.

to 172 countries for the consumption of alcohol and obesity, considering the 183 countries for which the incidence of gastric cancer was available in the GLOBOCAN database.

A visual analysis of the scatter plots of the incidence of gastric cancer and the study variables suggested a weak or nonexistent correlation, as exemplified by Figure 1.

By the selection of extreme incidences of gastric cancer according to percentile distribution [under the 25<sup>th</sup>

percentile (< P25) and above the 75<sup>th</sup> percentile (> P75)], a different view of the association was possible. The countries categorized in the extreme incidence ranges are presented in the world map in Figure 2.

The Mann-Whitney test showed differences in measures of certain risk factors between the extreme incidence ranges, as summarized in Table 2.

Countries in the > P75 range of the incidence of gastric cancer had a significantly higher average HDI (0.76019 vs 0.52404) than countries in the < P25 range.

The consumption of alcohol was associated with higher incidences of gastric cancer, with an average consumption of 10.60 L/person/year, which is significantly higher than the consumption in countries with incidences below the 25<sup>th</sup> percentile (3.75 L/person/year).

The consumption of tobacco was also associated with the highest incidences of gastric cancer, with an average of 30.03% of the population being consumers of any tobacco product, compared with an average of 14.47% among countries with incidences of cancer below the 25<sup>th</sup> percentile.

A higher consumption of fruits was related to higher incidences of gastric cancer. An average consumption of 222.9 g/d was observed among countries with incidences above the 75<sup>th</sup> percentile, and consumption of 145.8 g/d was observed among countries with incidences below the 25<sup>th</sup> percentile.

The same direction of association was found for vegetable consumption, with an average consumption of 318.7 g/d among countries with the highest incidences of gastric cancer and an average of 154.4 g/d among countries below the 25<sup>th</sup> percentile of the distribution of cancer incidence.

However, a higher consumption of legumes was significantly associated with lower incidences of gastric cancer.

The highest *per capita* consumption of meat was related to higher incidences of gastric cancer, as an average consumption of 157.1 g/d/person was found in countries grouped above the 75<sup>th</sup> percentile of the distribution of the incidence of gastric cancer, in comparison with an average of 63.3 g/person/d in countries below the 25<sup>th</sup> percentile.

In contrast, no significant association was observed for between the average consumption of fish and the population rates of female obesity.

Regarding the national percentage of obese male adults, the difference was significant, as the highest rates of obesity were associated with higher incidences of gastric cancer.

These results were reinforced by the analysis of box-plot diagrams shown in Figure 3.

## DISCUSSION

The prevention and treatment of stomach cancer, which is currently the fourth most common malignancy worldwide, are still major challenges<sup>[2-4]</sup>.

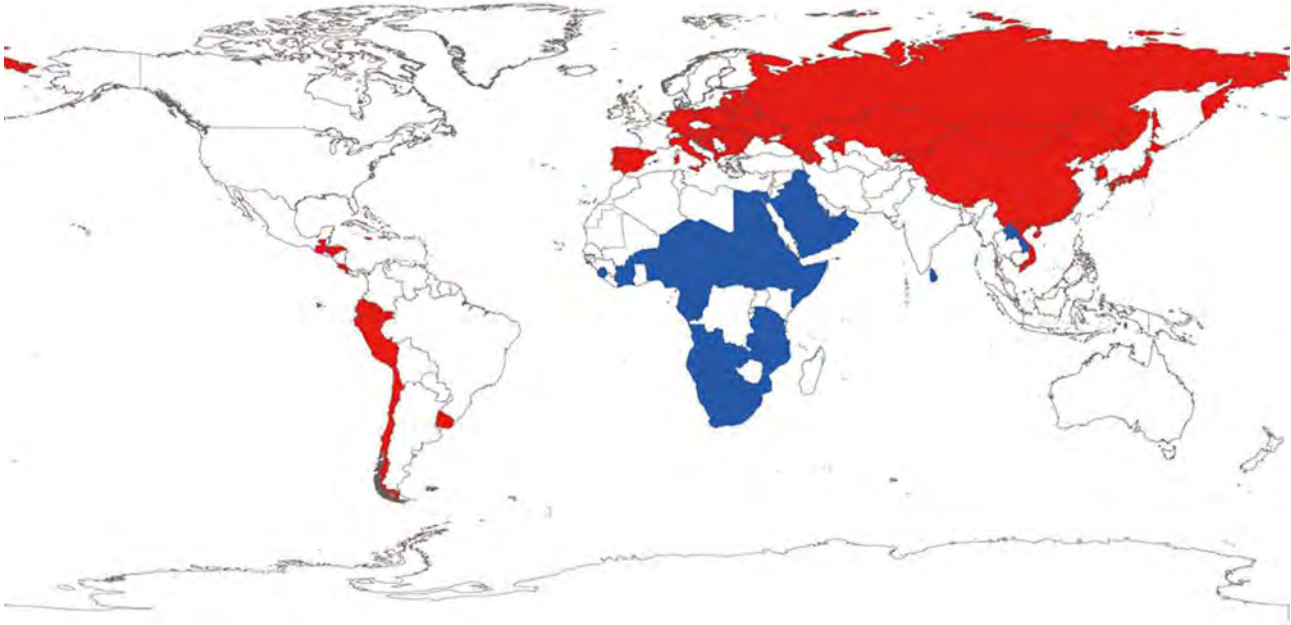


Figure 2 World map of countries with extreme incidences of gastric cancer. Blue: Countries categorized as < P25; Red: Countries categorized as > P75.

**Table 2 Association between the incidence of gastric cancer and risk/protection factors based on the Mann-Whitney test**

Risk/protective factors	<i>n</i>	<i>n</i> (< P25)	Average	SD	<i>n</i> (> P75)	Average	SD	<i>P</i> value
HDI	171	46	0.52	0.14	43	0.76	0.09	0.000
Alcohol, liters of alcohol/inhabitant per year	172	45	3.74	3.42	43	10.59	5.17	0.000
Tobacco, % of smoking population	132	36	14.47	7.27	35	30.03	7.11	0.000
Fruits, g/person per day	165	42	145.84	109.74	40	222.9	89.92	0.001
Vegetables, g/person per day	165	42	154.35	151.55	40	318.66	167.25	0.000
Legumes, g/person per day	165	42	23.08	19.88	41	8.42	8.91	0.000
Meat, g/person per day	165	42	63.26	51.07	42	157.05	63.16	0.000
Fish, g/person per day	162	42	42.56	74.83	38	44.93	42.14	0.260
Obesity in males, % men > 20 yr and BMI > 30 kg/m <sup>2</sup>	172	46	9.56	102.11	42	17.03	67.73	0.000
Obesity in females, % women > 20 yr and BMI > 30 kg/m <sup>2</sup>	172	46	18.94	150.44	42	21.66	81.28	0.100

HDI: Human development index; BMI: Body mass index.

There are geographic and ethnic differences in the distribution of the incidence of gastric cancer worldwide and changing trends in each population over time, which hinder a better understanding of this cancer's etiology.

It is assumed that the incidence has been decreasing in most industrialized countries over the past three decades and that the incidence patterns observed in immigrant groups move toward the patterns in the countries of origin. These changes suggest a close association of gastric cancer with modifiable factors<sup>[5-11]</sup>.

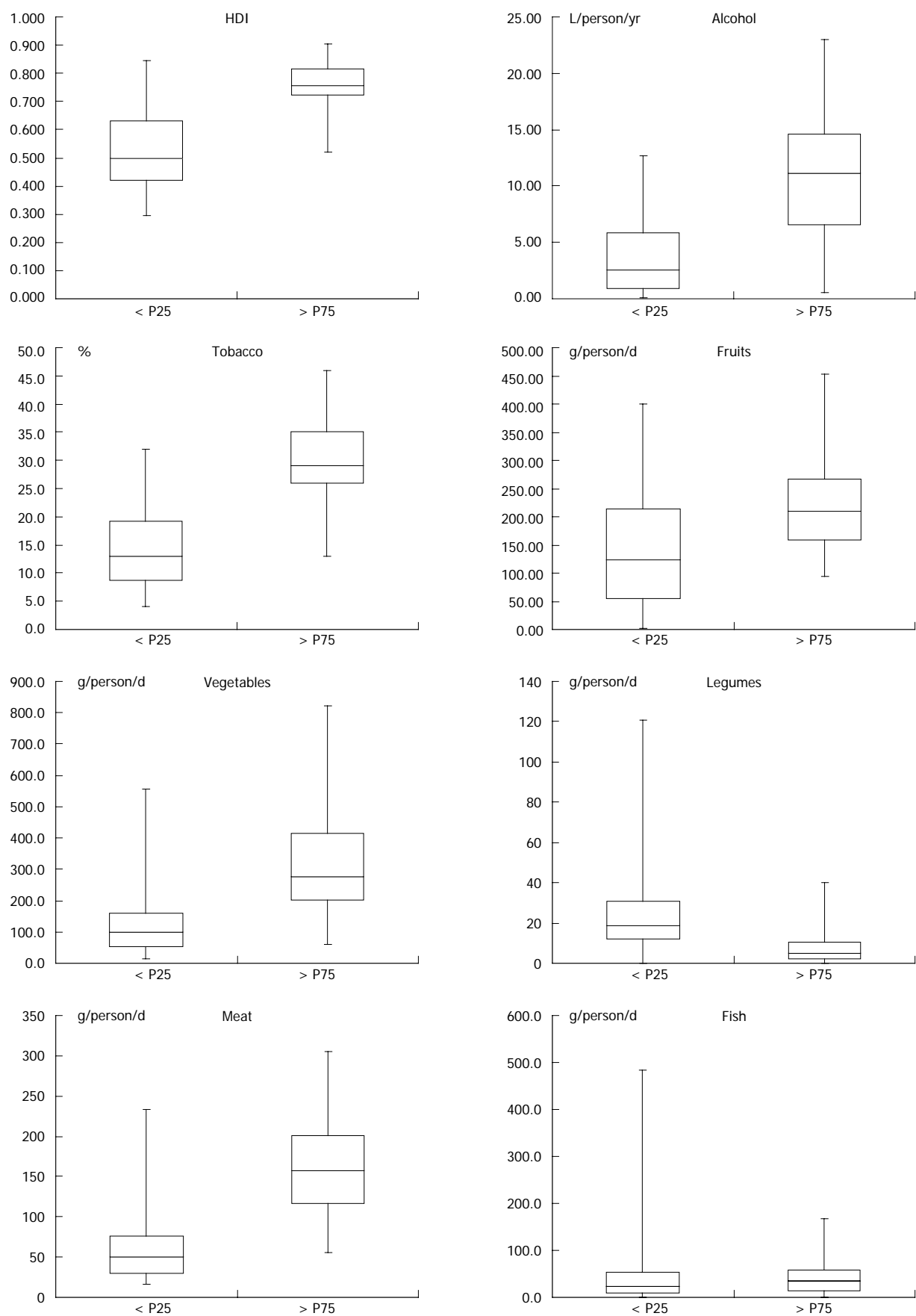
During the development of this study, when we first analyzed the crude data from all countries, we could not detect significant associations between protective/risk factors and gastric cancer due to the weak correlations observed. However, when studying the extreme incidences, we observed evidence of several of the associations already described in the literature between dietary/behavioral factors and the incidence of gastric cancer.

The IARC has presented fruits and vegetables as probable protective factors in the development of stom-

ach cancer. Therefore, the World Cancer Research Fund recommends a daily intake of vegetables/fruits greater than 400 g for a protective effect. This association was not observed in the current study. Moreover, although gastric cancer is considered to be a multifactorial disease, we observed that several of the countries with higher incidences, such as Korea, had a *per capita* consumption of fruits and vegetables above the suggested protective intake.

Part of this result may be justified by specific dietary components and certain cooking practices that are also associated with an increased risk of gastric cancer through the formation of *N*-nitroso compounds and polycyclic aromatic hydrocarbons. These practices include grilling, baking, curing, drying in the sun, smoking, cooking, frying in open ovens and salting. Certain foods also have natural nitrate concentrations (cabbage, cauliflower, carrots, celery, radishes, beets and spinach), or these compounds may be added during preservation. In addition, the nitrate content in soil fertilizers and water





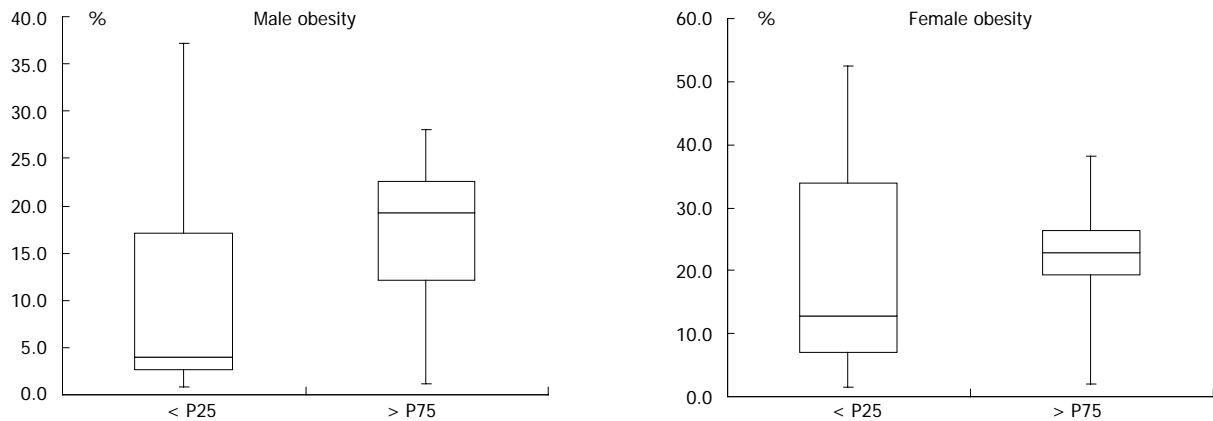


Figure 3 Boxplot diagrams of human development index values. the consumption of alcohol, tobacco, fruits, vegetables, legumes, meat and fish; and the prevalence of obesity in males and females among countries with gastric cancer incidences < P25 and > P75.

also contributes to the level of dietary nitrate<sup>[12]</sup>.

When evaluating obesity in males and the consumption of meat, vegetables, tobacco and alcohol, the results were consistent with findings described in the literature, reinforcing the concept that a modification of lifestyle represents a practical strategy for preventing gastric cancer, especially in middle-aged or elderly people<sup>[12-14]</sup>.

Currently, more than 80% of cases of gastric cancer may be associated with infection by *H. pylori*. In general, both cancer and infection tend to affect more individuals of lower socioeconomic classes, presumably due to poor education and sanitary conditions<sup>[15-21]</sup>. Due to the unavailability of a worldwide database representing this factor, we could not study its association with gastric cancer incidence.

It is known that the incidence rates of gastric cancer can vary up to 10 times worldwide and that nearly two-thirds of stomach cancers occur in developing countries. Even so, Japan and Korea, countries with high levels of development, have the highest rates of gastric cancer in the world<sup>[22-27]</sup>.

One of the possible explanations for this finding might be the large difference not only in the etiology but also in the programs for early detection, specialized treatment and prevention in these countries. Furthermore, there is evidence that in areas of low incidence, genetic and biological characteristics seem to have a greater influence on the development of the disease. For example, the incidence of cancer in Africa is the lowest among all developing and developed countries, ranging from 2 to 5.6/100000<sup>[5,28]</sup>.

It is important to remember that proximal tumors are more common in developed countries and in higher socioeconomic classes and have shown a progressive increase in incidence<sup>[29]</sup>. Distal tumors remain predominant in Japan, the country with the highest incidence, in contrast to the rest of the world<sup>[14,29]</sup>.

The multifactorial etiology of gastric cancer imposes an additional challenge on the understanding and development of effective prevention and monitoring programs. The most appropriate epidemiological studies

that are focused on factors associated with the disease require long and careful monitoring because these studies are based on observing individual cases, exposure to factors and cancer development, which makes these studies extremely expensive. The present study, with no claim to challenge epidemiological data or to create a method to replace epidemiological studies, proposes the implementation of a simple, low-cost methodology for the evaluation of the relationship between risk factors and outcome.

Ecological studies are valued in research on seasonal changes or geographical variations of events, especially under adverse social or territorial conditions that make it impossible to study every citizen. However, such studies have limitations. By shifting the focus from the subject to the population, one can lose sight of the direct relationship between a risk/protective factor and individual development of the disease. However, as exposure is measured in an ecological way, it is assumed that an average variation in the incidence of gastric cancer in a particular country also reflects a variation in the average exposure of each individual residing there<sup>[30-33]</sup>. This concept can be taken into consideration when evaluating individual consumption of food, tobacco products or alcohol.

The quality of a database depends on the properties of the components used in its formulation and the accuracy of the information systems used in its construction, including the database's integrity (completeness) and internal consistency (data consistent and not contradictory). The systematic application of operational definitions and standardized procedures for measuring and calculating allows inferences about and predictions of unavailable data<sup>[30,31]</sup>.

Although we did not spare efforts to identify the best data needed for our research, the fact that we worked with secondary data implies a limitation of this study, especially given a lack of access to the primary measures. This lack of data disallowed the proper analysis of major risk factors, such as salt intake and exposure to *H. pylori*.

The attempt to create a database for the prevalence of infection by *H. pylori* from data from published studies

to which we had access resulted in very small number of evaluated countries, which could have affected the final outcome. Thus, we disregarded the results of this analysis in our conclusions. Due to its importance in the genesis of gastric cancer, we consider the creation of a global database for the prevalence of *H. pylori* infection in each country to be important.

The inaccuracy of cancer incidence data for certain countries due to the methodology used by GLOBOCAN, which is considered to be a relatively reliable and stable source, being widely used in technical and scientific papers, should be noted. However, the collection of data from GLOBOCAN incorporates data measured by national health agencies and data derived from approximations of incidence based on the known frequencies of all types of cancer. In addition, 34 countries do not have local data, leading to the use of data available for neighboring countries in the same geographic region<sup>[4]</sup>.

The data collected from the FAO of the United Nations include global information from national statistical offices with internationally recognized definitions, concepts and classifications. Time series statistics have been compiled, processed and stored by each country since 1961, and the database contains the records of more than 245 countries and territories. The data are provided by governments through national publications and FAO questionnaires (paper or electronic). To make the data coverage as complete as possible, the official data are occasionally supplemented with data from unofficial sources.

The ecological study of gastric cancer based on public databases proved to be feasible and promising, and this method can be used to monitor the global behavior of similar diseases.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The multifactorial etiology of gastric cancer imposes an additional challenge on the understanding and development of effective prevention and monitoring programs. Ecological studies are valued in research on seasonal changes or geographical variations of events, especially under adverse social or territorial conditions that make it impossible to study every citizen.

### Research frontiers

The results of ecological studies can provide the opportunity for more carefully designed studies based on the initial observations. The use of databases is a simple, low-cost methodology for the evaluation of the relationship between risk factors and outcome, using data that are generally already available. The quality of a database is crucial to the analysis results.

### Innovations and breakthroughs

An ecological study on gastric cancer based on public databases proved to be feasible and promising, and this method can be used to monitor the behavior of the disease globally. The results of this study indicated a higher level of development, coupled with the highest prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables, and meat, among the countries with the highest incidences of gastric cancer. In contrast, a high

consumption of vegetables was associated with a lower disease incidence in other countries.

## Applications

The study's results suggest that ecological studies using populational databases can be used to monitor the global behavior of the disease. For this purpose, it is important to create a global database for the prevalence of *Helicobacter pylori* (*H. pylori*) infection in each country.

## Peer review

This ecological study on gastric cancer, based on public databases, provides a useful contribution to the dimension of gastric cancer protection, and the method can be used to monitor the behavior of the disease globally, although the study implies a limitation of using secondary data. The results of this study indicated a higher level of development, coupled with the highest prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables and meat, among the countries with the highest incidences of gastric cancer. In contrast, a high consumption of vegetables was associated with a lower disease incidence in other countries. The concern in this study is the exclusion of *H. pylori*, which is a very important risk factor for gastric cancer, from the analysis.

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## Clinical significance of white gastric crypt openings observed *via* magnifying endoscopy

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### Abstract

**AIM:** To evaluate the relationship between *Helicobacter pylori* (*H. pylori*)-induced gastritis and white gastric mucosal crypt openings (COs) in the gastric corpus.

**METHODS:** A total of 175 consecutive patients (including 69 patients with gastric cancer) were enrolled in this study. We used magnifying endoscopy (ME) to observe the mucosa microsurface of the lesser and greater curvature of the gastric corpus (350 areas in all). We focused on areas with a round pit microstructure (primarily observed in non-atrophied areas) and evaluated the white openings of these gastric pits. We classified the whiteness of the COs as the "white-edged dark spot" type (consisting of a dark spot bordered by white); the "white" type (pure white with no dark spot); and the "dense white pit (DWP)" type (dense white, resembling a snowball). Gastritis was also histologically

evaluated according to the updated Sydney System.

**RESULTS:** We detected round COs using ME in 246 of the 350 areas examined. The histological examination showed significantly more mononuclear cells and neutrophil infiltration in the "white" and "DWP" types than the "white-edged dark spot" type ( $P < 0.001$ ). Furthermore, significantly high-grade inflammation and evidence of active *H. pylori*-induced gastritis was observed in the "DWP" type ( $P < 0.001$ ). Significant differences were observed in the whiteness of COs between *H. pylori*-positive ( $n = 139$ ) and negative ( $n = 36$ ) patients ( $P < 0.001$ ). The sensitivity and specificity of the "white" and "DWP" types for predicting *H. pylori* infection were 78.5% and 81.7%, respectively. Of the patients with gastric cancer, 22.5% (18/80) had "white-edged dark spots", 51.3% (41/80) had "white" COs, and 26.3% (21/80) had "DWP"-type COs. "DWPs" were frequently observed among patients with undifferentiated gastric cancer [45.7% (16/35)].

**CONCLUSION:** CO whiteness detected *via* ME was associated with histological evidence of gastritis and helps to predict the severity of inflammation and *H. pylori*-induced activity.

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**Key words:** Magnifying endoscopy; *Helicobacter pylori*; Gastritis; Gastric cancer; Inflammation

**Core tip:** Recent studies have reported that advances in magnifying endoscopy (ME) have led to better correlations between histopathological findings and the ME features of *Helicobacter pylori* (*H. pylori*)-induced gastritis. However, the ME findings regarding *H. pylori*-induced severe inflammation are insufficient. Therefore, we evaluated the relationship between *H. pylori*-induced gastritis and the whiteness of gastric mucosal crypt openings (COs) in the gastric corpus using ME.

Our results showed that mononuclear cell and neutrophil infiltration differed significantly among the CO subtypes. CO whiteness detected *via* ME was associated with histological evidence of gastritis and helps to predict the severity of inflammation or activity induced by *H. pylori* in the gastric corpus.

Kawamura M, Sekine H, Abe S, Shibuya D, Kato K, Masuda T. Clinical significance of white gastric crypt openings observed *via* magnifying endoscopy. *World J Gastroenterol* 2013; 19(48): 9392-9398 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9392.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9392>

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) infection causes acute and chronic inflammation accompanied by neutrophil or lymphocyte infiltration. This type of persistent inflammation can result in gastric mucosal changes such as glandular atrophy, intestinal metaplasia, dysplasia, and eventually carcinoma<sup>[1-3]</sup>. Previous studies using conventional standard endoscopy have reported correlations between *H. pylori*-induced gastritis and endoscopic findings. Endoscopic atrophy is correlated with the grade of glandular atrophy and intestinal metaplasia<sup>[6]</sup>. With regard to severe grades of *H. pylori*-induced inflammation, nodular gastritis in the antral area is an endoscopic marker for the early phase of *H. pylori* infection and an exaggerated immune response<sup>[7-10]</sup>.

Advances in magnifying endoscopy (ME) and narrow-band imaging have enabled the real-time observation of the microsurface structure and microvascular architecture of the gastric mucosa. Recent studies have reported that these advances have led to stronger correlations between histopathological findings and the ME features of *H. pylori*-induced gastritis compared with data obtained using standard endoscopy<sup>[11-17]</sup>. Using ME, one can observe the microsurface structure of the gastric mucosa change from a round pit pattern to vertical long pits, tubular and granular patterns after the start of *H. pylori*-induced gastritis.

However, many investigators have reported that the morphological changes identified using ME are closely associated with histological glandular atrophy or intestinal metaplasia. The data regarding the characteristics of the ME findings in *H. pylori*-induced severe inflammation, such as nodular gastritis in the antral area, are insufficient. Therefore, we used high-resolution ME to investigate the characteristics of *H. pylori*-induced inflammation of the gastric corpus in *H. pylori*-negative and *H. pylori*-positive patients.

## MATERIALS AND METHODS

### Patients and methods

This observational study was performed in the endos-

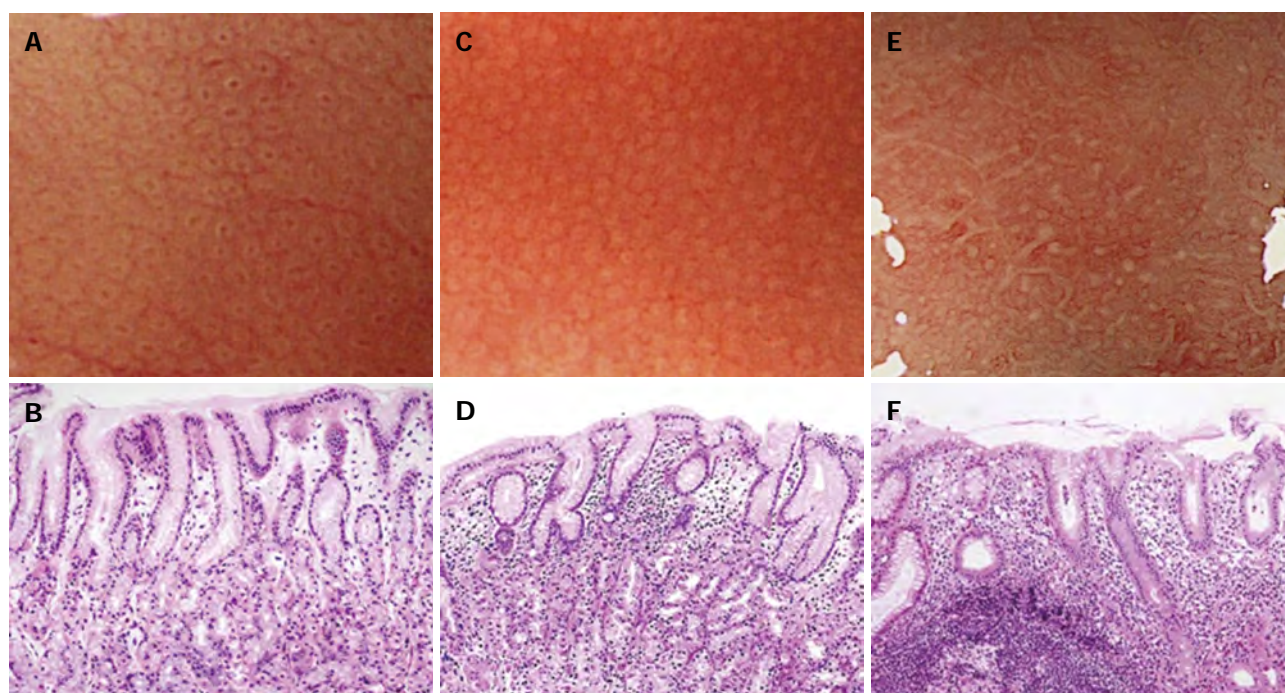
copy unit of a city hospital (JR Sendai Hospital, Sendai, Mi., Japan). Between September 2007 and November 2010, 175 consecutive patients who had undergone ME in our hospital as part of their annual health checks to investigate digestive symptoms (or as an additional pre-treatment examination) were enrolled. Patients were excluded if they had severe systemic disease; had a history of upper digestive tract surgery; had been treated with nonsteroidal anti-inflammatory drugs, antiplatelet agents, or anticoagulants within 7 d of endoscopy; or had active bleeding, advanced gastric cancer, or other non-gastric malignancies. Patients who received *H. pylori* eradication therapy were also excluded. *H. pylori* infection was diagnosed using a rapid urease test, which examined the histology of biopsy specimens obtained from the greater curvature of the gastric antrum and body, and the urea breath test. Patients who tested positive on any of these tests were considered positive for *H. pylori* infection. Written informed consent was obtained from each patient, and the institutional review board of JR Sendai Hospital approved the study protocol.

### Endoscopic procedure

Magnifying endoscopy was performed using either a CV-240 or CV-260 video system (Olympus Optical, Tokyo, Japan) and a magnifying endoscope (Model Q240Z or H260Z, Olympus). To obtain a clear view using ME, a black rubber attachment (MB-46 or MB-162, Olympus) was fitted to the tip of the videoendoscope to ensure an appropriate distance between the lens and the mucosal surface. A single experienced endoscopist (Masashi Kawamura) performed all procedures. A videoendoscope was inserted into the patient's stomach, and the diseased and uninvolved gastric mucosa were visualized using standard and magnified views. The microsurface structures of the non-cancerous areas in the greater and lesser curvatures of the upper gastric corpus were evaluated for the presence of patterns such as round pit, long pit, tubular, or granular. If areas with round pit microstructures were observed under maximum magnification, then we evaluated the whiteness of the gastric pit crypt openings (COs). Then, the specific area that had just been magnified was biopsied under magnification. The whiteness of each round CO was classified into one of the following three categories: A round dark spot bordered by white was classified as a "white-edged dark spot" CO; pure white COs without a dark spot were classified as "white" COs; and densely white COs resembling snowballs were classified as "dense white pit" ("DWP") COs (Figure 1).

### Histological assessment

Biopsy specimens were fixed with buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. An expert pathologist who was unaware of the endoscopic findings assessed the grade of histological gastritis in each biopsy sample. The degree of mononuclear cell and neutrophil infiltration, atrophy, and intestinal metaplasia was assessed and graded as normal, mild, moderate, or marked using a visual analog scale in-



**Figure 1** The whiteness of crypt openings (HE,  $\times 100$ ). A: "White-edged dark spot" crypt openings (COs) (a round dark spot bordered by white); B: Histological image of "white-edged dark spot" COs; C: "White" COs (pure white COs without a dark spot); D: Histological image of "white" COs; E: "Dense white pit" ("DWP") COs (densely white COs resembling snowballs); F: Histological image of "DWP" COs.

cluded in the updated Sydney System<sup>[18]</sup>. Gastric carcinomas were classified as differentiated or undifferentiated based on the degree of glandular structure formation among the tumor cells (the Japanese Classification was proposed by the Japanese Research Society for Gastric Cancer)<sup>[19]</sup>. These types match the intestinal and diffuse types of gastric carcinoma, respectively, described in the Lauren classification<sup>[20]</sup>.

### Statistical analysis

The results are presented as the mean  $\pm$  SD. Kruskal-Wallis and Mann-Whitney *U* tests were used to evaluate the relationship between the whiteness of the COs and the histological findings. The differences between the *H. pylori*-negative and *H. pylori*-positive groups with regard to CO type were compared using the Mann-Whitney *U* test. Data analyses were performed using R Version 3.0.1 (The R Foundation for Statistical Computing, Vienna, Austria); *P* values  $< 0.05$  were considered significant.

## RESULTS

Of the 175 enrolled patients, 116 were men and 59 women; their mean age was 63.9 years. The diagnoses included 46 patients with differentiated-type gastric cancer, 23 with undifferentiated-type gastric cancer, 22 with active duodenal ulcers, and eight with active gastric ulcers. A total of 76 patients had only gastritis or normal findings. The ME observations of the lesser and greater curvatures revealed round pit patterns in 246 of the 350 areas examined (Table 1).

Regarding the whiteness of the round COs, 89 had the "white-edged dark spot", 114 were "white", and

43 were "DWP" COs. Figure 2 shows the relationship between CO whiteness and the severity of gastritis diagnosed histologically based on the updated Sydney System. In both "white" and "DWP" type COs, the histological examination tended to show moderate or marked mononuclear cell and neutrophil infiltration accompanied by normal-to-mild glandular atrophy and intestinal metaplasia. These variables were mostly classified as normal to mild among "white-edged dark spot" COs. We evaluated the histological findings according to the updated Sydney system, which uses 4 classes (none, mild, moderate and marked) for each parameter [inflammation (mononuclear cell infiltration), activity (neutrophil infiltration), atrophy (glandular atrophy), and intestinal metaplasia]. Significant differences ( $P < 0.001$ ) were found between the three CO types in the parameters of inflammation and activity; however, the degree of glandular atrophy and intestinal metaplasia did not differ significantly across CO type. The grades of inflammation and activity were higher among "DWP" COs compared with "white" COs ( $P < 0.001$ ).

In this study, 139 patients were positive and 36 were negative for current *H. pylori* infection. Of the 186 round pit areas found in *H. pylori*-positive patients, 21.5% (40/186) were "white-edged dark spot" COs, 55.4% (103/186) were "white", and 23.1% (43/186) were "DWP" COs. Of the 60 round pit areas found among *H. pylori*-negative patients, 81.7% (49/60) were "white-edged dark spot" COs, 18.3% (11/60) were "white", and none (0/60) were "DWP" COs. Significant differences were found between the "white" and "DWP" COs in *H. pylori*-positive patients, and "white-edged dark spot" COs were found among *H. pylori*-negative patients ( $P < 0.001$ ).



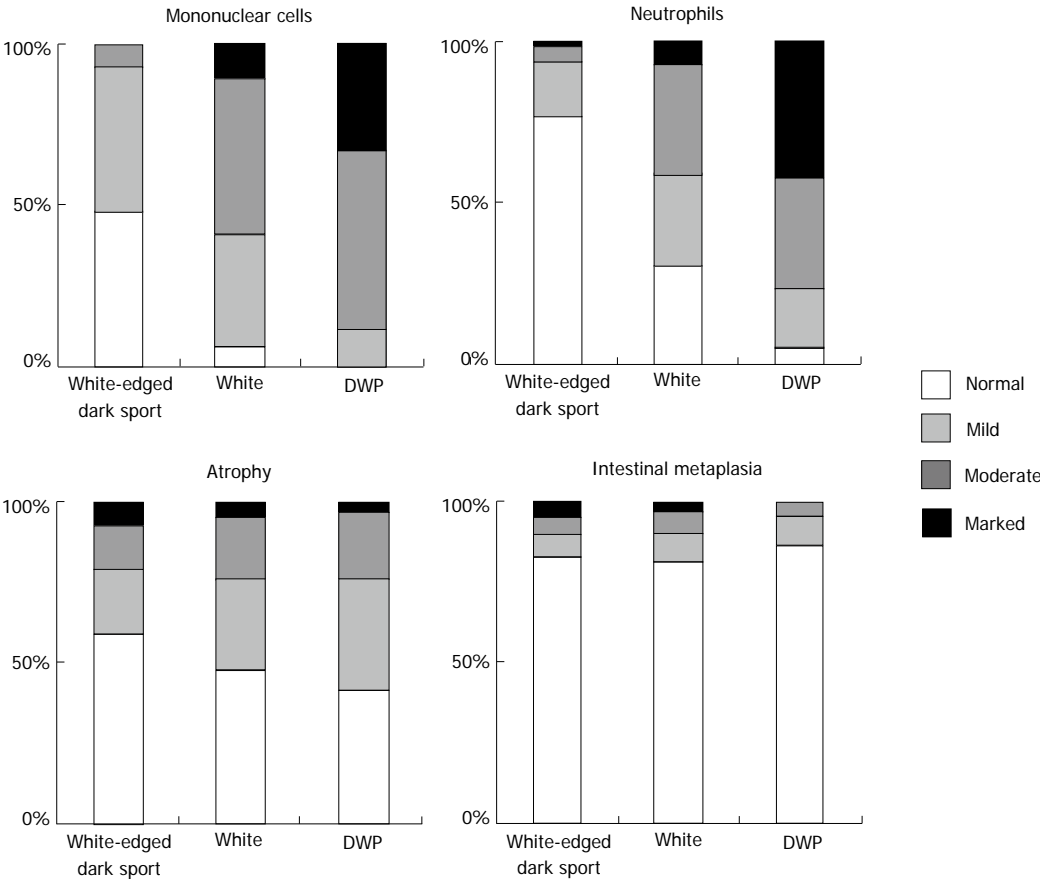


Figure 2 Whiteness of crypt opening and grade of gastritis according to the updated Sydney System. Kruskal-Wallis test showed significant correlations between grades of histological inflammation ( $P < 0.001$ ) and activity ( $P < 0.001$ ) and whiteness of crypt openings (COs). Glandular atrophy and intestinal metaplasia were not significantly correlated with whiteness of COs. DWP: Dense white pit.

Table 1 Characteristics of study patients <i>n</i> (%)					
	Diff-GC	Undiff-GC	GU	DU	Gastritis and normal
Number of patients	46	23	8	22	76
Age (yr, mean $\pm$ SD)	70.6 $\pm$ 9.2	65.5 $\pm$ 8.2	62.6 $\pm$ 10.6	49.2 $\pm$ 12.4	63.7 $\pm$ 12.5
Sex, male/female	36/10	7/16	6/2	17/5	50/26
Current <i>Helicobacter pylori</i> infection	40 (87.0)	21 (91.3)	8 (100)	22 (100)	48 (63.2)
Endoscopic degree of atrophy (mild/moderate/severe)	3/14/29	9/12/2	2/2/4	20/2/0	41/15/20
Round pits in LC	12 (35.3)	12 (52.2)	3 (37.5)	22 (100)	48 (63.2)
Round pits in GC	33 (71.7)	23 (100)	8 (100)	22 (100)	63 (82.9)

Diff-GC: Differentiated-type gastric cancer; DU: Duodenal ulcer; GC: Greater curvature of corpus; GU: Gastric ulcer; LC: Lesser curvature of corpus; Undiff-GC: Undifferentiated-type gastric cancer.

Table 2 Correlation between current disease and whiteness of crypt openings in round pit areas <i>n</i> (%)			
Whiteness of COs	White-edged dark spot	White	DWP
Diff-GC	16 (35.6)	24 (53.3)	5 (11.1)
Undiff-GC	2 (5.7)	17 (48.6)	16 (45.7)
GU	2 (18.2)	6 (54.5)	3 (27.3)
DU	13 (29.5)	26 (59.1)	5 (11.4)
Gastritis and normal	56 (50.5)	41 (36.9)	14 (12.6)

COs: Crypt openings; Diff-GC: Differentiated-type gastric cancer; DU: Duodenal ulcer; DWP: Dense white pit; GU: Gastric ulcer; Undiff-GC: Undifferentiated-type gastric cancer.

COs used to predict *H. pylori* infection were 78.5% and 81.7%, respectively.

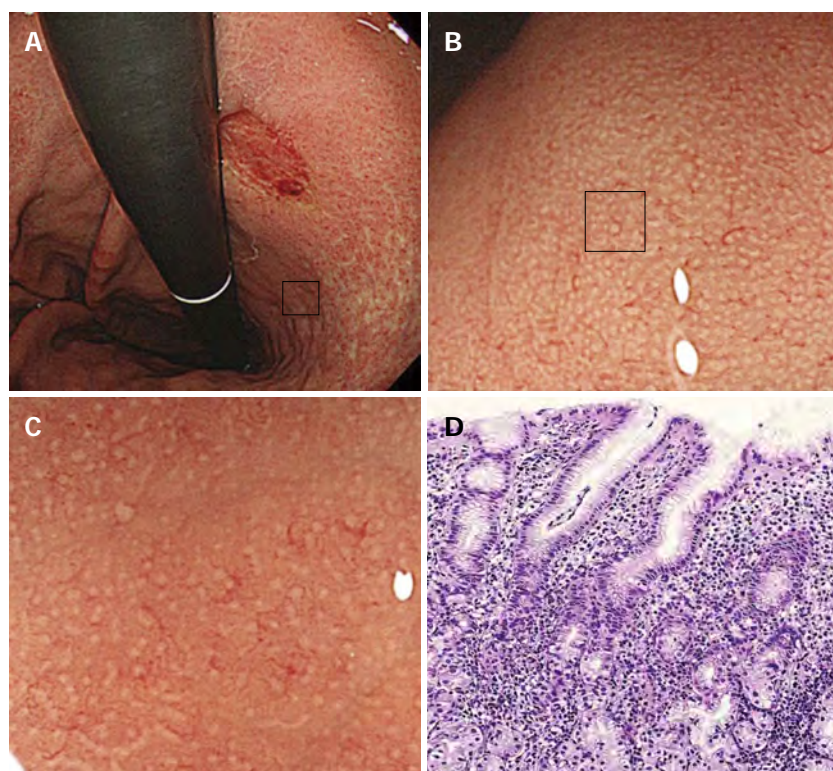
In total, 22.5% (18/80) of the COs among patients with *H. pylori*-related disease and gastric cancer were “white-edged dark spot” COs, 51.3% (41/80) were “white”, and 26.3% (21/80) were “DWP” COs (Table 2). The prevalence of “DWP” COs was higher [45.7% (16/35)] among patients with undifferentiated-type gastric cancer (Figure 3).

## DISCUSSION

This study is the first to investigate the relationship be-

The sensitivity and specificity of the “white” and “DWP”





**Figure 3** A case with an early undifferentiated-type gastric cancer in the middle of the corpus. A: A 67-year-old woman was found to have a depressed undifferentiated-type gastric cancer about 20 mm in diameter in an area found endoscopically to be non-atrophic; B: Magnifying endoscopy image showing minute round pits in uninvolved corpus mucosa; C: The whiteness of the crypt openings is "dense white pit" type; they resemble snowballs; D: Microscopic examination of uninvolved corpus mucosa revealed marked lymphocyte and neutrophil infiltration in the lamina propria and neutrophil infiltration within the foveolar lumens (HE stain,  $\times 100$ ).

tween CO whiteness type and *H. pylori*-induced gastritis. We categorized CO color in gastric corpus areas with round pit patterns, and we observed less inflammation and activity in "white-edged dark spot" COs than in other types of COs. The round dark spots observed in the ME images might correspond to tangential views of the foveolar gland COs, whereas the white portions that surround the dark spots might be tangential views of the epithelial cells that surround the ductal lumen. This CO type is the typical microsurface mucosal pattern of the gastric corpus among patients without *H. pylori* infection. A histological examination of the areas with "white" type COs revealed degenerated and hypertrophic surface epithelial cells accompanied by lymphocyte and neutrophil infiltration into the lamina propria. These histological changes might cause the dark spots to disappear and be replaced by a white color. The "DWP" COs were accompanied by a high degree of lymphocyte and neutrophil infiltration.

Several studies have evaluated the relationship between *H. pylori*-induced gastritis and the microsurface or microvascular structure of the gastric mucosa *via* ME. In 2007, Yagi *et al.*<sup>[21]</sup> modified their former Z classification and created the A-B classification based on the combination of microsurface and microvascular patterns (type B-0 consists of pinhole pits, the network of true capillaries, and the regular arrangement of collecting venules; type B-1 consists of round pits and a network of capillaries; type B-2 consists of white pits and sulci; and type B-3 consists of dilated white pits with surrounding microvessels). Although this classification was considered useful for predicting the grade of *H. pylori*-induced gastritis, we often observed other combinations of microstructure

and microvessel changes than those described in the A-B classification (*e.g.*, pinhole pits without capillaries). The current study classified our observations into three types of ME findings based on the color of the gastric pits and found strong correlations with histological *H. pylori*-induced inflammation and activity. Our classification is advantageous because it is simple and easy to understand and does not include variations in the combination of microstructure and microvessels.

Several reports have described that the prevalence, distribution, and grade of *H. pylori*-induced gastritis varies among individuals<sup>[22,23]</sup>. We previously reported that the ME findings of the gastric mucosa in *H. pylori*-infected patients are also heterogeneous in the stomach<sup>[15]</sup>. The present study investigated each case at two sites (the greater and lesser curvature of the upper corpus) because a multipoint evaluation of gastritis is important for assessing its status. Our results indicated that most round pit areas existed in the endoscopic non-atrophied area; furthermore, more were found in the greater curvature of the corpus. These results are in agreement with those of a previous report showing that gastric atrophy starts at the lower portion of the lesser curvature in the corpus, then extends to the upper portion and laterally involves the greater curvature<sup>[24]</sup>. The diagnosis for the CO whiteness grade seemed to be homogenous under magnified observations (see the figures). We diagnosed using maximum magnification at all sites; therefore, the CO whiteness types were recognized as a homogenous pattern using these narrow fields of view (approximately 2-mm squares).

The present study showed that the CO whiteness type had higher sensitivity and specificity than that reported

for conventional endoscopy<sup>[25,26]</sup> but lower sensitivity and specificity for detecting *H. pylori* infections than have been previously reported for ME assessment<sup>[13,14]</sup>. This discrepancy might have been caused by the differences in the *H. pylori* strain or immune responses. Our CO whiteness classification system was advantageous because it helped to predict the severity of inflammation and the *H. pylori*-induced activity in the gastric corpus.

Many reports have suggested that a relationship exists between differentiated-type gastric carcinoma and the severe atrophic gastritis caused by persistent *H. pylori* infection<sup>[2,23]</sup>. Conversely, undifferentiated-type gastric cancer is associated with *H. pylori*-induced active gastritis<sup>[23,27]</sup>. The present report showed that “DWP”-type COs that were accompanied by a histologically high grade of inflammation and activity were frequently observed in the gastric corpus of patients with undifferentiated-type gastric cancer; however, additional investigations are needed to clarify the relationship between CO whiteness and gastric cancer in a large population study.

The current study did not detect an area with round pits in 104/350 of the areas examined. As noted in earlier reports<sup>[12,21]</sup>, the microsurface pattern changes a round pit pattern to vertical long pits, tubular and granular patterns with continuous *H. pylori* inflammation. Thus, in the case of severe endoscopic atrophy, tubular and granular patterns (but not pit patterns) were often observed *via* ME. Given the difficulty of assessing histological inflammation (which differs from histological glandular atrophy) under endoscopic observations, our results suggest that ME is a useful method for predicting *H. pylori* inflammation in detail. However, our ME classification might not be acceptable in cases with severe atrophy.

Another limitation of this study is its small number of patients. An analysis of patients with other *H. pylori*-related diseases is needed. In addition, assessments of the inter- and intra-observer variability with regard to the classification of CO whiteness are required to generalize the diagnostic ability of our findings. Another limitation is that we did not investigate the nature of the white substance in “DWP”-type COs. As described in the updated Sydney System<sup>[11]</sup>, the marked neutrophil infiltration of foveolar lumens induced by *H. pylori*-infection might cause the formation of “pit abscesses”. We speculate that the appearance of “DWP”-type COs is attributable to the severe degenerative and hypertrophic changes in surface epithelial cells that are accompanied by lymphocyte and neutrophil infiltration; additional analyses are needed to investigate this possibility.

In conclusion, we found that CO whiteness in ME images of the gastric corpus was correlated with histological findings of inflammation and activity. ME observation of CO whiteness might facilitate the histological diagnosis of the inflammation and activity induced by *H. pylori*.

## ACKNOWLEDGMENTS

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tection Center, Miyagi Cancer Society for their technical assistance.

## COMMENTS

### Background

Magnifying endoscopy (ME) can be used for approximately x 80 magnified observation. Advances in ME have enabled the real-time observation of the microsurface structure and microvascular architecture of the gastric mucosa.

### Research frontiers

*Helicobacter pylori* (*H. pylori*) infection causes chronic inflammation of the gastric mucosa. This persistent inflammation leads to morphological changes in gastric mucosa as observed *via* ME.

### Innovations and breakthroughs

ME provides a detailed observation of *H. pylori*-induced gastritis, and the relationship between ME findings and histological gastritis was reported. Although many investigators have reported that the morphological changes identified *via* ME are closely associated with histological glandular atrophy and intestinal metaplasia, the data regarding the characteristics of these ME findings in *H. pylori*-induced gastritis are insufficient in cases of severe inflammation and activity. The current results indicate that the white gastric mucosa crypt openings observed *via* ME are useful for assessing histological inflammation and activity.

### Applications

*H. pylori* infection causes chronic gastritis, gastric or duodenal ulcer, and carcinoma. Conventional standard endoscopy has shown a poor relationship between *H. pylori* infection and active *H. pylori* gastritis. The results enable the assessment of histological activity and inflammation without a biopsy.

### Peer review

This article is well-written and well-designed for publication. It gives very new information for gastroenterologist.

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## Stapled gastro/duodenojejunostomy shortens reconstruction time during pylorus-preserving pancreaticoduodenectomy

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### Abstract

**AIM:** To investigate whether a stapled technique is superior to the conventional hand-sewn technique for gastro/duodenojejunostomy during pylorus-preserving pancreaticoduodenectomy (PpPD).

**METHODS:** In October 2010, we introduced a mechanical anastomotic technique of gastro- or duodenojejunostomy using staplers during PpPD. We compared clinical outcomes between 19 patients who underwent PpPD with a stapled gastro/duodenojejunostomy (stapled anastomosis group) and 19 patients who underwent PpPD with a conventional hand-sewn duodenojejunostomy (hand-sewn anastomosis group).

**RESULTS:** The time required for reconstruction was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group ( $186.0 \pm$

$29.4$  min *vs*  $219.7 \pm 50.0$  min,  $P = 0.02$ ). In addition, intraoperative blood loss was significantly less ( $391.0 \pm 212.0$  mL *vs*  $647.1 \pm 482.1$  mL,  $P = 0.03$ ) and the time to oral intake was significantly shorter ( $5.4 \pm 1.7$  d *vs*  $11.3 \pm 7.9$  d,  $P = 0.002$ ) in the stapled anastomosis group than in the hand-sewn anastomosis group. There were no differences in the incidences of delayed gastric emptying and other postoperative complications between the groups.

**CONCLUSION:** These results suggest that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

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**Key words:** Pylorus-preserving pancreaticoduodenectomy; Stapled anastomosis; Gastrojejunostomy; Duodenojejunostomy; Delayed gastric emptying

**Core tip:** The operative procedure of pylorus-preserving pancreaticoduodenectomy (PpPD) includes reconstruction of the pancreatic, biliary, and digestive systems, thus requiring a significant amount of time. We compared clinical outcomes between 19 patients who underwent PpPD with a stapled gastro/duodenojejunostomy and 19 patients who underwent PpPD with a conventional hand-sewn duodenojejunostomy. We demonstrate that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

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## INTRODUCTION

Pancreaticoduodenectomy (PD) remains one of the major and challenging operations associated with a relatively high mortality and morbidity rate. The PD operative procedure includes reconstruction of the pancreatic, biliary, and digestive systems, thus requiring a significant amount of time. Because prolonged operative time has been demonstrated to be a risk factor for mortality and postoperative complications<sup>[1-3]</sup>, efforts should be made to shorten the operative time by improving surgical skills and techniques.

The introduction of mechanical suture/stapling devices has provided surgeons with options for simple and sophisticated reconstruction methods in the field of gastrointestinal surgery. Recently, anastomotic techniques using staplers have been increasingly used for operations of the esophagus, stomach, and colorectum, particularly since the advent of laparoscopic surgery. In general, stapled anastomoses require less operative time and provide equal or better results in terms of the rate of leakage compared with hand-sewn anastomoses<sup>[4]</sup>.

Although stapled anastomoses can be used for reconstruction of the alimentary tract in virtually all operations, only a few studies have described such a method in the setting of pancreatic resection<sup>[5,6]</sup>. We introduced a mechanical anastomotic technique of gastro- or duodenojejunostomy using staplers during pylorus-preserving pancreaticoduodenectomy (PpPD). In an attempt to investigate the feasibility and efficacy of stapled gastro/duodenojejunostomy, we compared the outcomes between the stapled and conventional hand-sewn anastomotic techniques in patients undergoing PpPD.

## MATERIALS AND METHODS

### Patients

The study included 38 patients (25 men and 13 women with a mean age of 66 years) who underwent PpPD for cancers of the pancreatic head, ampulla of Vater, lower bile duct, and gallbladder; cystic neoplasms of the pancreas; neuroendocrine tumors; and others (chronic pancreatitis and duodenal submucosal tumor) at our institution between January 2009 and March 2012. Patients who underwent classical pancreaticoduodenectomy (Whipple operation), subtotal stomach-preserving pancreaticoduodenectomy (SSPPD), and laparoscopy-assisted pancreaticoduodenectomy were excluded from this study. In October 2010, we altered the technique of alimentary tract reconstruction (gastro/duodenojejunostomy and Braun anastomosis) from the conventional hand-sewn technique

to a mechanical anastomosis technique using staplers. The patients were divided into two groups according to the method of alimentary tract reconstruction: 19 patients who underwent hand-sewn duodenojejunostomy (hand-sewn anastomosis group) and 19 patients who underwent stapled gastro/duodenojejunostomy (stapled anastomosis group).

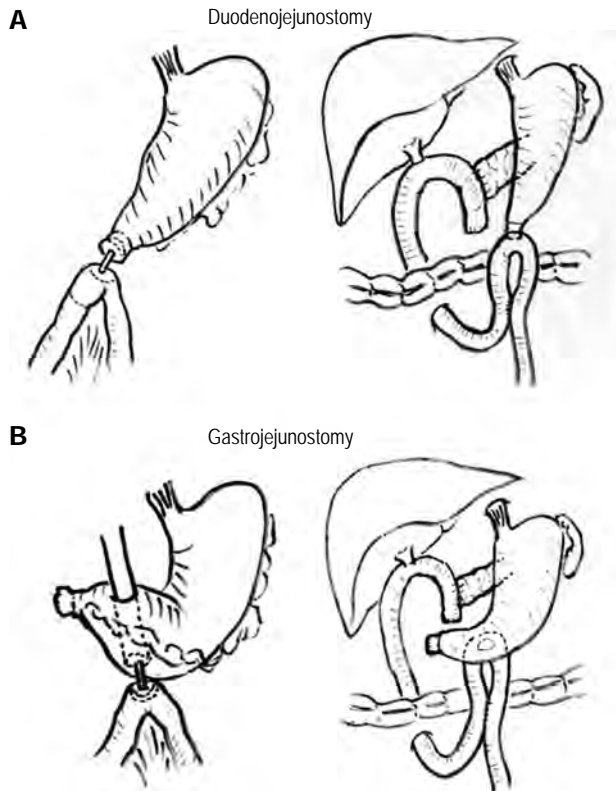
### Operative procedure

The detailed PpPD operative procedure was previously described elsewhere<sup>[7]</sup>. We routinely use the modified Child method for reconstruction. After removal of the pancreatic head, the anal stump of the jejunum was lifted through the mesocolon right to the middle colic artery. The pancreaticojejunostomy was performed using a modified Kakita's method<sup>[8]</sup>. A mucosa-to-mucosa anastomosis of the pancreaticojejunostomy was performed with interrupted sutures using 5-0 monofilament absorbable sutures (PDS, Ethicon Inc., Tokyo, Japan). A pancreatic tube was placed from the jejunum to the main pancreatic duct. The hepaticojejunostomy was performed by interrupted sutures using 4-0 monofilament absorbable sutures (PDS II, Ethicon Inc.), and the biliary tube was placed from the jejunal lumen to the hepatic duct of the liver. The biliary and pancreatic tubes were taken from the jejunal stump to the outside of the body.

The conventional hand-sewn duodenojejunostomy (end-to-side anastomosis) was performed by a two-layer Albert-Lembert method (whole layer, running sutures of 4-0 PDS, and seromuscular layer, interrupted sutures of 4-0 silk). A Braun anastomosis (side-to-side jejunojejunostomy) was also performed using the same method.

The stapled gastro/duodenojejunostomy was performed using a circular stapler (CDH25, Ethicon Inc.). For duodenojejunostomy, the anvil was inserted into the stomach through a small gastrotomy incision, moved to the duodenum, and fixed at the duodenal stump by a purse-string suture. A circular stapler was inserted into the jejunal loop through a small opening and connected to the anvil to complete the anastomosis (Figure 1A). For gastrojejunostomy, the anvil was inserted into and fixed at the jejunum. A circular stapler was inserted into the stomach through a small opening made in the anterior wall of the antrum and connected to the anvil to complete the anastomosis at the posterior wall of the stomach (Figure 1B). The openings made in the stomach or jejunum were closed by either running or interrupted sutures. A Braun anastomosis (side-to-side jejunojejunostomy) was also performed mechanically using a linear stapler (GIA, Covidien Japan, Tokyo, Japan). The gastro/duodenojejunostomy was made *via* an antecolic route in both the hand-sewn and stapled techniques.

Drainage tubes were placed at the posterior aspect of the hepaticojejunostomy and the anterior side of the pancreaticojejunostomy. They were drained to the outside of the abdomen. Biliary and pancreatic tubes were placed from the stump of the jejunum using Witzel's method.



**Figure 1** Schema of stapled gastro/duodenojejunostomy. A: Stapled duodenojejunostomy. The anvil was inserted into the stomach through a small gastrotomy incision, moved to the duodenum, and fixed at the duodenal stump by a purse-string suture. A circular stapler was inserted into the jejunal loop through a small opening and connected to the anvil for completion of anastomosis; B: Stapled gastrojejunostomy. The anvil was inserted into and fixed at the jejunum. A circular stapler was inserted into the stomach through a small opening made in the anterior wall of the antrum and connected to the anvil for completion of anastomosis at the posterior wall of the stomach.

All procedures were performed by one of the authors (Yamaguchi K).

### Postoperative management

The nasogastric tube was removed when the amount of drainage fluid was less than 200 mL/d and the nature of the fluid was not bloody. Liquid oral intake was resumed after gas passage if there was no evidence of pancreatic fistula. Delayed gastric emptying (DGE) was defined based on the International Study Group on Pancreatic Surgery classification<sup>[9]</sup>. Grade B (unable to tolerate solid oral intake by POD 14 with/without vomiting) or C (unable to tolerate solid oral intake by POD 21 with/without vomiting) was considered clinically relevant. Requirement of nasogastric tube reinsertion after POD 7 was also considered DGE.

### Statistical analysis

All statistical analyses were performed using JMP 10 software (SAS Institute Inc., Cary, NC). Categorical variables were analyzed using Fisher's exact probability test, and continuous variables were analyzed using the Mann-Whitney *U*-test. A *P* value of less than 0.05 was consid-

**Table 1** Patient characteristics in the stapled anastomosis group and hand-sewn anastomosis group

	Stapled anastomosis group	Hand-sewn anastomosis group	<i>P</i> value
Age (yr, mean $\pm$ SD)	67.2 $\pm$ 11.7	65.2 $\pm$ 11.2	0.59
Gender (M/F)	11/8	14/5	0.50
ASA			
1	4	1	
2	10	16	
3	5	2	0.11
Comorbidities <i>n</i> (%)	8 (42)	8 (42)	1.00
Diabetes <i>n</i> (%)	5 (26)	6 (32)	1.00
Previous history of upper abdominal surgery	2 (11)	2 (11)	1.00
Preoperative albumin level (g/dL, mean $\pm$ SD)	3.96 $\pm$ 0.35	3.74 $\pm$ 0.44	0.10
Disease			
Pancreatic cancer	6	4	0.08
Ampullary cancer	3	6	
Lower bile duct cancer	2	2	
Gallbladder cancer	1	0	
IPMN	3	3	
SCN	0	1	
PNET	3	0	
Duodenal GIST	0	1	
Mass-forming pancreatitis	0	1	
Others	1	1	

ASA: American Society of Anesthesiologists; IPMN: Intraductal papillary mucinous neoplasms; SCN: Solid cystic neoplasms; PNET: Pancreatic neuroendocrine tumor; GIST: Gastrointestinal stromal tumor.

ered statistically significant.

## RESULTS

### Patient characteristics in the stapled anastomosis group and hand-sewn anastomosis group

The patient characteristics in the stapled anastomosis group and the hand-sewn group are shown in Table 1. There were no significant differences in age, gender, American Society of Anesthesiologists (ASA) score, comorbidities, history of diabetes mellitus, previous history of upper abdominal surgery, preoperative level of serum albumin (as a nutritional status), or disease distribution between the groups (Table 1).

### Operative variables and postoperative outcomes in the stapled anastomosis and hand-sewn anastomosis groups

The operative variables were compared between the groups (Table 2). The mean operative time tended to be shorter in the stapled anastomosis group than in the hand-sewn group (500 min *vs* 530 min), although the difference was not statistically significant. However, the time required for reconstruction (removal of the pancreatic head to completion of surgery) was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group (186.0  $\pm$  29.4 min *vs* 219.7  $\pm$  50.0 min, *P* = 0.02). The total amount of blood loss during

**Table 2** Operative and postoperative outcomes in the stapled anastomosis group and hand-sewn anastomosis group

	Stapled anastomosis group	Hand-sewn anastomosis group	<i>P</i> value
Total operative time (min)	500 ± 68.3	530 ± 88	0.33
Reconstruction time (min)	186 ± 29.4	219.7 ± 50	0.02
Intraoperative blood loss (mL)	391 ± 212.3	647.1 ± 482.1	0.03
Duration of nasogastric tube insertion (d)	1.42 ± 1.22	1.3 ± 0.58	0.67
Resuming liquid oral intake (POD)	5.4 ± 1.7	11.3 ± 7.89	0.002
Starting solid diet (POD)	13.8 ± 8.56	17.7 ± 11.2	0.26
Postoperative complications	6 (31.6)	12 (63.2)	0.10
Delayed gastric emptying	1 (5.3)	3 (15.8)	0.60
Pancreatic anastomotic leakage/pancreatic fistula	1 (5.3)	3 (15.8)	0.60
Intraabdominal abscess	1 (5.3)	4 (21.1)	0.34
Postoperative hospital stay (d)	35.8 ± 12	39.4 ± 15.4	0.67

Values shown are mean ± SD or *n* (%).

surgery was significantly less in the stapled anastomosis group than in the hand-sewn group (391.0 ± 212.0 mL *vs* 647.1 ± 482.1 mL, *P* = 0.03).

We next compared the postoperative outcomes between the groups (Table 2). Although there was no difference in the duration of nasogastric tube insertion between the groups, the time from surgery to resuming liquid oral intake was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group (5.4 ± 1.7 d *vs* 11.3 ± 7.9 d, *P* = 0.002). Overall, postoperative complications occurred in 18 patients, including 6 patients (31.6%) in the stapled anastomosis group and 12 patients (63.2%) in the hand-sewn group (not significant). Among the complications, pancreatic fistula/anastomotic leakage occurred in 1 patient (5.3%) in the stapled anastomosis group and in 3 patients (15.8%) in the hand-sewn anastomosis group (not significant). Intra-abdominal abscess was observed in 1 patient (5.3%) in the stapled anastomosis group and in 4 patients (21.1%) in the hand-sewn anastomosis group (not significant). No patient in either group developed leakage of the gastro/duodenojejunostomy. DGE was observed in 1 patient (5.3%; grade C) in the stapled anastomosis group and in 3 patients (15.8%; grade B in 1 patient and grade C in 2 patients) in the hand-sewn anastomosis group (not significant). There was no difference in the duration of postoperative hospital stay between the groups. No 30-d postoperative mortality was observed in either group.

## DISCUSSION

In this study, we compared clinical outcomes between the stapled and conventional hand-sewn alimentary tract anastomotic techniques in a total of 38 patients undergoing PpPD. The major findings obtained were as follows: (1) the reconstruction time was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group; (2) intraoperative blood loss was significant-

ly less and the time from the operation to resuming oral intake was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group; and (3) there were no differences in the incidences of delayed gastric emptying and other postoperative complications between the groups. These findings suggest that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

Despite the disseminated use of mechanical suture/stapling devices in the field of gastrointestinal surgery, the application of these devices to reconstruction in PD remains uncommon. To date, only one Japanese group has described the method of gastro/duodenojejunostomy using staplers during PD<sup>[5,6]</sup>. In their method of stapled reconstruction, an antecolic gastrojejunostomy or duodenojejunostomy was performed by Roux-en-Y reconstruction using a linear or circular stapler<sup>[5]</sup>, which is slightly different from our technique in terms of dividing the jejunum for Roux-en-Y loop in their technique. The authors also demonstrated that the incidence of delayed gastric emptying was significantly lower in patients who underwent stapled gastro/duodenojejunostomy than in those who underwent hand-sewn reconstruction<sup>[6]</sup>. In the present study, we also found that the time to resuming oral intake was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group. Although the exact mechanism for improved oral intake and DGE by mechanical anastomosis is unknown, one possible explanation is that edema around the anastomotic site can be prevented by stapled anastomosis, particularly in the early postoperative period.

It has been reported that prolonged operative time is associated with an increased incidence of postoperative mortality and morbidity after PD<sup>[1-3]</sup>. According to a recent study in a total of 4817 patients undergoing PD<sup>[1]</sup>, longer operative time was linearly associated with increased 30-d morbidity (*P* < 0.001) and mortality (*P* < 0.01). Therefore, it is important for surgeons to avoid prolonged operative time by improving surgical techniques. With an aim to shorten the operative time, we introduced a technique of stapled gastro/duodenojejunostomy and Braun anastomosis. Although the difference in total operative time did not reach statistical significance, the reconstruction time was significantly shorter (by approximately 30 min) in the stapled anastomosis group than in the hand-sewn group. Importantly, intraoperative blood loss was significantly less in the stapled anastomosis group than in the hand-sewn anastomosis group (a mean volume of 391 mL *vs* 647 mL). Because a variety of factors can affect the volume of intraoperative blood loss, this difference is unlikely to be attributable solely to the different reconstruction techniques used. Furthermore, because of the small number of patients in each group, the mean volume of blood loss can be affected by a small number of patients with an unexpectedly large intraoperative blood loss. However, the reduced reconstruction time observed using rapid stapling devices may



have played a role, at least in part, in the reduced blood loss observed during surgery.

One concern that might be raised against our technique of stapled gastrojejunostomy is the significance of preserving the pylorus because food may not pass the pylorus in this anastomosis. By analyzing the plasma motilin concentration and phase III activity of the migrating motor complex of the stomach, it has been shown that preservation of the duodenum is important to maintain gastric motility and to prevent so-called “gastroparesis”<sup>[10,11]</sup>. In contrast to these findings, several lines of evidence have suggested that PD with pylorus resection (SSPPD) is comparable or even superior to that with pylorus preservation (PpPD) in terms of dietary intake and DGE<sup>[12-15]</sup>. Therefore, the clinical relevance of pylorus preservation requires further investigation.

Our study had several limitations. First, this study was a retrospective and historical cohort analysis; therefore, the possibility of bias cannot be eliminated. Second, the small number of patients in each anastomosis group may have underpowered our statistical evaluation. Third, we were unable to perform a cost comparison between the groups because of a lack of information. Therefore, to precisely determine the exact benefits of stapled gastro/duodenojejunostomy during PpPD, a prospective randomized trial, including an analysis of cost effectiveness, should be performed in the future.

In conclusion, our preliminary results suggest that stapled gastro/duodenojejunostomy is a feasible technique that could shorten the reconstruction and operative time during PpPD without increasing the incidence of postoperative complications including delayed gastric emptying. More recently, a mechanical anastomosis technique using a circular stapler has been applied to the hepaticojejunostomy during PD in selected patients with a dilated bile duct<sup>[16]</sup>. Thus, the introduction and standardization of these stapled anastomosis techniques can shorten the reconstruction time during PD and ultimately reduce the incidence of postoperative mortality and morbidity.

## COMMENTS

### Background

The operative procedure of pylorus-preserving pancreaticoduodenectomy (PpPD) includes reconstruction of the pancreatic, biliary, and digestive systems, thus requiring a significant amount of time.

### Research frontiers

Although stapled anastomosis can be applied to the reconstruction of alimentary tract in virtually all operations, only a few studies have described the reconstruction method of alimentary tract using staplers in the setting of pancreatic resection.

### Innovations and breakthroughs

The authors compared clinical outcomes between 19 patients who underwent PpPD with a stapled gastro/duodenojejunostomy and 19 patients who underwent PpPD with a conventional hand-sewn duodenojejunostomy.

### Applications

This study showed that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

### Peer review

This is a very novel report about the pylorus-preserving pancreaticoduode-

nectomy.

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## Sphincterotomy by triple lumen needle knife using guide wire in patients with Billroth II gastrectomy

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### Abstract

**AIM:** To investigate the usefulness of a guide wire and triple lumen needle knife for removing stones in Billroth II (B-II) gastrectomy patients.

**METHODS:** Endoscopic sphincterotomy in patients with B-II gastrectomy is challenging. We used a new guide wire technique involving sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy. This technique was performed in nine patients between August 2010 and June 2012. Sphincterotomy as described above was performed. Adequate sphincterotomy, successful stone removal, and complications were investigated prospectively.

**RESULTS:** Sphincterotomy by triple lumen needle knife using guide wire was successful in all nine patients. Sphincterotomy started towards the 4-5 o'clock direction

and continued to the upper margin of the papillary roof. Complete stone removal in one session was achieved in all patients. There were no procedure related complications, such as bleeding, pancreatitis, or perforation.

**CONCLUSION:** In patients with B-II gastrectomy, guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy seems to be an effective and safe procedure for the removal of common bile duct stones.

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**Key words:** Billroth II gastrectomy; Endoscopic sphincterotomy; Forward-viewing endoscopy; Guide wire; Triple lumen needle knife

**Core tip:** Guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy seems to be a safe, easy, and effective method for removing common bile duct stones in patients with B-II gastrectomy.

Park SB, Kim HW, Kang DH, Choi CW, Yoon KT, Cho M, Song BJ. Sphincterotomy by triple lumen needle knife using guide wire in patients with Billroth II gastrectomy. *World J Gastroenterol* 2013; 19(48): 9405-9409 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9405.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9405>

### INTRODUCTION

Endoscopic sphincterotomy (EST) is essential for the endoscopic removal of common bile duct (CBD) stones. However, EST is more difficult in an altered anatomy, such as Billroth II (B-II) gastrectomy, in which the major papillae are inverted<sup>[1]</sup>. To overcome this problem, several techniques

and specialized accessories have been devised, including Soehendra sphincterotome<sup>[2]</sup>, Sohma sphincterotome<sup>[3]</sup>, needle knife sphincterotomy guided by a biliary endoprosthesis<sup>[4,5]</sup>, S-shape sphincterotome<sup>[6]</sup>, and papillary balloon dilation<sup>[7,8]</sup>. These methods may be more difficult to control and frequently produce complications<sup>[5-7]</sup>.

Large balloon papillary dilation after minor EST with newly developed papillotomes, such as rotatable papillotome<sup>[9]</sup> and scissors papillotome<sup>[10]</sup>, has been reported for the removal of CBD stones in B-II gastrectomy. The triple lumen needle knife, which is capable of accepting a guidewire in one channel while simultaneously injecting and/or cutting in other lumens, is used primarily for pre-cut sphincterotomy or fistulotomy in difficult cases of deep cannulation of the bile duct. These characteristics allow the simultaneous maintenance of the guide wire in CBD and sphincterotomy without the assistance of other accessories. Additionally, a guide wire positioned in the CBD can make sphincterotomy by triple lumen needle knife easier.

Herein, we detail our technique and present experiences of guide wire sphincterotomy using a triple lumen needle knife through forward-viewing endoscopy in patients with B-II gastrectomy.

## MATERIALS AND METHODS

### Patients

From August 2010 to June 2012, endoscopic retrograde cholangiopancreatography (ERCP) for removal of CBD stones was performed on 469 patients. Of these patients, 20 with B-II gastrectomy underwent ERCP. Eleven patients who had previous EST procedures ( $n = 5$ ), failure to reach major papilla ( $n = 4$ ), and needle knife fistulotomy due to difficult cannulation ( $n = 2$ ) were excluded. The remaining nine patients (6 men and 3 women; mean age 66.3 years) were enrolled. The same endoscopist performed all procedures. All patients provided written informed consent for their participation and the study was approved by the Institutional Review Board of Pusan National University Yansan Hospital.

### Technique

All ERCP procedures were performed under conscious sedation and coverage using prophylactic antibiotics with a model GIF-H260 cap-attached forward-viewing endoscopy apparatus (Olympus Optical, Tokyo, Japan). A transparent cap (Distal Attachments D-201-11804; Olympus) was attached to the tip of the endoscope. Selective cannulation of the CBD was achieved using a cannulation catheter with a straight tip. A 0.025-inch guide wire (Jagwire; Boston Scientific, Natick, MA) was advanced through the catheter into the CBD. The catheter was then removed, and a triple lumen needle knife (Microknife™ XL; Boston Scientific) was introduced into the major papilla over the guide wire. The needle tip was controlled with a length of 2-3 mm (Figure 1A), and sphincterotomy was performed along the guide wire

as a guidance mark directed at 4-5 o'clock with sophisticated maneuver of the needle knife and endoscopy (Figure 1B). The model PSD-30 electrosurgical unit (Olympus) was used at a setting of blended one current with a power setting of 30 W/s for both the cutting and coagulation currents (cut: coagulation ratio of 3:1). During sphincterotomy, the pattern of cutting in the current method closely resembles the action of a pair of cutting scissors. Although the direction of the needle tip was toward the 1-2 o'clock direction, sphincterotomy toward the 4-5 o'clock direction was possible because the guide wire acted as a guide when the needle knife approached the bile duct pathway. At this time, the cap enabled us to perform safe and effective sphincterotomy by keeping a visual field during the procedure. After sphincterotomy to the upper margin of the papillary roof (Figure 1C), a stone retrieval basket or balloon catheter was used to extract CBD stones (Figure 1D). In cases when EST was insufficient in extracting the stone, papillary balloon dilation or mechanical lithotripsy was used in an attempt to remove the stone. To assess the efficacy and safety of this technique, we evaluated the sphincterotomy of the desired direction, use of balloon dilation or mechanical lithotripsy, successful stone removal, and complications after ERCP.

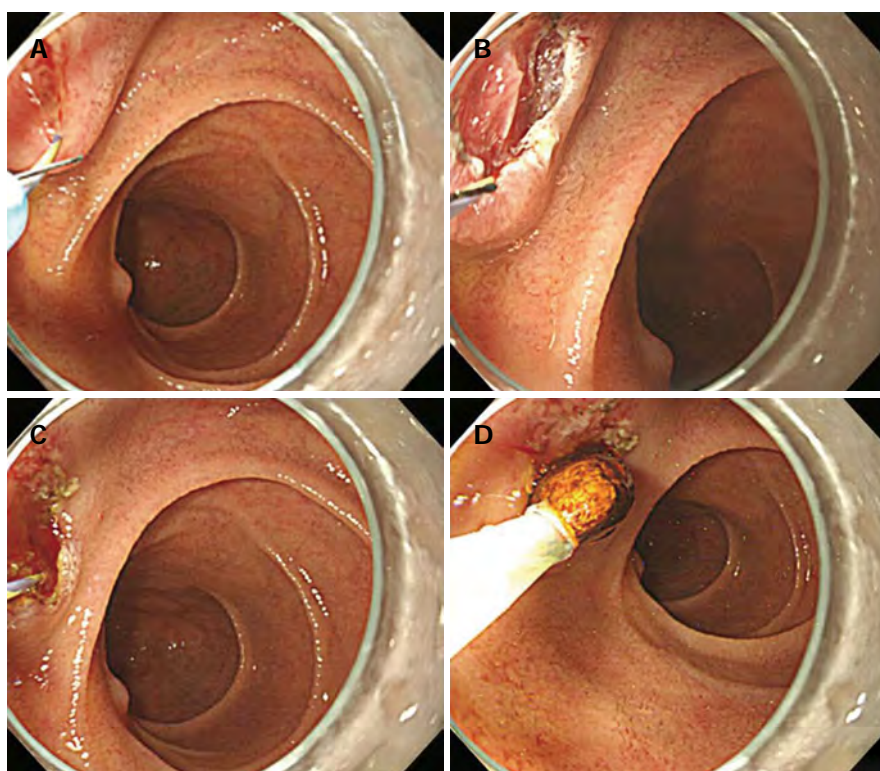
## RESULTS

ERCP with B-II gastrectomy was performed in 20 patients by the same endoscopist. Nine patients (6 men and 3 women) underwent EST using a guide wire and triple lumen needle knife sphincterotomy. The mean age of patients was 66.3 (range, 56-85) years. The mean size and number of stones were 7.89 (range, 5-12) and 1.78 (range, 1-4) mm, respectively (Table 1).

Sphincterotomy in the 4-5 o'clock direction and to the upper margin of the papillary roof were successful in all nine patients (100%). Complete endoscopic stone removal was achieved in a single session in all patients. Papillary balloon dilatation was performed in only one patient owing to the large CBD stones (12 mm diameter) and stenosis in the distal CBD they exhibited. No mechanical lithotripsy was performed in any patient (Table 1). Serum amylase and lipase were measured before and after the procedure (4 and 24 h, respectively). Complete blood count and a liver function test were performed the morning after the procedure. There were no complications, such as bleeding, pancreatitis, or perforation.

## DISCUSSION

Diagnostic and therapeutic ERCP in patients with B-II gastrectomy can be hindered by difficulties in the identification and intubation of the afferent loop, negotiation of abrupt turns in the afferent loop, cannulation, and adequate sphincterotomy of papilla due to inverted position<sup>[11-14]</sup>. In particular, the standard pull-type sphincterotome cannot cut toward the 6 o'clock position<sup>[15,16]</sup>. To



**Figure 1** Guide wire using sphincterotomy by triple lumen needle knife. A: A triple lumen needle knife was introduced into the major papilla over the guidewire and the tip of needle was then controlled with 2-3 mm length; B: Sphincterotomy was performed along the guide wire as a guidance mark directed in the 4-5 o'clock direction with sophisticated maneuver of needle knife and endoscopy; C: Sphincterotomy to the upper margin of the papillary roof was performed; D: A stone was removed by basket without balloon dilation or mechanical lithotripsy.

**Table 1** Baseline characteristics of patients and treatment outcomes

Patient No.	Age (yr)	Sex	Stone		Procedure sessions	Balloon dilation (size, mm)	Stone removal	Complications
			Size, mm	No.				
1	63	F	12	4	1	12	Success	None
2	69	M	5	2	1	0	Success	None
3	56	M	6	2	1	0	Success	None
4	68	F	10	2	1	0	Success	None
5	67	M	5	1	1	0	Success	None
6	65	M	8	1	1	0	Success	None
7	65	M	10	1	1	0	Success	None
8	59	F	10	2	1	0	Success	None
9	85	M	5	1	1	0	Success	None

overcome this problem, several devices and techniques have been developed using the push-type papillotome, such as the Sohma<sup>[3]</sup> and Soehendra sphincterotomes<sup>[2]</sup>. These refinements allow proper orientation of the wire for the CBD and needle knife with guided techniques using a nasobiliary drain<sup>[17]</sup>, cannula, or endoprosthesis<sup>[5]</sup>. However, effective push-type sphincterotomes or needle knives are, as yet, not as readily available as the standard pull-type sphincterotome.

Recently devised methods to resolve these problems rely on large balloon papillary dilation after minor EST with newly developed papillotomes<sup>[18-21]</sup>, such as the rotatable papillotome<sup>[9]</sup> and scissors papillotome<sup>[10]</sup>. These methods are user-friendly, but difficult to use in performing major EST.

Another problem is the difficulty of handling the side-viewing duodenoscope through the afferent loop in a retrograde maneuver; the result is a high rate of failed procedures and serious complications that include perforation of the small bowel<sup>[22-24]</sup>. The side-viewing duodenoscope has the advantages of allowing an en-face view of papilla and an elevator to adjust the direction of accessories. However, the apparatus is not useful in B-II anastomosis and increases the risk of perforation while passing the tortuous jejunum<sup>[14]</sup> and in cases of previous jejunal enteroanastomosis (Braun's anastomosis)<sup>[25]</sup>.

This method contrasts with existing methods in three ways. First, a triple lumen needle knife was used instead of a push-type sphincterotome or rotatable papillotome. The obvious advantage of this knife is the simultaneous



use of the needle knife and guide wire through the same device, which provides an indication of where and how deeply to cut, as well as avoiding blind or inappropriate cutting under direct vision of the cutting device and the presence of a clear guide. This enables a large sphincterotomy that can still be safely performed even when done by a less experienced endoscopist. Actually, sphincterotomy in all patients reached the upper margin of the papillary roof and most CBD stones were removed without papillary balloon dilatation or mechanical lithotripsy, although most were small. These findings clearly showed different results compared with other studies<sup>[9,10]</sup>. In the current study, papillary large balloon dilation was performed in one patient as a rescue method due to a large CBD stone and distal CBD stricture, but mechanical lithotripsy was not necessary. Additionally, bleeding, pancreatitis and perforation after sphincterotomy did not occur in all patients.

Secondly, conventional guide wire was used instead of nasobiliary drain, a cannula, or endoprosthesis. A guide wire already inserted in the bile duct can reduce the time of removal and subsequent reintroduction of the endoscope or insertion of a plastic stent. Also, this technique is cost-effective compared with endoprosthesis-guided sphincterotomy using a plastic stent, as a guide wire permits directed movement in the bile duct and control of the depth of incision, similar to the role of a plastic stent in endoprosthesis-guided sphincterotomy.

Thirdly, a forward-viewing endoscope was used instead of the side-viewing version. The forward-viewing endoscope in patients with Billroth II gastrectomy makes selective bile duct cannulation easier, as the endoscope and cannula are in line with the CBD<sup>[13,26]</sup>. Therefore, the lack of an elevator in forward-viewing endoscopes is only a slight disadvantage, and not a major factor in determining the success rate of cannulation and subsequent procedure. Also, a forward-viewing endoscope makes it easier to introduce the afferent loop and find the correct route, as well as making it easier to control for negotiating the acute angles of the anastomoses than side-viewing endoscope. These advantages were related with low complication rates in another study<sup>[14]</sup>, and no complications, such as perforation, were noted in the current study.

Despite the several advantages of the current method, some problems remain. If the endoscopic approach to major papilla is difficult, biliary cannulation and sphincterotomy are also difficult. Therefore, for effective application of this technique, the endoscopy tip must be approached near the major papilla.

In patients with B-II gastrectomy, we do not yet know which type of sphincterotomy is superior. The several techniques and accessories each have their own drawbacks. Currently, treatment strategy guided by personal preference or level of experience with specific techniques may be necessary. Our results support the use of the guide wire and triple lumen needle knife technique previously described as one option in patients with B-II gastrectomy. The technique is especially attractive when less experienced endoscopists perform sphincterotomy in patients with B-II gastrectomy.

In conclusion, guide wire using sphincterotomy by triple

lumen needle knife through a forward-viewing endoscopy seems to be a safe, easy, and effective method for removing CBD stones in patients with B-II gastrectomy. Further studies may be needed to compare the safety and efficacy of the technique in order to confirm our findings.

## COMMENTS

### Background

Endoscopic sphincterotomy is more difficult in an altered anatomy; the authors suggested a new technique by guide wire and triple lumen needle knife.

### Research frontiers

Recently introduced methods have their limitations due to their difficulty in being controlled. Guide wire technique involving sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy can be used more easily.

### Innovations and breakthroughs

The authors demonstrated the effectiveness and safety of removing common bile duct (CBD) stones in patients with Billroth II (B-II) gastrectomy, although those that were removed were small in size.

### Applications

Guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy are a feasible and effective intervention for removing CBD stones in patients with B-II gastrectomy.

### Peer review

This is a clinical study to evaluate the efficiency of a new technique for removing common bile duct stones in patients with gastrectomy. This is an important and novel topic in clinics.

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## Improvement of type 2 diabetes mellitus after gastric cancer surgery: Short-term outcome analysis after gastrectomy

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### Abstract

**AIM:** To evaluate the effect of gastrectomy on diabetes control in patients with type 2 diabetes mellitus and early gastric cancer.

**METHODS:** Data from 64 patients with early gastric cancer and type 2 diabetes mellitus were prospectively collected. All patients underwent curative gastrectomy (36 subtotal gastrectomy with gastroduodenostomy, 16 subtotal gastrectomy with gastrojejunostomy, 12 total

gastrectomy) and their physical and laboratory data were evaluated before and 3, 6 and 12 mo after surgery.

**RESULTS:** Fasting blood glucose (FBS), HbA1c, insulin, C-peptide, and homeostasis model assessment-estimated insulin resistance were significantly improved 3 mo after surgery, regardless of operation type, and the significant improvement in all measured values, except HbA1c, was sustained up to 12 mo postoperatively. Approximately 3.1% of patients stopped diabetes medication and had HbA1c < 6.0% and FBS < 126 mg/dL. 54.7% of patients decreased their medication, and had reduced FBS or HbA1c. In multivariate analysis, good diabetic control was not associated with operation type, but was associated with diabetes duration.

**CONCLUSION:** Diabetes improved in more than 50% of patients during the first year after gastric cancer surgery. The degree of diabetes control was related to diabetes duration.

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**Key words:** Type 2 diabetes mellitus; Gastrectomy; Gastric cancer; Short-term outcome; Glucose control

**Core tip:** Diabetes mellitus is one of the most important health problems and has an impact on the quality of life of gastric cancer patients as well as ordinary individuals. In this study, we evaluated the impact of conventional gastric cancer surgery on type 2 diabetes. Gastric cancer surgery led to a significant improvement in type 2 diabetes during the first year after surgery, and the degree of diabetes control was related to diabetes duration.

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type 2 diabetes mellitus after gastric cancer surgery: Short-term outcome analysis after gastrectomy. *World J Gastroenterol* 2013; 19(48): 9410-9417 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9410.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9410>

## INTRODUCTION

Gastric cancer is a leading cause of cancer death worldwide and is one of the most common cancers in Korea<sup>[1,2]</sup>. During the last several decades, there has been notable progress in the field of gastric cancer diagnosis and treatment, as indicated by the increasing proportion of early gastric cancers and improved survival rate<sup>[3,4]</sup>. Therefore, postoperative quality of life as well as the appropriate surgical treatment for a cure has become very important.

However, the increase in older patients due to an aging population and the increased incidence of lifestyle-related diseases including diabetes, hypertension, cardiovascular disease, and hypercholesterolemia make postoperative healthcare more difficult and complicated. Diabetes mellitus (DM) is one of the most difficult health problems worldwide as it is a multi-factorial chronic disease. In Korea, the prevalence of diabetes has increased dramatically from less than 1.5% in the 1970s to approximately 10% in the 2000s, and it is currently the 5<sup>th</sup> most common cause of death<sup>[5,6]</sup>. Although the prevalence of diabetes in gastric cancer patients has not been reported, it may be similar to that of the general population. After gastric cancer surgery, many surgeons focus on improving the nutritional status of patients rather than controlling diabetes as the main problem after gastric cancer surgery is weight loss. In addition, the beneficial effects of weight loss often lead to improvement in hyperglycemia, hypercholesterolemia, and hypertension.

Recently, metabolic surgery has become an appealing treatment option for patients with type 2 DM. The effects of metabolic surgery and the mechanism of action have been reported in several studies<sup>[7-9]</sup>. Although the purpose of metabolic surgery and gastric cancer surgery is completely different, there is a connection between the two procedures clinically and technically. In line with this thinking, the organized evaluation of the impact of conventional gastric cancer surgery on diabetes appears to be necessary. Such an evaluation will allow surgeons to select a favorable reconstruction type after gastrectomy in gastric cancer patients with diabetes. Therefore, in this study, we investigated the short-term effect of three types of routine gastric cancer surgery on type 2 DM.

## MATERIALS AND METHODS

### Patients

We analyzed the data from 64 early gastric cancer patients with type 2 DM who underwent curative gastrectomy for primary gastric cancer between 2009 and 2010. All of the patients had been diagnosed with type 2 DM after 40

years of age and were taking medication before the diagnosis of early gastric cancer. All the data were collected prospectively. Patients with the following conditions were excluded: (1) other malignancies; (2) pre- and post-operative chemotherapy or chemoradiotherapy; (3) other endocrine disorders such as thyroid or adrenal disease; (4) moderate to severe cardiovascular, pulmonary or renal disease; and (5) active infection. This study was reviewed and approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, and written informed consent was obtained from all patients prior to surgery.

### Surgical procedures

Subtotal or total gastrectomy was performed according to the tumor location. Billroth I or II reconstruction was carried out after subtotal gastrectomy and Roux-en-Y esophagojejunostomy after total gastrectomy. In Billroth I reconstruction, the duodenum was transected 1 cm distal to the pyloric ring and gastroduodenostomy was performed using a circular stapler. In Billroth II reconstruction, the length from the ligament of Treitz was approximately 20 cm and gastrojejunostomy was performed using a linear stapler. After total gastrectomy, the length of the esophagojejunostomy to jejunojunctionostomy was approximately 45 cm, and the length of the ligament of Treitz to jejunojunctionostomy was 20-25 cm. Esophagojejunostomy was performed using a circular stapler. Generally, D1+ $\beta$  or D2 lymph node dissection was performed according to the guidelines of the Japanese Gastric Cancer Association<sup>[10]</sup>.

### Evaluation of clinical variables and biochemical data during follow-up

All of the clinical and laboratory data were collected and recorded prospectively at each point of the routine follow-up. Blood samples were obtained after an overnight fast. Patients visited the hospital at 3-, 6- and 12-month time points during the first year after gastrectomy for a physical examination, laboratory tests, imaging, and/or endoscopy. The variables for evaluating the status of glucose control included body weight, body mass index, biochemical data [serum glucose, HbA1c, insulin, C-peptide, homeostasis model assessment-estimated insulin resistance (HOMA-IR), low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride], and medication status, and were recorded preoperatively and 3, 6 and 12 mo after surgery. All of the patients completed the study.

The degree of diabetes control was divided into three groups: (1) Remission: No medication and FBS < 126 mg/dL and HbA1c < 6.0%; (2) Improved: Reduced medication and one of the following: FBS or HbA1c reduction; and (3) Stationary: No change of medication, or patients excluded from the improved and remission categories.

### Statistical analysis

Statistical analysis was carried out using SPSS® version



**Table 1 Patient demographics *n* (%)**

Variables	<i>n</i> = 64
Age (yr)	62.7 ± 8.6
Range	45-77
Sex	
Male	43 (67.2)
Female	21 (32.8)
Body mass index (kg/m <sup>2</sup> )	24.7 ± 3.4
Range	18.6-38.1
Smoking history	
No	36 (56.2)
Yes	28 (43.8)
Alcohol history	
No	38 (59.4)
Yes	26 (40.6)
Family history of DM	
No	52 (81.2)
Yes	12 (18.8)
Operation type	
STG B I	36 (56.2)
STG B II	16 (25.0)
TG	12 (18.8)
Surgical approach	
Open surgery	28 (43.7)
Laparoscopic surgery	36 (56.3)
Duration of DM (yr)	6.6 ± 6.4
Range	0.5-25
DM medication	
Oral hyperglycemic agents	60 (93.7)
Insulin only	1 (1.6)
Both	3 (4.7)

Data are expressed as absolute numbers (percentage) or mean ± SD. DM: Diabetes mellitus; STG B I: Subtotal gastrectomy with Billroth I anastomosis; STG B II: Subtotal gastrectomy with Billroth II anastomosis; TG: Total gastrectomy.

15.0 for Windows® (SPSS, Chicago, IL, United States). Categorical variables were compared using the chi-square or Fisher exact test, and continuous data were compared by the Mann-Whitney *U* test. The Kruskal-Wallis test was used to compare biochemical data among the three surgical groups at the same evaluation time. The paired *t* test was used to compare preoperative and postoperative 12-mo biochemical data. Data are presented as mean ± SD. Binary logistic regression analysis was used to identify the independent variables associated with the degree of diabetic control. *P* values < 0.05 were considered statistically significant.

## RESULTS

### Patient demographics

The preoperative patient demographics are shown in Table 1. The mean body mass index (BMI) was 24.7 ± 3.4 kg/m<sup>2</sup>. After subtotal gastrectomy, gastroduodenostomy (STG B I) was performed in 36 patients and gastrojejunostomy (STG B II) in 16 patients. Twelve patients underwent total gastrectomy with Roux-en-Y esophagojejunostomy (TG). Of the patients in this study, 18.8% had a family history of diabetes in first degree relatives, 93.7% were taking oral hyperglycemic agents, and 6.3% were taking insulin with or without oral agents.

### Changes in biochemical data after surgery

All of the patients completed 12-mo follow-up. BMI, FBS, HbA1c, insulin, C-peptide, HOMA-IR, triglyceride, LDL-cholesterol, and HDL-cholesterol were determined preoperatively and 3, 6 and 12 mo after surgery (Table 2).

In the same operation type, data on preoperative day and postoperative 12 mo were compared. In addition, at the same follow-up points, variables of each operation type were compared. Figure 1 shows the changes in mean value of the biochemical data. BMI rapidly decreased during the first 3 mo after surgery and was maintained up to 12 mo (Figure 1A). In all operation types, the 12-mo postoperative BMI value significantly decreased to approximately 90% of the preoperative value. At the same follow-up point, BMI level showed no significant difference according to operation type. Despite this, the degree of weight loss tended to be greater after total gastrectomy than after subtotal gastrectomy.

The FBS levels at 3, 6 and 12 mo after surgery were lower than preoperative levels, and the difference in FBS levels at the preoperative time point and 12 mo after STG B I (*P* = 0.001) was statistically significant. As shown in Figure 1B, FBS levels decreased markedly up to 3 or 6 mo after surgery and then slowly declined or increased again up to 12 mo. There was no difference in the FBS level according to operation type at the same time points.

HbA1c levels improved 3 mo after each type of surgery, but increased 12 mo after subtotal gastrectomy and were maintained after total gastrectomy (Figure 1C). Therefore, there were no significant differences between preoperative and postoperative 12-mo HbA1c levels following the three types of surgery. This may be associated with the stabilization of BMI and FBS levels between 3 and 12 mo after surgery, which would result from an increase in food intake. Insulin levels rapidly decreased 3 mo after all types of surgery and then slowly decreased up to 12 mo (Figure 1D).

There were significant differences in insulin and C-peptide levels (Figure 1E) between 3 and 12 mo after surgery, but there were no differences according to operation type at the same follow-up points. HOMA-IR levels consistently improved at 3, 6 and 12 mo after all types of surgery (Figure 1F) and the levels at 12 mo after surgery were significantly lower than the preoperative levels. The HOMA-IR level at 6 mo follow-up after total gastrectomy was significantly lower than that after subtotal gastrectomy.

The lipid profile which included triglyceride, LDL, and HDL did not show significant differences between preoperative and postoperative 12-mo levels, with the exception of HDL level in the STG B I group (Figure 1G-I).

### Diabetes control after surgery

Patients were divided into three groups based on their diabetes status: stationary, improved and remission (Table 3). Among the 64 patients, 35 patients (54.7%) improved and 2 patients (3.1%) went into remission. In the STG B I group, 58.3% of patients had improved 12 mo after

**Table 2** Changes in biochemical data after surgery according to the follow up period and operation type

	Operation type	Preoperative	PO 3 mo	PO 6 mo	PO 12 mo	<sup>1</sup> P pre-12 mo
BMI (kg/m <sup>2</sup> )	STG B I	24.3 ± 2.9	22.5 ± 2.6	22.3 ± 2.8	21.9 ± 2.7	< 0.001
	STG B II	24.7 ± 3.1	22.6 ± 2.9	22.9 ± 2.1	22.1 ± 1.9	0.002
	TG	25.7 ± 5.2	23.8 ± 5.7	22.5 ± 4.6	22.9 ± 4.4	0.004
	<sup>2</sup> P	0.793	0.965	0.616	0.780	
BMI	STG B I	100%	92.9% ± 5.2%	92.2% ± 5.3%	92.0% ± 7.0%	< 0.001
	STG B II	100%	91.6% ± 6.7%	93.3% ± 6.9%	90.3% ± 11.0%	0.004
	TG	100%	90.9% ± 6.4%	87.8% ± 5.7%	87.1% ± 8.1%	0.003
	<sup>2</sup> P	1.000	0.661	0.050	0.333	
Glucose (mg/dL)	STG B I	147.3 ± 44.1	125.0 ± 30.3	122.3 ± 38.4	114.4 ± 21.7	0.001
	STG B II	155.7 ± 40.0	136.4 ± 56.6	122.5 ± 31.9	126.4 ± 39.1	0.061
	TG	145.9 ± 37.5	114.3 ± 22.2	117.0 ± 28.0	115.4 ± 27.3	0.234
	<sup>2</sup> P	0.682	0.489	0.931	0.773	
HbA1c	STG B I	7.2% ± 1.1%	6.8% ± 0.6%	7.0% ± 0.7%	7.1% ± 0.9%	0.834
	STG B II	7.3% ± 1.3%	6.9% ± 1.4%	7.1% ± 1.3%	7.1% ± 1.6%	0.626
	TG	7.1% ± 0.8%	6.5% ± 0.7%	6.5% ± 0.5%	6.5% ± 0.6%	0.119
	<sup>2</sup> P	0.981	0.227	0.201	0.201	
Insulin (μIU/mL)	STG B I	21.3 ± 20.7	8.6 ± 11.2	8.1 ± 11.3	5.1 ± 4.7	< 0.001
	STG B II	22.5 ± 29.0	9.6 ± 8.8	8.9 ± 3.5	7.7 ± 7.1	0.041
	TG	18.1 ± 10.1	7.5 ± 5.7	4.0 ± 2.1	3.7 ± 1.9	0.02
	<sup>2</sup> P	0.679	0.652	0.050	0.350	
C-peptide (ng/mL)	STG B I	3.4 ± 2.3	2.1 ± 1.6	2.3 ± 1.2	1.5 ± 0.8	< 0.001
	STG B II	3.3 ± 1.8	2.0 ± 1.4	2.4 ± 0.9	2.1 ± 1.5	0.028
	TG	2.8 ± 1.3	2.1 ± 1.2	1.5 ± 1.3	1.6 ± 0.6	0.02
	<sup>2</sup> P	0.877	0.778	0.597	0.523	
HOMA-IR	STG B I	8.7 ± 10.1	2.9 ± 4.7	2.6 ± 7.9	1.3 ± 0.9	0.001
	STG B II	9.3 ± 13.5	3.3 ± 3.4	2.9 ± 2.0	2.5 ± 2.5	0.045
	TG	7.1 ± 4.1	2.1 ± 1.6	0.8 ± 0.2	1.1 ± 0.6	0.015
	<sup>2</sup> P	0.660	0.484	0.036	0.176	
TG (mg/dL)	STG B I	126.1 ± 80.8	100.1 ± 51.6	115.9 ± 62.6	101.4 ± 41.7	0.341
	STG B II	144.8 ± 102.6	118.5 ± 75.9	124.3 ± 53.7	105.2 ± 44.6	0.114
	TG	143.5 ± 88.9	130.5 ± 71.4	72.3 ± 36.6	96.6 ± 37.3	0.082
	<sup>2</sup> P	0.831	0.503	0.130	0.937	
LDL (mg/dL)	STG B I	96.8 ± 34.7	89.0 ± 27.9	81.3 ± 23.5	90.8 ± 29.2	0.522
	STG B II	97.4 ± 28.7	97.9 ± 33.5	94.4 ± 29.7	101.6 ± 37.5	0.515
	TG	113.6 ± 28.9	99.8 ± 28.0	63.5 ± 32.0	98.5 ± 32.0	0.023
	<sup>2</sup> P	0.299	0.556	0.192	0.594	
HDL (mg/dL)	STG B I	43.9 ± 10.6	48.3 ± 11.4	49.4 ± 15.1	52.0 ± 15.1	0.002
	STG B II	43.2 ± 9.2	42.6 ± 8.2	46.2 ± 10.6	46.5 ± 8.6	0.173
	TG	42.4 ± 10.5	45.0 ± 7.3	48.7 ± 3.2	46.3 ± 8.1	0.073
	<sup>2</sup> P	0.953	0.161	0.757	0.552	

<sup>1</sup>Paired *t*-test, mean ± SD; <sup>2</sup>Kruskal-Wallis test was used to evaluate the difference by operation type at the same follow up period. Significant values are indicated in bold face. BMI: Body mass index, BMI (%) refers to percentage of BMI at each follow-up compared to preoperative BMI. STG B I: Subtotal gastrectomy with Billroth I anastomosis; STG B II: Subtotal gastrectomy with Billroth II anastomosis; TG: Total gastrectomy; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

surgery and 16.7% of patients stopped their medication. However, no patients went into remission. In the STG B II group, one (6.2%) of 16 patients went into remission and 9 (56.2%) were improved 12 mo after surgery. In the TG group, 5 (41.7%) patients were improved and one (8.3%) was in remission 12 mo after surgery. Three (25%) patients stopped medication.

#### Factors for diabetes control 12 mo after surgery

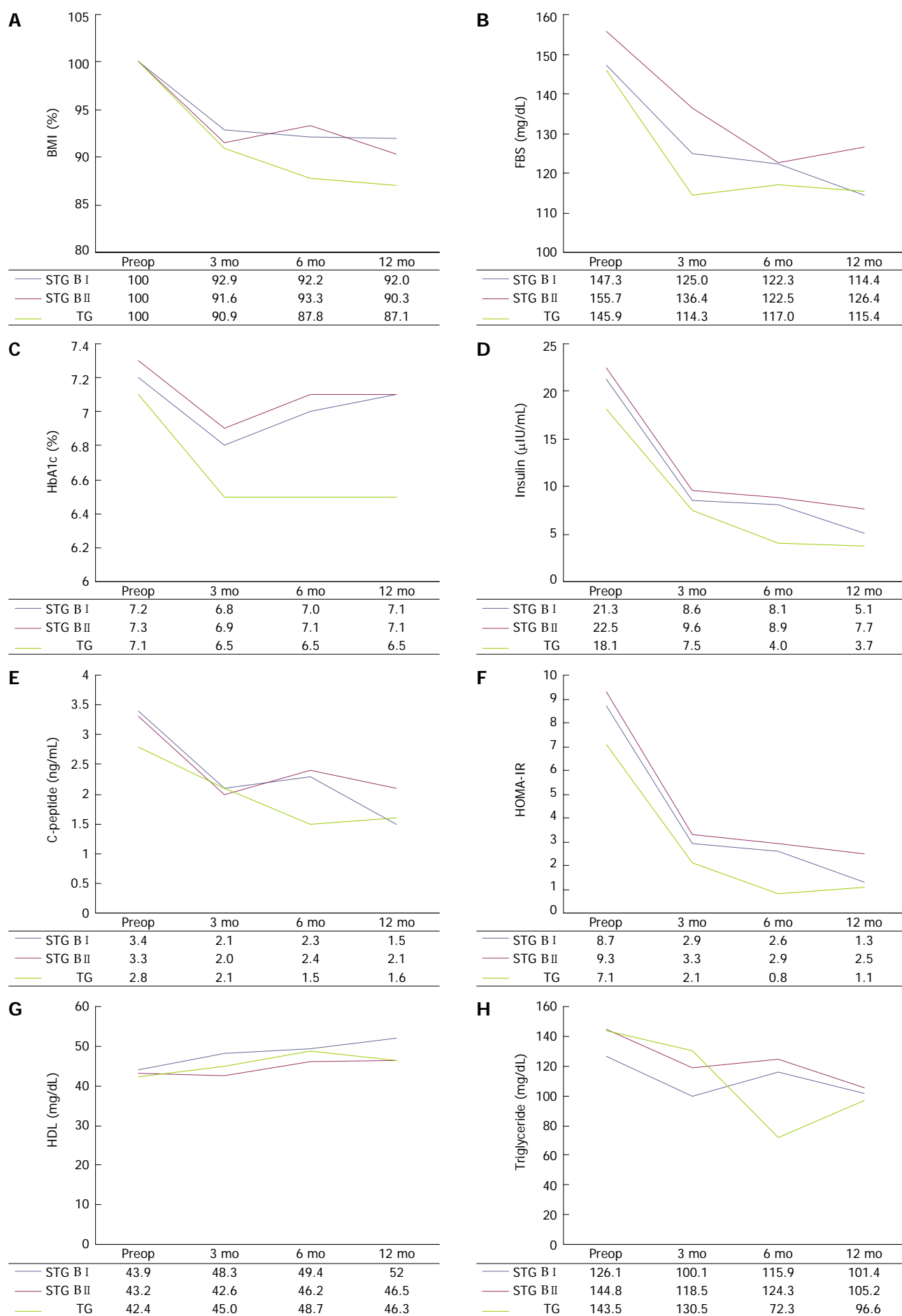
We compared the improved and in remission patients to those who were stationary to identify predictive factors for diabetes control. Age, sex, change in BMI, smoking, alcohol history, familial history (1<sup>st</sup> degree relatives) of type 2 DM, operation type, preoperative fasting blood glucose, HbA1c, insulin, C-peptide, HOMA-IR, triglyceride, LDL, and HDL were not associated with the degree of diabetes control 12 mo after gastrectomy (Table 4).

Postoperative BMI changes, smoking history, and the duration of type 2 DM were predictive factors for diabetes control after surgery. BMI levels 3-, 6- and 12-mo after surgery were lower, the incidence of non-smokers was higher, and the duration of DM was shorter in patients satisfying improved or remission criteria than those in the stationary group.

In multivariate analysis, the duration of DM was the only significant factor associated with postoperative diabetic control (Table 5).

## DISCUSSION

In recent studies which evaluated diabetes resolution after gastric cancer surgery, diabetes remitted in 15.1%-19.7% of gastric cancer patients<sup>[11,12]</sup>. However, because these studies involved a retrospective review of medical re-



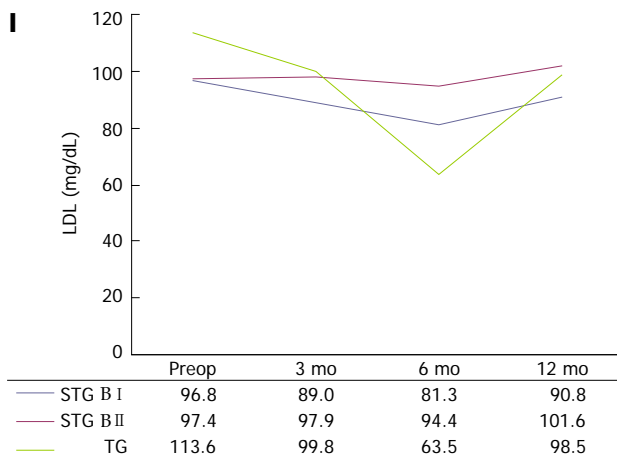


Figure 1 Changes in body mass index and serum biochemical data after gastric cancer surgery according to the follow-up periods and operation type. A: Body mass index; B: Fasting blood glucose level; C: HbA1c; D: Insulin; E: C-peptide; F: Homeostasis model assessment-estimated insulin resistance; G: Triglyceride; H: Low-density lipoprotein-cholesterol; I: High-density lipoprotein-cholesterol.

Table 3 Degree of diabetes mellitus control after surgery *n* (%)

	STG B I ( <i>n</i> = 36)			STG B II ( <i>n</i> = 16)			TG ( <i>n</i> = 12)		
	3 mo	6 mo	12 mo	3 mo	6 mo	12 mo	3 mo	6 mo	12 mo
Stationary	16 (44.4)	16 (44.4)	15 (41.7)	8 (50)	8 (50)	6 (37.5)	6 (50)	7 (58.3)	6 (50)
Improved	19 (52.8)	20 (62.5)	21 (58.3)	8 (50)	8 (50)	9 (56.2)	6 (50)	4 (33.3)	5 (41.7)
Remission	1 (2.8)	0	0	0	0	1 (6.2)	0	1 (8.3)	1 (8.3)
Medication stopped	6 (16.7)	6 (16.7)	6 (16.7)	2 (12.5)	2 (12.5)	4 (25)	3 (25)	3 (25)	3 (25)

Stationary: No change in medication, or patients except improved and remission criteria; Improved: Reduced medication and a reduction in fasting blood glucose (FBS) or HbA1c; Remission: No medication and FBS < 126 mg/dL and HbA1c < 6.0%. STG B I: Subtotal gastrectomy with Billroth I anastomosis; STG B II: Subtotal gastrectomy with Billroth II anastomosis; TG: Total gastrectomy.

cords or interviewing, the available laboratory and physical parameters were limited. Although data analysis of the present study was performed retrospectively, all of our data were collected prospectively including laboratory data, body weight change, medical and familial history, and medication status. The low rate of diabetic remission in our study (3.1%) may be due to the strict evaluation of parameters reflecting diabetic control status at an exact time point, 12 mo after surgery.

The BMI of patients decreased by approximately 10% in the first 3 mo after surgery and was maintained or slightly decreased until the 12-mo evaluation. The FBS level showed rapid improvement at 3 mo and then slowed or was maintained up to 12 mo. HbA1c levels decreased at 3 mo and then increased or maintained between 3 and 12 mo after surgery. These patterns were similar in all three types of surgery and may be associated with the increased calorie intake and general recovery that occurs 3 mo after gastrectomy. These results suggest that weight loss is an important factor for diabetes improvement after gastric cancer surgery. Because one of the main treatments for type 2 diabetes is reduced calorie intake and gastrectomy is a type of restrictive surgery, our results are not unexpected<sup>[13]</sup>.

However, serum insulin, C-peptide, and HOMA-IR continuously improved to at least 12 mo after surgery, even if the rate of improvement slowed 3 mo post-

operatively. As shown in Table 2 and Figure 1, insulin, C-peptide, and HOMA-IR levels were significantly lower at the 12-mo evaluation compared to preoperative levels in all operation types. This suggests that insulin resistance continued to improve after surgery up to 12 mo, although body weight, FBS, and HbA1c did not. Gastric cancer surgery, including gastric resection with or without bypass procedures of a short segment of the proximal small bowel, appears to have a beneficial metabolic influence on diabetes control. However, considering that the pattern of biochemical data was similar in the STG B I and STG B II group, incomplete bypass of a short segment of proximal small bowel (B II) did not seem to provide significant additional benefits in terms of glucose metabolism.

The need for and amount of diabetes medication, FBS levels, and HbA1c levels are convenient tools for evaluating the impact of gastric cancer surgery on glucose control in clinical practice. We divided patients into three groups (stationary, improved, and remission) based on the severity of diabetes after surgery. Thirty three (51.6%) of 64 patients had improved glycemic control 3 mo after surgery and this increased to 54.7% 12 mo after surgery. One patient went into remission 3 mo after STG B I, however, this patient did not satisfy remission criteria at the 12 mo evaluation time. Finally, only 2 patients were in remission 12 mo after surgery: one (6.2%) in the



**Table 4** Factors for diabetic control at postoperative 12 mo *n* (%)

	Stationary ( <i>n</i> = 27)	Improved or remission ( <i>n</i> = 37)	<sup>1</sup> <i>P</i> (univariate)
Age (yr)	64.3 ± 7.2	62.2 ± 8.4	0.582 <sup>2</sup>
Sex			0.789
Male	19 (44.2)	24 (55.8)	
Female	8 (38.1)	13 (61.9)	
BMI, preop (kg/m <sup>2</sup> )	24.5 ± 4.0	24.9 ± 3.0	0.434 <sup>2</sup>
BMI			
3 mo	94.4% ± 5.0%	91.2% ± 5.3%	0.018 <sup>2</sup>
6 mo	93.7% ± 6.8%	90.4% ± 5.7%	0.036 <sup>2</sup>
12 mo	93.8% ± 9.1%	88.9% ± 7.8%	0.041 <sup>2</sup>
Smoking			0.043
Yes	16 (57.1)	12 (42.9)	
No	11 (30.6)	25 (69.4)	
Alcohol history			0.132
No	13 (34.2)	25 (65.8)	
Yes	14 (53.8)	12 (46.2)	
DM duration (yr)			0.013
> 10	10 (76.9)	3 (23.1)	
5-10	8 (40.0)	12 (60.0)	
< 5	9 (29.0)	22 (71.0)	
Family history of DM			1.000
No	22 (42.3)	30 (57.7)	
Yes	5 (41.7)	7 (58.3)	
Operation type			0.799
STG B I	15 (41.7)	21 (58.3)	
STG B II	6 (37.5)	10 (62.5)	
TG	6 (50.0)	6 (50.0)	
Preop FBS	152.1 ± 43.6	145.6 ± 40.3	0.624 <sup>2</sup>
Preop HbA1c	6.9 ± 0.8	7.4 ± 1.2	0.313 <sup>2</sup>
Preop Insulin	21.7 ± 26.3	19.0 ± 17.8	0.804 <sup>2</sup>
Preop C-peptide	3.1 ± 2.2	3.3 ± 1.9	0.509 <sup>2</sup>
Preop HOMA-IR	9.2 ± 12.8	7.3 ± 8.0	0.835 <sup>2</sup>

<sup>1</sup> $\chi^2$ ; <sup>2</sup>Mann-Whitney *U* test, mean ± SD. Significant values are indicated in bold face. BMI: Body mass index, BMI (%) refers to the percentage of BMI at postoperative follow-up. DM: Diabetes mellitus; STG B I: Subtotal gastrectomy with Billroth I anastomosis; STG B II: Subtotal gastrectomy with Billroth II anastomosis; TG: Total gastrectomy; FBS: Fasting blood glucose; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.

STG B II group and the other (8.3%) in the TG group. It seems to be difficult to adequately control diabetes with gastric cancer surgery to stop medication.

As shown in Table 5, the predictive factor for diabetes control 12 mo after surgery was the duration of diabetes. In univariate analysis, the rate of BMI change, smoking history, and diabetes duration were associated with diabetes control 12 mo after surgery. In multivariate analysis, diabetes was controlled in gastric patients with a shorter duration of diabetes. This result was similar to previous reports of type 2 DM patients who took oral hypoglycemic agents and for those who received bariatric surgery<sup>[14,15]</sup>. It is possible that islet cell function is less impaired in patients with a shorter duration of diabetes than in patients with a longer duration. Therefore, diabetes control would be more effective in gastric cancer patients with a short history of diabetes. The operation type which reflects the extent of gastric resection and the presence of bypass of a short segment of proximal jejunum, were not associated with diabetes control 12 mo after surgery. Although we failed to identify a differ-

**Table 5** Multivariate analysis of predictive factors for diabetic control at postoperative 12 mo

Variables	Odds ratio	95%CI	<sup>1</sup> <i>P</i>
Sex			
Male			
Female	0.168	0.018-1.529	0.113
Smoking			
Yes			
No	12.636	0.946-124.216	0.068
BMI (%)			
3 mo	0.869	0.690-1.094	0.232
6 mo	0.839	0.626-1.125	0.240
12 mo	1.055	0.893-1.246	0.526
DM duration (yr)			
> 10			
5-10	27.505	2.174-347.988	0.010
< 5	10.583	0.808-138.670	0.072
Operation type			
STG B I			
STG B II	0.547	0.086-3.493	0.523
TG	0.088	0.006-1.365	0.088

<sup>1</sup>Binary logistic regression. STG B I: Subtotal gastrectomy with Billroth I anastomosis; STG B II: Subtotal gastrectomy with Billroth II anastomosis; TG: Total gastrectomy.

ence in the efficacy of diabetes control based on the type of gastric cancer surgery, we did find that gastric cancer surgery positively affected diabetes. Although only 3.1% of the 64 patients went into remission, 57.8% showed improved glycemic control after surgery. Considering that approximately 10% of the general population suffers from diabetes and the incidence of diabetes in gastric cancer patients is similar to that of the general population, an improvement in diabetes after gastric cancer surgery would reduce health-care costs and improve the quality of life of these individuals.

Because more than half of the patients in this study were not obese, the impact of gastrectomy described should not be interpreted as if resulting from bariatric surgery. This study was initially planned to help surgeons select the most effective gastric cancer surgery for gastric cancer patients with diabetes. In other retrospective studies, diabetic resolution rates after TG ranged from 27.3%-50%, which were much higher than those after STG B I and B II. However, because the pattern of clinical and laboratory data were similarly changed in all three groups, we could not identify a difference between the STG B I and STG B II groups or between the STG and TG groups. Considering that the gap in HbA1c, insulin, C-peptide, and HOMA-IR levels between STG and TG widened at postoperative 6 mo, TG seemed to have more potential for better and more persistent glucose control than STG. However, they showed no significant difference at postoperative 12 mo and we cannot clarify whether the extent of gastrectomy or bypass length was more important in this study. We did not include patients who had STG with Roux-en-Y reconstruction which can provide a longer and complete bypass length of proximal jejunum than STG B I and B II. As the extent of gastrectomy mainly depends on the tumor location and extent, and the length of bypass of proximal small bowel is very short in conventional gastric cancer surgery, the

modification of bypass length of proximal small bowel would offer a better outcome for diabetic control. Therefore, a study that includes a larger number of patients and other types of surgery will be necessary to identify any differences between the types of gastric cancer surgery. In addition, we did not determine postprandial glucose and insulin level, thus we calculated only the insulin sensitivity index using a fasting-based formula. Therefore, further studies are needed to investigate the effect of gastric cancer surgery on gastric cancer patients with type 2 DM using an insulin sensitivity index derived from the oral glucose tolerance test.

In conclusion, gastric cancer surgery led to weight loss and a significant improvement in type 2 DM during the first year after surgery. The degree of diabetes control was related to diabetes duration in each patient. However, the impact of operation type in conventional gastric cancer surgery, such as the extent of gastric resection and current reconstruction methods, on diabetes remains to be determined.

## COMMENTS

### Background

Due to the increased incidence of early gastric cancers and improved survival, postoperative quality of life has become very important after gastric cancer surgery. Diabetes mellitus (DM) is an important health problem worldwide and in gastric cancer patients with DM.

### Research frontiers

After gastric cancer surgery, many surgeons focus on improving the nutritional status of patients rather than controlling diabetes as the main problem after gastric cancer surgery is weight loss. Therefore, the effect of gastric cancer surgery on diabetic control in gastric cancer patients with type 2 DM has not yet been fully evaluated. In this study, the authors demonstrated the short-term effect of three types of routine gastric cancer surgery on type 2 DM.

### Innovations and breakthroughs

In this study, the organized serial evaluation of the status of diabetic control was prospectively performed according to surgical extent and reconstruction type in conventional gastric cancer surgery.

### Applications

This study will allow surgeons to select a favorable reconstruction type after gastrectomy in gastric cancer patients with diabetes.

### Peer review

The study showed that diabetes mellitus was improved in more than 50% of patients during the first year after gastric cancer surgery and the degree of diabetes control was related to diabetes duration and not with the surgical type. However, the effect of gastric cancer surgery type on diabetic control should be further evaluated. These results are very original and support the possibilities that gastric surgery may be an alternative in the treatment of type 2 diabetes mellitus.

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## Two surgical procedures for esophagogastric variceal bleeding in patients with portal hypertension

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### Abstract

**AIM:** To determine the clinical value of a splenorenal shunt plus pericardial devascularization (PCVD) in portal hypertension (PHT) patients with variceal bleeding.

**METHODS:** From January 2008 to November 2012, 290 patients with cirrhotic portal hypertension were treated surgically in our department for the prevention of gastroesophageal variceal bleeding: 207 patients received a routine PCVD procedure (PCVD group), and 83 patients received a PCVD plus a splenorenal shunt procedure (combined group). Changes in hemodynamic parameters, rebleeding, encephalopathy, portal vein thrombosis, and mortality were analyzed.

**RESULTS:** The free portal pressure decreased to  $21.43 \pm 4.35$  mmHg in the combined group compared with  $24.61 \pm 5.42$  mmHg in the PCVD group ( $P < 0.05$ ). The changes in hemodynamic parameters were more

significant in the combined group ( $P < 0.05$ ). The long-term rebleeding rate was 7.22% in the combined group, which was lower than that in the PCVD group (14.93%), ( $P < 0.05$ ).

**CONCLUSION:** Devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT. It should be recommended as a first-line treatment for preventing bleeding in PHT patients when surgical interventions are considered.

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**Key words:** Comparative study; Portal hypertension; Splenorenal shunt; Devascularization; Esophagogastric variceal bleeding

**Core tip:** A comparison of two surgical techniques for esophagogastric variceal bleeding in patients with cirrhotic portal hypertension was performed. Pericardial devascularization and shunt are the main surgical strategies for the prevention of esophagogastric variceal bleeding in patients with portal hypertension (PHT). In this study, we found that devascularization plus splenorenal shunt was an effective and safe strategy for controlling esophagogastric variceal bleeding in PHT patients. This surgical technique should be recommended as a first-line treatment for the prevention of bleeding in PHT patients when surgical interventions are considered.

Yang L, Yuan LJ, Dong R, Yin JK, Wang Q, Li T, Li JB, Du XL, Lu JG. Two surgical procedures for esophagogastric variceal bleeding in patients with portal hypertension. *World J Gastroenterol* 2013; 19(48): 9418-9424 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9418.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9418>

## INTRODUCTION

In China, portal hypertension (PHT) is a major health threat in patients with hepatitis-related cirrhosis. PHT usually leads to multiple complications including splenomegaly, ascites, hepatorenal syndrome, encephalopathy, and even variceal hemorrhage. Of these complications, the main complication associated with mortality risk is variceal hemorrhage, along with a high rate of recurrence<sup>[1]</sup>. Variceal hemorrhage develops in half of patients with cirrhosis, and bleeding occurs in approximately 20%. Patients with large varices have a 30% risk of bleeding over 2 years<sup>[2]</sup>. The risk of rebleeding without intervention is 65% over 2 years<sup>[3,4]</sup>. Although 70%-80% patients can be treated effectively with a non-cardioselective  $\beta$ -blocker and band ligation, 20%-30% of them will fail in such treatment and require further therapy<sup>[2]</sup>. In recent years, with the advent of alternative treatments, particularly the widespread use of endoscopy and transjugular intrahepatic portosystemic shunt (TIPS), the use of surgery in the acute management of active variceal bleeding has decreased<sup>[5]</sup>.

Although the results of endoscopic procedures are satisfactory, the rate of hemostasis failure is almost 10%-20%, and mortality is approximately 60% if a second unsuccessful endoscopic treatment is performed without further intervention<sup>[6,7]</sup>. TIPS is currently one of the most commonly used therapies to decrease the free portal pressure (FPP) and stop bleeding. Although the rate of encephalopathy is higher (30%) with a high mortality rate<sup>[8,9]</sup>, it results in better control of rebleeding, but no change in mortality<sup>[2]</sup>. TIPS is also a useful method for patients who are immediate candidates for liver transplantation, which is the only treatment to significantly prolong long-term survival in patients with cirrhosis.

However, in China, the majority of patients with refractory variceal bleeding do not have the opportunity of liver transplantation due to the high cost and a shortage of donor livers. Moreover, there are many patients with Child-Pugh class A disease who may not require transplantation for many years and TIPS is not recommended. Thus, in these cases, surgical intervention is necessary, and may be the only effective treatment to control rebleeding. Devascularization and shunts are two widely accepted surgical techniques for the management of portal hypertension. Although pericardial devascularization (PCVD) and the shunt procedure have their advantages, they also have disadvantages, such as a significant rebleeding rate following PCVD and a high encephalopathy rate following shunt procedures<sup>[10-12]</sup>.

Hence, over the last two decades, we have performed a new combined operation (splenorenal shunt plus devascularization) to manage variceal esophageal bleeding resulting from portal hypertension secondary to cirrhosis. The aim of this operation is to combine the advantages of devascularization and the shunt, and to reduce the disadvantages of both techniques. In addition, we aimed to identify a more suitable treatment for those

patients who have no other conditions or are unsuitable for liver transplantation or TIPS.

## MATERIALS AND METHODS

### *Patients and exclusion criteria*

From January 2008 to November 2012, 290 patients with PHT secondary to cirrhosis were hospitalized in our department. The patients were divided into two groups who received either the combined operation of PCVD and splenorenal shunt or PCVD only for esophageal and gastric varices. Exclusion criteria for the combined group were as follows: (1) thrombosis of the splenic vein; (2) the splenic vein was not suitable for a shunt; (3) extensive bleeding in the upper digestive tract associated with poor liver condition; (4) FPP lower than 30 mmHg after splenectomy; (5) patients with Child-Pugh class B or less, and poor condition; (6) regional portal hypertension; and (7) emergency surgery. Otherwise, patients received the combined operation. However, the final decision was often made during surgery.

Portal hypertension was due to liver cirrhosis in all patients, and these patients underwent routine preoperative clinical, biochemical and radiological evaluations, computed tomography scanning, and endoscopy. Prior to surgery, all patients were grouped according to Child-Pugh classification.

### *PCVD*

PCVD was carried out using the modified Hassab procedure<sup>[13]</sup> first described by Qiu<sup>[10]</sup>. Briefly, we made a left transabdominal incision, and after the abdomen was opened, we measured the FPP *via* catheterization of the right gastroepiploic vein, and performed a splenectomy and PCVD. We used sequential ligation to devascularize the upper two-third vessels of both the lesser and greater curvatures of the stomach, including the left gastroepiploic vein, short gastric vein, and left gastric vein. The retrogastric venous collaterals running from the upper border of the pancreas to the gastroesophageal junction were meticulously divided and ligated. The lower 5 cm of the esophagus was devascularized *via* the transhiatal approach by sequential ligation.

### *PCVD plus a splenorenal shunt*

In the combined group, after the PCVD was performed, a modified proximal splenocaval shunt was carried out. The tail and body of the pancreas, and the splenic vein and tributaries were carefully dissociated and placed to the right *via* the transverse mesocolon. The splenic vein was dissociated to a length of 2-3 cm and the infrarenal inferior vena cava was freed to a length of 4-5 cm for the preparation of a splenocaval anastomosis, and the splenocaval shunt was then performed. The diameter of the anastomotic stomas ranged from 6 to 8 mm, and the tail of the pancreas was fixed to the connective tissue surrounding the inferior vena cava to reduce the tension



**Table 1 Clinical characteristics**

Characteristics	PCVD group (n = 207)	Combined group (n = 83)
Mean age (yr)	45.79	43.72
SD	11.35	8.03
Range	17-77	30-63
Gender		
Male	130	55
Female	77	28
Etiology		
HBV-related	142	58
HCV-related	28	10
HBV and HCV	3	1
Other causes	14	4
Alcohol-related	20	10
Child-Pugh		
Grade A	134	73
Grade B	67	10
Grade C	6	0
Grade of varices		
Grade I - II	41	15
Grade III - IV	143	56
Bleeding site		
Esophageal	55	25
Fundus varices	10	6

PCVD: Pericardial devascularization; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

of the anastomotic stoma. During both operations, the FPP was measured *via* the right gastroepiploic vein after opening the abdomen, ligation of the splenic artery, removal of the spleen, and PCVD, respectively. The FPP was also measured in the combined group after the shunt procedure.

All patients underwent color Doppler ultrasound before and after the operation to measure the portal and splenic vein diameters, maximum velocity, flow direction, and to determine the presence of thrombosis in the portal system. A follow-up visit was scheduled after the patients were discharged from hospital. The postoperative mortality (defined as death in the perioperative period), the rate of complications, the incidence of rebleeding, the rate of encephalopathy, and survival were recorded.

### Statistical analysis

The data were analyzed by SPSS 19.0 statistical software. All results are presented as mean  $\pm$  SD. The Mann-Whitney *U* test and the  $\chi^2$  test were used appropriately. The Kaplan-Meier method (log rank test) was used to analyze long-term complications appropriately.  $P < 0.05$  was considered statistically significant. This study was exempt from IRB review after institutional IRB review.

## RESULTS

### Clinical characteristics

Patient age, sex ratio and Child-Pugh classification were statistically similar between the two groups ( $P > 0.05$ ). Of 290 patients, 207 underwent PCVD, and the remaining 83 patients underwent the combined operation. No emergency surgery was carried out in either of the two

**Table 2 Intra- and post-operative clinical characteristics n (%)**

Clinical characteristics	PCVD group	Combined group	P value
Intraoperative			
Operative time (min)	246 $\pm$ 71	307 $\pm$ 68	< 0.01
Blood loss (mL)	936 $\pm$ 1627	744 $\pm$ 832	< 0.01
Blood transfusion (mL)	843 $\pm$ 1237	760 $\pm$ 583	0.010
Postoperative			
Fever	56	10	< 0.01
Ascites	941 $\pm$ 833	759 $\pm$ 695	0.24
Rebleeding	12 (5.80%)	2 (2.41%)	0.04
Long-term complications			
Congestive gastropathy	35 (17.41%)	2 (2.41%)	< 0.01
Encephalopathy	3 (1.45%)	2 (2.41%)	0.58
Portal vein thrombosis	16 (7.96%)	3 (3.61%)	0.04
Rebleeding	30 (14.93%)	6 (7.22%)	< 0.05

PCVD: Pericardial devascularization.

groups (Table 1).

### Hemorrhage during the operation and operation time

During the operation, the average blood loss in the PCVD group was 936  $\pm$  1627 mL compared with 744  $\pm$  832 mL in the combined group ( $P < 0.05$ ). Moreover, the operation time was significantly shorter in the PCVD group compared with the combined group (246  $\pm$  71 min *vs* 307  $\pm$  68 min,  $P < 0.05$ ) (Table 2).

### Complications in the perioperative period

In the perioperative period, the main complications were ascites and postoperative fever. After surgery, there were no significant differences in the mean amount of postoperative ascites, which was 941  $\pm$  833 mL in the PCVD group and 759  $\pm$  695 mL in the combined group (Table 2). However, the incidence of postoperative fever in the PCVD group (27.86%, 56 of 201 patients) was significantly higher than that in the combined group (12.04%, 10 of 83 patients) ( $P < 0.05$ ) (Table 2).

### Changes in free portal pressure

In both groups, the postoperative free portal pressure (FPP) was significantly lower than that preoperatively ( $P < 0.01$ ). However, there was no difference in the first measured FPP at abdominal opening, 29.23  $\pm$  4.58 mmHg in the PCVD group *vs* 29.81  $\pm$  3.83 mmHg in the combined group. However, after PCVD and shunt surgery, a significant decrease in the combined group (21.43  $\pm$  4.35 mmHg) was observed compared to the PCVD group (24.61  $\pm$  5.42 mmHg) ( $P < 0.01$ ) (Table 3).

### Changes in hemodynamic parameters

Hemodynamic parameters of the portal vein (PV) were measured preoperatively and postoperatively (Table 4). There were no significant differences in the inner diameter, blood flow velocity and venous flow preoperatively, however, significant changes were found after surgery. In the PCVD group and the combined group, the postoperative inner diameter, blood flow velocity and venous flow of the PV were significantly decreased ( $P < 0.01$ ),

**Table 3** Changes of free portal pressure in the two groups (mmHg)

	PCVD group	Combined group	Z	P value
Abdominal opening	29.23 ± 4.58	29.81 ± 3.83	-0.36	0.72
Splenectomy	22.32 ± 5.33	24.60 ± 5.01	-2.91	< 0.05
PCVD	24.61 ± 5.42	22.06 ± 4.03	-3.08	< 0.05
Shunt		21.43 ± 4.35		

PCVD: Pericardial devascularization.

and the *D* values were also significantly different ( $P < 0.01$ ), respectively. Similar results for the splenic vein (SV) and the superior mesenteric vein (SMV) are also shown in Table 4.

### Rebleeding rate

The postoperative rebleeding rates in the PCVD and combined groups were 5.80% (12/207) and 2.41% (2/83), respectively. In the PCVD group, 10 of 12 patients had bleeding before the operation. Compared with the PCVD group, 2 patients had preoperative bleeding in the combined group (Table 2). The data in Table 2 also show the long-term results of rebleeding. In the 284 survived patients (6 died in the perioperative period), the overall incidence of rebleeding in the PCVD group and combined group was 14.93% (30/201) and 7.22% (6/83), respectively ( $P < 0.05$ ). Twenty-one of 30 patients in the PCVD group had postoperative rebleeding, and 23 patients had preoperative bleeding, while 6 patients in the combined group had either preoperative bleeding or postoperative rebleeding.

### Long-term results of complications

The incidence of congestive gastropathy in the PCVD group was 17.41% (35/201), which was significantly higher than that in the combined group, with only 2 patients (2.41%) affected ( $P < 0.05$ ). The main long-term liver disease-related complications were encephalopathy and thrombosis. The incidence of encephalopathy was 1.45% (3/201) and 2.41% (2/83) in the PCVD group and combined group, respectively ( $P = 0.58$ ). In each group, one patient died of progressive liver failure at 10 mo postoperatively due to severe encephalopathy. Portal vein thrombosis was found in 16 (7.96%) patients in the PCVD group and 3 (3.61%) patients in the combined group ( $P = 0.04$ ), (Table 2).

### Postoperative mortality and survival

As a result of the small number of deaths in both groups, we analyzed the cause of death and did not use statistical methods to evaluate mortality and survival. In contrast to the combined group with no death during the perioperative period, 6 of the 207 patients in the PCVD group died during the perioperative period. Two of these patients died due to rebleeding, 3 due to hepatic failure, and one due to multiple system organ failure (MSOF) caused by gastric fistula. The 3-year survival rate was 95.52% (192/201) in the PCVD group, 2 died

due to rebleeding, 3 due to hepatic failure, 2 due to primary hepatic cancer, 1 due to cerebral hemorrhage, and 1 due to other reasons. The 3-year survival rate in the combined group was 96.39% (80/83), 1 died due to hepatic failure, 1 due to primary hepatic cancer, and 1 due to other reasons.

## DISCUSSION

The main aims of the treatments used in PHT patients are to control variceal bleeding and to prevent rebleeding. In addition, it is necessary to maintain enough portal hepatopetal perfusion and protect the limited liver function. In China, PCVD and distal splenorenal shunt are favored and widely accepted by surgeons, and have prevailed until now. Although these two favorable procedures are based on two different hemodynamic theories, they achieve reliable effects by controlling variceal bleeding. However, a high rebleeding rate caused by recurrent varices or a high rate of residual varices and changes in the gastric mucosa following PCVD have been observed<sup>[14-16]</sup>. In addition, it was reported that the shunt can preserve hepatopetal perfusion to support liver function and improve the microcirculation of gastric mucosa<sup>[17-19]</sup>, however, the incidence of hepatic encephalopathy needs to be reduced.

Thus, in order to combine the advantages of these two operations, we integrated these two different surgical procedures into one operation. Although there are limited reports on this combined operation, a comprehensive analysis is needed to prove its rationality. In this study, we retrospectively analyzed clinical data to determine the clinical value of the combined procedure in patients with cirrhotic portal hypertension and variceal bleeding. A total of 290 patients with portal hypertension of cirrhotic origin were enrolled in this study and received either PCVD only or the combined operation, respectively.

The primary end point of this study was variceal rebleeding. According to reports in the literature, the rebleeding rate of patients who underwent devascularization was 7.1%-37%<sup>[20-22]</sup>, and following distal splenorenal shunt the rebleeding rate was approximately 5%-15%<sup>[2,22-25]</sup>. In China, the rebleeding rate after prolonged follow-up in patients who underwent the combined operation was 5%-10%, in contrast to 10%-30% in patients who underwent PCVD<sup>[26-29]</sup>. Furthermore, after the PCVD procedure, the postoperative venous pressure in the gastric wall increased and exacerbated pathologic changes and congestive conditions in the gastric mucosa, therefore increasing the risk of congestive gastropathy. It has been reported that the rate of postoperative rebleeding caused by congestive gastropathy is 20%-83%<sup>[21,30,31]</sup>. However, the rates of rebleeding and the incidence of congestive gastropathy in our study were lower than those reported in the literature. In addition, the rebleeding rate in our combined group was significantly lower than that following TIPS which was reported to be 10.5%<sup>[2]</sup>.

**Table 4** Comparison of hemodynamics in the two groups pre- and post-operatively

		Inner diameter (cm)				Blood flow velocity (cm/s)			
		Pre-op	Post-op	D value	P value	Pre-op	Post-op	D value	P value
PV	PCVD	1.42 ± 0.21	1.24 ± 0.26	0.15 ± 0.17	< 0.01	15.28 ± 4.69	13.27 ± 4.76	2.01 ± 3.01	< 0.01
	Combined	1.39 ± 0.26	1.01 ± 0.30	0.38 ± 0.25	< 0.01	16.52 ± 4.67	11.33 ± 3.78	5.19 ± 3.42	< 0.01
	P	0.57	< 0.01	< 0.01		0.06	< 0.05	< 0.01	
SV	PCVD	1.21 ± 0.24	1.05 ± 0.21	0.18 ± 0.13	< 0.01	17.65 ± 5.53	14.10 ± 5.58	3.55 ± 0.92	< 0.01
	Combined	1.25 ± 0.22	0.81 ± 0.22	0.43 ± 0.20	< 0.01	18.76 ± 5.76	13.10 ± 5.38	5.65 ± 3.00	< 0.01
	P	0.25	< 0.01	< 0.01		0.17	0.21	< 0.01	
SMV	PCVD	0.95 ± 0.70	0.81 ± 0.21	0.14 ± 0.17	< 0.01	13.17 ± 4.61	11.00 ± 4.76	2.13 ± 2.33	< 0.01
	Combined	0.92 ± 0.19	0.66 ± 0.23	0.26 ± 0.27	< 0.01	14.69 ± 5.23	10.40 ± 4.46	4.29 ± 3.07	< 0.01
	P	0.3	< 0.01	< 0.01		< 0.05	0.33	< 0.01	

PV: Portal vein; SV: Splenic vein; SMV: Superior mesenteric vein; PCVD: Pericardial devascularization.

The lower rebleeding rate and incidence of gastropathy in the combined group resulted from the following mechanisms. First, it has been demonstrated that extensive PCVD of at least 6–8 cm under the mediastinal esophagus and the dissociation of the uppermost gastric vessels<sup>[32]</sup> are necessary for the disappearance of esophageal varices<sup>[33]</sup>. Second, congestive gastropathy can be attenuated by an effective shunt, which can reduce the blood flow to the gastroesophageal mucosa and improve mucosal microcirculation in the stomach. Third, portal hypertension is relieved to some extent after splenocaval shunt surgery, and this may delay the reformation of gastroesophageal varices.

The changes in FPP and hemodynamics in the present study had significant clinical implications. In both groups, the postoperative FPP decreased significantly, however, in the PCVD group, it was still higher than normal. The postoperative FPP in patients who underwent the combined procedure decreased to a normal level. Furthermore, we also found that in both groups the PV, SV, and SMV parameters after the treatments were lower than preoperative levels. However, these changes were more significant in the combined group. These findings suggested that: (1) the combined operation efficiently reduced hypertensive congestion in the portal system; (2) a splenorenal shunt reduced the FPP more and markedly decreased hypertensive congestion in the portal system, and the combined procedure maintained PV patency and prevented a significant decrease in the pressure and blood flow of the PV; (3) the combined operation not only decreased PVF, but also resulted in good control of rebleeding. However, PCVD alone did not achieve control of rebleeding; (4) the postoperative FPP was normal in the combined group, and may have contributed to the complementary action of the hypotensive effect of the shunt and the hypertensive effect of PCVD; and (5) a significant decrease in the postoperative inner diameter and blood flow velocity in the combined group indicated that the PCVD plus shunt resulted in better hemodynamics.

These changes in hemodynamics may also maintain blood flow to the liver and prevent hepatic failure. Therefore, in our combined procedure we restricted the anastomotic stoma to maintain the hemodynamics in the

appropriate range. Our historical experience showed that if we restricted the stoma more than 1.0 cm the FPP would decrease faster following PCVD, and the stoma would diminish due to an excessive drop in the FPP. In our department, we restrict the anastomotic stoma to 6–8 mm. This procedure also reduces the rate of encephalopathy. Based on previous experience, all types of shunts have been shown to have high encephalopathy rates due to a sharp drop in the PV and a reduction in portal pressure. In our study, the rate of encephalopathy in the two groups was similar, indicating that PCVD plus shunt did not increase the risk of encephalopathy.

During both procedures, splenectomy was performed in all patients due to splenomegaly associated with hypersplenism. However, after surgery the platelet count was elevated, which resulted in a high-coagulation status and injury to the inner mucosa of vessels, and blood flow velocity was reduced. As a result, thrombosis occurred in the portocaval stoma and portal vein. The rate of thrombosis is an important prognostic factor in patients with portal hypertension and cirrhosis. In our future studies, more effective efforts will be made to prevent thrombosis.

According to the results from our study, we can conclude that splenorenal shunt plus devascularization is an effective choice in patients with esophagogastric variceal bleeding due to PHT. The clinical characteristics in the combined group were better than those in the PCVD group. Although the surgical risk in the combined group was equal to the PCVD group, the combined procedure resulted in a lower rate of complications. Furthermore, the combined procedure maintained liver function, which is beneficial in patients who may have the opportunity of future liver transplantation.

In the present study, we compared the outcomes following treatment with PCVD and the combined operation. We hope that a comparative study of this combined procedure and other treatments in cirrhotic PHT patients can be carried out in the future.

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their support of this research.

## COMMENTS

### Background

In China, portal hypertension (PHT) is a major threatening event due to hepatitis-related cirrhosis. In recent years, with the advent of alternative treatments, the role of surgery in the acute management of active variceal bleeding caused by PHT has decreased. However, devascularization and shunts are still two widely accepted surgeries for the management of portal hypertension. In this study, the authors investigated the clinical value of a splenorenal shunt plus pericardial devascularization (PCVD) in PHT patients with variceal bleeding.

### Research frontiers

Liver transplantation is the major treatment for portal hypertension and upper gastrointestinal bleeding; however, due to the shortage of liver source and high cost, it is not acceptable extensively. It is important to find an effective method to control the complication of portal hypertension and prolong the survival time of the patients.

### Innovations and breakthroughs

The authors performed a combined operation (devascularization plus splenorenal shunt) over the past two decades to manage variceal esophageal bleeding which results from the portal hypertension secondary to cirrhosis. They evaluated the clinical value of this combined surgery, and found that the devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT patients. It is superior to the traditional surgeries.

### Applications

The devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT patients. It could be recommended as a first-line treatment for preventing bleeding in PHT patients when surgical interventions are considered.

### Terminology

In the combined group, after the splenectomy and PCVD, a modified proximal splenocaval shunt was performed. The tail and body of the pancreas, and the splenic vein and tributaries were carefully dissociated and were turned right via the transverse mesocolon. The splenic vein was dissociated to a length of 2-3 cm and the infrarenal inferior vena cava was freed to a length of 4-5 cm for the preparation of a splenocaval anastomosis, and then the splenocaval shunt was performed. The diameter of the anastomotic stomas ranged from 6 to 8 mm, and the tail of the pancreas was fixed to the connective tissue surrounding the inferior vena cava to reduce the tension of the anastomotic stoma.

### Peer review

This is a very interesting topic and has puzzled the surgeons for many decades. It still remains controversial in China. The authors investigated the surgical outcomes of patients with PHT who underwent PCVD alone or splenorenal shunt plus PCVD. They conclude that the devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT patients. Strengths of the study are the large number of cases, good follow-up and excellent annotation with clinical data.

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## Overexpression of kallikrein gene 10 is a biomarker for predicting poor prognosis in gastric cancer

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**RESULTS:** *KLK10* was found to be highly expressed in 57/80 (70%) of gastric cancer samples, while its expression was very low in normal gastric tissues. Positive relationships between *KLK10* expression and lymph node metastasis ( $P = 0.048$ ), depth of invasion ( $P = 0.034$ ) and histology ( $P = 0.015$ ) were observed. Univariate survival analysis revealed that gastric cancer patients with positive *KLK10* expression had an increased risk for relapse/metastasis and death ( $P = 0.005$  and  $0.002$ , respectively). Cox multivariate analysis indicated that *KLK10* was an independent prognostic indicator of disease-free survival and overall survival in patients with gastric cancer.

**CONCLUSION:** *KLK10* expression is an independent biomarker of unfavorable prognosis in patients with gastric cancer.

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**Key words:** Kallikrein gene 10; Gastric cancer; Survival analysis; Prognostic biomarkers

### Abstract

**AIM:** To analyze the expression of kallikrein gene 10 (*KLK10*) in gastric cancer and to determine whether *KLK10* has independent prognostic value in gastric cancer.

**METHODS:** We studied *KLK10* expression in 80 histologically confirmed gastric cancer samples using real-time quantitative reverse transcription-PCR and hK10 expression using immunohistochemistry. Correlations with clinicopathological variables (lymph node metastasis, depth of invasion and histology) and with outcomes (disease-free survival and overall survival) during a median follow-up period of 31 mo were assessed. Gastric cancer tissues were then classified as *KLK10* positive or negative.

**Core tip:** The study examined the clinicopathologic and prognostic significance of kallikrein gene 10 (*KLK10*) expression in gastric cancer. Based on collective findings, we hypothesize that *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with gastric cancer. *KLK10* expression is an independent biomarker for predicting unfavorable prognosis in patients with gastric cancer.

Jiao X, Lu HJ, Zhai MM, Tan ZJ, Zhi HN, Liu XM, Liu CH, Zhang DP. Overexpression of kallikrein gene 10 is a biomarker for predicting poor prognosis in gastric cancer. *World J Gastroenterol* 2013; 19(48): 9425-9431 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9425.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9425>

## INTRODUCTION

Gastric cancer is the fourth most common cancer, and the second leading cause of cancer death worldwide<sup>[1]</sup>. Mortality due to gastric cancer has risen in China over the past 20 years, especially in rural areas and in aging populations<sup>[2,3]</sup>. Although the increased use of screening for early disease diagnosis and the widespread administration of systemic adjuvant therapies have led to a decline in mortality rates, the incidence and mortality of gastric cancer are still second only to lung cancer<sup>[4,5]</sup>.

The kallikrein gene family of secreted serine proteases, consisting of 15 genes, is localized in tandem on chromosome 19q13.4 and shows significant homologies at both the nucleotide and the protein levels<sup>[6,7]</sup>. Kallikrein-related peptidase 10 is a member of the kallikrein family and has been shown in numerous reports to be upregulated in ovarian cancer<sup>[8,9]</sup>. The human kallikrein (*KLK*) gene 10 encodes human kallikrein gene 10 (*KLK10*) protein. Recent studies have shown that human *KLKs* are involved in human carcinogenesis and that several *KLKs* are promising biomarkers of prostate, ovarian, testicular and breast cancer<sup>[10,11]</sup>. For instance, prostate-specific antigen (PSA/hK3) which is encoded by the *KLK3* gene is used as a cancer-specific marker for male population screening, early diagnosis and monitoring of prostate cancer<sup>[12]</sup>. In addition, quantification of *KLK5* expression is critical for both the discovery of early cellular and molecular alterations in breast cancer, as well as the identification of novel diagnostic and prognostic biomarkers<sup>[13]</sup>. Many other *KLKs* are also expected to act as tumor biomarkers<sup>[14-17]</sup>. More recent evidence also implicates the *KLKs* in many cancer-related processes, including cell growth regulation, angiogenesis, invasion and metastasis<sup>[17]</sup>. Several authors have reported that *KLK10* mRNA was highly expressed in ovarian cancer tissue and that hK10 may be a useful serum biomarker for the diagnosis and management of ovarian cancer<sup>[18]</sup>. However, few studies have focused on *KLK10* expression in human gastric cancer.

In the present study, we examined the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer. Based on collective findings, we hypothesize that *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with this malignancy.

## MATERIALS AND METHODS

### Study population

Tumor specimens from 80 consecutive patients undergoing surgical treatment for primary gastric cancer at the Department of General Surgery, Tianjin First Central Hospital (Tianjin, China) were analyzed in this study. Patient age ranged from 35 to 74 years, with a median of 51 years (Table 1). All tumor specimens and matched control samples taken from normal tissues at the incision edge were snap-frozen in liquid nitrogen and stored at -80 °C for subsequent RNA extraction. Investigations were carried out in accordance with the ethical standards of the

**Table 1** Data of the study population

Variable	No. of patients	mean $\pm$ SE	Range
Age (yr)	80	51 $\pm$ 0.81	35-74
Lymph nodes <sup>1</sup>	80	30 $\pm$ 3.11	0-63
Follow-up (mo) <sup>2</sup>	80	31 $\pm$ 1.98	7-52

<sup>1</sup>Number of lymph nodes removed during surgery; <sup>2</sup>Follow-up time after surgery.

**Table 2** Associations between kallikrein gene 10 status and other variables in 80 patients with gastric cancer

Variable	Total	<i>KLK10</i> -negative	<i>KLK10</i> -positive	<i>P</i> value <sup>1</sup>
Sex				
Male	56	14	42	0.258
Female	24	9	15	
Age (yr)				
< 60	31	10	21	0.581
$\geq$ 60	49	13	36	
Depth of invasion <sup>2</sup>				
T1	27	12	15	0.034
T2-T3	23	7	16	
T4	30	4	26	
Lymph node metastasis <sup>2</sup>				
N1	21	4	17	0.048
N2	35	15	20	
N3	24	4	20	
Differentiation <sup>2</sup>				
Well	18	10	8	0.015
Moderate	37	7	30	
Poor	25	6	19	

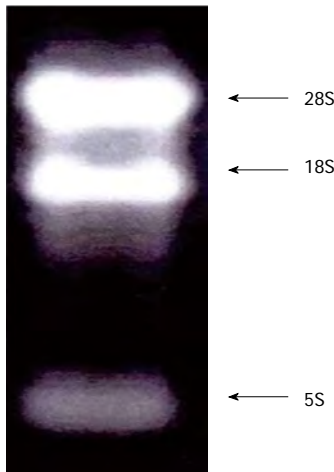
<sup>1</sup> $\chi^2$  test; <sup>2</sup>TNM stage system of American Joint Committee on Cancer of 2012. *KLK10*: Kallikrein gene 10.

1975 Helsinki Declaration, as revised in Tokyo 2004. The patients had not received hormonal therapy or chemotherapy prior to surgery. After surgery, all patients were treated with oxaliplatin-based chemotherapy regimens based on a platinum compound, alone or in combination with other drugs; grade 1 and stage I patients received no further treatment. Follow-up information (median follow-up period of 31 mo) was available for 80 patients (Table 1). Two time-to-event outcomes after surgery were recorded: disease-free survival (DFS) and overall survival (OS). DFS in each case was defined as the time interval between the date of primary cancer removal and the date of the first documented evidence of relapse. OS was defined as the time interval between the date of surgery and the date of death, or the date of last follow-up for those who were alive at the end of the study.

Clinical and pathological information documented at the time of surgery included clinical stage, histology, depth of invasion and lymph node metastasis (Table 2). All pathological factors were established as described by the 2010 National Comprehensive Cancer Network Guideline.

### Ethics

The study protocol was approved by the Ethics Com-



**Figure 1** Confirmation of the integrity of total RNA.

mittee of the hospital and written informed consent was obtained from each patient.

### Immunohistochemistry

Immunohistochemical studies of hK10 were carried out using the avidin-biotin-peroxidase method (LSAB2 kit, Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded surgical specimens from patients with gastric cancer. All sections were counterstained with hematoxylin. Primary goat polyclonal antibodies against hK10 (Santa Cruz, United States) were used at dilutions of 1:700.

All sections were independently examined by two researchers (Xin Jiao, Mi-Mi Zhai). The expression of hK10 was scored as positive when the carcinoma cell cytoplasm was stained brown. We examined hK10 protein expression in tumor tissues and corresponding normal tissues from 80 gastric cancer cases.

### Total RNA extraction and reverse transcription

Tumor tissues of 100 mg were minced on dry ice using a scalpel and immediately transferred to 2 mL polypropylene tubes. Total RNA was isolated from these samples using TRI-reagent (Ambion Inc., Austin, TX, United States) following the manufacturer's instructions. Total RNA concentration and quality were determined spectrophotometrically at 260 and 280 nm, and RNA integrity was evaluated using agarose gel electrophoresis. Reverse transcription of the mRNA molecules into first-strand cDNA was carried out using 1 µg of total RNA from each tissue specimen, M-MuLV Reverse Transcriptase RNase H (Finnzymes Oy, Espoo, Finland) and an oligo(dT) oligonucleotide as a reverse transcription primer, according to the manufacturer's instructions. Confirmation of the integrity of total RNA is shown in Figure 1.

### Real-time quantitative reverse transcription-PCR

Based on the mRNA sequences from the NCBI Sequence database, gene specific primers were designed and synthesized for the target *KLK10* gene (NCBI Refer-

ence Sequence: NM\_002776) and HPRT1 (hypoxanthine phosphoribosyltransferase-1) endogenous reference gene (NCBI Reference Sequence: NM\_000194.2) using the Primer Express software (Applied Biosystems, CA, United States). A *KLK10* fragment was amplified using the primers: forward, 5'-CTCTGGCGAAGCTGCTG-3' and reverse, 5'-ATAGGCTTCGGGGTCCAA-3', whereas the primers for HPRT1 were: forward, 5'-TGGAAAGGGTGTTTATTCCTCAT-3' and reverse, 5'-ATGTAATCCAGCAGGTCAGCAA-3'.

Real-time PCR assays of *KLK10* mRNA expression levels were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States), and the reagents TaqMan Fast Universal PCR Master Mix (29) (Applied Biosystems, United States) according to the manufacturer's instructions. With an initial polymerase activation step at 95 °C for 10 min, the amplification conditions of the 40 cycles consisted of denaturation at 95 °C for 15 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 30 s. The products were then subjected to a temperature gradient from 55 °C to 95 °C at 0.1 °C/s with continuous fluorescence monitoring to produce a melting curve of the products. *KLK10* mRNA expression was calculated from the standard curve, and quantitative normalization of cDNA in each sample was performed using the expression of GAPDH mRNA as an internal control<sup>[19]</sup>. We classified the 80 cases into two groups using the mean expression level of *KLK10* mRNA in tumor tissues (0.03): *i.e.*, a positive-expression group ( $\geq 0.03$ ,  $n = 57$ ) and a negative-expression group ( $< 0.03$ ,  $n = 23$ ).

### Statistical analysis

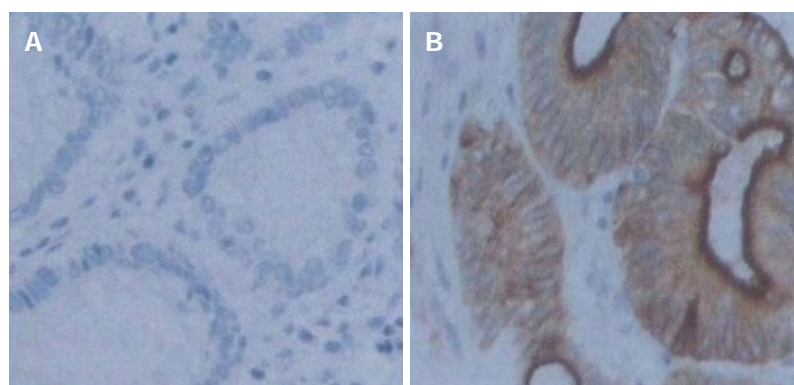
Statistical analysis were performed using SPSS for Windows version 19.0 (SPSS, Chicago, IL, United States). Associations between clinicopathological parameters, such as depth of invasion, lymph node metastasis, histology and *KLK10* expression were analyzed by the Chi-square test or Fisher's exact test, where appropriate. Survival analysis were performed by constructing Kaplan-Meier DFS and OS curves and differences between curves were evaluated by the log-rank test (Mantel, 1966), and by estimating the relative risks for relapse and death using the Cox proportional hazards regression model (Cox, 1972). Cox analysis was conducted at both univariate and multivariate levels. Only the patients with known status of all variables were included in the multivariate regression models, which incorporated *KLK10* and all other variables, for which the patients were characterized.

## RESULTS

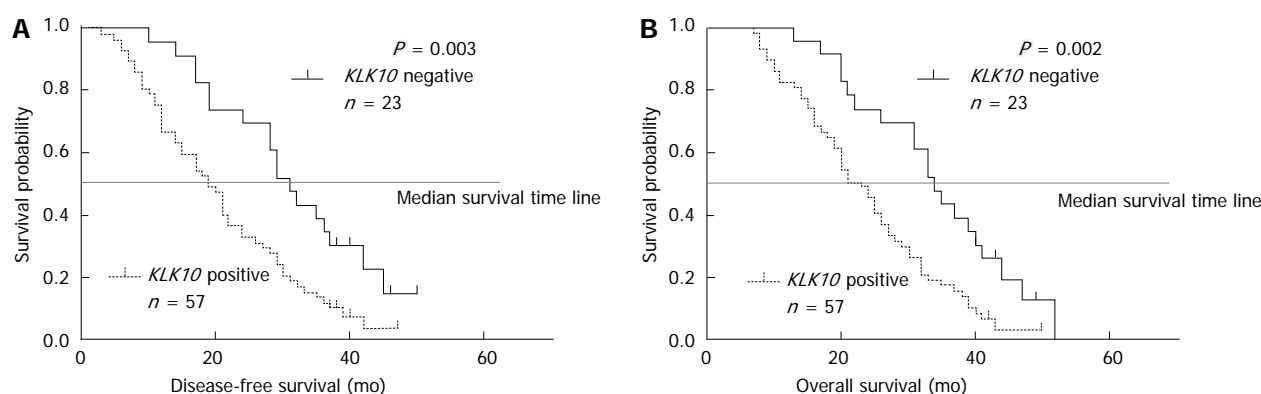
### Relationship between *KLK10* expression and other parameters

Of the 80 patients included in this study, 57 (70 %) were positive for *KLK10* expression in gastric cancer tissues. In normal gastric tissues, the level of *KLK10* was undetectable or low. Table 2 shows the distribution of *KLK10*





**Figure 2** Immunohistochemical analysis of hK10 protein expression in normal gastric tissues and gastric tumor tissues. A: hK10 protein expression in normal gastric tissues; B: hK10 protein expression in gastric tumor tissues.



**Figure 3** Kaplan-Meier survival analysis of disease-free survival (A) and overall survival (B) in gastric cancer patients who were either kallikrein gene 10 positive or kallikrein gene 10 negative. *KLK10*: Kallikrein gene 10.

expression (positive or negative) in gastric cancer tissues in relation to age, sex, lymph node metastasis, depth of invasion and histology. Patients with *KLK10*-positive gastric cancer more frequently had more lymph node metastasis ( $P = 0.048$ ), greater depth of invasion ( $P = 0.034$ ) and poorer histology ( $P = 0.015$ ). No significant associations between *KLK10* expression and age ( $P = 0.581$ ) and sex ( $P = 0.258$ ) were found.

### Immunohistochemistry

In 55 of the 57 patients who were positive for *KLK10* mRNA expression, specific expression of hK10 protein was only found in cancer tissues, but not in the corresponding normal tissues (Figure 2). In 23 cases with negative expression of *KLK10* mRNA, 20 exhibited negative or weak expression of hK10 in cancer tissues. In contrast, 2 cases with high *KLK10* mRNA expression exhibited negative or poor hK10 protein expression in cancer tissues.

### Clinicopathologic significance of *KLK10* mRNA expression in gastric cancer

The clinicopathologic factors analyzed in relation to *KLK10* mRNA expression in tumor tissues are shown in Table 2. The level of lymphatic invasion was significantly higher ( $P = 0.048$ ) in the positive-expression group than in the negative-expression group. The depth of gastric wall invasion was greater ( $P = 0.034$ ) in the positive-expression group than in the negative-expression group.

The histotype also correlated with these groups ( $P = 0.015$ ). In contrast, no significant difference was observed regarding age and sex. The 3-year actuarial OS rates in patients with gastric cancer and positive *KLK10* mRNA expression and in patients with negative *KLK10* mRNA expression were 20% and 42%, respectively (Figure 3). The survival difference between these two groups was statistically significant ( $P = 0.002$ ; log-rank test).

### Univariate and multivariate survival analysis

The degree of association between each clinicopathological variable and DFS and OS is shown in Table 3. In univariate analysis, patients with *KLK10*-positive gastric cancer had a significantly increased risk of relapse (decreased DFS) and death (decreased OS) (hazards ratios of 0.46 and 0.43;  $P = 0.005$  and 0.002, respectively). Nevertheless, positive *KLK10* expression was weakly associated with an increased risk of death (decreased OS) (hazards ratio of 0.55;  $P = 0.03$ ) in multivariate analysis compared with univariate analysis, while no significant association was found between the positive *KLK10* expression and relapse (decreased DFS) in patients with gastric cancer in multivariate analysis ( $P = 0.06$ ).

Depth of invasion and histotype were the strongest independent indicators of poor prognosis ( $P < 0.05$ , except for depth of invasion for OS in multivariate analysis). As expected, Kaplan-Meier survival curves (Figure 3) indicated that patients with *KLK10*-positive gastric cancer had shorter DFS ( $P = 0.003$ ) and OS ( $P = 0.002$ ) com-

**Table 3** Univariate and multivariate analysis of disease-free survival and overall survival

Survival variable	Disease-free survival			Overall survival		
	95%CI <sup>2</sup>	P value	HR <sup>1</sup>	95%CI <sup>2</sup>	P value	HR <sup>1</sup>
Univariate analysis						
<i>KLK10</i>						
Negative			1.00			1.00
Positive	0.25-0.74	0.002	0.46	0.26-0.79	0.005	0.43
Age	0.97-1.01	0.46	0.99	0.97-1.01	0.49	0.99
Sex	0.55-1.53	0.75	0.90	0.54-1.51	0.69	0.92
Depth of invasion	1.55-2.81	< 0.001	2.13	1.57-2.89	< 0.001	2.08
Lymph node metastasis	0.97-1.97	0.07	1.42	0.99-2.02	0.051	1.38
Histology	6.42-22.46	< 0.001	10.25	5.73-18.32	< 0.001	12.01
Multivariate analysis						
<i>KLK10</i>						
Negative		1.00			1.00	
Positive	0.31-0.96	0.03	0.58	0.33-1.02	0.06	0.55
Age	0.95-1.00	0.02	0.97	0.95-0.99	0.04	0.97
Sex	0.77-2.22	0.31	1.33	0.77-2.28	0.30	1.31
Depth of invasion	0.99-1.90	0.06	1.42	1.02-1.98	0.03	1.37
Lymph node metastasis	0.84-1.59	0.35	1.21	0.87-1.67	0.24	1.16
Histology	6.55-25.41	0.03	11.11	5.87-21.02	< 0.001	12.90

<sup>1</sup>Hazard ratio (HR) estimated from Cox proportional hazard regression model; <sup>2</sup>Confidence interval of the estimated HR. *KLK10*: Kallikrein gene 10.

pared with *KLK10*-negative patients.

## DISCUSSION

Gastric cancer is a common malignant tumor of the gastrointestinal tract. The optimal management of patients with gastric cancer involves a multidisciplinary approach: diagnosis, surgery, and chemotherapy, including the use of biological markers. Several authors have reported that *KLK10* mRNA is highly expressed in human cancer tissues<sup>[20-23]</sup>. In the current study, we found a significant relationship between *KLK10* mRNA expression and lymph node metastasis, depth of invasion and histology in patients with gastric cancer, as shown in Table 1. These findings indicate that the overexpression of *KLK10* was significantly associated with both an increased incidence of lymphatic invasion and poor histology in patients with gastric cancer. These results suggest that enhanced expression of *KLK10* may play an important role in various pathologic processes of gastric cancer. The results obtained in this study are in agreement with previous studies which examined the association between *KLK10* expression status and the clinicopathological features of patients with gastric cancer<sup>[24]</sup>.

Some members of the KLK family have been identified as potential biological markers of prognosis, including KLK5, KLK14 and KLK7<sup>[25]</sup>. For example, KLK5 expression is an indicator of poor prognosis in ovarian cancer<sup>[26]</sup>. Furthermore, stratifying patients based on the presence or absence of such markers may result in a different prognosis in individuals. For example, both KLK8 mRNA and KLK6 mRNA are highly expressed in human breast cancer tissues, however, it is unknown whether a breast cancer patient with high expression of KLK8 has a good/poor prognosis compared to a breast cancer patient with high expression of KLK6.

In this study, we identified *KLK10* as a new biomarker of poor prognosis in gastric cancer. Patients with *KLK10*-positive tumors were more likely to have poor histology and advanced stage disease. Our findings demonstrate that *KLK10* expression can reduce DFS in patients with gastric cancer in univariate, but not in multivariate analysis (Table 2). In addition, when assessing *KLK10* expression to predict survival outcomes, we found an increased risk of death in patients with *KLK10*-positive tumors in both univariate and multivariate analysis (Table 2). This indicates that *KLK10*-positivity may be an independent prognostic factor in patients with gastric cancer. That is, *KLK10* may induce gastric cancer cell growth and proliferation. However, the function of the *KLK10* signaling pathway is unclear. Some reports indicate that serine proteinases (the KLK gene family of secreted serine proteases includes 15 genes) participate in tumor growth and invasion by cleaving and activating proteinase-activated receptors (PARs: PAR-1 and PAR-2)<sup>[27-29]</sup>. A recent study demonstrated that KLK4 is aberrantly expressed in colon cancer and capable of inducing PAR-1 signaling in cancer cells<sup>[30]</sup>. KLK4 is a tumorigenic factor. Another report showed that KLK14 induced significant extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation and HT29 cell proliferation, presumably by activating PAR-2. A PAR-2 cleavage and activation-blocking antibody markedly reduced KLK14-induced ERK1/2 signaling<sup>[31]</sup>. Our lack of knowledge of *KLK10* function and regulation in gastric cancer tissues does not allow us to formulate reasonable hypothesis to explain these observations. More studies with a larger group of patients are necessary to substantiate these findings.

The current study indicates that *KLK10* mRNA was significantly overexpressed in gastric cancer tissues and high *KLK10* expression levels were associated with lymphatic invasion, tumor invasion and poor patient prog-

nosis. hK3 has been well documented to be an excellent tumor marker for prostate cancer. Moreover, hK10 is a promising serum biomarker for ovarian cancer. Therefore, studies are now underway to investigate whether hK10 may also be a useful biomarker for gastric cancer using serum samples from the patients treated at our hospital.

## COMMENTS

### Background

Gastric carcinoma is one of the most common tumors worldwide. The expression of kallikreins is involved in cancer cell formation. Abnormal expression of kallikrein gene 10 (*KLK10*) is associated with carcinogenesis, and it is a promising serum biomarker for cancers.

### Research frontiers

Several authors have reported that *KLK10* mRNA is highly expressed in ovarian cancer tissue and that hK10 could be a useful serum biomarker for the diagnosis and management of ovarian cancer. However, there is little information on *KLK10* expression in human gastric cancer.

### Innovations and breakthroughs

This study assessed the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer. Furthermore, based on the collective findings, this study investigated whether *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with gastric cancer.

### Applications

By exploring the relation between the expression of *KLK10* and clinicopathology in gastric cancer, this study may provide a strategy for predicting the prognosis of gastric cancer patients.

### Peer review

It is a well written paper. The authors investigated the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer, which helps understand the pathogenesis and predict the prognosis of gastric cancer. The experimental procedure is quite well performed.

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## Cystatin C is a biomarker for predicting acute kidney injury in patients with acute-on-chronic liver failure

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### Abstract

**AIM:** To investigate serum cystatin C level as an early biomarker for predicting acute kidney injury (AKI) in patients with acute-on-chronic liver failure (ACLF).

**METHODS:** Fifty-six consecutive patients with hepatitis B virus-related ACLF who had normal serum creatinine (Cr) level ( $< 1.2$  mg/dL in men, or  $< 1.1$  mg/dL in women) were enrolled in the Liver Failure Treatment and Research Center of Beijing 302 Hospital between August 2011 and October 2012. Thirty patients with chronic hepatitis B (CHB) and 30 healthy controls in the same study period were also included. Measurement of serum cystatin C (CysC) was performed by a particle-

enhanced immunonephelometry assay using the BN Prospec nephelometer system. The ACLF patients were followed during their hospitalization period.

**RESULTS:** In the ACLF group, serum level of CysC was  $1.1 \pm 0.4$  mg/L, which was significantly higher ( $P < 0.01$ ) than those in the healthy controls ( $0.6 \pm 0.3$  mg/L) and CHB patients ( $0.7 \pm 0.2$  mg/L). During the hospitalization period, eight ACLF patients developed AKI. Logistic regression analysis indicated that CysC level was an independent risk factor for AKI development (odds ratio = 1.8; 95%CI: 1.4-2.3,  $P = 0.021$ ). The cutoff value of serum CysC for prediction of AKI in ACLF patients was 1.21 mg/L. The baseline CysC-based estimated glomerular filtration rate (eGFR<sub>CysC</sub>) was significantly lower than the creatinine-based eGFR (eGFR<sub>Cr</sub> and eGFR<sub>MDRD</sub>) in ACLF patients with AKI, suggesting that baseline eGFR<sub>CysC</sub> represented early renal function in ACLF patients while the Cr levels were still within the normal ranges.

**CONCLUSION:** Serum CysC provides early prediction of renal dysfunction in ACLF patients with a normal serum Cr level.

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**Key words:** Acute-on-chronic liver failure; Cystatin C; Creatinine; Acute kidney injury; Prediction

**Core tip:** Severe renal dysfunction often occurs in patients with acute-on-chronic liver failure (ACLF) due to circulatory abnormalities and inflammation. New biomarkers with higher reliability and specificity for monitoring renal function are required. Fifty-six patients with ACLF and normal serum creatinine (Cr) were enrolled. Our results showed that patients who developed acute kidney injury during hospitalization had significantly higher basal serum cystatin C (CysC) levels.

CysC-based estimated glomerular filtration rate more accurately represented renal function in ACLF patients. CysC can be used as an early biomarker for detection of renal dysfunction in patients with ACLF before any increase in serum Cr is detected.

Wan ZH, Wang JJ, You SL, Liu HL, Zhu B, Zang H, Li C, Chen J, Xin SJ. Cystatin C is a biomarker for predicting acute kidney injury in patients with acute-on-chronic liver failure. *World J Gastroenterol* 2013; 19(48): 9432-9438 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9432.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9432>

## INTRODUCTION

Acute-on-chronic liver failure (ACLF) encompasses patients with previously well-compensated liver disease in whom acute decompensation of liver function occurs because of a precipitating event<sup>[1]</sup>. In China, hepatitis B virus (HBV)-infected ACLF patients account for > 80% of ACLF patients, due to a high incidence of chronic HBV infection<sup>[2,3]</sup>. The progressive nature of ACLF affects many organ systems. Kidney dysfunction is a common complication of advanced liver disease and associated with a high mortality<sup>[4-6]</sup>.

Acute tubular necrosis and hepatorenal syndrome (HRS) may account for the majority of cases of severe renal dysfunction in patients with ACLF due to underlying circulatory abnormalities and inflammation<sup>[4,7]</sup>. Recently, the Acute Kidney Injury Network (AKIN) proposed a new term for acute renal dysfunction, namely, acute kidney injury (AKI), which can represent the entire spectrum of acute renal dysfunction<sup>[8,9]</sup>. The definition of AKI is based on changes in serum creatinine (Cr). Unfortunately, Cr is an unreliable indicator during acute changes in kidney function because it is highly dependent on extrarenal factors during the estimation<sup>[10]</sup>. Serum Cr concentrations may not change until approximately 50% of kidney function has already been lost<sup>[11]</sup>. In addition, elevated serum bilirubin in ACLF patients can interfere with the measurement of serum Cr using the Jaffe method<sup>[12]</sup>. Therefore, a Cr-based estimation of the glomerular filtration rate (GFR) may overestimate renal function in patients with ACLF. Thus, new biomarkers with higher reliability and specificity for estimation of renal function are required.

Serum cystatin C (CysC) is currently being investigated for the prediction of AKI in patients with cardiac surgery<sup>[13]</sup>, advanced liver diseases<sup>[14]</sup>, and patients undergoing liver transplantation<sup>[15]</sup>. CysC is a ubiquitous protein that is freely filtered by the kidney and then metabolized by the tubules. Unlike Cr level, CysC level is independent of muscle mass, age or sex, and is not influenced by inflammatory conditions or malignancy<sup>[16,17]</sup>. CysC significantly outperforms both Cr and endogenous creatinine clearance rate and detects impairment of GFR earlier

than Cr does<sup>[18]</sup>. Several reports have suggested that increased CysC levels are more sensitive for prediction of HRS development in patients with cirrhosis<sup>[14,19,20]</sup>, but the data using CysC levels in ACLF patients are lacking.

The purposes of this study were to investigate whether serum CysC levels are increased in ACLF patients by comparing with chronic hepatitis B (CHB) patients and healthy controls, and to further determine whether CysC can be used as an early biomarker for predicting AKI in ACLF patients.

## MATERIALS AND METHODS

### Ethics

The protocol was approved by the Ethical Committee of Beijing 302 Hospital. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from each patient before entering the study protocol.

### Patients and controls

Fifty-six consecutive patients with HBV-related ACLF and normal serum creatinine level (male: < 1.2 mg/dL, female: < 1.1 mg/dL) were admitted to the Liver Failure Treatment and Research Center of Beijing 302 Hospital between August 2011 and October 2012. ACLF was diagnosed based on a recent increase in jaundice (serum total bilirubin > 171.0  $\mu$ mol/L) and decreasing plasma prothrombin activity (< 40%)<sup>[21]</sup>. Thirty patients with CHB were enrolled during the same study period. CHB was diagnosed according to the criteria recommended by the Chinese Society of Infectious Diseases, and the Chinese Society of Hepatology<sup>[22]</sup>. Serum samples from 30 age- and sex-matched healthy volunteers were used to determine the normal values of the indicators. The ACLF patients were followed during their hospitalization. Patients with intrinsic renal disease, spontaneous bacterial peritonitis, sepsis or gastrointestinal bleeding at enrollment were excluded from the study.

### Laboratory and clinical parameters

Consecutive serum samples from ACLF patients were collected upon admission and throughout hospitalization (every 3 d). The serum samples of healthy controls were collected when they came to the Health Examination Centre. The serum samples of CHB patients were collected on admission to our center. All samples were stored within 2 h at -20 °C until analysis. Biochemical tests, including blood urea nitrogen (BUN), sodium, albumin, and bilirubin, were routinely performed. Serum Cr levels were determined using the modified Jaffe method (Beckman, Hamburg, Germany). Serum CysC measurements were performed by the particle-enhanced immunonephelometry assay using the BN Prospec Nephelometer system (Dade Behring, Newark, DE, United States). The model for end-stage liver disease (MELD) score was

**Table 1** Clinical characteristics of the study population at admission

Parameter	ACLF ( <i>n</i> = 56)	CHB ( <i>n</i> = 30)	Control ( <i>n</i> = 30)
Male/female	40/16	21/9	14/6
Age (yr)	44 ± 11	40 ± 10	39 ± 8
Alanine aminotransferase (IU/L)	145 ± 189	71 ± 61	21 (10-31)
Total bilirubin (mg/dL)	20.2 ± 5.6	2.2 ± 1.3	0.7 ± 0.2
Albumin (g/L)	30.4 ± 4.8	33.5 ± 5.2	35.6 ± 4.9
BUN (mmol/L)	4.4 ± 2.1	4.1 ± 1.8	4.8 ± 1.5
Plasma sodium (mEq/L)	134.8 ± 4.5	135.6 ± 5.7	137.4 ± 3.8
Cr (mg/dL)	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CysC (mg/L)	1.1 ± 0.4	0.7 ± 0.2	0.6 ± 0.3
International normalized ratio	1.9 ± 0.4	1.1 ± 0.2	0.9 ± 0.1
HBV DNA log10 (IU/mL)	4.75 ± 2.11	6.21 ± 2.78	
Ishak score	ND	4 (3-5) <sup>1</sup>	
MELD score	24 ± 3	8 ± 4	

<sup>1</sup>Histopathological data from 20 chronic hepatitis B (CHB) patients. ND: Not determined; BUN: Blood urea nitrogen; Cr: Creatinine; CysC: Serum cystatin C; HBV: Hepatitis B virus; MELD: Model for end-stage liver disease.

calculated as:  $3.8 \ln(\text{total bilirubin in mg/dL}) + 11.2 \ln(\text{INR}) + 9.6 \ln(\text{Cr in mg/dL}) + 6.4$ . In addition, two methods of Cr-based estimated GFR (eGFR) were used: (1) the formula of Cockcroft and Gault<sup>[23]</sup> (eGFR<sub>CG</sub>); and (2) the modification of the diet in renal disease (MDRD) equation (eGFR<sub>MDRD</sub>)<sup>[24]</sup>. CysC-based GFR estimation was calculated using the Hoek formula (eGFR<sub>CysC-1</sub>)<sup>[25]</sup> and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (eGFR<sub>CysC-2</sub>)<sup>[26]</sup>.

AKI was diagnosed as follows<sup>[9]</sup>: an abrupt reduction in kidney function as manifested by an absolute increase in serum Cr by  $\geq 0.3$  mg/dL, equivalent to a percentage increase in serum Cr by  $\geq 50\%$  ( $\geq 1.5$  folds from baseline) without any evidence of pre-existing kidney disease.

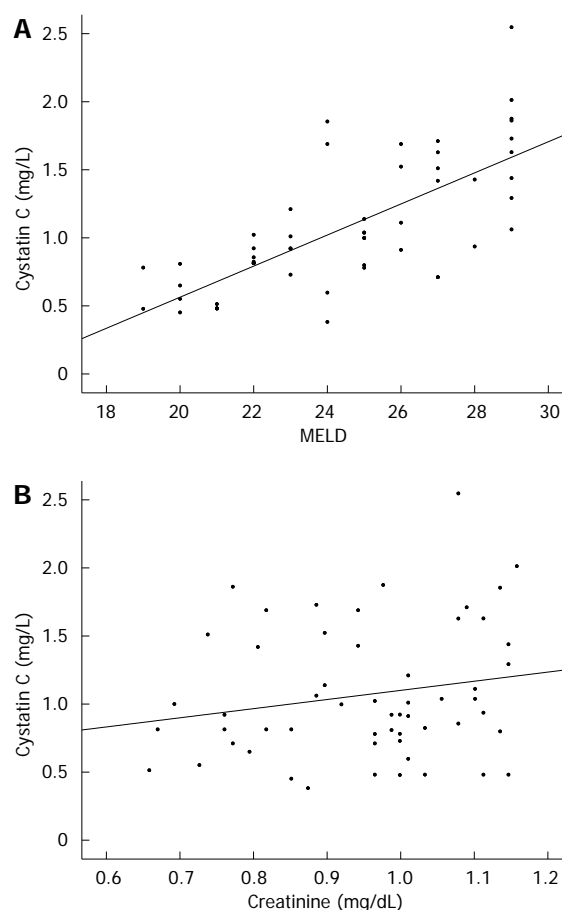
### Statistical analysis

The results are expressed as mean  $\pm$  SD or the number of patients. Data processing was carried out using SPSS for Windows version 17.0 (SPSS, Chicago, IL, United States). Continuous variables were determined using Student's *t* test. Parameters with non-normal distribution were compared using the Mann-Whitney *U* test. Categorical data were compared by the  $\chi^2$  test. Spearman's correlation analysis was used to assess relationships between two parameters. Receiver operating characteristic curves (ROCs) were formed to detect sensitivity and specificity of CysC, Cr, BUN and serum sodium for predicting the development of AIK, using Medcalc 12.7.7 software. Multivariate analysis with logistic regression was used to determine independent factors.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Clinical characteristics of enrolled patients

A total of 86 patients with chronic HBV infection, including 56 with ACLF and 30 with CHB, and 30 healthy



**Figure 1** Scatter plots. A: Serum cystatin C (CysC) level vs model for end-stage liver disease (MELD) score; B: Serum CysC level vs serum creatinine (Cr) level.

volunteers were enrolled in this study. The basal clinical characteristics of patients at admission are summarized in Table 1. Patients with ACLF comprised 40 men (71.4%) and 16 women (28.6%) with a mean age of  $44 \pm 11$  years. The serum level of sodium in ACLF patients was  $134.8 \pm 4.5$  mEq/L and hyponatremia (serum sodium level  $< 130$  mEq/L) was documented in six patients (10.7%). The average level of Cr was  $0.9 \pm 0.1$  mg/dL in ACLF patients. As shown in Table 1, the baseline serum level of CysC was  $1.1 \pm 0.4$  mg/L in ACLF patients, which was significantly higher ( $P < 0.01$ ) than those in CHB patients ( $0.7 \pm 0.2$  mg/L) and healthy controls ( $0.6 \pm 0.3$  mg/L). A moderate increase ( $P > 0.05$ ) in CysC was found in CHB patients in comparison with healthy controls. Meanwhile, in ACLF patients, the serum CysC level showed a significant positive correlation with the MELD score ( $r = 0.746$ ,  $P < 0.001$ ) (Figure 1A). However, the serum CysC level was not correlated with the serum Cr level ( $r = 0.193$ ,  $P = 0.155$ ) (Figure 1B).

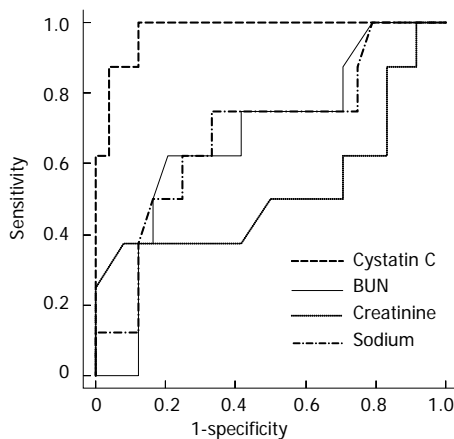
### Development of AKI

Patients with ACLF were followed during their hospitalization. The average hospitalization duration was  $36 \pm 10$  d. During this period, eight (14.3%) of 56 patients developed AKI. The baseline clinical and laboratory charac-

**Table 2** Comparison of baseline clinical characteristics in patients with or without acute kidney injury during hospitalization

Parameter	Without AKI (n = 48)	With AKI (n = 8)	P (univariate)	P (multivariate)	OR (95%CI)
Age (yr)	41 ± 9	55 ± 7	< 0.001		
Alanine aminotransferase (IU/L)	156 ± 158	62 ± 40	0.202		
Albumin (g/L)	30.1 ± 5.1	30.2 ± 2.1	0.965		
BUN (mmol/L)	4.2 ± 2.0	5.7 ± 1.1	0.065		
Sodium (mEq/L)	135.3 ± 4.2	132.1 ± 3.8	0.064		
Total bilirubin (mg/dL)	19.8 ± 5.6	21.3 ± 4.9	0.506		
Cr (mg/dL)	0.9 ± 0.1	1.0 ± 0.2	0.792		
International normalized ratio	1.9 ± 0.4	2.0 ± 0.3	0.367		
MELD score	24 ± 3	26 ± 2	0.094		
CysC (mg/L)	0.9 ± 0.3	1.8 ± 0.4	< 0.001	0.021	1.8 (1.4-2.3)

AKI: Acute kidney injury; BUN: Blood urea nitrogen; Cr: Creatinine; CysC: Serum cystatin C; MELD: Model for end-stage liver disease.



**Figure 2** Receiver operating characteristic curve. Receiver operating characteristic curve analysis was performed to compare the efficacy of serum cystatin C, creatinine, blood urea nitrogen (BUN) and serum sodium level in predicting acute kidney injury.

teristics of patients with or without AKI are summarized in Table 2. Univariate analysis showed that patients who developed AKI were older ( $55 \pm 7$  years *vs*  $41 \pm 9$  years,  $P < 0.001$ ) and had a higher level of CysC ( $1.8 \pm 0.4$  mg/L *vs*  $0.9 \pm 0.3$  mg/L,  $P < 0.001$ ). The indicators (age, sodium, MELD score and CysC) which had  $P < 0.1$  for patients with or without AKI were included in multivariate regression analysis. The results revealed that CysC level was the only independent predictive factor for the development of AKI in ACLF patients [odds ratio (OR) = 1.8; 95%CI: 1.4-2.3,  $P = 0.021$ ].

ROC curve analysis was performed to compare the efficacy of CysC, Cr, BUN and serum sodium levels in predicting development of AKI during hospitalization (Figure 2). As shown in Table 3, the area under the curve (AUC) for CysC, Cr, BUN and serum sodium levels was 0.975, 0.526, 0.674 and 0.687, respectively. The results indicated that the AUC for CysC level had a better predictive value for development of AKI in ACLF patients ( $P < 0.01$ , DeLong's method for ROC curve comparison) in comparison with Cr, BUN and serum sodium. With an optimal cutoff value of 1.21 mg/L, the sensitivity and specificity of CysC for predicting the development of AKI were 100% and 87.5%, respectively ( $P < 0.0001$ ).

### Comparison of methods for estimating GFR using Cr- or CysC-based formulae

The methods for measuring eGFR included: Cr-based eGFR (eGFR<sub>CG</sub> and eGFR<sub>MDRD</sub>), and CysC-based eGFRs (eGFR<sub>CysC-1</sub> and eGFR<sub>CysC-2</sub>). These methods were compared between patients with or without AKI development (Figure 3). The four baseline eGFRs were not significantly different in patients without AKI (Figure 3A). In patients with AKI, baseline eGFR<sub>CysC-1</sub> when using the Hoek formula was  $40.8 \pm 9.7$  mL/min, while the eGFR<sub>CysC-2</sub> from the Chronic Kidney Disease Epidemiology Collaboration equation was  $40.3 \pm 10.5$  mL/min, which indicated a similar eGFR value using the two formulae. These two baseline eGFR<sub>CysC</sub> were significantly lower than eGFR<sub>CG</sub> ( $80.8 \pm 19.6$  mL/min,  $P < 0.01$ ) and eGFR<sub>MDRD</sub> ( $79.8 \pm 14.3$  mL/min,  $P < 0.01$ ) in patients with AKI (Figure 3A). The baseline eGFR<sub>CysC-1</sub> and eGFR<sub>CysC-2</sub> were significantly decreased in patients with AKI compared with those without AKI ( $P < 0.001$ ). The baseline eGFR<sub>CG</sub> and eGFR<sub>MDRD</sub> were similar between patients with or without AKI. When AKI was diagnosed in ACLF patients, the eGFR<sub>CG</sub>, eGFR<sub>MDRD</sub>, eGFR<sub>CysC-1</sub> and eGFR<sub>CysC-2</sub> were  $29 \pm 3.3$ ,  $30.5 \pm 5.1$ ,  $34.2 \pm 5.7$  and  $33.3 \pm 5.2$  mL/min, respectively, suggesting no significant differences in the four eGFRs (Figure 3B). The results indicated that either Cr or CysC-based eGFRs reflected severe renal dysfunction when AKI occurred in ACLF patients. However, baseline eGFR<sub>CysC</sub> represented renal function of ACLF patients early during mild-to-moderate renal dysfunction, while the Cr levels were still within the normal ranges.

## DISCUSSION

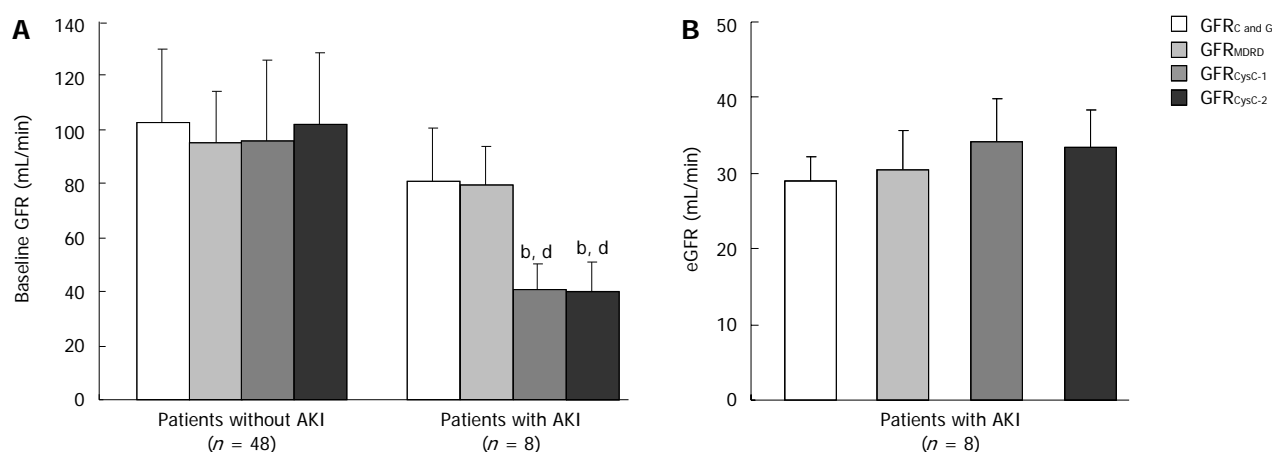
Patients with ACLF have immunological defects that are comparable to those in patients with sepsis. The clinical picture of both ACLF and septic shock is strikingly similar, and characterized by progressive vasodilatory shock and multiple organ failure<sup>[27]</sup>. Inflammation and oxidative stress also induce production of NO, which mediates the circulatory and renal disturbances of liver failure<sup>[28]</sup>. Recent reports from the European Association for the Study of the Liver (EASL) showed that the kidney failure



**Table 3** Area under the curve for receiver operating characteristics and cutoff values for predicting acute kidney injury in acute-on-chronic liver failure patients

Parameter	Cutoff value	AUC (95%CI)	Sensitivity	Specificity	P value
CysC (mg/L)	1.21	0.974 (0.846-1.000)	100%	87.5%	< 0.0001
Cr (mg/dL)	1.1	0.526 (0.343-0.704)	37.5%	91.7%	0.828
BUN (mmol/L)	4.9	0.674 (0.487-0.829)	62.5%	79.2%	0.124
Serum sodium (mEq/L)	131	0.687 (0.500-0.839)	50%	87.5%	0.113

BUN: Blood urea nitrogen; Cr: Creatinine; CysC: Serum cystatin C; AUC: Area under the curve.



**Figure 3** Performance of four equations for measuring estimated glomerular filtration rate in patients with acute-on-chronic liver failure. A: Baseline estimated glomerular filtration rate (eGFR) between patients with or without acute kidney injury (AKI); B: Comparison of four eGFR values in patients with AKI. eGFR<sub>CG</sub>: The Cockcroft and Gault formula; eGFR<sub>MDRD</sub>: The modification of the diet in renal disease equation; eGFR<sub>CysC-1</sub>: Cystatin C-based Hoek estimate; eGFR<sub>CysC-2</sub>: Chronic Kidney Disease Epidemiology Collaboration cystatin C equation. <sup>b</sup>*P* < 0.01 vs eGFR<sub>CG</sub> in patients without AKI; <sup>d</sup>*P* < 0.01 vs eGFR<sub>CG</sub>, and eGFR<sub>MDRD</sub> in patients with AKI.

was clearly a risk factor for mortality in ACLF patients<sup>[6]</sup>.

The progression of renal dysfunction in the presence of liver failure may be insidious and rapid or it may present as a mild or severe disturbance. Serum Cr is an easily measurable and widely used marker of renal function. However, Cr is an insensitive marker of kidney injury, and is usually maintained within the normal range until renal function is severely impaired, as in patients with cirrhosis and liver failure<sup>[29]</sup>. Several studies have reported that CysC is more useful for the assessment of renal function in patients with cirrhosis<sup>[14,19,20]</sup>. Assessment of CysC levels could be valuable in the early detection of renal dysfunction because they increase faster, as the GFR decreases, than do Cr levels<sup>[30]</sup>. However, data concerning CysC levels in ACLF patients are unavailable. All of the patients in the ACLF group had normal serum Cr level, with an average of  $0.9 \pm 0.1$  mg/dL. Meanwhile, the average level of serum CysC was significantly higher in ACLF patients in comparison with healthy controls and CHB patients. Our results suggest that mild-to-moderate renal dysfunction may occur in ACLF patients who have a normal Cr level.

Patients with ACLF may have renal dysfunction other than HRS, due to the underlying circulatory abnormalities and sepsis<sup>[4,27,28]</sup>. Recently, the term AKI was proposed by AKIN, which may accurately represent the entire spectrum of acute renal dysfunction in ACLF patients.

In our study, eight out of 56 ACLF patients (14.3%) developed AKI during hospitalization. Our results showed that CysC levels were significantly higher in patients who developed AKI during hospitalization. Our findings indicated that CysC could be used for the early detection of renal dysfunction in patients with ACLF before any increase in serum Cr levels is detected. Our multivariate analysis showed that age, Cr, sodium, and MELD score were not useful for predicting the development of AKI. The only independent predictive factor for AKI was CysC (OR = 1.8), which suggested that CysC represented renal function status more accurately in ACLF patients than did Cr. The results also indicated that CysC level might have accurately and rapidly reflected abnormalities in the renal handling of sodium and solute-free water before reduction in serum sodium level was detected. The diagnostic cutoff value of CysC for AKI prediction was 1.21 mg/L, which was different from that in cirrhosis patients reported previously<sup>[19,20]</sup>.

It has been suggested that a Cr-based assessment of eGFR will overestimate the renal function in nonazotemic patients with cirrhosis and with moderate renal dysfunction (GFR < 60 mL/min)<sup>[31-33]</sup>. Two Cr-based and two CysC-based eGFR values were calculated in ACLF patients. The baseline Cr-based eGFR was similar in patients with or without AKI; however, baseline CysC-based eGFR was significantly lower in patients with AKI.

The results indicated that CysC-based eGFR was better in assessing kidney dysfunction in ACLF patients with a normal Cr level. In ACLF patients with an established diagnosis of AKI, both Cr-based and CysC-based eGFR were decreased to the same level, suggesting that these eGFR calculations were accurate in the case of severe renal dysfunction. Direct measurement of GFR using exogenous markers [Tc-99m diethylene-triamine-penta-acetic acid (DTPA) or inulin clearance]<sup>[34,35]</sup> remains the standard for assessment of renal function. Unfortunately, direct GFR assessment was not performed in our study because of the disease severity in ACLF patients. Demirtas *et al.*<sup>[33]</sup> showed a correlation between CysC and 99mTc-DTPA clearance ( $r = -0.522$ ,  $P = 0.006$ ). They suggested that CysC assay, which has good analytical performance, could measure eGFR in patients with cirrhosis. That previous study, as well as our present study, suggests that CysC assay could replace Cr measurement for GFR assessment in patients with cirrhosis or ACLF.

In conclusion, CysC can be used as an early biomarker for the detection of renal dysfunction in patients with ACLF before any increase in the serum Cr levels can be detected. CysC-based eGFR calculation is more early represented renal function in ACLF patients during the period of mild-to-moderate renal dysfunction. The number size of patients for AKI development was small in this retrospective study. A prospective, large cohort study is ongoing in our research center to resolve this issue.

## COMMENTS

### Background

Kidney dysfunction is a common complication of advanced liver disease and associated with a high mortality. Serum creatinine (Cr) is an easily measurable and widely used marker of renal function. Unfortunately, Cr is an unreliable indicator during acute changes in kidney function because it highly depends on extrarenal factors such as muscle mass, gender, age and protein intake during the estimation. New biomarkers with higher reliability and specificity for estimation of renal function are required. Several reports have suggested that increased cystatin C levels are more sensitive for prediction of hepatorenal syndrome (HRS) development in patients with cirrhosis. However, data concerning serum cystatin C (CysC) levels in acute-on-chronic liver failure (ACLF) patients are unavailable.

### Research frontiers

CysC is a ubiquitous protein that is freely filtered by the kidney and then metabolized by the tubules. Unlike Cr level, CysC level is independent of muscle mass, age or sex, and is not influenced by inflammatory conditions or malignancy. CysC detects impairment of glomerular filtration rate (GFR) earlier than Cr. The research hotspot is to investigate whether CysC can be used as an early biomarker for the detection of renal dysfunction in patients with ACLF before any increase in the serum Cr levels is detected.

### Innovations and breakthroughs

Serum CysC is currently being investigated in the prediction of acute kidney injury (AKI) following cardiac surgery, advanced liver diseases, and undergoing liver transplantation, but the data using CysC levels in ACLF patients are lacking. Previous studies have reported that CysC was useful for the assessment of HRS in patients with cirrhosis. However, acute tubular necrosis and HRS may account for the majority of cases of severe renal dysfunction in patients with ACLF due to underlying circulatory abnormalities and inflammation. AKI which can represent the entire spectrum of acute renal dysfunction in ACLF patients was introduced in this paper.

### Applications

CysC can be used as an early biomarker for the detection of renal dysfunction

in patients with ACLF before any increase in the serum Cr levels. CysC-based eGFR more early represented renal function of ACLF patients during the period of mild-to-moderate renal dysfunction.

### Terminology

AKI: a abrupt reduction in kidney function manifested by an absolute increase in serum creatinine by 0.3 mg/dL or more, equivalent to a percentage increase in serum creatinine by 50% or more ( $\geq 1.5$  folds from baseline) without any evidence of preexisting kidney disease.

### Peer review

The authors presented the finding that, in case of acute-on-chronic liver failure, the predictive performance of serum CysC and eGFR calculated from CysC is superior to that of serum creatinine and the other parameters. The prospective observation is excellent. The data collected in this study contribute to our understanding of common rule in the ACLF.

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## Oxidative stress induces gastric submucosal arteriolar dysfunction in the elderly

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### Abstract

**AIM:** To evaluate human gastric submucosal vascular dysfunction and its mechanism during the aging process.

**METHODS:** Twenty male patients undergoing subtotal gastrectomy were enrolled in this study. Young and elderly patient groups aged 25-40 years and 60-85 years, respectively, were included. Inclusion criteria were: no clinical evidence of cardiovascular, renal or diabetic diseases. Conventional clinical examinations were carried out. After surgery, gastric submucosal arteries were immediately dissected free of fat and connective tissue. Vascular responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were measured by isolated vascular

perfusion. Morphological changes in the gastric mucosal vessels were observed by hematoxylin and eosin (HE) staining and Verhoeff van Gieson (EVG) staining. The expression of xanthine oxidase (XO) and manganese-superoxide dismutase (Mn-SOD) was assessed by Western blotting analysis. The malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined according to commercial kits.

**RESULTS:** The overall structure of vessel walls was shown by HE and EVG staining, respectively. Disruption of the internal elastic lamina or neointimal layers was not observed in vessels from young or elderly patients; however, cell layer number in the vessel wall increased significantly in the elderly group. Compared with submucosal arteries in young patients, the amount of vascular collagen fibers, lumen diameter and media cross-sectional area were significantly increased in elderly patients. ACh- and SNP-induced vasodilatation in elderly arterioles was significantly decreased compared with that of gastric submucosal arterioles from young patients. Compared with the young group, the expression of XO and the contents of MDA and H<sub>2</sub>O<sub>2</sub> in gastric submucosal arterioles were increased in the elderly group. In addition, the expression of Mn-SOD and the activities of SOD and GSH-Px in the elderly group decreased significantly compared with those in the young group.

**CONCLUSION:** Gastric vascular dysfunction and senescence may be associated with increased oxidative stress and decreased antioxidative defense in the aging process.

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**Key words:** Aging; Vascular dysfunction; Gastric blood flow; Oxidative stress; Human



**Core tip:** Aging is usually accompanied by a high risk of gastric disease. It is currently thought that adequate mucosal blood flow plays an important role in maintaining mucosal integrity. This study showed that oxidative stress induces gastric submucosal vascular structure dysfunction during the aging process. Vascular aging of gastric mucosa may lead to blood supply insufficiency, and thus increase the incidence of gastric diseases.

Liu L, Liu Y, Cui J, Liu H, Liu YB, Qiao WL, Sun H, Yan CD. Oxidative stress induces gastric submucosal arteriolar dysfunction in the elderly. *World J Gastroenterol* 2013; 19(48): 9439-9446 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9439.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9439>

## INTRODUCTION

Although it is difficult to define the term “aging” in medical fields, it usually means the progressive accumulation of irreversible degenerative changes leading to loss of homeostasis. It is thought that there is also a modest decline in the structure and function of several digestive organs.

The most common stomach diseases in elderly individuals are atrophic gastritis and peptic ulcer disease<sup>[1]</sup>. The former is significantly associated with *Helicobacter pylori* infection and reduced acid secretion<sup>[2]</sup>. Hyposecretion of gastric acid reduces the absorption of vitamin B12, iron and calcium, and these deficits can lead to megaloblastic or iron-deficiency anemia and a higher frequency of osteoporosis<sup>[3]</sup>. Peptic ulcers in older patients are often caused by the use or overuse of nonsteroidal anti-inflammatory drugs<sup>[4]</sup>.

It is currently thought that an adequate mucosal blood flow plays an important role in maintaining mucosal integrity. The blood supplies oxygen, nutrients and gastrointestinal hormones to support the correct structure, function and turnover of gastric mucosa. Blood flow is also important in the production and secretion of mucus and helps to maintain the mucosal barrier. In addition, the blood circulating in the surface mucosa removes waste materials and back-diffusing hydrogen ions and maintains the secretion of bicarbonate ions, protecting the mucosa by maintaining the neutral status of regional mucosa<sup>[5]</sup>. Numerous experimental studies have demonstrated the importance of mucosal blood flow in the defense of gastric mucosa against injury<sup>[6-13]</sup>.

The structure and function of gastric blood vessels are important for determining blood flow, which plays an important role in maintaining mucosal integrity. There is considerable evidence showing that vascular aging is associated with an increased production of reactive oxygen species (ROS)<sup>[14]</sup>. There is a balance between the generation and elimination of ROS in vessels<sup>[15]</sup>. If the balance is destroyed, excess ROS will be produced, resulting in

cellular dysfunction and vascular aging<sup>[16,17]</sup>. In the present study, human gastric submucosal arterioles from subtotal gastrectomy specimens were used to determine the relationship between gastric vascular dysfunction and oxidative stress, and to further explain the reason for the higher risk of gastric diseases in the elderly.

## MATERIALS AND METHODS

### Drugs and reagents

Acetylcholine (ACh) and sodium nitroprusside (SNP) were purchased from Sigma (St. Louis, MO, United States). The kits for assessing lipid oxidation injury including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu Province, China). Polyclonal antibodies for xanthine oxidase (XO), manganese-superoxide dismutase (Mn-SOD) and the alkaline phosphorylase tagged goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology (CA, United States). The 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium blue chloride (BCIP/NBT) kit was purchased from Promega (Madison, WI, United States).

### Harvesting of samples

The study was approved by the Ethics Committee of Xuzhou Medical College. Twenty male patients undergoing subtotal gastrectomy for gastric cancer in the Affiliated Hospital of Xuzhou Medical College were enrolled in this study. Patients with diabetes, hypertension or other cardiovascular diseases were excluded. All enrolled patients underwent conventional clinical examinations, including fasting blood glucose, total cholesterol, triglycerides and blood pressure, which were all found within normal ranges for clinic.

The patients were divided into two groups according to age: 10 patients aged 25-40 years were included in the young group and 10 patients aged 60-85 years were included in the elderly group. Gastric tissues, which were confirmed by pathology to be normal tissue, were obtained a distance from the cancer tissue and were undamaged due to surgical instruments. Gastric submucosal arterioles were immediately dissected free of fat and connective tissue in order to measure vascular function and changes in biochemistry and molecular biology.

### Conventional clinical examinations in enrolled patients

Body mass index was calculated using body weight in kilograms divided by the square of the height in meters (kg/m<sup>2</sup>). Fasting plasma total cholesterol, triglyceride concentration, systolic blood pressure, diastolic blood pressure and fasting plasma glucose concentration were measured. Arterial blood pressure was measured over the brachial artery during supine rest using a semiautomatic device (Dynamap XL, Johnson and Johnson). Fasting plasma metabolic parameters and oxidized low-density

lipoprotein were determined by standard assays. Plasma samples were also analyzed for oxidized low-density lipoprotein<sup>[18]</sup>.

### Histological staining

After surgery, sections of arteries were placed in phosphate buffered formaldehyde (4%) overnight, then stored in ethanol and embedded in paraffin. Cross sections (4  $\mu\text{m}$ ) were stained with hematoxylin and eosin (HE) staining and Verhoeff van Gieson (EVG) staining.

### Arteriolar response to acetylcholine and sodium nitroprusside

Similar to our previous study<sup>[19]</sup>, gastric submucosal arterioles, approximately 200  $\mu\text{m}$  in maximal diameter and approximately 10 mm in length, were isolated and cannulated in a water-jacketed (37 °C) perfusion device, intravascular pressure was maintained at 80 mmHg, and the changes in vascular diameter were recorded using a video monitor system. Dilations due to ACh ( $10^{-7}$  to  $10^{-5}$  mol/L) and SNP ( $10^{-7}$  to  $10^{-5}$  mol/L) were assessed in the arterioles from the young and elderly groups. Vasodilation responses were expressed as the percentage of basal diameter at 80 mmHg.

### ROS determination

Gastric arteries were isolated and crushed with liquid nitrogen and a homogenate was prepared. The homogenate was centrifuged, and the supernatant was used for biochemical analyses. The protein concentration in the supernatant was determined by the bicinchoninic acid assay (BCA assay, Nanjing Jiancheng Bioengineering Institute).

The MDA concentration in the homogenate was determined using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on thiobarbituric acid (TBA) reactivity. Briefly, after mixing trichloroacetic acid with the homogenate and centrifuging, a supernatant was obtained, and TBA was added. The developed red color of the resulting reaction was measured at 532 nm with a spectrophotometer. Other procedures were carried out following the manufacturer's protocols.

The content of  $\text{H}_2\text{O}_2$  in gastric submucosal arteries was assessed using a commercially available kit (Nanjing Jiancheng Bioengineering Institute).  $\text{H}_2\text{O}_2$  bound with molybdenic acid to form a complex, which was measured at 405 nm and the content of  $\text{H}_2\text{O}_2$  was then calculated.

### Antioxidant activity assay

SOD activity in gastric submucosal arteries was assessed using a commercially available kit (Nanjing Jiancheng Bioengineering Institute) based on the auto-oxidation of hydroxylamine. The developed blue color was measured at 550 nm.

GSH-Px activity was determined by the velocity method using a GSH-Px kit (Nanjing Jiancheng Bioengineering Institute). The reaction was initiated by the addition of  $\text{H}_2\text{O}_2$ . A series of enzymatic reactions was activated by

GSH-Px in the homogenate which subsequently led to the conversion of GSH (reduced glutathione) to oxidized glutathione (GSSG). The change in absorbance during the conversion of GSH to GSSG was recorded spectrophotometrically at 412 nm.

### Western blotting analysis

Gastric submucosal arteries from young and elderly patients were isolated and pooled in liquid nitrogen, respectively. Samples were solubilized in lysis buffer containing 1% protease inhibitor cocktail (Sigma) in ice for 30 min, followed by sonication for 1 min which was carried out twice at a 5-min interval. The supernatants were collected after centrifugation at  $10000 \times g$  for 15 min at 4 °C. Protein concentrations were determined using the BCA protein assay kit. Samples (50  $\mu\text{g}$  protein) were separated on 10% SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane. The blots were incubated with 5% bovine serum albumin in TBST (10 mmol/L Tris, pH 7.5; 150 mmol/L NaCl, 0.05% Tween-20) at room temperature for 2 h, then incubated with primary antibodies (anti-Mn-SOD, polyclonal antibody 1:500, anti-XO polyclonal antibody 1:500) at 4 °C overnight. After washing with TBST, the blots were incubated with secondary antibody for 2 h and were determined using a BCIP/NBT assay kit.  $\beta$ -actin was used to normalize loading variations.

### Statistical analysis

Data were expressed as mean  $\pm$  SE. Comparisons between two groups were made using the Student's *t* test. Statistical analyses were performed using SPSS for Windows version 13.0.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Conventional clinical examinations in enrolled patients

Patients with diabetes, hypertension or other cardiovascular diseases which could affect the structure and function of vessels were excluded from the study. The results of conventional clinical examinations in the young and elderly patients are shown in Table 1. Fasting plasma total cholesterol, triglyceride concentrations, fasting plasma glucose concentrations, systolic blood pressure, diastolic blood pressure and mean arterial pressure were higher in the elderly group compared with the young group ( $P < 0.05$ ). However, these values were within normal ranges for clinic. There were no differences in body mass index between the two groups.

### Changes in morphology of gastric submucosal arteries

The overall structure of vessel walls was examined by HE and EVG staining, respectively (Figure 1). Disruption of the internal elastic lamina or neointimal layers was not observed in young or aged vessels; however, the cell layer number in the vessel walls increased significantly in the elderly group (Figure 1A and B). Compared with submucosal arteries in young patients, the amount of vascular collagen fibers, lumen diameter and media cross-sectional

**Table 1 Clinical characteristics of young and elderly patients with isolated gastric submucosal arteries**

	Young group	Elderly group
No. of patients	10	10
Age, yr	34.2 ± 4	71.6 ± 7 <sup>a</sup>
Body mass index, kg/m <sup>2</sup>	21.5 ± 2.6	20.5 ± 3
Fasting blood glucose, mmol/L	4.2 ± 0.7	5.4 ± 0.6 <sup>a</sup>
Total cholesterol, mmol/L	3.87 ± 0.3	4.89 ± 0.17 <sup>a</sup>
Triglycerides, mmol/L	1.03 ± 0.01	1.23 ± 0.11 <sup>a</sup>
Systolic blood pressure, mmHg	112.2 ± 9.6	126.8 ± 8.6 <sup>a</sup>
Diastolic blood pressure, mmHg	67.7 ± 3.7	77.9 ± 5.5 <sup>a</sup>
Mean arterial pressure, mmHg	82.53 ± 2.98	94.2 ± 5.05 <sup>a</sup>

Data are mean ± SE. <sup>a</sup>*P* < 0.05 vs young group.

area were significantly increased in the elderly group (Figure 1C and D).

### ACh- and SNP-induced arteriolar response

ACh ( $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  mol/L)- and SNP ( $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  mol/L)-induced dilations were compared in gastric submucosal arterioles. Basal diameter and passive diameter at 80 mmHg of intravascular pressure in the arterioles from young and elderly patients were  $142.8 \pm 3.6$  and  $144.8 \pm 2.8$   $\mu$ m, and  $192.8 \pm 4.1$  and  $189.0 \pm 2.27$   $\mu$ m, respectively. As shown in Figure 2, dilation due to ACh, which stimulates NO synthesis and release from endothelium, was greatly reduced in the vessels from the elderly group compared with the young group (*P* < 0.05). Endothelium-independent vasodilatation was determined using SNP (a NO donor). Dilation due to SNP was also reduced in the vessels from the elderly group (*P* < 0.05), but the magnitude of this reduction was less.

### Changes in MDA, H<sub>2</sub>O<sub>2</sub>, SOD and GSH-Px in gastric submucosal arteries

To determine whether oxidative stress participates in the aging process, we measured the contents of MDA and H<sub>2</sub>O<sub>2</sub> and the activities of GSH-Px and SOD in gastric submucosal arteries, these are key markers of oxidative stress. Compared with the young group, there was a significant increase in MDA and H<sub>2</sub>O<sub>2</sub> content (*P* < 0.05), and a decrease in SOD and GSH-Px activities (*P* < 0.05) in the elderly group (Figure 3).

### Changes in XO and SOD protein expression in gastric submucosal arteries

Gastric submucosal arteries were isolated and the expression of XO and Mn-SOD was assessed. Figure 4 shows a representative Western blotting of XO and Mn-SOD in arteries. The XO protein was significantly increased (Figure 4A), whereas the expression of Mn-SOD (Figure 4B) was significantly reduced in the elderly group compared with the young group (*P* < 0.05).

## DISCUSSION

Vascular aging is a key factor in accelerating the aging

process and increasing the incidence of disease in humans. In this study, we demonstrated a marked deterioration in the structure and function of gastric blood vessels during aging. The underlying mechanisms may be associated with increased oxidative stress and decreased anti-oxidative defense, which induce an imbalance in oxidative stress during the aging process, thus accelerating vascular dysfunction and senescence in the elderly.

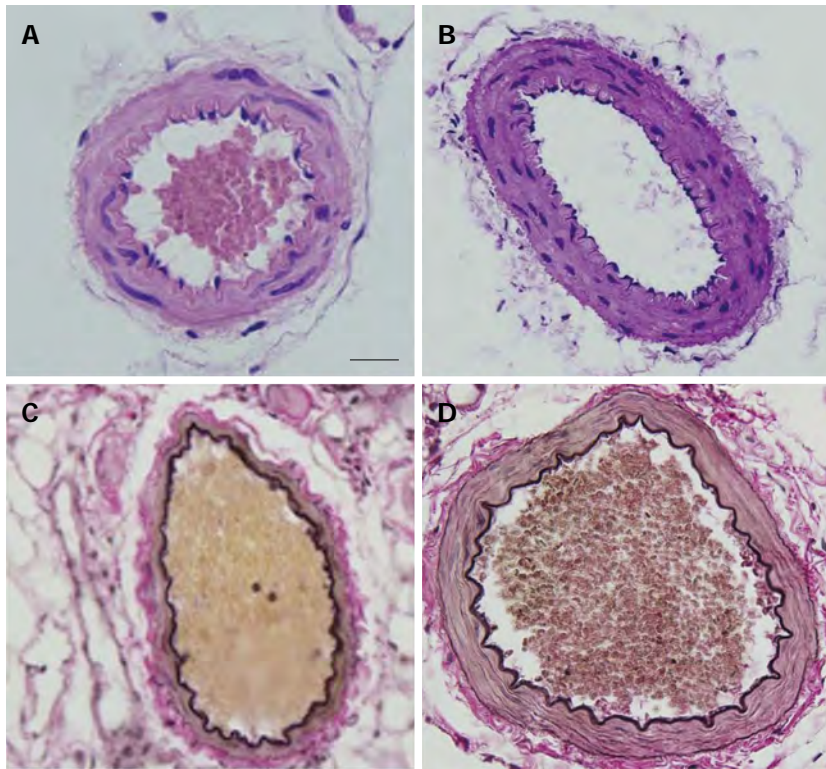
Aging is considered the leading cause of morbidity and mortality worldwide, and the proportion of elderly people is steadily growing<sup>[20]</sup>. With increasing age, the vasculature undergoes functional and structural impairment. Vascular changes during aging are manifested in various ways in many experimental animals; the most obvious changes noted are thickening of less compliant vessel walls<sup>[21]</sup>. In the present study, we evaluated the walls of gastric submucosal arteries from elderly patients, which were much thicker than the arteries from young patients. In addition, the collagen content was increased in aged gastric submucosal arteries (Figure 1). Aging can radically transform the endothelial layers lining the vessel wall in response to shear and stretch stress<sup>[22]</sup>, and prompt them to thicken, as observed in many vascular models<sup>[23,24]</sup>. The changes in gastric submucosal arteries may be due to the recruitment of vascular smooth muscle cells for increased synthesis of interstitial, extra-cellular matrix proteins<sup>[25,26]</sup>.

As expected, endothelium-dependent and -independent dilation were significantly attenuated in aged gastric submucosal arterioles compared with arterioles from young patients (Figure 2). Endothelial cell dysfunction has been observed in the elderly<sup>[27]</sup>, and the production of endothelium-derived vasodilator substances is decreased<sup>[28,29]</sup>. In the present study, ACh-induced dilation of the gastric submucosal arteries from elderly patients was reduced, which was related to endothelial dysfunction during the aging process. Endothelium-independent dilation was also detected. SNP (a NO donor) induced dilation in the arterioles of elderly patients was reduced compared with that in arterioles from young patients. This change may be due to the thick vessel walls and increased collagen content. The changes in gastric submucosal vascular structure and function may lead to gastric blood supply insufficiency in the elderly.

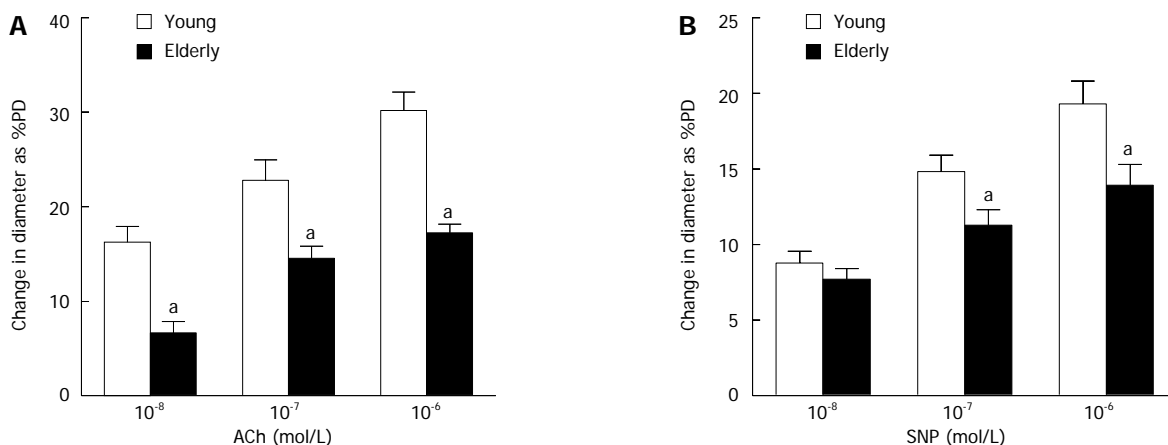
Adequate mucosal blood flow plays an important role in maintaining mucosal integrity. Decreased gastric blood flow causes acute gastric mucosal lesions in animals and humans<sup>[9]</sup>. Gastric blood flow is important in the development of gastric ulceration and healing. Furthermore, the speed of ulcer healing is affected by the speed of blood flow at the ulcer edge<sup>[30]</sup>. Our group has recently shown that aspirin-induced injury of gastric mucous membrane was inhibited by increasing gastric mucosal blood flow<sup>[31]</sup>. The changes in gastric submucosal vascular structure and function which induced blood supply insufficiency may be one of the most important reasons for the higher incidence of gastric diseases in elderly subjects.

The mechanism involved in these changes in vascular structure and function in the elderly was also investigated





**Figure 1** Changes in vascular structure of gastric submucosal arteries isolated from young and elderly patients shown by hematoxylin and eosin and Verhoeff van Gieson staining. A: Young, hematoxylin and eosin (HE); B: Elderly, HE; C: Young, Verhoeff van Gieson (EVG); D: Elderly, EVG. Images were obtained at  $\times 400$ , Bar 50  $\mu\text{m}$ .



**Figure 2** Effects of age on relaxant response to acetylcholine (A) and sodium nitroprusside (B) in gastric arterioles from young and elderly patients. Data are mean  $\pm$  SE,  $n = 6$ . <sup>a</sup> $P < 0.05$  vs young group. ACh: Acetylcholine; SNP: Sodium nitroprusside.

in the present study. MDA is a marker of free radical species-related injury,  $\text{H}_2\text{O}_2$  is the main reactive oxygen species produced, and these are by-products of mitochondrial respiration. In our experiments, MDA and  $\text{H}_2\text{O}_2$  were significantly increased in aged arteries compared to arteries from young patients. Age-related increases in oxidative stress may result in changes in vascular structure and function in aged gastric submucosal arteries.

Oxidative stress is associated with increased production of oxidizing species or a significant decrease in antioxidant defense capability<sup>[32]</sup>. XO is an important

potential source of superoxide generation, it catalyzes the conversion reactions of hypoxanthine to xanthine and xanthine to uric acid, the last reaction in purine catabolism, with the byproduct of toxic superoxide radical<sup>[33]</sup>. XO protein levels in gastric submucosal arteries were significantly higher in elderly patients than in young patients. Increased expression of oxidases is the main source of reactive oxygen species in humans<sup>[34]</sup>.

SOD, a major antioxidant enzyme, contributes to the destruction of free superoxide radicals and other reactive oxygen species, and blocks free radical-induced damage



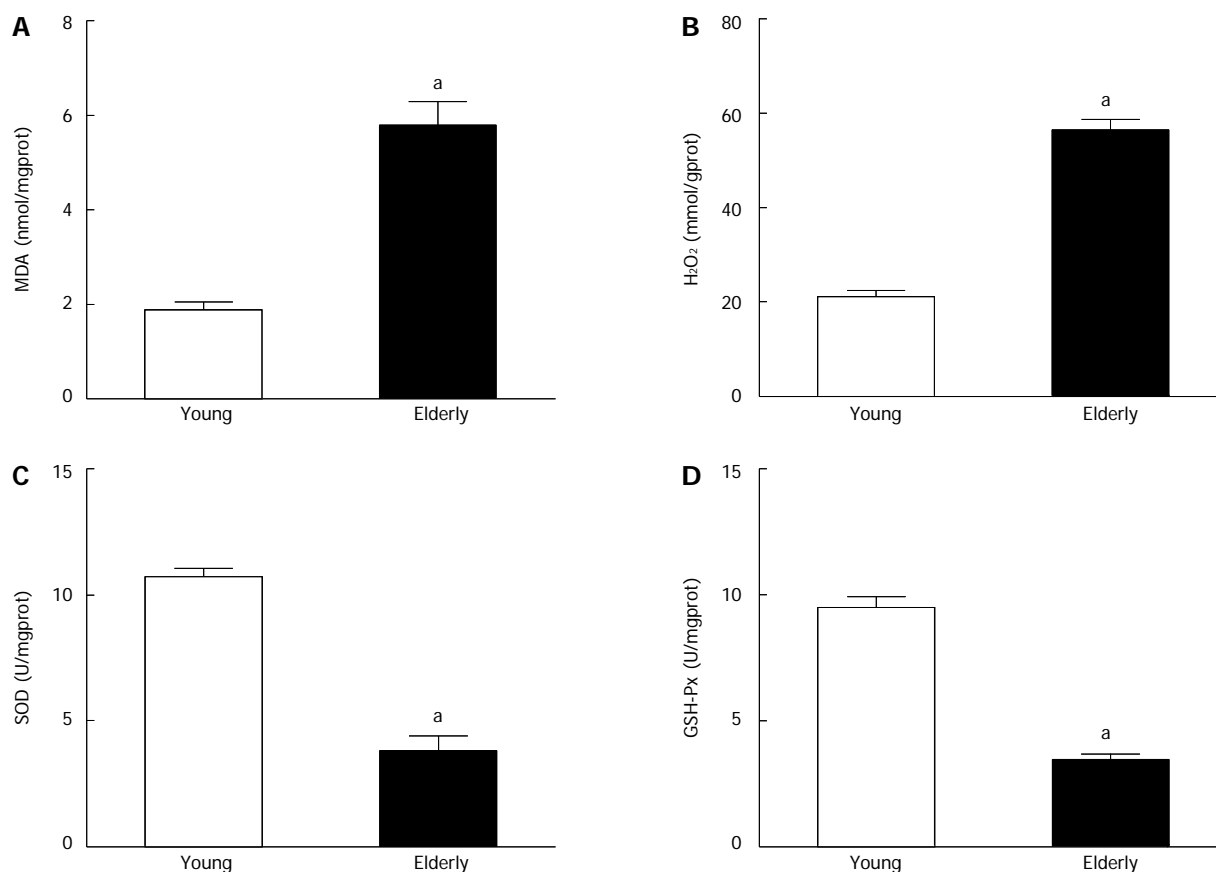


Figure 3 Changes in malondialdehyde (A) and hydrogen peroxide (B) and the activities of superoxide dismutase (C) and glutathione peroxidase (D) in gastric submucosal arteries isolated from young and elderly patients. Data are mean  $\pm$  SE,  $n = 10$ . \* $P < 0.05$  vs young group. MDA: Malondialdehyde; H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase.

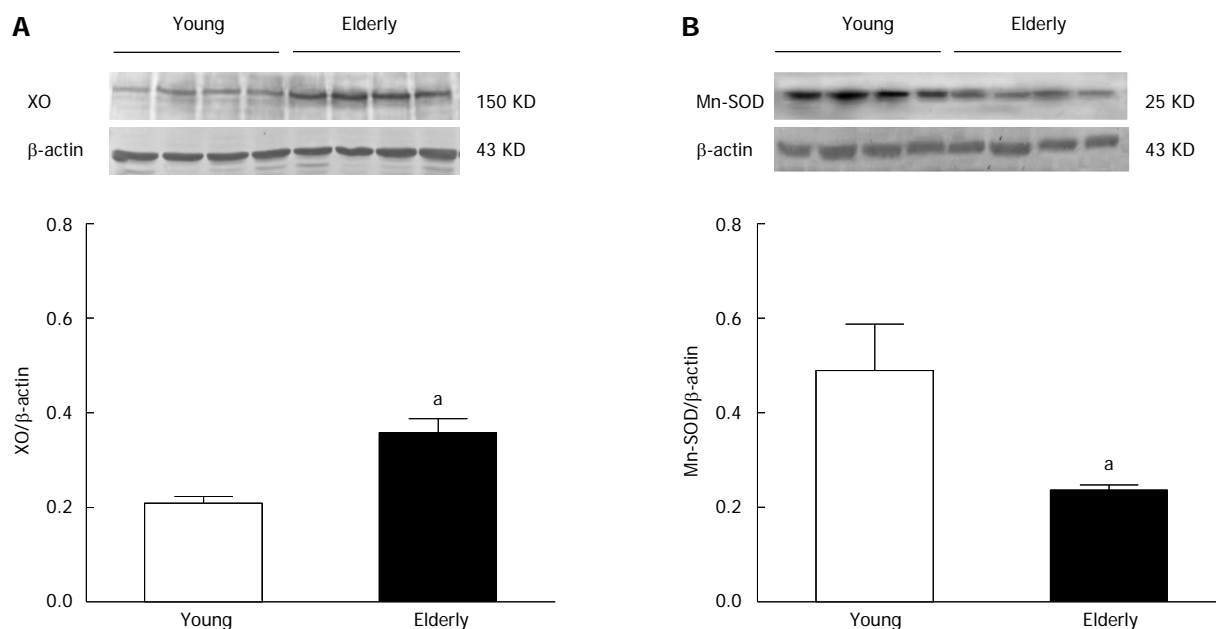


Figure 4 Expression of xanthine oxidase (A) and manganese-superoxide dismutase (B) protein in gastric mucosa substratum arteries isolated from young and elderly patients. \* $P < 0.05$  vs young group.  $\beta$ -actin was used to normalize loading variations (each bar represents the mean  $\pm$  SE of 4 independent experiments). XO: Xanthine oxidase; Mn-SOD: Manganese-superoxide dismutase.

in the body<sup>[35,36]</sup>. There are three isoforms of SOD, cytosolic SOD or copper zinc SOD (CuZn-SOD or SOD-1),

mitochondrial SOD or manganese SOD (Mn-SOD or SOD-2), and extracellular CuZn-SOD (EC-SOD or

SOD-3). Mn-SOD, which is found in mitochondria, plays an important role in the maintenance of vascular function. Previous animal studies found that the antioxidant enzyme content was decreased, while the expression of oxidative enzymes was significantly increased with aging<sup>[37]</sup>. The Mn-SOD protein level (Figure 4B) and SOD activity (Figure 3C) were significantly decreased in this study, which was consistent with previously reported findings.

GSH-Px is a free radical scavenging enzyme similar to SOD, which converts H<sub>2</sub>O<sub>2</sub> to water independently<sup>[38]</sup>. In the present study, GSH-Px and total SOD were significantly lower in the elderly group compared with the young group. These results suggest that elderly patients have a higher risk of oxidative stress than younger patients and consequently greater vulnerability for chronic disease in old age. The decreased expression and activity of antioxidant enzymes will accelerate oxidative stress damage in aging vessels.

In summary, we demonstrated that oxidative stress and a decreased antioxidative defense induce vascular aging and enhance vascular dysfunction. Vascular aging of gastric mucosa may lead to blood supply insufficiency and an increased incidence of gastric diseases. This research has provided theoretical evidence suggesting that a decrease in oxidative stress during the aging process and improvement in the function of gastric submucosal vessels may be beneficial in the treatment of gastric disease in the elderly.

## COMMENTS

### Background

Aging is usually accompanied by a higher risk of gastric disease. Current opinion suggests that adequate mucosal blood flow plays an important role in maintaining mucosal integrity. The structure and function of gastric submucosal arteries are important for regulating gastric blood flow.

### Research frontiers

Mucosal blood flow plays an important role in maintaining mucosal structure, function and turnover of gastric mucosa. Numerous experimental studies have demonstrated the importance of mucosal blood flow in the defense of gastric mucosa against injury. However, few studies have directly studied the structural and functional changes in gastric submucosal vessels.

### Innovations and breakthroughs

This study showed that oxidative stress and a decreased antioxidative defense induce gastric vascular aging and enhance vascular dysfunction in the elderly. The structure and function of gastric submucosal arteries are important for regulating gastric blood flow. Vascular aging of gastric mucosa leads to blood supply insufficiency and an increase in the incidence of gastric disease.

### Applications

This research provides theoretical evidence to suggest that a decrease in oxidative stress during the aging process and improvement in the function of gastric submucosal arteries may be beneficial in the treatment of gastric diseases in the elderly.

### Terminology

Vascular aging involves vascular structure changes and dysfunction during the aging process. The aging of submucosal arteries leads to changes in mucosal blood flow which plays an important role in maintaining mucosal integrity. Inadequate blood flow will increase the incidence of gastric diseases.

### Peer review

The authors demonstrated that aging induces vascular dysfunction through increasing oxidative stress in isolated human gastric submucosal arterioles. This study has provided theoretical evidence that a decrease in oxidative stress dur-

ing the aging process and improvement in the function of gastric submucosal arterioles may be beneficial in the treatment of gastric disease in the elderly.

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## Nedaplatin concurrent with three-dimensional conformal radiotherapy for treatment of locally advanced esophageal carcinoma

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### Abstract

**AIM:** To evaluate the efficacy and toxicity of nedaplatin (NDP) concurrent with radiotherapy in the treatment of locally advanced esophageal carcinoma.

**METHODS:** Sixty-eight patients with locally advanced esophageal carcinoma were randomized into either a NDP group ( $n = 34$ ) or a cisplatin (DDP) group ( $n = 34$ ). The NDP group received NDP 80-100 mg/m<sup>2</sup> *iv* on day 1 + leucovorin (CF) 100 mg/m<sup>2</sup> *iv* on days 1-5 + 5-fluorouracil (5-FU) 500 mg/m<sup>2</sup> *iv* on days 1-5. The DDP group received DDP 30 mg/m<sup>2</sup> *iv* on days 1-3 + CF 100 mg/m<sup>2</sup> on days 1-5 + 5-FU 500 mg/m<sup>2</sup> *iv* on days 1-5. The treatment was repeated every 4 wk in both groups. Concurrent radiotherapy [60-66 Gy/(30-33 f)/(6-7 wk)] was given during chemotherapy.

**RESULTS:** There was no significant difference in the short-term response rate between the NDP group and

DDP group (90.9% *vs* 81.3%,  $P = 0.528$ ). Although the 1- and 2-year survival rates were higher in the NDP group than in the DDP group (75.8% *vs* 68.8%, 57.6% *vs* 50.0%), the difference in the overall survival rate was not statistically significant between the two groups ( $P = 0.540$ ). The incidences of nausea, vomiting and nephrotoxicity were significantly lower in the NDP group than in the DDP group (17.6% *vs* 50.0%,  $P = 0.031$ ; 11.8% *vs* 47.1%,  $P = 0.016$ ; 8.8% *vs* 38.2%,  $P = 0.039$ ). There was no significant difference in the incidence of myelosuppression, radiation-induced esophagitis or radiation-induced pneumonia between the two groups.

**CONCLUSION:** NDP-based concurrent chemoradiotherapy is effective and well-tolerated in patients with locally advanced esophageal carcinoma. NDP-based regimen has comparable efficacy to DDP-based regimen but is associated with lower incidences of gastrointestinal and renal toxicity.

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**Key words:** Esophageal carcinoma; Chemoradiotherapy; Nedaplatin; Cisplatin

**Core tip:** This paper describes patients with locally advanced esophageal carcinoma who underwent nedaplatin (NDP) concurrent with radiotherapy. The survival and local control as well as the side effects during follow-up were analyzed by comparing with cisplatin (DDP). We found that NDP-based concurrent chemoradiotherapy is effective and well-tolerated. Compared with DDP, NDP-based concurrent chemoradiotherapy exhibits favorable efficacy with lower toxicity.

Shen ZT, Wu XH, Li B, Shen JS, Wang Z, Li J, Zhu XX. Nedaplatin concurrent with three-dimensional conformal radiotherapy



for treatment of locally advanced esophageal carcinoma. *World J Gastroenterol* 2013; 19(48): 9447-9452 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9447.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9447>

## INTRODUCTION

Radiotherapy is one of the main treatments for esophageal carcinoma, especially for patients with locally advanced esophageal cancer who have no indications for surgery. However, the 5-year survival rate for patients with non-early esophageal carcinoma after radiotherapy alone is only 8% to 17%<sup>[1]</sup>. Approximately 70%-80% of cases of radiotherapy failure are due to uncontrolled or recurrent localized disease. Chemotherapy given concurrently with radiotherapy can improve the efficacy of radiotherapy in esophageal carcinoma. Concurrent chemoradiotherapy has been recommended as the standard treatment for locally advanced esophageal carcinoma in some countries, and conventional fractionated radiotherapy plus cisplatin (DDP) and 5-fluorouracil (5-FU) has been advocated as a standard regimen for this malignancy<sup>[2,3]</sup>. However, the risk of gastrointestinal and renal toxicity associated with DDP-based PF regimen (DDP + 5-FU) limits its use. In the present study, we designed a randomized controlled phase II trial to compare the efficacy, acute adverse reactions and late toxicity of three-dimensional conformal radiotherapy plus nedaplatin (NDP) and 5-FU versus plus the PF regimen in the treatment of locally advanced esophageal carcinoma, with an aim to find a regimen that has fewer adverse reactions and better efficacy than the PF regimen.

## MATERIALS AND METHODS

### Test drug

NDP injection (trade name, Jiebaishu, 10 mL) was provided by Simcere Pharmaceutical (Nanjing, China).

### Subjects

Sixty-eight patients who were pathologically proven to have locally advanced esophageal squamous cell carcinoma by gastroesophagoscopy from March 2007 to September 2009 in Department of Radiation Oncology of the Nanjing General Hospital of Nanjing Military Region and had evaluable tumor lesions were included in the study. There were 38 males and 30 females, and their median age was 54 years (range, 26 to 72 years). According to the 1997 Union for International Cancer Control esophageal cancer staging system, 29 patients had stage II disease and 39 had stage III disease. The patients were randomly divided into either a NDP group ( $n = 34$ ) or a DDP group ( $n = 34$ ) to receive NDP + leucovorin (CF) + 5-FU and DDP + CF + 5-FU, respectively. In the NDP group, 14 patients had stage II disease and 20 had stage III disease. In the DDP group, 15 patients had stage II disease and 19 had stage III disease. The average age of patients in the

**Table 1 Clinical data for patients in the nedaplatin group and the cisplatin group**

	NDP group	DDP group	$\chi^2$	P value
Case	34	34		
Gender				
Male	18	20	0.239	0.625
Female	16	14		
Age				
Range	27-72	26-70		
Median	54	53		
Clinical stage (Union for International Cancer Control)				
II a	4	6	0.478	0.787
II b	10	9		
III	20	19		
Tumor length				
< 5 cm	14	17	0.534	0.465
≥ 5 cm	20	17		
Cervical	5	3		
Location in the esophagus				
Upper	12	15	1.130	0.770
Middle	14	12		
Lower	3	4		
Medullary	20	22		
Fungoid	6	5		
Pathology				
Ulcer type	5	5	0.386	0.943
Sclerotic type	3	2		
General status (Eastern Cooperative Oncology Group score)				
0-1	24	21	0.591	0.442
2	10	13		

NDP: Nedaplatin; DDP: Cisplatin.

NDP and DDP groups was 55 and 53 years old, and the median age was 54 and 53 years old, respectively. Clinical data for patients in both groups are shown in Table 1.

Inclusion criteria are (1) previously untreated, histologically or pathologically proven locally advanced esophageal carcinoma, with at least one measurable lesion ( $\geq 2$  cm); (2) Eastern Cooperative Oncology Group performance status score  $\leq 2$ ; (3) expected survival for three months or more; (4) age between 26 and 72 years; (5) basically normal heart, lung, liver, kidney functions; (6) no previous thoracic radiotherapy or chemotherapy, and no significant chemotherapy contraindications; (7) no other malignancy; or (8) willing to provide signed informed consent.

Exclusion criteria included (1) participation in other drug trial or receiving anti-tumor therapy within 4 wk; (2) other serious complications that made the patient not to fit to the study; (3) pregnant or lactating women; and (5) allergy to the test drug.

Withdrawal criteria included (1) serious adverse reactions during treatment, such as life-threatening bleeding due to thrombocytopenia, life-threatening infections for leukopenia, and grade III or more liver and kidney adverse reactions; (2) not being able to complete the treatment; (3) not willing to continue the trial; or (4) disease progression during treatment.

### Treatments

The NDP group received NDP 80-100 mg/m<sup>2</sup> *iv* on day 1 + CF 100 mg/m<sup>2</sup> *iv* on days 1-5 + 5-FU 500 mg/m<sup>2</sup> *iv*

**Table 2** Short-term response in the two groups *n* (%)

Group	<i>n</i>	CR	PR	SD	PD	RR	$\chi^2$	<i>P</i> value
NDP group	33	6 (18.2)	24 (72.7)	3 (9.1)	0 (0)	90.9%	1.276	0.528
DDP group	32	5 (15.6)	21 (65.6)	6 (18.8)	0 (0)	81.3%		

RR = (PR+CR)/*n*. NDP: Nedaplatin; DDP: Cisplatin; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; RR: Response rate.

on days 1-5. The DDP group received DDP 30 mg/m<sup>2</sup> *iv* on days 1-3 + CF 100 mg/m<sup>2</sup> on days 1-5 + 5-FU 500 mg/m<sup>2</sup> *iv* on days 1-5. The treatment was repeated every 4 wk in both groups. Before chemotherapy, prophylactic antiemetic therapy with 5-HT<sub>3</sub> receptor antagonist was given. Granulocyte colony-stimulating factor (G-CSF) was administered when grade 3/4 neutropenia occurred. When anemia and grade 3/4 thrombocytopenia occurred, erythropoietin (EPO) and recombinant human interleukin-11 (IL-11) or therapeutic plateletpheresis were given, and the dose of main chemotherapy drugs was reduced by 25% in the next cycle or the interval between two cycles was extended. Concurrent radiotherapy was given during chemotherapy in both groups.

Three-dimensional conformal radiotherapy (3D-CRT) was adopted, with high-energy X-ray beams (6 MV) produced by a linear accelerator. Gross tumor volume (GTV) boundaries were determined by esophageal X-ray, barium meal, CT, and esophagoscopy. The upper and lower boundaries for clinical target volume (CTV) were defined as upper and lower boundaries for GTV plus 3 cm. The lateral boundaries for CTV were defined as the lateral boundaries for tumors plus 0.8 cm. Planning target volume (PTV) was defined as CTV plus 0.5 cm.

Efficacy was evaluated using the 2000 RECIST criteria based on physical examination and imaging data (X-ray, barium meal, chest CT). Imaging data were assessed independently by two professional radiologists. Patients were rated as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). The response rate (RR) was defined as (number of cases with CR + number of cases with PR)/total number of cases (*n*). Acute radiation injury was assessed using the Radiation Therapy Oncology Group (RTOG) acute radiation morbidity criteria (grades 0 to 4). Chemotherapy-associated adverse reactions were assessed using the U.S. National Cancer Institute common toxicity criteria (NCI-CTC), version 3.0 (grades 0-4).

### Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Rates or percentages between two groups were compared using the chi-square test and Fisher exact test. Survival was analyzed using the Kaplan-Meier method. Survival curves were compared using the Log-rank significance test. Survival was defined as the period from the date of diagnosis to death. Two-tailed *P*-values <0.05 were considered statistically significant.

## RESULTS

### Short-term response

In the NDP group, 33 of 34 patients completed two or more cycles of treatment and were evaluable for efficacy and toxicity. In the DDP group, 32 patients completed two or more cycles of chemotherapy and can be evaluated for efficacy and toxicity, and the remaining two cases discontinued the treatment after one cycle of chemotherapy (one for intolerable side effects and the other for poor compliance) but can be evaluated for toxicity. Short-term responses in the two groups are shown in Table 2.

### Survival rate and causes of death

The 1-year overall survival rate was 75.8% (25/33) for the NDP group and 68.8% (22/32) for the DDP group, and the 2-year overall survival rate was 57.6% (19/33) and 50.0% (16/32), respectively. Although the overall survival rate was higher in the NDP group than in the DDP group, the difference was not statistically significant ( $\chi^2 = 0.375$ , *P* = 0.504).

During the follow-up period, 19 patients survived and 14 died in the NDP group. Of 14 dead patients, 9 died of distant metastasis, 3 of local control failure, and 2 of distant metastasis plus local control failure. Of 16 dead patients in the DDP group, 4 died of local control failure, 7 of local control failure plus distant metastasis, and 5 of distant metastasis. These findings suggest that distant metastasis was the main cause of death in both groups. The percentage of patients who died of distant metastasis showed no significant difference between the NDP group and DDP group (78.6% *vs* 75.0%,  $\chi^2 = 0.053$ , *P* = 0.818) (Figure 1).

### Toxicity

Toxicity could be evaluated in all cases. In the NDP group, grades I-IV decreased hemoglobin developed in 20 patients (58.8%), grades I-IV leukopenia in 21 patients (61.8%), and grades I-IV thrombocytopenia in 19 patients (55.9%); the corresponding figures in the DDP group were 18 (52.9%), 19 (55.9%) and 14 cases (41.2%). The incidences of decreased hemoglobin, leukopenia and thrombocytopenia showed no significant differences between the two groups (*P* = 0.990, 0.805, 0.540). Although the incidence of hepatic dysfunction did not differ significantly between the two groups (*P* = 0.565), the incidence of renal toxicity was significantly higher in the DDP group (38.2% *vs* 8.8%, *P* = 0.039). The incidences

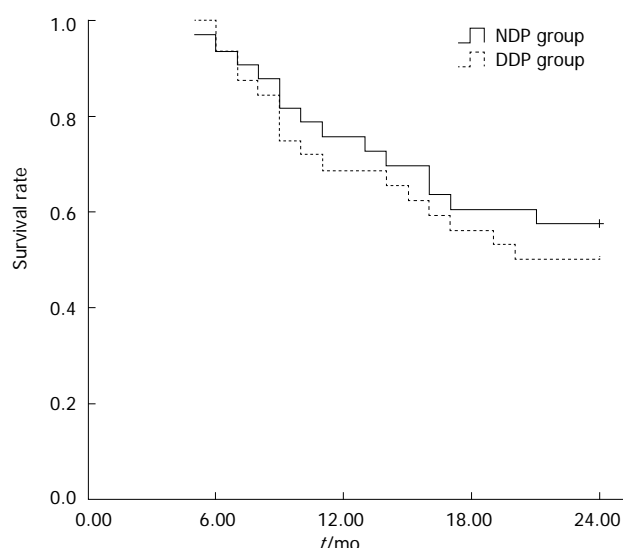


Figure 1 Survival curves for the two groups. NDP: Nedaplatin; DDP: Cisplatin.

of nausea and vomiting were significantly lower in the NDP group than in the DDP group (17.6% *vs* 50.0%, 11.8% *vs* 47.1%,  $P = 0.031$ , 0.016) (Table 3).

Late grade 4 esophageal toxicity was noted in one patient in the DDP group, but no patient developed late grade 3 or more esophageal toxicity in the NDP group. The incidences of late esophageal and lung toxicities showed no significant difference between the two groups ( $P > 0.05$  for both). Serious late radiation toxicities such as radiation-induced myelitis and pericarditis were not observed (Table 4).

## DISCUSSION

Esophageal carcinoma is one of the most common malignancies in China. Surgical excision is the standard treatment for esophageal carcinoma. Because most patients with esophageal carcinoma are diagnosed at the advanced stage, most of them have missed the chance of radical surgery. For patients without indications for surgery or those with localized disease after surgical resection, radiotherapy is another possible cure. However, both surgery alone and radiotherapy alone can not significantly improve the five-year survival rate in patients with esophageal carcinoma. To overcome this problem, worldwide scholars have tried a variety of comprehensive treatment from the 1970s to improve the therapeutic effect against this malignancy<sup>[4-8]</sup>. Chemotherapy combined with radiotherapy has yielded encouraging results. Particularly, the RTOG8501 trial conducted by Cooper *et al*<sup>[5]</sup> has provided convincing evidence to support the effectiveness of concurrent chemoradiotherapy in the management of esophageal carcinoma.

Compared to radiotherapy alone, concurrent chemoradiotherapy will further increase the incidence of side effects. Main side effects include radiation-induced esophagitis, pneumonia, bone marrow suppression, nausea, and vomiting. Seung *et al*<sup>[9]</sup> reported that the

incidences of grade 2 and 3 esophagitis were 89% and 39%, respectively. Severe radiation-induced esophagitis is difficult to manage and often affects the implementation of treatment regimens or extends the total treatment time, thereby affecting therapeutic effects. Many studies have shown that the most commonly used PF regimen plus concurrent radiotherapy is associated with a high incidence of esophagitis. DDP is the main factor causing toxicity and is intolerable in some patients. Therefore, researchers have been seeking more efficient drugs or regimens with lower toxicity.

NDP (cis-diam-mincgly-colatoplatinum; formula,  $C_2H_8N_2O_3Pt$ ; molecular mass, 303.18 kD) is a second-generation anti-cancer platinum derivative developed by Japanese pharmaceutical company Shionogi and approved for marketing in Japan in June 1995. Clinical studies have demonstrated that NDP is effective in esophageal carcinoma, head and neck cancer, lung cancer, cervical cancer, ovarian cancer, bladder cancer, testicular cancer and other solid tumors. It can be used alone or in combination with other chemotherapeutic drugs or radiotherapy to improve efficacy and reduce side effects. The mechanism of action of NDP is the same as that of DDP; they bind to DNA by forming platinum-nucleoside complexes and inhibit DNA replication<sup>[10]</sup>. The solubility of NDP is about 10 times that of DDP, and there exists certain cross-resistance between DDP and NDP<sup>[11]</sup>. NDP does not require hydration, has low renal and gastrointestinal toxicity, and shows a good synergistic effect when being used with other chemotherapy drugs. There is no complete cross-resistance between CDDP and NDP<sup>[12]</sup>. Although NDP has a high therapeutic index, its side effects are low. The dose-limiting toxicity of NDP is myelosuppression-induced thrombocytopenia, and its renal and gastrointestinal toxicity is low<sup>[13]</sup>. In recent years, many foreign clinical studies have demonstrated that the response rate of NDP-based regimens is above 50% in patients with advanced esophageal carcinoma, which is higher than or similar to those of conventional DDP-based regimens, but adverse reactions could be expected and well tolerated<sup>[14]</sup>. A similar study has also been reported in China<sup>[15]</sup>. Watanabe *et al*<sup>[16]</sup> reported the use of NDP and 5-FU with concurrent radiotherapy for advanced esophageal carcinoma. Kato *et al*<sup>[14]</sup> reported that NDP and 5-FU combined with radiotherapy achieved an overall response rate of 77%, a 1-year survival rate of 30.7%, a 2-year survival rate of 10.2%, and the median survival time of 10.1 mo in patients with unresectable advanced esophageal squamous cell carcinoma.

The present study showed that the short-term response rate and the 1- and 2-year survival rates were higher in the NDP group than in the DDP group (90.9% *vs* 81.3%, 75.8% *vs* 68.8%, 57.6% *vs* 50.0%), although the differences were not statistically significant. These findings suggest that NDP-based regimen has a trend to improve the short- and long-term response rates in locally advanced esophageal carcinoma, and that the efficacy of NDP-based concurrent chemoradiotherapy regimen is

**Table 3** Acute adverse events in the two groups

Acute adverse reactions	NDP group (n = 34)						DDP group (n = 34)						$\chi^2$	P value
	0	I	II	III	IV	Incidence	0	I	II	III	IV	Incidence		
Hemoglobin	14	9	5	5	1	58.80%	16	8	5	4	1	52.90%	0.303	0.990
Leukopenia	13	8	7	6	0	61.80%	15	8	6	4	1	55.90%	1.62	0.805
Platelet	15	7	6	5	1	55.90%	20	4	7	3	0	41.20%	3.109	0.540
Bilirubin	29	4	1	0	0	14.70%	30	3	1	0	0	11.80%	0.16	0.923
Transaminase	25	8	1	0	0	26.50%	27	7	0	0	0	20.60%	1.144	0.565
Urea nitrogen	30	4	0	0	0	11.80%	29	3	2	0	0	14.70%	2.16	0.340
Creatinine	31	2	1	0	0	8.80%	21	9	3	1	0	38.20%	8.378	0.039
Nausea	28	4	1	1	0	17.60%	17	7	6	4	0	50.00%	8.878	0.031
Vomiting	30	2	1	1	0	11.80%	18	6	4	6	0	47.10%	10.371	0.016
Esophagitis	8	18	7	1	0	76.50%	4	19	9	1	1	88.20%	2.61	0.625
Pneumonia	18	14	2	0	0	47.10%	12	17	4	1	0	64.70%	3.157	0.368

NDP: Nedaplatin; DDP: Cisplatin.

**Table 4** Late adverse events in the two groups n (%)

Late adverse event	NDP group	DDP group	$\chi^2$	P value
Late esophageal injury				
0	18 (52.9)	13 (38.2)	2.299	0.681
I	10 (29.4)	12 (35.3)		
II	4 (11.8)	5 (14.7)		
III	2 (5.9)	3 (8.8)		
IV	0 (0)	1 (2.9)		
Late lung injury				
0	24 (70.6)	20 (58.8)	1.43	0.698
I	7 (20.6)	8 (23.5)		
II	2 (5.9)	4 (11.8)		
III	1 (2.9)	2 (5.9)		
IV	0 (0)	0 (0)		

NDP: Nedaplatin; DDP: Cisplatin.

not lower, or slightly higher than that of traditional CD-DP-based concurrent chemoradiotherapy regimen. With regard to adverse effects, the incidences of nausea and vomiting were significantly lower in the NDP group than in the DDP group (17.6% *vs* 50.0%, 11.8% *vs* 47.1%,  $P < 0.05$  for both). The majority of cases of nausea and vomiting in the NDP group were grades I - II and could be easily managed using antiemetic therapy with 5-HT<sub>3</sub> receptor antagonist, while the incidences of grades II - III nausea and vomiting were relatively high in the DDP group. The incidence of renal toxicity, mainly grades I - II, was significantly lower in the NDP group than in the DDP group (8.8% *vs* 38.2%,  $P < 0.05$ ). There was no significant difference in the incidence of liver toxicity between the two groups ( $P > 0.05$ ). The incidence of leukopenia, mainly grades I - II, was slightly higher in the NDP group than in the DDP group, but the difference was not statistically significant ( $P > 0.05$ ). The incidence of thrombocytopenia (grades I - II: 38.2%; grades III - IV: 17.6%) was also slightly higher in the NDP group. Thrombocytopenia occurred mainly 7 to 10 d after treatment and resolved in all cases 14 d after treatment. These results indicate that the incidence of gastrointestinal reactions such as nausea and vomiting was significantly lower in the NDP group. The liver and kidney toxicity was

mild. The main dose-limiting toxicity was myelosuppression, especially thrombocytopenia, which can be managed by symptomatic and supportive treatment or dosage adjustment.

In conclusion, NDP is an effective drug for treatment of esophageal carcinoma. NDP combined with 5-FU is superior to DDP plus 5-FU in terms of reducing the incidences of gastrointestinal and renal toxicity and improving clinical tolerance. Since the sample size is small in the present study, further large-sample trials are required to evaluate the long-term efficacy and toxicity of NDP-based regimens.

## COMMENTS

### Background

Radiotherapy given concurrently with chemotherapy can improve the efficacy of radiotherapy in esophageal carcinoma. Concurrent chemoradiotherapy has been recommended as the standard treatment for locally advanced esophageal carcinoma, and conventional fractionated radiotherapy plus cisplatin (DDP) and 5-fluorouracil (5-FU) has been advocated as a standard regimen for this malignancy. However, the risk of gastrointestinal and renal toxicity associated with DDP-based PF regimen (DDP + 5-FU) limits its use.

### Research frontiers

In the present study, the authors designed a randomized controlled phase II trial to compare the efficacy, acute adverse reactions and late toxicity of three-dimensional conformal radiotherapy plus nedaplatin (NDP) and 5-FU *vs* plus the PF regimen in the treatment of locally advanced esophageal carcinoma, with an aim to find a regimen that has fewer adverse reactions and better efficacy than the PF regimen.

### Innovations and breakthroughs

The survival and local control as well as the side effects during follow-up were analyzed by comparing with cisplatin. The authors found NDP-based concurrent chemoradiotherapy is effective and well-tolerated. Compared with DDP, NDP-based concurrent chemoradiotherapy exhibits favorable efficacy with lower toxicity.

### Applications

The study results suggest that NDP-based concurrent chemoradiotherapy is a potential therapeutic regimen that could be used in locally advanced esophageal carcinoma.

### Terminology

Cisplatin, a common chemotherapeutic drug, has been one of doctors' first lines of defense against tumors, especially those of the lung, ovary, testes and locally advanced esophageal carcinoma. Nedaplatin is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical



cal antitumor activity against various solid tumors with lower nephrotoxicity and gastrointestinal reactions.

# Peer review

This is a good clinical study in which the authors evaluated the efficacy and safety of three-dimensional conformal radiotherapy plus NDP and 5-FU versus plus the PF regimen in the treatment of locally advanced esophageal carcinoma. The results suggest that NDP-based concurrent chemoradiotherapy exhibits favorable efficacy with lower toxicity.

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## Endoscopic sphincterotomy plus large-balloon dilation vs endoscopic sphincterotomy for choledocholithiasis: A meta-analysis

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### Abstract

**AIM:** To perform a meta-analysis of large-balloon dilation (LBD) plus endoscopic sphincterotomy (EST) vs EST alone for removal of bile duct stones.

**METHODS:** Databases including PubMed, EMBASE, the Cochrane Library, the Science Citation Index, and important meeting abstracts were searched and evaluated by two reviewers independently. The main outcome measures included: complete stone removal, stone removal in the first session, use of mechanical lithotripsy, procedure time, and procedure-related complications. A fixed-effects model weighted by the Mantel-Haenszel method was used for pooling the odds ratio (OR) when heterogeneity was not significant among the studies. When a  $Q$  test or  $I^2$  statistic indicated substantial heterogeneity, a random-effects model weighted by the DerSimonian-Laird method was used.

**RESULTS:** Six randomized controlled trials involving 835 patients were analyzed. There was no significant heterogeneity for most results; we analyzed these using a fixed-effects model. Meta-analysis showed EST plus LBD caused fewer overall complications than EST alone (OR = 0.53, 95%CI: 0.33-0.85,  $P = 0.008$ ); sub-

category analysis indicated a significantly lower risk of perforation in the EST plus LBD group (Peto OR = 0.14, 95%CI: 0.20-0.98,  $P = 0.05$ ). Use of mechanical lithotripsy in the EST plus LBD group decreased significantly (OR = 0.26, 95%CI: 0.08-0.82,  $P = 0.02$ ), especially in patients with a stone size larger than 15 mm (OR = 0.15, 95%CI: 0.03-0.68,  $P = 0.01$ ). There were no significant differences between the two groups regarding complete stone removal, stone removal in the first session, post-endoscopic retrograde cholangiopancreatography pancreatitis, bleeding, infection of biliary tract, and procedure time.

**CONCLUSION:** EST plus LBD is an effective approach for the removal of large bile duct stones, causing fewer complications than EST alone.

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**Key words:** Balloon dilation; Cholangiopancreatography; Endoscopic retrograde; Choledocholithiasis; Endoscopic sphincterotomy; Meta-analysis

**Core tip:** This meta-analysis demonstrates that endoscopic sphincterotomy (EST) plus large-balloon dilation (LBD) is an effective approach for the removal of large bile duct stones. Specifically, when compared with the outcomes of EST alone, the combined technique is associated with fewer complications. Furthermore, use of mechanical lithotripsy in the EST plus LBD group decreased significantly, especially in patients with a stone size larger than 15 mm. However, more well-designed trials are required to clarify whether this combined technique is preferable.

Yang XM, Hu B. Endoscopic sphincterotomy plus large-balloon dilation vs endoscopic sphincterotomy for choledocholithiasis: A meta-analysis. *World J Gastroenterol* 2013; 19(48): 9453-9460

## INTRODUCTION

During endoscopic retrograde cholangiopancreatography (ERCP), endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) is the standard method of enlarging the papillary orifice before stone retrieval. However, the extent of orifice dilation with conventional EST or EPBD is limited<sup>[1-3]</sup>, and the use of other methods such as mechanical lithotripsy, intraductal shock-wave lithotripsy, extracorporeal shock-wave lithotripsy or, if those fail, biliary stent placement with repeated ERCP or even surgery may be required in patients with difficult (usually large) stones<sup>[1]</sup>. These methods are not widely available, and a larger opening of the orifice by large-balloon dilation (LBD) seems to be necessary. Ersoz *et al.*<sup>[4]</sup> first reported the use of LBD after sphincterotomy for large common bile duct stones and achieved a high stone clearance rate of up to 89%-95% without mechanical lithotripsy. Since then, a number of case series have also suggested that the combination technique facilitated large stone extraction and reduced dependence on mechanical lithotripsy, contributing to higher stone clearance in a single endoscopic session with an acceptable risk of complications<sup>[5-9]</sup>. However, the comparison of EST plus LBD and EST alone for removal of choledocholithiasis has given inconsistent results.

To the best of our knowledge, the only systematic review on the topic has been published by Liu *et al.*<sup>[10]</sup>. This included non-randomized controlled trials (non-RCTs); two eligible abstracts<sup>[11,12]</sup> which were regarded as non-randomized in the review were in fact randomized; this was validated by contacting the authors. More recently, a well-arranged trial has been published and some conflicting results have emerged<sup>[13]</sup>. Therefore, we believe that an updated meta-analysis is required.

## MATERIALS AND METHODS

### Search strategy

A literature search was performed to identify all relevant studies that compared EST plus LBD and EST alone for removal of bile duct stones. The PubMed, EMBASE, Cochrane Library databases, and the Science Citation Index were searched systematically for all articles published up to May 2013, without language restriction, using the following terms in their titles, abstracts, or keyword lists: "balloon dilation," "sphincteroplasty," "sphincterotomy," "bile duct stone," and "choledocholithiasis." The references in retrieved articles were also screened manually. The abstracts of the United European Gastroenterology Week and Digestive Disease Week, from 2004 to 2012, were also searched systematically. An attempt to contact the first author was made when information was not ex-

tractable from potentially eligible published abstracts.

### Study selection

Papers selected from this initial search were then screened for eligibility using the following criteria: (1) RCTs that evaluated a comparison of EST plus LBD (larger than 12 mm in balloon size) and EST alone in the removal of large common bile duct stones (larger than 10 mm in diameter); and (2) Outcomes of interest included complete stone removal, use of mechanical lithotripsy and complications. If reports came from the same study center, we only included data from the publication with the largest population. Comments, reviews, case reports, and guideline articles were excluded.

### Data extraction

Data from eligible studies were extracted independently by two reviewers (Yang XM and Hu B) using standard forms, and consensus was reached on all items. Data were extracted on: first author, year of publication, country of origin, study setting, number, age and sex of patients, stone size, balloon diameter, complete stone removal, stone removal in the first session, use of mechanical lithotripsy, procedure time, and procedure-related complications.

### Assessment of study quality

Two independent reviewers (Yang XM and Hu B) assessed the quality score of primary trials according to the Jadad scale<sup>[14]</sup>. Total scores ranged from 0 to 5. The Cochrane Collaboration's tool for assessing risk of bias was also used to address potential bias (Table 1). We defined studies with a Jadad score of 3 or more points and a low risk of bias as high quality in this meta-analysis. Disagreements were discussed by the reviewers and resolved through consensus.

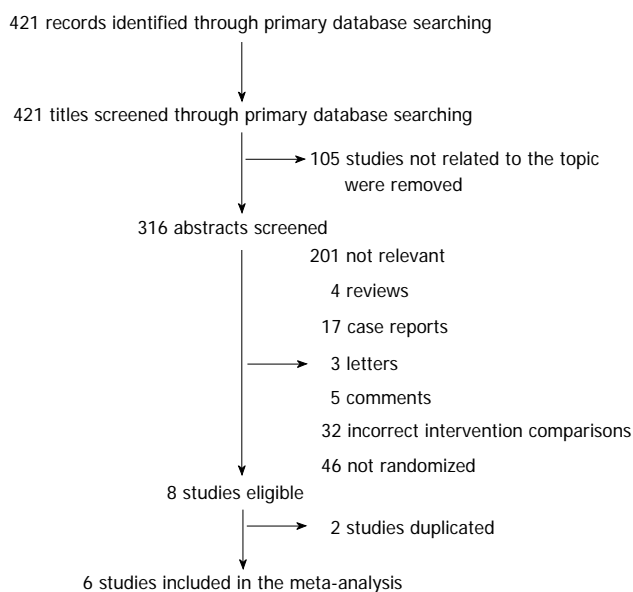
### Statistical analysis

For summary statistics in meta-analysis, the odds ratio (OR) is recommended for dichotomous data, and the weighted mean difference is recommended for continuous data. Complete stone removal, stone removal in the first session, use of mechanical lithotripsy and overall complications were summarized as OR with 95%CI. Peto OR with 95%CI was used for separate complications, including post-ERCP pancreatitis, bleeding, infection of biliary tract (including cholangitis and cholecystitis), and perforation, since it could generate the least biased pooled results of studies with zero event in both groups<sup>[15]</sup>. *P* values of less than 0.05 were considered significant.

Heterogeneity was assessed by visual inspection of a Forest plot, the Cochran *Q* test, and the *I*<sup>2</sup> statistic. Heterogeneity was considered significant by the Cochran *Q* test when *P* < 0.1 or *I*<sup>2</sup> > 50%<sup>[16,17]</sup>. A fixed-effects model weighted by the Mantel-Haenszel method was used for pooling the OR when heterogeneity was not significant among the studies<sup>[18]</sup>. When a *Q* test or *I*<sup>2</sup> statistic indicated substantial heterogeneity, a random-effects model

**Table 1** Characteristics of the included randomized controlled trials (according to the Cochrane Collaboration's tool for assessing risk of bias)

Ref.	Sequence generation	Allocation concealment	Blinding of participants	Incomplete outcome	Selective outcome	Other sources of bias
Heo <i>et al</i> <sup>[22]</sup> 2007	Computer random number generator	Sealed envelope	Outcome assessment blinded	No missing outcome data	All prespecified outcomes reported	No
Hong <i>et al</i> <sup>[11]</sup> 2009	Unclear	Not reported	Unclear	No missing outcome data	All prespecified outcomes reported	No
Kim <i>et al</i> <sup>[23]</sup> 2009	The order of the procedure	Not reported	Unclear	No missing outcome data	All prespecified outcomes reported	No
Kim <i>et al</i> <sup>[12]</sup> 2009	Unclear	Not reported	Unclear	No missing outcome data	All prespecified outcomes reported	No
Stefanidis <i>et al</i> <sup>[24]</sup> 2011	Random number table	Sealed envelope	Outcome assessment blinded	No missing outcome data	All prespecified outcomes reported	No
Teoh <i>et al</i> <sup>[13]</sup> 2013	Computer random number generator	Sealed envelope	Outcome assessment blinded	No missing outcome data	All prespecified outcomes reported	No

**Figure 1** Flow chart of included and excluded trials.

weighted by the DerSimonian-Laird method was used<sup>[19]</sup>. We performed a sensitivity analysis by removing each study in turn from the overall data to evaluate the influence of a single study on the pooled analysis and by restricting the meta-analysis to high-quality studies. We also assessed the potential for publication bias through visual inspection of funnel plot asymmetry and evaluated the statistical significance of differences according to the methods of Begg *et al*<sup>[20]</sup> and Egger *et al*<sup>[21]</sup>. Statistical analyses were performed using Review Manager software (version 5.1 for Windows, Cochrane Collaboration, Oxford, United Kingdom).

## RESULTS

### Identification of eligible studies

The literature search yielded 316 abstracts for review, and 308 were excluded for the reasons shown in Figure 1. The results of two studies were conflated because they were from the same trial. Thus, six studies<sup>[11-13,22-24]</sup> were

included, four of which were available as full texts and were high quality studies. The combined studies enrolled 835 patients who had been randomly allocated to the EST plus LBD group or the EST alone group. The characteristics of the included trials are listed in Tables 1 and 2, and the outcome data are shown in Table 3.

### Efficacy

Six studies reported complete stone removal. Heterogeneity among these studies was not significant ( $P = 0.28$ ,  $I^2 = 22\%$ , Figure 2A). Thus, we used the fixed-effects model and found that there was no significant difference in complete stone removal between EST plus LBD and EST alone (OR = 1.41, 95%CI: 0.63-3.17,  $P = 0.40$ , Figure 2A). Sensitivity analysis by removing each study in turn from the overall data or by restricting the meta-analysis to high-quality studies showed that the result was robust. Four RCTs<sup>[12,13,22,23]</sup> reported stone removal in the first session, and there was no significant difference in stone clearance between the two methods (OR = 1.02, 95%CI: 0.65-1.61,  $P = 0.92$ ). A comparison of EST plus LBD and EST alone in patients with stones larger than 15 mm was carried out, and five studies<sup>[11,13,22-24]</sup> with 377 patients were included. Meta-analysis showed that there was no significant difference in the complete stone removal rate according to the fixed-effects model (OR = 0.99, 95%CI: 0.35-2.81,  $P = 0.98$ , Figure 2B).

### Use of mechanical lithotripsy

Six studies reported the use of mechanical lithotripsy during the stone removal process. The trials were heterogeneous ( $P < 0.001$ ,  $I^2 = 87\%$ ), and a random-effects model analysis was performed. The results indicated a significantly reduced dependence on mechanical lithotripsy in the EST plus LBD group (OR = 0.26, 95%CI: 0.08-0.82,  $P = 0.02$ ). We conducted a sensitivity analysis by excluding the study by Stefanidis *et al*<sup>[24]</sup>, as no mechanical lithotripsy was used in the LBD group in this trial, and the result did not change (OR = 0.42, 95%CI: 0.18-0.98,  $P = 0.05$ ). However, after removing the two eligible abstracts<sup>[11,12]</sup>, there was no significant difference in the use of mechanical lithotripsy between EST plus



**Table 2** Characteristics of the included randomized controlled trials

Ref.	Format	Country	Center involved	EST plus LBD, EST				Balloon diameter (mm)	Jadad score
				Number (n)	Male, female	Mean age (yr)	Stone size (mm)		
Heo <i>et al</i> <sup>[22]</sup> 2007	Full text	Korea	1	100	48, 52	64	16.0 ± 0.7 <sup>1</sup>	12-20	4
Hong <i>et al</i> <sup>[11]</sup> 2009	Abstract	Korea	1	100	50, 50	63	15.0 ± 0.7 <sup>1</sup>	15 or 20	1
				70	Not reported	Not reported	> 15		
Kim <i>et al</i> <sup>[23]</sup> 2009	Full text	Korea	1	65	12, 15	70	> 15	15, 16.5 or 18	3
				27			15-38.3		
Kim <i>et al</i> <sup>[12]</sup> 2009	Abstract	Korea	1	28	14, 14	70	15-48	12-20	1
				104	53, 51	70	> 10		
Stefanidis <i>et al</i> <sup>[24]</sup> 2011	Full text	Greece	1	100	49, 51	69	> 10	15, 18 or 20	4
				45	24, 21	69	12-20		
Teoh <i>et al</i> <sup>[13]</sup> 2013	Full text	Hong Kong	2	45	22, 23	68	12-20	13-15	4
				73	32, 41	72	≥ 13		
				78	40, 38	73	≥ 13		

<sup>1</sup>Values are mean ± SD. EST: Endoscopic sphincterotomy; LBD: Large-balloon dilation.**Table 3** Outcome data derived from the included randomized controlled trials *n* (%)

Ref.	Intervention	Complete stone removal	Stone removal in the first session	Mechanical lithotripsy	Overall complications	Pancreatitis	Bleeding	Infection of biliary tract	Perforation
Heo <i>et al</i> <sup>[22]</sup> 2007	Small EST plus LBD	97/100 (97)	83/100 (83)	8/100 (8)	5/100 (5)	4/100 (4)	0/100 (0)	1/100 (1)	0/100 (0)
	Full EST	98/100 (98)	87/100 (87)	9/100 (9)	7/100 (7)	4/100 (4)	2/100 (2)	1/100 (1)	0/100 (0)
Hong <i>et al</i> <sup>[11]</sup> 2009	Small EST plus LBD	70/70 (100)	Not reported	13/70 (19)	8/70 (11)	4/70 (6)	4/70 (6)	0/70 (0)	0/70 (0)
	Conventional EST	65/65 (100)		47/65 (72)	19/65 (29)	9/65 (14)	10/65 (15)	0/65 (0)	0/65 (0)
Kim <i>et al</i> <sup>[23]</sup> 2009	Small EST plus LBD	27/27 (100)	23/27 (85)	9/27 (33)	0/27 (0)	0/27 (0)	0/27 (0)	0/27 (0)	0/27 (0)
	Conventional EST	28/28 (100)	23/28 (82)	9/28 (32)	0/28 (0)	0/28 (0)	0/28 (0)	0/28 (0)	0/28 (0)
Kim <i>et al</i> <sup>[12]</sup> 2009	Small EST plus LBD	100/104 (96)	89/104 (86)	8/104 (8)	11/104 (11)	10/104 (10)	1/104 (1)	0/104 (0)	0/104 (0)
	Conventional EST	92/100 (92)	82/100 (82)	17/100 (17)	10/100 (10)	9/100 (9)	0/100 (0)	0/100 (0)	1/100 (1)
Stefanidis <i>et al</i> <sup>[24]</sup> 2011	Full EST plus LBD	44/45 (98)	Not reported	0/45 (0)	2/45 (4)	1/45 (2)	1/45 (2)	0/45 (0)	0/45 (0)
	Full EST plus ML	41/45 (91)		45/45 (100)	9/45 (20)	1/45 (2)	1/45 (2)	6/45 (13)	1/45 (2)
Teoh <i>et al</i> <sup>[13]</sup> 2013	Small EST plus LBD	71/73 (97)	65/73 (89)	21/73 (29)	5/73 (7)	2/73 (3)	1/73 (1)	2/73 (3)	0/73 (0)
	Full EST	78/78 (100)	69/78 (88)	36/78 (46)	8/78 (10)	3/78 (4)	0/78 (0)	3/78 (4)	2/78 (3)

EST: Endoscopic sphincterotomy; LBD: Large-balloon dilation.

LBD and EST alone (OR = 0.26, 95%CI: 0.05-1.48, *P* = 0.13). A subgroup analysis in patients with a stone size larger than 15 mm demonstrated that the use of mechanical lithotripsy in the EST plus LBD group decreased significantly (OR = 0.15, 95%CI: 0.03-0.68, *P* = 0.01, Figure 3A).

### Safety

Six RCTs evaluated the safety in both groups (Table 4). The statistical results showed that EST plus LBD caused fewer overall complications than EST alone (OR = 0.53, 95%CI: 0.33-0.85, *P* = 0.008), and the result did not change by restricting the meta-analysis to the four high-quality studies (OR = 0.48, 95%CI: 0.24-0.99, *P* = 0.05). Subcategory analysis indicated that patients undergoing

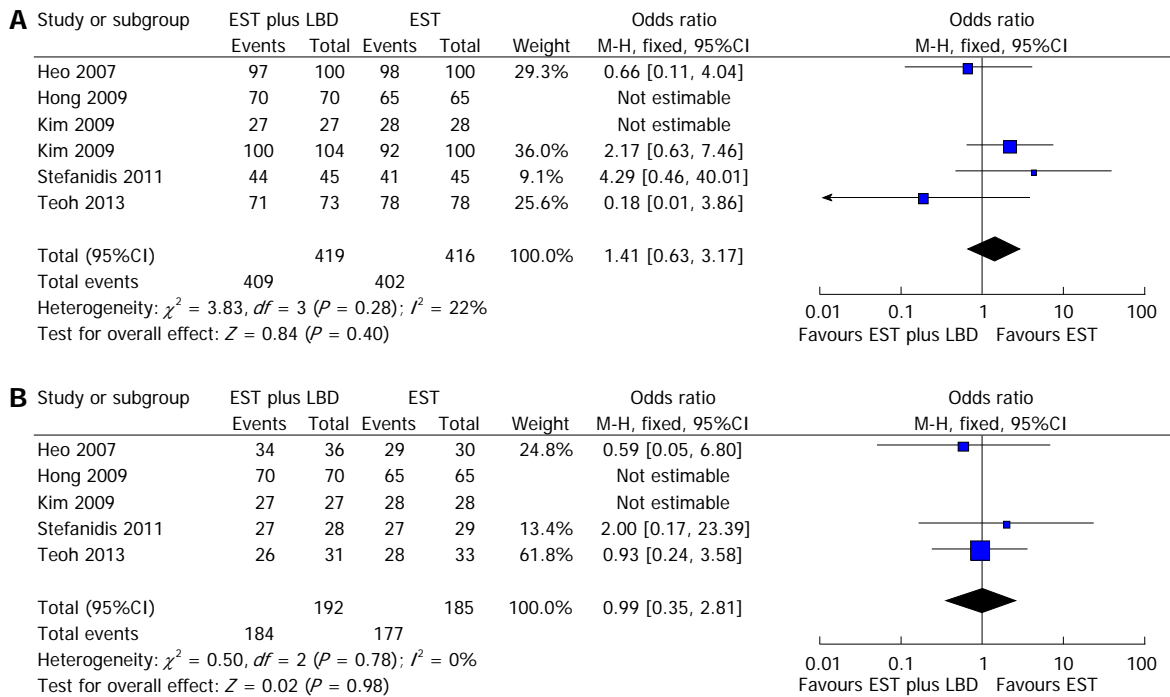
EST plus LBD had a lower risk of perforation (OR = 0.14, 95%CI: 0.20-0.98, *P* = 0.05). No significant difference was found in terms of post-ERCP pancreatitis (OR = 0.77, 95%CI: 0.43-1.39, *P* = 0.39), bleeding (OR = 0.50, 95%CI: 0.20-1.23, *P* = 0.13), and infection of the biliary tract (OR = 0.34, 95%CI: 0.11-1.02, *P* = 0.05).

### Procedure time

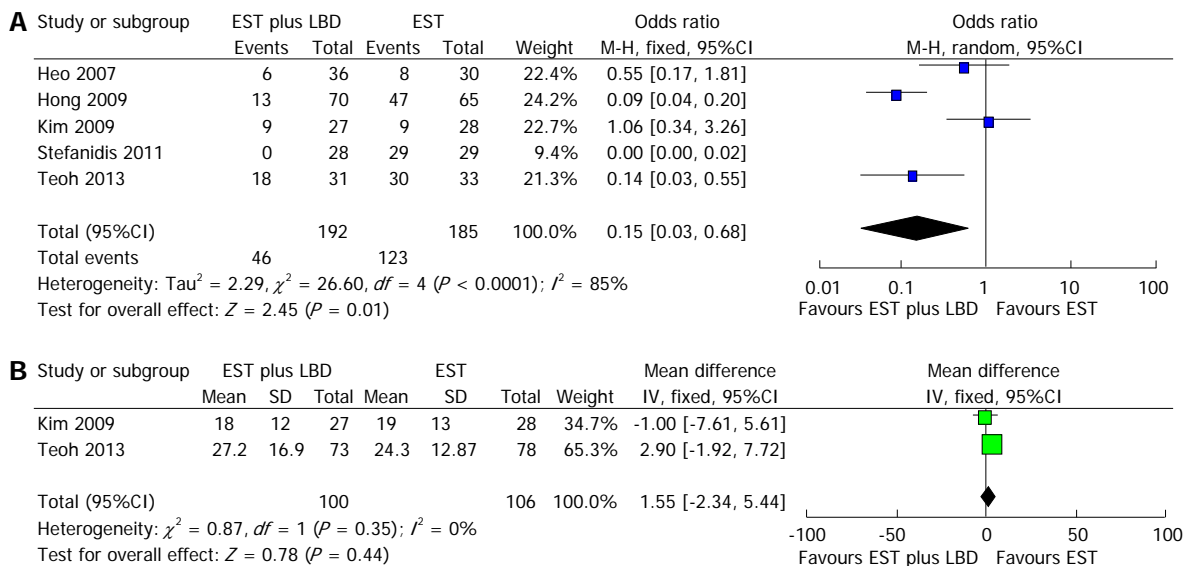
Only two studies reported the total procedure time<sup>[13,23]</sup>. Meta-analysis showed no difference in ERCP duration between EST plus LBD and EST alone (OR = 1.55, 95%CI: -2.34-5.44, *P* = 0.44, Figure 3B).

### Publication bias

The funnel plot did not show an asymmetrical pattern



**Figure 2** Forest plot demonstrating no significant difference in complete stone removal between endoscopic sphincterotomy plus large-balloon dilation and endoscopic sphincterotomy alone and in patients with stone size larger than 15 mm. A: Endoscopic sphincterotomy (EST) plus large-balloon dilation (LBD) and EST alone; B: EST plus LBD and EST alone and in patients with stone size larger than 15 mm.



**Figure 3** Forest plot demonstrating. A: The use of mechanical lithotripsy in the endoscopic sphincterotomy (EST) plus large-balloon dilation (LBD) group decreased significantly in patients with stone size larger than 15 mm; B: No significant difference in procedure time between EST plus LBD and EST alone.

(Figure 4). In addition, neither the Begg test nor the Egger test revealed significant publication bias ( $P = 0.148$  and  $P = 0.426$ , respectively).

## DISCUSSION

We performed this meta-analysis mainly to investigate whether EST plus LBD was feasible and safe for the removal of large stones. Theoretically, a large enough opening to the papilla may facilitate the extraction of large

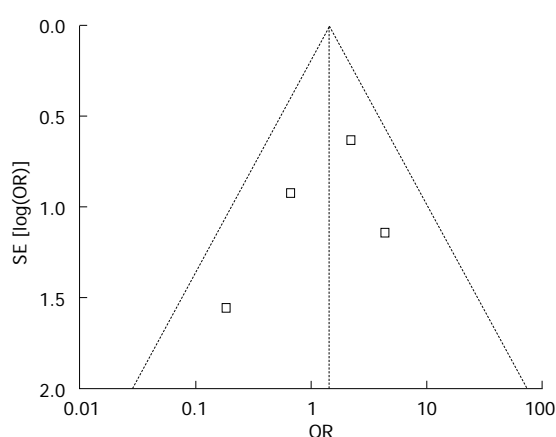
bile duct calculi. Our meta-analysis suggested that EST plus LBD achieved an equivalent success rate in stone clearance to that of EST alone. The use of mechanical lithotripsy in the EST plus LBD group decreased significantly, especially in patients with a stone size larger than 15 mm. Mechanical lithotripsy is a challenging technique and may create many stone fragments that are then difficult to clear<sup>[25]</sup>, thus it is worth reducing dependence on mechanical lithotripsy.

Recent data has suggested that LBD does not cause

**Table 4** Analyses of procedure-related complications *n* (%)

Complications	All 6 studies					Four full-text studies (high-quality)				
	Incidence EST plus LBD, EST	OR/Peto OR (95%CI)	P value	Heterogeneity $I^2$	P value	Incidence EST plus LBD, EST	OR/Peto OR (95%CI)	P value	Heterogeneity $I^2$	P value
Overall	31/419 (7.4) 53/416 (12.7)	0.53 (0.33-0.85)	0.008	28%	0.24	12/245 (4.9) 24/251 (9.6)	0.48 (0.24-0.99)	0.05	0%	0.37
Pancreatitis	21/419 (5.0) 26/416 (6.3)	0.77 (0.43-1.39)	0.39	0%	0.74	7/245 (2.9) 8/251 (3.2)	0.89 (0.32-2.49)	0.83	0%	0.95
Bleeding	7/419 (1.7) 13/416 (3.1)	0.50 (0.20-1.23)	0.13	22%	0.27	2/245 (0.8) 3/251 (1.2)	0.68 (0.12-3.93)	0.66	31%	0.24
Infection of biliary tract	3/419 (0.7) 10/416 (2.4)	0.34 (0.11-1.02)	0.05	28%	0.25	3/245 (1.2) 10/251 (4.0)	0.34 (0.11-1.02)	0.05	28%	0.25
Perforation	0/419 (0.0) 4/416 (1.0)	0.14 (0.02-0.98)	0.05	0%	1.00	0/245 (0.0) 3/251 (1.2)	0.14 (0.01-1.35)	0.09	0%	0.98

EST: Endoscopic sphincterotomy; LBD: Large-balloon dilation.

**Figure 4** Funnel plot did not show publication bias.

serious complications such as severe pancreatitis and bile duct perforation if performed strictly under established guidelines<sup>[5,6,22]</sup>. Similarly, the current meta-analysis demonstrated that the incidence of overall complications was significantly lower in the EST plus LBD group. When standard EST is performed to remove large stones, a full or large incision may be made, possibly leading to bleeding or perforation. Our review showed that perforation occurred in four patients in the EST alone group, and in none in the EST plus LBD group. Furthermore, bleeding was rarer when balloon dilation was performed (1.7% *vs* 3.1%) after limited sphincterotomy, although no significant difference was observed. We presume that this may be due to the small incision made before LBD.

Many concerns have been raised about post-ERCP pancreatitis with increasing balloon size, especially for those over 15 mm. However, our meta-analysis showed that LBD did not increase pancreatitis. Theoretically, the initial sphincterotomy may orientate the direction of subsequent dilation, leading to a resultant tear away from the pancreatic orifice, which might decrease the risk of pancreatitis. Post-ERCP pancreatitis may also be associated with other factors such as cannulation time and stone removal time. Only two studies reported the total procedure time<sup>[13,23]</sup>, and meta-analysis showed no difference in ERCP duration between the two groups. We cannot

estimate the effect of procedure duration on the risk of pancreatitis.

Only the study by Teoh *et al.*<sup>[13]</sup> compared the direct cost of the procedures between the two groups. A significant reduction in overall cost was noted in the EST plus LBD group [USD \$5025 (interquartile range, \$4140-\$5235) *vs* \$6005 (interquartile range, \$4462-\$5441), *P* = 0.034]. Whether this combined technique is less expensive requires clarification by conducting further trials.

Our findings are similar to those of the previous meta-analysis by Liu *et al.*<sup>[10]</sup>. This previous meta-analysis included three RCTs<sup>[22,23,26]</sup>, and summarized the results of RCTs and non-RCTs separately. One trial included in the previous meta-analysis which performed dilation using a small (8 mm) balloon<sup>[26]</sup> was excluded in our review. A well-arranged trial was excluded in the previous meta-analysis because mechanical lithotripsy was used in all the patients in the EST group, but in none of the patients in the EST plus LBD group<sup>[24]</sup>, which did not accurately reflect the use of mechanical lithotripsy. We conducted a sensitivity analysis by excluding this study, and the result did not change. By contacting the authors, we found two eligible abstracts<sup>[11,12]</sup> regarded as non-randomized in the previous meta-analysis, which were in fact randomized. Furthermore, our meta-analysis included a recently published well-designed trial by Teoh *et al.*<sup>[13]</sup>. The previous meta-analysis showed a significant reduction in the use of mechanical lithotripsy and overall complications for non-RCTs, but not for RCTs. However, our meta-analysis showed that EST plus LBD caused fewer overall complications than EST alone, and the result did not change by restricting the meta-analysis to high-quality studies. In addition, our meta-analysis showed that the use of mechanical lithotripsy in the EST plus LBD group decreased significantly, especially in patients with a stone size larger than 15 mm.

This meta-analysis also has some limitations. Firstly, it included two low-quality trials. It has been well documented that in RCTs and meta-analyses, low-quality studies are vulnerable to bias and may lead to exaggerated results. However, subgroup analysis of high-quality studies was also significant, which strengthened the results.

Secondly, only a few studies were included, which might decrease the robustness of the analysis and mask publication bias. Our meta-analysis showed that the significant reduction in perforations in the EST plus LBD group was marginal ( $P = 0.05$ ), this was probably attributable to the small number of subjects with perforation ( $n = 4$ , all in the EST alone group).

In conclusion, large-balloon dilation following limited sphincterotomy appears to be an effective approach for large stone extraction. This method may cause fewer complications and reduce dependence on mechanical lithotripsy. However, it warrants more well-designed studies to clarify whether this combined technique is outweighed.

## COMMENTS

### Background

Endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) is the standard method for stone retrieval. However, the extent of orifice dilation with conventional EST or EPBD is limited, and the use of other methods, such as mechanical lithotripsy, may be required in patients with large stones. A larger opening of the orifice by large-balloon dilation (LBD) may facilitate stone removal. For the past few years, LBD following limited EST appears to be an alternative to EST alone for removing large bile duct stones. However, which one is predominant remains controversial.

### Research frontiers

The current meta-analysis was carried out to comparatively assess LBD plus EST and EST alone for removal of large bile duct stones. The main outcome measurements included complete stone removal, stone removal in first session, use of mechanical lithotripsy, procedure time, and procedure-related complications.

### Innovations and breakthroughs

The current meta-analysis demonstrated that EST plus LBD is an effective approach for the removal of large bile duct stones, causing fewer complications than EST alone. Furthermore, this combined technique may decrease dependence on mechanical lithotripsy during stone extraction.

### Applications

The results from this meta-analysis suggest that LBD following limited EST is an effective alternative to EST alone for removing large bile duct stones, warranting routine clinical use.

### Peer review

This is an interesting and well performed meta-analysis addressing the efficacy of EST plus LBD vs EST alone for removal of bile duct stones. The research design is solid, and its results have clinical relevancy as they demonstrate that EST plus LBD decreases complications and the usage of mechanical lithotripsy.

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## *TNF- $\alpha$ -308* polymorphism and risk of digestive system cancers: A meta-analysis

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### Abstract

**AIM:** To evaluate the association between the tumour necrosis factor alpha-308 (*TNF- $\alpha$ -308*) gene polymorphism and the risk of digestive system cancers.

**METHODS:** All eligible case-control studies published up to December 2012 were identified by searching PubMed, Web of Science, Embase and China National Knowledge Internet without language restrictions. The risk of digestive system cancers associated with the *TNF- $\alpha$ -308* polymorphism was estimated for each study using odds ratio (OR) together with its 95%CI, respectively. Cochrane Collaboration RevMan 5.1 was used to perform the analysis. A  $\chi^2$ -test-based *Q* statistic test and an *I*<sup>2</sup> test were performed to assess the between-study heterogeneity. When the *Q* test was significant (*P* < 0.05) or *I*<sup>2</sup> > 50%, the random effects model was used, otherwise the fixed effects model was used.

**RESULTS:** Fifty-eight studies from fifty-five publications with a total of 9986 cancer patients and 15511

healthy controls were included. Overall, a significant association was found between the *TNF- $\alpha$ -308* polymorphism and the risk of digestive system cancers [dominant model: OR = 1.23, 95%CI: 1.09-1.39, (G/A) vs (G/G): OR = 1.15, 95%CI: 1.02-1.28, (A/A) vs (G/G): OR = 1.44, 95%CI: 1.19-1.73, recessive model: OR = 1.38, 95%CI: 1.15-1.66]. Furthermore, when the analysis was stratified by ethnicity, similar results were observed in both the Asian and Caucasian populations, except for the dominant model and heterozygote comparisons in the Asian population [dominant model: OR = 1.24, 95%CI: 0.99-1.56, (G/A) vs (G/G): OR = 1.09, 95%CI: 0.96-1.24]. When the cancer type subgroups were examined, similar results were detected in gastric and hepatocellular carcinomas; however, no significant association was observed among other digestive system cancers.

**CONCLUSION:** The *TNF- $\alpha$ -308* gene polymorphism may be significantly associated with the risk of gastric and hepatocellular carcinomas, but not colorectal, pancreatic, or oesophageal cancer, in the Asian population.

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**Key words:** Tumour necrosis factor alpha; rs1800629; Polymorphism; Digestive system cancer; Meta-analysis; Association

**Core tip:** Genetic polymorphisms contribute to the risk of human malignant tumours. Many studies have reported the relationship between the tumour necrosis factor alpha-308 (*TNF- $\alpha$ -308*) gene polymorphism and risk of digestive system cancers. However, the results of these studies are inconsistent and contradictory. In this meta-analysis, our results suggest that the *TNF- $\alpha$ -308* polymorphism is significantly associated with the risk of gastric and hepatocellular carcinomas in the Asian

population (dominant model: 95%CI: 1.02-1.34,  $P < 0.05$  and 95%CI: 1.20-2.54,  $P < 0.05$ , respectively). This finding indicates that certain polymorphisms and mutations at *TNF- $\alpha$ -308* may increase susceptibility to digestive system cancers.

Guo XF, Wang J, Yu SJ, Song J, Ji MY, Cao Z, Zhang JX, Wang J, Dong WG. *TNF- $\alpha$ -308* polymorphism and risk of digestive system cancers: A meta-analysis. *World J Gastroenterol* 2013; 19(48): 9461-9471 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9461.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9461>

## INTRODUCTION

Digestive system cancers are the most common malignant tumours worldwide, with 3.4 million new cases each year, and their mortality rates have increased gradually over the past decade<sup>[1,2]</sup>. Molecular epidemiology has confirmed that carcinogenesis is a complex, multifactorial and multistep event, in which the interaction of environmental triggers and genetic susceptibility may play an important role. However, the exact mechanism of carcinogenesis is still not fully understood.

Tumour necrosis factor- $\alpha$  (*TNF- $\alpha$* ), which is mainly produced by macrophages, is a multifunctional cytokine that plays an important role in the pathogenesis of inflammatory, autoimmune, and malignant diseases<sup>[3]</sup>. The *TNF- $\alpha$*  gene is located in the major histocompatibility complex class III region on the short arm of chromosome six. Several polymorphisms in the promoter region of the *TNF- $\alpha$*  gene have been identified and are implicated in the regulation of *TNF- $\alpha$*  transcription<sup>[4-5]</sup>. The *TNF- $\alpha$ -308* polymorphism (rs1800629) is the most extensively studied polymorphism in digestive system cancers<sup>[6-9]</sup>. However, the results of the studies on *TNF- $\alpha$ -308* have been inconclusive or inconsistent. Therefore, we conducted a meta-analysis to evaluate the association between the *TNF- $\alpha$ -308* polymorphism and susceptibility to digestive system cancers.

## MATERIALS AND METHODS

### Search strategy

A literature search was conducted using PubMed, Web of Science, Embase and CNKI for studies that were published up to December 2012 without language restrictions. The relevant studies were identified using the following terms: ["tumour necrosis factor alpha or *TNF* alpha or *TNF- $\alpha$* "] AND ["genetic polymorphism or polymorphisms or variant"] AND ["digestive system cancer or gastric cancer or colorectal cancer or hepatocellular carcinoma or pancreatic cancer or oesophageal cancer"]. The search was restricted to humans. Additional studies were identified by a manual search of references of original or review articles on this topic. If more

than one cancer type was reported in one study, the data for each type was extracted separately. If data or data subsets were published in more than one article, only the publication with the largest sample size was included.

### Inclusion and exclusion criteria

Studies were included if they met the following criteria: (1) studies that evaluated the association between the *TNF- $\alpha$ -308* polymorphism and digestive system cancer risk; (2) studies with a case-control study design; and (3) studies with detailed genotype frequencies for cases and controls or text that allowed for the calculation of these values. The major exclusion criteria were: (1) case-only studies, case reports, or review articles; (2) studies without raw data for the *TNF- $\alpha$ -308G/A* genotype; and (3) studies that compared the *TNF- $\alpha$ -308G/A* variants in precancerous lesions and other cancers.

### Data extraction and quality assessment

Two investigators (Guo XF and Wang J) independently extracted the data and reached a consensus on each item. If the two investigators generated different results, they would check the data again and have a discussion to come to an agreement. If they could not reach an agreement, an expert (Dong WG) was invited to the discussion. The data extracted from the selected articles included the first author's name, year of publication, country of origin, ethnicity, cancer type, genotyping methods, and number of cases and controls. The ethnicities were categorised as Asian or Caucasian. The cancer types were categorised as gastric, colorectal, hepatocellular, pancreatic, or oesophageal.

### Statistical analysis

The meta-analysis was performed using the Cochrane Collaboration RevMan 5.1 software (Copenhagen, 2008). The association between the risk of digestive system cancers and the *TNF- $\alpha$ -308* polymorphism was estimated for each study using the odds ratio (OR) and 95%CI. A  $\chi^2$  test-based calculation of the  $Q$  statistic was performed to assess the between-study heterogeneity<sup>[10]</sup>. We also quantified the effect of heterogeneity with an  $I^2$  test. When the  $Q$  test was significant ( $P < 0.05$ ) or  $I^2 > 50\%$ , indicating heterogeneity across studies, the random effects model was used<sup>[11]</sup>; otherwise, the fixed effects model was used<sup>[12]</sup>. Before estimating the relationship between the *TNF- $\alpha$ -308* polymorphism and digestive system cancer risk, we tested whether the genotype frequencies of the controls were in Hardy-Weinberg equilibrium (HWE) using a  $\chi^2$  test. We first estimated this relationship with the dominant model [*G/A* (GA) + *A/A* (AA) vs *G/G* (GG)] and the recessive model (AA vs GA + GG) and then with the co-dominant model (GA vs GG and AA vs GG). To evaluate the ethnicity-specific and cancer type-specific effects, we performed stratification analyses with respect to ethnicity and cancer type. Sensitivity analysis was performed to evaluate the stability of the results. Funnel plots were used to evaluate publication bias.

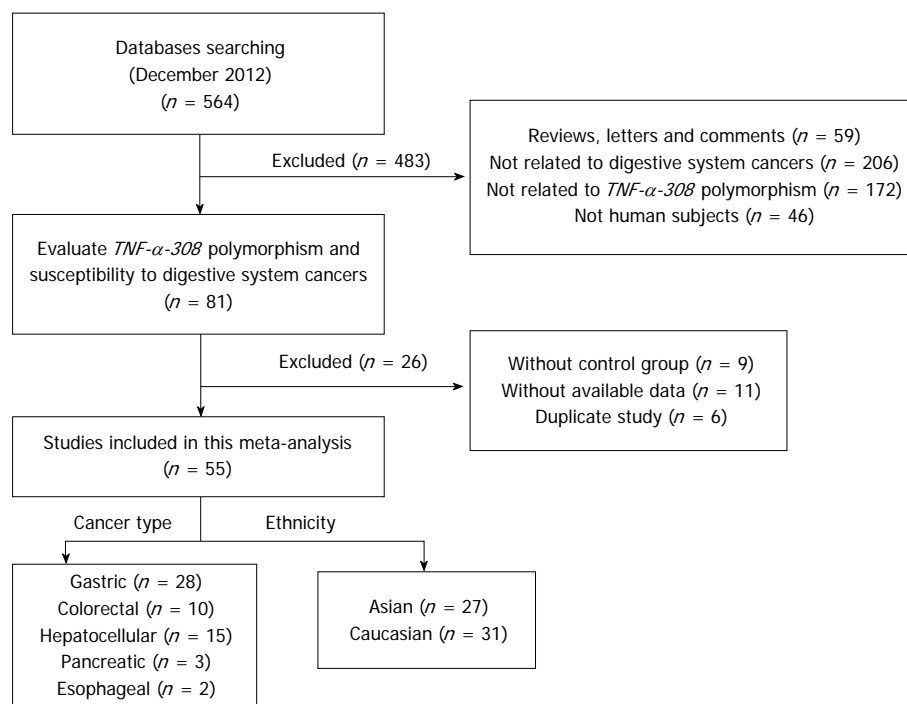


Figure 1 Flow chart showing study selection procedure. TNF- $\alpha$ : Tumour necrosis factor- $\alpha$ .

## RESULTS

### Study characteristics

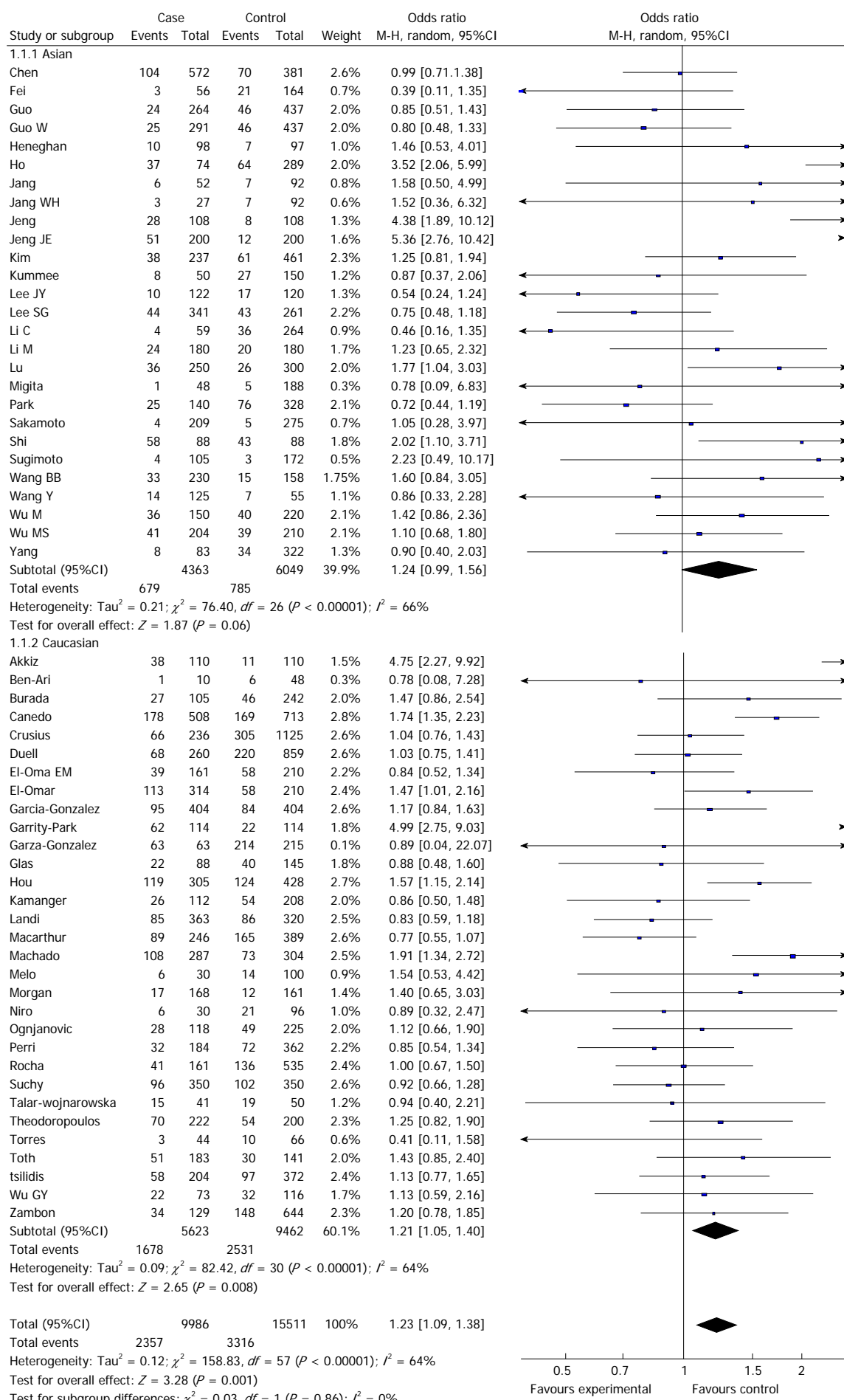
The search strategy retrieved 564 potentially relevant studies. According to the inclusion criteria, 55 studies with full-text were included in this meta-analysis and 509 studies were excluded. A flow chart of the study selection is shown in Figure 1. Because the studies of El-Omar *et al.*<sup>[9]</sup>, Guo *et al.*<sup>[13]</sup> and Jang *et al.*<sup>[14]</sup> each included separate analyses of two cancer types, we treated them separately in this meta-analysis<sup>[9,13,14]</sup>. Therefore, as shown in Table 1, there were 58 case-control studies from 55 publications on the *TNF- $\alpha$ -308* polymorphism with a total of 9986 cancer cases and 15511 controls. Two ethnicities were addressed: 27 studies focused on Asian populations, and 31 studies focused on Caucasian populations. Five cancer types were addressed: 28 studies focused on gastric cancer<sup>[6-9,13-36]</sup>, 10 studies on colorectal cancer<sup>[14,37-45]</sup>, 15 studies on hepatocellular carcinoma<sup>[46-60]</sup>, 3 studies on pancreatic cancer<sup>[61-63]</sup>, and 2 studies on oesophageal cancer<sup>[9,13]</sup>. The genotype distribution in the controls was consistent with HWE for all of the selected studies, except for four studies on gastric cancer<sup>[7,13,33-34]</sup>, one study on colorectal cancer<sup>[43]</sup>, six studies on hepatocellular carcinoma<sup>[47,49,51-52,55-56]</sup>, and one study on esophageal cancer<sup>[13]</sup>.

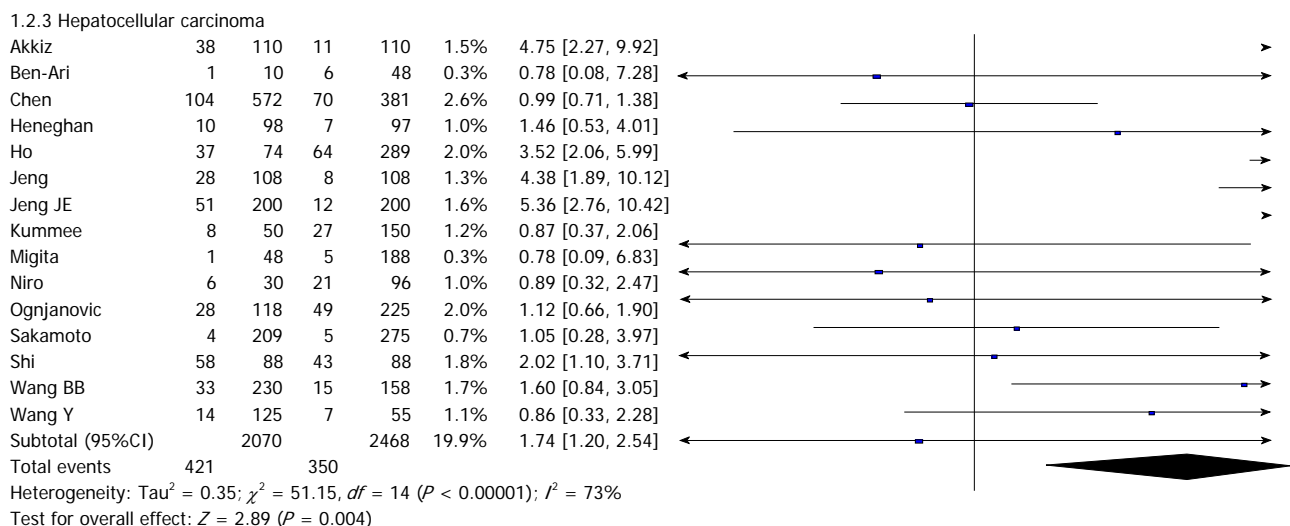
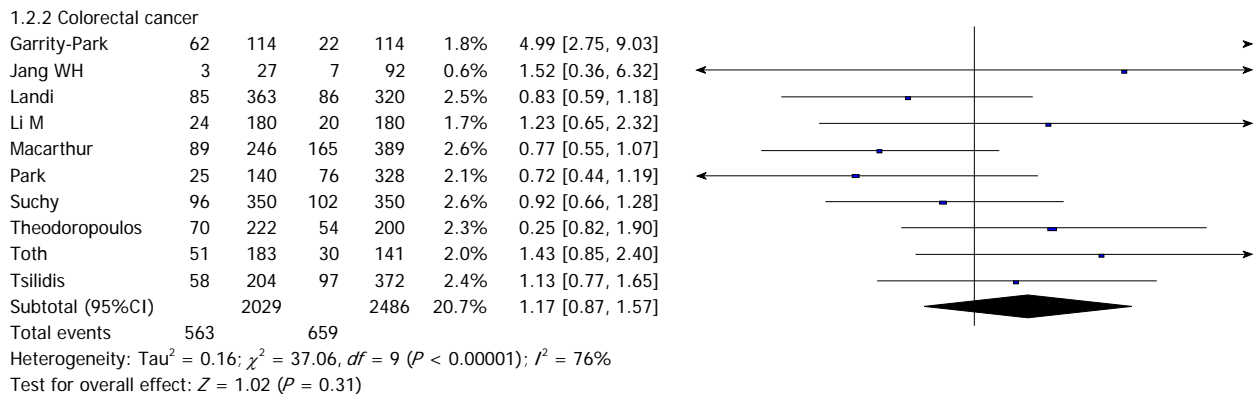
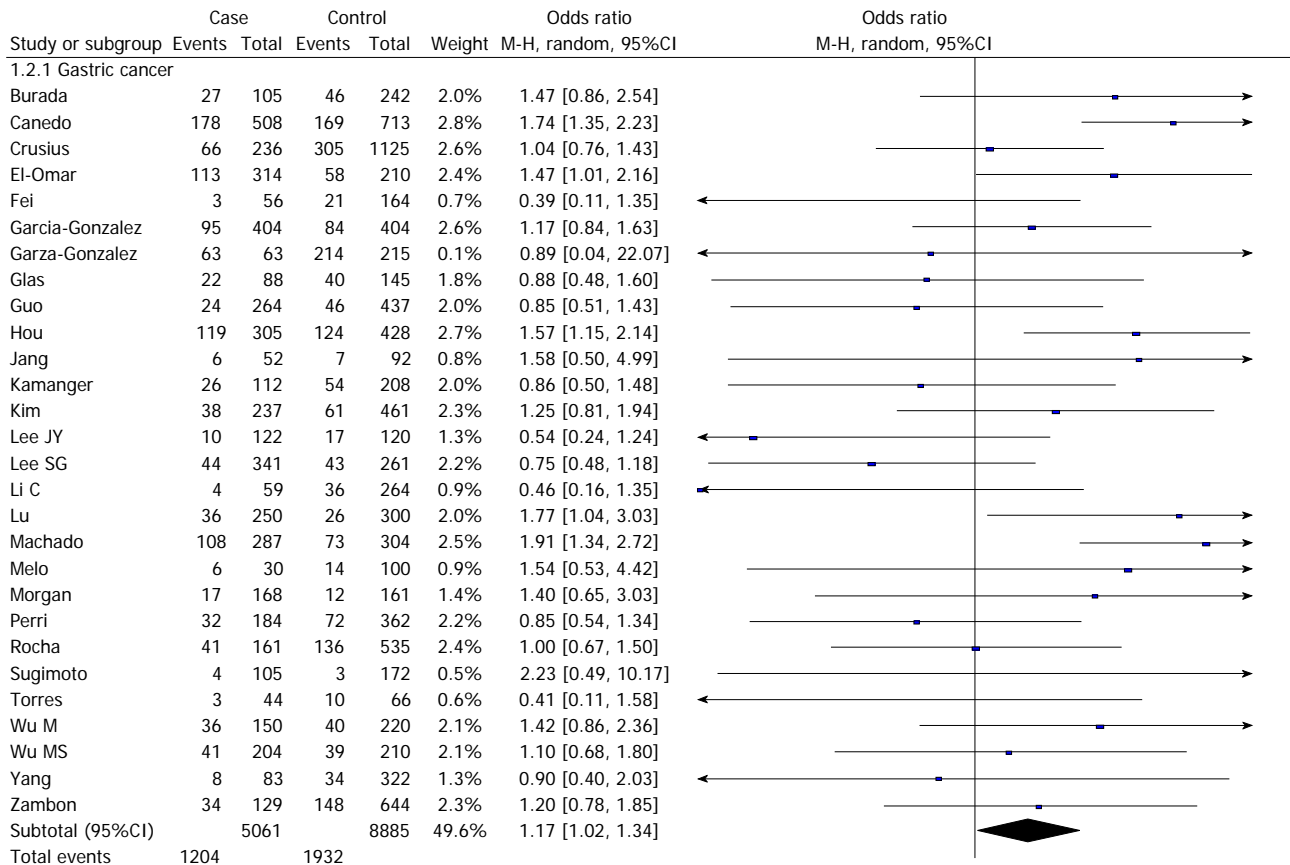
### Quantitative data synthesis

Overall, there was a significant difference in the *TNF- $\alpha$ -308G/A* genotype distribution between the digestive system cancer patients and the controls (dominant model: OR = 1.23, 95%CI: 1.09-1.39,  $P < 0.00001$ ; GA *vs* GG: OR = 1.15, 95%CI: 1.02-1.28,  $P < 0.0001$ ; AA *vs* GG: OR = 1.44, 95%CI: 1.19-1.73,  $P = 0.23$ ; recessive model:

OR = 1.38, 95%CI: 1.15-1.66,  $P = 0.50$ ) (Table 2, Figure 2). In the analysis of the ethnic subgroups, similar results were observed in the Caucasian population; but in the Asian population, we found that there was no significant association between the *TNF- $\alpha$ -308* polymorphism and the risk of digestive system cancers in the dominant model and heterozygote comparisons (GA + AA *vs* GG: OR = 1.24, 95%CI: 0.99-1.56, GA *vs* GG: OR = 1.09, 95%CI: 0.96-1.24) (Table 2, Figure 2). When stratified by cancer type, similar results were detected for gastric and hepatocellular carcinomas; however, no significant association was observed among the other digestive system cancer types (Table 2, Figure 3). Furthermore, we found that there was significant heterogeneity for the dominant model and heterozygote comparisons both overall and in the stratified analyses:  $I^2 = 64\%$  and  $52\%$  in the overall population,  $I^2 = 66\%$  and  $45\%$  ( $P = 0.008$ ) in the Asian population,  $I^2 = 64\%$  and  $58\%$  in the Caucasian population,  $I^2 = 76\%$  and  $70\%$  in colorectal cancer, and  $I^2 = 73\%$  and  $66\%$  in hepatocellular carcinoma. In addition, there was evidence of heterogeneity in gastric cancer (dominant model:  $P = 0.009$ ). Thus, the random effects model was employed in the OR calculations. Then, sensitivity analyses were conducted to determine whether modification of the inclusion criteria of the meta-analysis affected the final results. We examined the influence of these studies on the pooled OR by repeating the meta-analysis while excluding the study that was not in HWE. The estimated pooled OR did not show a significant change (Table 2), indicating that our results are statistically robust. The shapes of the funnel plots did not reveal any evidence of asymmetry, suggesting that there was no publication bias among the studies (Figure 4).



Figure 2  
Subgroup analysis of tumour necrosis factor  $\alpha$ -308 polymorphism by ethnicity (dominant model).



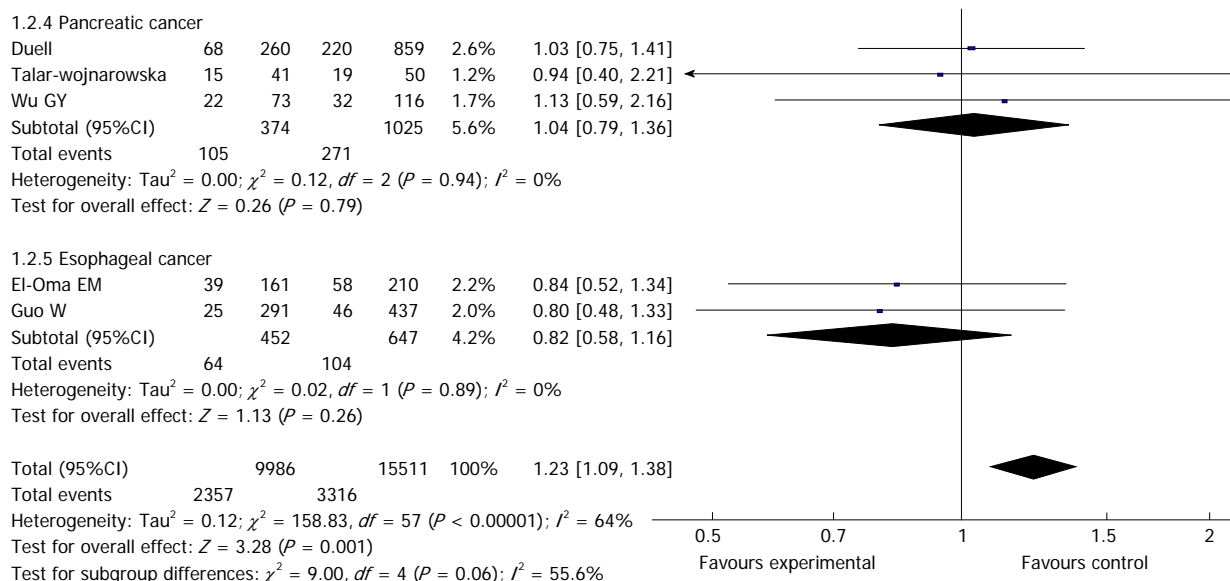
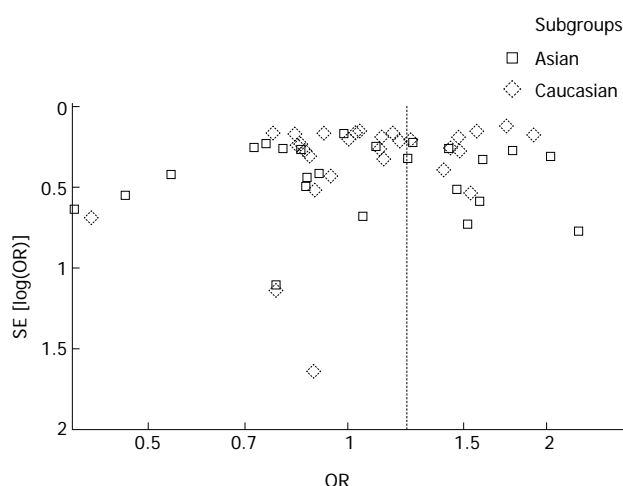
Figure 3 Subgroup analysis of tumor necrosis factor  $\alpha$ -308 polymorphism by cancer type (dominant model).

Figure 4 Funnel plots analysis to detect publication bias. Each point represents an independent study for the indicated association.

## DISCUSSION

TNF, an important pro-inflammatory cytokine, plays an important role in the regulation of cell differentiation, proliferation and death as well as in inflammation and the innate and adaptive immune response. TNF has also been implicated in a wide variety of human diseases. The presence of DNA sequence variations in the regulatory region might interfere with transcription of the *TNF* gene, influencing the circulating level of TNF and thus increasing susceptibility to human diseases, such as cancer<sup>[64]</sup>. The TNF enhancer polymorphism has been implicated in several diseases, and the *TNF- $\alpha$ -308* polymorphism has been described as the most important TNF polymorphism in human disease susceptibility. The significance of these polymorphisms reflects their possible influence on the transcription of the *TNF* gene. However, the results of studies in this area are inconsistent. Canedo *et al*<sup>[7]</sup> found

that the *TNF- $\alpha$ -308G/A* polymorphism increases the risk of gastric carcinoma. However, some studies have reported that no statistically significant association exists between the *TNF- $\alpha$ -308G/A* polymorphism and cancer risk<sup>[14,20]</sup>.

The current meta-analysis, which included 58 case-control studies and 25497 subjects, was conducted to explore the association of the *TNF- $\alpha$ -308* polymorphism with digestive system cancer risk. Overall, a significant association was identified between the *TNF- $\alpha$ -308* polymorphism and the risk of digestive system cancers. When the analysis was stratified by ethnicity, we found a statistically significant association between this polymorphism and the risk of these cancers in the Caucasian population. However, no significant association was observed in the dominant model and heterozygote comparisons in the Asian population, which could be due to ethnic differences. When the analysis was stratified by cancer type, we found a significant association between this polymorphism and gastric and hepatocellular carcinoma risk under all four genetic models, but no significant association was observed among colorectal, pancreatic or oesophageal cancer.

Heterogeneity is a potential problem when interpreting the results of meta-analyses. In this meta-analysis, heterogeneity was found in the dominant model and heterozygote comparisons in both the overall and subgroup analyses; thus, the random effects model was used. Sensitivity analyses were also conducted by excluding the study that was not in HWE. With this exclusion, the estimated pooled OR did not change significantly, strengthening our confidence in our results. This finding suggests that the population selection and the study that was not in HWE were not sources of heterogeneity. Alternatively, lifestyle, environment and other unknown factors may be sources of heterogeneity. Moreover, no publication bias was shown, suggesting that our results

Table 1 Characteristics of studies included in the meta-analysis

Ref.	Year	Country	Ethnicity	Cancer type	Genotyping method	Case				Control				P
						Total	GG	GA	AA	Total	GG	GA	AA	
Burada <i>et al</i> <sup>[6]</sup>	2012	Romania	Caucasian	Gastric	TaqMan	105	78	26	1	242	196	44	2	0.78
Canedo <i>et al</i> <sup>[7]</sup>	2008	Portugal	Caucasian	Gastric	TaqMan	508	330	178 <sup>1</sup>		713	544	169 <sup>1</sup>		NA
Crusius <i>et al</i> <sup>[8]</sup>	2008	Spain	Caucasian	Gastric	Real-time PCR	236	170	64	2	1125	820	274	31	0.17
El-Omar <i>et al</i> <sup>[9]</sup>	2003	United States	Caucasian	Gastric	TaqMan	314	201	87	26	210	152	52	6	0.55
Guo <i>et al</i> <sup>[13]</sup>	2005	China	Asian	Gastric	PCR-RFLP	264	240	20	4	437	391	40	6	<0.01
Jang <i>et al</i> <sup>[14]</sup>	2001	South Korea	Asian	Gastric	PCR-RFLP	52	46	4	2	92	85	7	0	0.70
Fei <i>et al</i> <sup>[15]</sup>	2004	China	Asian	Gastric	PCR	56	53	3	0	164	143	20	1	0.74
Garcia-Gonzalez <i>et al</i> <sup>[16]</sup>	2007	Spain	Caucasian	Gastric	TaqMan	404	309	84	11	404	320	77	7	0.35
Garza-Gonzalez <i>et al</i> <sup>[17]</sup>	2005	Mexico	Caucasian	Gastric	PCR-RFLP	63	0	8	55	215	1	35	179	0.61
Glas <i>et al</i> <sup>[18]</sup>	2004	Germany	Caucasian	Gastric	PCR-RFLP	88	66	19	3	145	105	36	4	0.67
Hou <i>et al</i> <sup>[19]</sup>	2007	Poland	Caucasian	Gastric	TaqMan	305	186	98	21	428	304	109	15	0.19
Kamangar <i>et al</i> <sup>[20]</sup>	2006	Finland	Caucasian	Gastric	TaqMan	112	86	23	3	208	154	52	2	0.29
Kim <i>et al</i> <sup>[21]</sup>	2006	South Korea	Asian	Gastric	PCR-RFLP	237	199	34	4	461	400	59	2	0.91
Lee <i>et al</i> <sup>[22]</sup>	2004	South Korea	Asian	Gastric	PCR	341	297	43	1	261	218	42	1	0.49
Lee <i>et al</i> <sup>[23]</sup>	2005	South Korea	Asian	Gastric	PCR-RFLP	122	112	10	0	120	103	17	0	0.40
Li <i>et al</i> <sup>[24]</sup>	2005	China	Asian	Gastric	PCR-RFLP	59	55	4	0	264	228	34	2	0.56
Lu <i>et al</i> <sup>[25]</sup>	2005	China	Asian	Gastric	PCR-DHPLC	250	214	36	0	300	274	24	2	0.08
Machado <i>et al</i> <sup>[26]</sup>	2003	Portugal	Caucasian	Gastric	PCR-SSCP	287	179	105	3	304	231	69	4	0.65
Melo <i>et al</i> <sup>[27]</sup>	2009	Brazil	Caucasian	Gastric	PCR-RFLP	30	24	5	1	100	86	13	1	0.53
Morgan <i>et al</i> <sup>[28]</sup>	2006	Honduras	Caucasian	Gastric	TaqMan	168	151	17	0	161	149	12	0	0.62
Perri <i>et al</i> <sup>[29]</sup>	2005	Italy	Caucasian	Gastric	PCR-RFLP	184	152	30	2	362	290	65	7	0.15
Rocha <i>et al</i> <sup>[30]</sup>	2005	Brazil	Caucasian	Gastric	PCR-RFLP	161	120	37	4	535	399	123	13	0.34
Sugimoto <i>et al</i> <sup>[31]</sup>	2007	Japan	Asian	Gastric	PCR-RFLP	105	101	4	0	172	169	3	0	0.91
Torres <i>et al</i> <sup>[32]</sup>	2004	Colombia	Caucasian	Gastric	PCR	44	41	3	0	66	56	10	0	0.51
Wu <i>et al</i> <sup>[33]</sup>	2002	China	Asian	Gastric	Direct sequencing	150	114	27	9	220	180	27	13	<0.01
Wu <i>et al</i> <sup>[34]</sup>	2004	China	Asian	Gastric	Direct sequencing	204	163	29	12	210	171	26	13	<0.01
Yang <i>et al</i> <sup>[35]</sup>	2009	South Korea	Asian	Gastric	SNaPshot	83	75	8	0	322	288	34	0	0.32
Zamboni <i>et al</i> <sup>[36]</sup>	2005	Italy	Caucasian	Gastric	TaqMan	129	95	31	3	644	496	138	10	0.91
Garrity-Park <i>et al</i> <sup>[37]</sup>	2008	Ireland	Caucasian	Colorectal	PCR, sequencing	114	52	49	13	114	92	20	2	0.46
Jang <i>et al</i> <sup>[14]</sup>	2001	South Korea	Asian	Colorectal	PCR-RFLP	27	24	3	0	92	85	7	0	0.70
Landi <i>et al</i> <sup>[38]</sup>	2003	Spain	Caucasian	Colorectal	TaqMan	363	278	80	5	320	234	76	10	0.22
Li M <i>et al</i> <sup>[39]</sup>	2011	China	Asian	Colorectal	PCR-RFLP	180	156	15	9	180	160	19	1	0.60
Macarthur <i>et al</i> <sup>[40]</sup>	2005	Scotland	Caucasian	Colorectal	TaqMan	246	157	74	15	389	224	145	20	0.58
Park <i>et al</i> <sup>[41]</sup>	1998	South Korea	Asian	Colorectal	PCR-RFLP	140	115	24	1	328	252	72	4	0.65
Suchy <i>et al</i> <sup>[42]</sup>	2008	Poland	Caucasian	Colorectal	PCR-RFLP	350	254	87	9	350	248	95	7	0.55
Theodoropoulos <i>et al</i> <sup>[43]</sup>	2006	Greece	Caucasian	Colorectal	PCR-RFLP	222	152	56	14	200	146	44	10	0.01
Toth <i>et al</i> <sup>[44]</sup>	2007	Hungary	Caucasian	Colorectal	PCR-SSP	183	132	48	3	141	111	30	0	0.16
Tsilidis <i>et al</i> <sup>[45]</sup>	2009	United States	Caucasian	Colorectal	TaqMan	204	146	55	3	372	275	90	7	0.91
Akkiz <i>et al</i> <sup>[46]</sup>	2009	Turkey	Caucasian	Hepatocellular	PCR-RFLP	110	72	35	3	110	99	11	0	0.58
Ben-Ari <i>et al</i> <sup>[47]</sup>	2003	United States	Caucasian	Hepatocellular	PCR-SSP	10	9	1 <sup>1</sup>		48	42	6 <sup>1</sup>		NA
Chen <i>et al</i> <sup>[48]</sup>	2005	China	Asian	Hepatocellular	TaqMan	572	468	95	9	381	311	67	3	0.77
Heneghan <i>et al</i> <sup>[49]</sup>	2003	China	Asian	Hepatocellular	ASO-PCR	98	88	10	0	97	90	6	1	0.03
Ho <i>et al</i> <sup>[50]</sup>	2004	China	Asian	Hepatocellular	PCR-RFLP	74	37	34	3	289	225	62	2	0.30
Jeng <i>et al</i> <sup>[51]</sup>	2007	China	Asian	Hepatocellular	PCR-SSO	108	80	28 <sup>1</sup>		108	100	8 <sup>1</sup>		NA
Jeng JE <i>et al</i> <sup>[52]</sup>	2009	China	Asian	Hepatocellular	PCR-SSO	200	149	51 <sup>1</sup>		200	188	12 <sup>1</sup>		NA
Kummee <i>et al</i> <sup>[53]</sup>	2007	Thailand	Asian	Hepatocellular	PCR-RFLP	50	42	8	0	150	123	26	1	0.77
Migita <i>et al</i> <sup>[54]</sup>	2005	Japan	Asian	Hepatocellular	PCR-SSP	48	47	1	0	188	183	5	0	0.85
Niro <i>et al</i> <sup>[55]</sup>	2005	Italy	Caucasian	Hepatocellular	Direct sequencing	30	24	6 <sup>1</sup>		96	75	21 <sup>1</sup>		NA
Ognjanovic <i>et al</i> <sup>[56]</sup>	2009	United States	Caucasian	Hepatocellular	TaqMan	118	90	28 <sup>1</sup>		225	176	49 <sup>1</sup>		NA
Sakamoto <i>et al</i> <sup>[57]</sup>	2008	Japan	Asian	Hepatocellular	PCR-RFLP	209	205	4	0	275	270	5	0	0.88
Shi <i>et al</i> <sup>[58]</sup>	2011	China	Asian	Hepatocellular	PCR-RFLP	88	30	43	15	88	45	35	8	0.75
Wang <i>et al</i> <sup>[59]</sup>	2003	Japan	Asian	Hepatocellular	Direct sequencing	125	111	13	1	55	48	6	1	0.16
Wang <i>et al</i> <sup>[60]</sup>	2010	China	Asian	Hepatocellular	PCR-SSO	230	197	30	3	158	143	15	0	0.53
Duell <i>et al</i> <sup>[61]</sup>	2006	United States	Caucasian	Pancreatic	PCR-RFLP	260	192	63	5	859	639	198	22	0.16
Talor-wojnarowska <i>et al</i> <sup>[62]</sup>	2009	Poland	Caucasian	Pancreatic	PCR-RFLP	41	26	12	3	50	31	17	2	0.86
Wu GY <i>et al</i> <sup>[63]</sup>	2010	Germany	Caucasian	Pancreatic	PCR-RFLP	73	51	20	2	116	84	30	2	0.72
El-Omar <i>et al</i> <sup>[9]</sup>	2003	United States	Caucasian	Esophageal	TaqMan	161	122	34	5	210	152	52	6	0.55
Guo <i>et al</i> <sup>[13]</sup>	2005	China	Asian	Esophageal	PCR-RFLP	291	266	21	4	437	391	40	6	<0.01

<sup>1</sup>Numbers of GA+AA.  $P_{HWE}$  was calculated by goodness-of fit  $\chi^2$ -test, and  $P_{HWE} < 0.05$  was considered statistically significant. PCR-DHPLC: Polymerase chain reaction-based denaturing high-performance liquid chromatography; HWE: Hardy-Weinberg equilibrium; NA: Not available; GG: Guanine/Guanine; GA: Guanine/Adenine; AA: Adenine/Adenine; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism.

are accurate.

Some limitations of this meta-analysis should be ad-

dressed. First, the number of published studies, especially for oesophageal and pancreatic cancers, was not suf-



**Table 2** Stratified analysis of the tumor necrosis factor alpha polymorphism and digestive system cancers risk

Group	GA + AA vs GG			GA vs GG			AA vs GG			AA vs GA + GG		
	n	OR (95%CI)	P <sup>1</sup>	n	OR (95%CI)	P <sup>1</sup>	n	OR (95%CI)	P <sup>1</sup>	n	OR (95%CI)	P <sup>1</sup>
Overall	58	1.23 (1.09, 1.38) <sup>2</sup>	< 0.00001	52	1.14 (1.01, 1.28) <sup>2</sup>	< 0.00001	44	1.43 (1.19, 1.73)	0.26	44	1.38 (1.15, 1.66)	0.55
Studies with HWE	46	1.18 (1.03, 1.34) <sup>2</sup>	< 0.00001	46	1.14 (1.00, 1.29) <sup>2</sup>	< 0.00001	38	1.54 (1.25, 1.90)	0.15	38	1.48 (1.20, 1.81)	0.40
Cancer type												
Gastric	28	1.23 (1.12, 1.34) <sup>2</sup>	0.009	27	1.15 (1.04, 1.27)	0.07	22	1.38 (1.06, 1.80)	0.63	22	1.33 (1.03, 1.72)	0.67
Colorectal	10	1.17 (0.87, 1.57) <sup>2</sup>	< 0.0001	10	1.10 (0.83, 1.45) <sup>2</sup>	0.0004	9	1.45 (0.76, 2.75) <sup>2</sup>	0.02	9	1.40 (0.99, 2.00)	0.07
Hepatocellular	15	1.74 (1.20, 2.54) <sup>2</sup>	< 0.00001	10	1.58 (1.05, 2.39) <sup>2</sup>	0.002	8	2.55 (1.38, 4.70)	0.49	8	2.15 (1.19, 3.90)	0.66
Pancreatic	3	1.04 (0.79, 1.36)	0.94	3	1.04 (0.79, 1.38)	0.88	3	0.99 (0.46, 2.14)	0.63	3	0.99 (0.46, 2.13)	0.60
Esophageal	2	0.82 (0.58, 1.16)	0.89	2	0.80 (0.55, 1.15)	0.89	2	1.01 (0.42, 2.43)	0.95	2	1.05 (0.44, 2.51)	0.92
Ethnicity												
Asian	27	1.24 (0.99, 1.56) <sup>2</sup>	< 0.00001	25	1.07 (0.94, 1.22) <sup>2</sup>	0.008	19	1.55 (1.11, 2.17)	0.43	19	1.47 (1.05, 2.06)	0.60
Caucasian	31	1.21 (1.05, 1.40) <sup>2</sup>	< 0.00001	27	1.17 (1.01, 1.35) <sup>2</sup>	< 0.0001	25	1.38 (1.10, 1.74)	0.18	25	1.34 (1.08, 1.67)	0.39

<sup>1</sup>Test for heterogeneity; <sup>2</sup>Random-effects model was used when the *P* for heterogeneity test was < 0.05. GG: Guanine/Guanine; GA: Guanine/Adenine; AA: Adenine/Adenine; HWE: Hardy-Weinberg equilibrium.

ficiently large for a comprehensive analysis, and some studies with small sample sizes may not have enough statistical power to prove authentic associations. Therefore, our analysis should be interpreted with caution, and more studies are needed. Second, our results were based on unadjusted estimates, and lack of information for the data analysis may cause serious confounding bias. Third, significant heterogeneity was found in some models, which may lead to failure to confirm marginal associations. In spite of these limitations, our meta-analysis had several advantages. First, a substantial number of cases and controls were pooled from different studies, which significantly increased the statistical power of the analysis. Second, the quality of the case-control studies included in the current meta-analysis was satisfactory and met our inclusion criteria. Third, we did not detect any publication bias, suggesting that the whole pooled result is unbiased.

In summary, this meta-analysis suggests that the *TNF- $\alpha$ -308* polymorphism increases susceptibility to digestive system cancers in the Caucasian population. The *TNF- $\alpha$ -308* AA genotype is closely related to the risk of digestive system cancers in people of Asian descent. The *TNF- $\alpha$ -308* polymorphism may be significantly associated with the risk of gastric and hepatocellular carcinomas, but not colorectal, pancreatic, or oesophageal cancer. Future studies should use standardised unbiased genotyping methods, examine homogeneous cancer patients and well-matched controls, and include multiethnic groups.

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## COMMENTS

### Background

Digestive system cancers are the most common malignant tumors worldwide. Tumor necrosis factor alpha-308 (*TNF- $\alpha$ -308*) polymorphism (rs1800629) is the most extensively studied polymorphism in digestive system cancers. However,

the results are different or even inconsistent.

### Research frontiers

Molecular epidemiology has confirmed that carcinogenesis is a complex, multifactorial, and multistep event, and genetic mutation play an important role in the process. Many studies have reported the association between the *TNF- $\alpha$ -308* polymorphism and human malignant tumors, but no agreements have been reached till now.

### Innovations and breakthroughs

This meta-analysis systemically assessed the association between *TNF- $\alpha$ -308* polymorphism and risk of digestive system cancers. Results show that *TNF- $\alpha$ -308* polymorphism may be significantly associated with the risk of gastric and hepatocellular carcinomas in Asians.

### Applications

This study results indicate that *TNF- $\alpha$ -308* polymorphism may be used as a detectable biomarker for gastric and hepatocellular carcinoma patients.

### Peer review

The authors present a meta-analysis study over the influence of a polymorphism of *TNF- $\alpha$*  on digestive system cancers. The manuscript is well written and interesting, especially because it is the first meta-analysis study on the subject.

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## Association of interleukin-10 polymorphisms with risk of irritable bowel syndrome: A meta-analysis

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### Abstract

**AIM:** To clarify the current understanding of the association between interleukin-10 (*IL-10*) polymorphisms and the risk of irritable bowel syndrome (IBS).

**METHODS:** We searched for studies in any language recorded in PubMed, Embase and Cochrane library before August 2013. The associations under allele contrast model, codominant model, dominant model, and recessive model were analyzed. The strengths of the association between *IL-10* polymorphisms and IBS risk were estimated using odds ratios (OR) with 95% confidence interval (CI). Fixed effects model was used to pool the result if the test of heterogeneity was not significant, otherwise the random-effect model was selected.

**RESULTS:** Eight case-control studies analyzing three

single-nucleotide polymorphisms rs1800870 (-1082 A/G), rs1800871 (-819C/T), and rs1800872 (-592A/C) of the *IL-10* gene, which involved 928 cases and 1363 controls, were eligible for our analysis. The results showed that rs1800870 polymorphisms were associated with a decreased risk of IBS (GG+GA vs AA: OR = 0.80, 95%CI: 0.66-0.96), (AA+GA vs GG: OR = 0.68, 95%CI: 0.52-0.90). Subgroup analysis revealed such association only existed in Caucasian ethnicity (AA+GA vs GG, OR = 0.70, 95%CI: 0.55-0.89). The rs1800872 polymorphisms were associated with an increased risk of IBS in Asian ethnicity (CC vs GG: OR = 1.29, 95%CI: 1.01-1.16). There were no associations between rs1800871 polymorphisms and the IBS risk.

**CONCLUSION:** The results suggest that *IL-10* rs1800870 confers susceptibility to the risk of IBS in Caucasian ethnicity, and the rs1800872 may associate with IBS risk in Asians. However, no significant associations are found between rs1800871 and IBS risk.

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**Key words:** Interleukin-10; Irritable bowel syndrome; Gene polymorphism; Case-control; Meta-analysis

**Core tip:** Interleukin-10 (*IL-10*) polymorphisms have been identified as a biomarker causally associated with occurrence of irritable bowel syndrome (IBS) and receives extensive interest. However, its relationship with IBS remains obscure. In this paper, after combing the data from 8 case-control studies with 928 cases and 1363 controls, the authors found that the *IL-10* rs1800870 confers susceptibility to the risk of IBS in Caucasian ethnicity, and the rs1800872 may associate with IBS risk in Asians. However, no significant associations are found between rs1800871 and IBS risk.

Qin SY, Jiang HX, Lu DH, Zhou Y. Association of interleukin-10 polymorphisms with risk of irritable bowel syndrome: A meta-analysis. *World J Gastroenterol* 2013; 19(48): 9472-9480 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9472.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9472>

## INTRODUCTION

Irritable bowel syndrome (IBS) is a type of functional gastrointestinal disorder that has a multi-factorial origin. The exact pathophysiology leading to the occurrence of IBS is largely unknown, although inflammatory reactions are believed to play an important role in its pathogenesis. In some animal studies, researchers have found that inflammatory responses can alter the function of gut smooth muscles, enteric nerves, and interstitial cells of Cajal<sup>[1-3]</sup>. Moreover, IBS patients show an increase in the number of inflammatory cells in the gut<sup>[4-6]</sup>. The clustering of IBS in families and the results from twin studies have also provided evidence for a role of hereditary factors in the propensity of developing IBS<sup>[7,8]</sup>.

Cytokines are important modulators of immune responses and inflammatory reactions and play a central role in intestinal inflammation<sup>[9]</sup>. The production of cytokines can be affected by genetic polymorphisms within the coding and promoter regions of cytokine genes<sup>[10,11]</sup>. Therefore, a genetic predisposition for the high or low production of a particular cytokine may affect disease susceptibility and clinical outcome<sup>[12,13]</sup>. Interleukin 10 (IL-10), also known as a human cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine capable of inhibiting the synthesis of proinflammatory cytokines, such as interferon- $\gamma$ , IL-2, IL-3, and tissue necrosis factor- $\alpha$ , which are produced by macrophages and regulatory T-cells<sup>[14]</sup>. Several studies have shown that serum IL-10 levels are significantly lower in IBS patients than in normal controls, suggesting that altered IL-10 levels may be involved in the pathogenesis of IBS and may be an IBS biomarker<sup>[15-17]</sup>.

Some reports<sup>[18-21]</sup> have also indicated a significant association between *IL-10* polymorphisms and IBS risk; however, other studies<sup>[13,22-24]</sup> have failed to find such associations. Generally, this disparity may be partly due to ethnic differences or to the limited numbers of subjects involved in the studies. Therefore, the relationship between IBS risk and *IL-10* polymorphisms is not confirmed and needs further study with a large, genetically homogenous sample. The current study is a comprehensive meta-analysis performed to further evaluate the associations between *IL-10* polymorphisms and the risk of IBS.

## MATERIALS AND METHODS

### Search strategy and study selection

All methods were based on the guidelines proposed by

the Human Genome Epidemiology Network for systematic reviews of genetic association studies, and followed the PRISMA guidelines<sup>[25]</sup>. A systematic literature search was performed using PubMed, Embase, the Cochrane Library, Google Scholar databases, Chinese National Knowledge Infrastructure (CNKI), and conference abstracts to identify published studies evaluating genetic association between *IL-10* polymorphisms and IBS risk published prior to August 2013; letters and abstracts were included. The Medical Subject Headings and text words used for the search were “interleukin-10” or “IL-10”, “polymorphism,” and “irritable bowel syndrome” or “IBS”. Search results were limited to human studies. All languages were searched, and the retrieved articles were translated, when necessary. The references of the identified publications were searched for additional studies, and the MEDLINE option for searching for related articles was used to examine all relevant articles.

### Inclusion and exclusion criteria

Studies were included if they (1) examined the association between *IL-10* polymorphisms and IBS risk; (2) had a case-control design; and (3) contained sufficient information on genotype frequency. To achieve adequate statistical power, only single-nucleotide polymorphisms (SNPs) reported in > 2 publications were selected. For studies describing results from the same or overlapping groups of subjects or controls, but reported in > 1 publication, only the largest published data set was included.

Studies were excluded if they did not evaluate the association between *IL-10* polymorphisms and the risk of IBS or if the genotype and allele frequency was inadequately reported, and such data could not be obtained by contacting the authors. Studies reporting associations with SNPs described by fewer than 3 publications were also excluded. In the event of duplicate publications, the smaller data set was excluded.

### Data extraction

Two investigators independently extracted data from the identified publications, including the first author's name, year of publication, source of publication, diagnostic criteria for IBS, method of genotyping, number of cases and controls, genotype frequency, and allele frequency. Discrepancies in data extraction were resolved by repeating the study review and discussing the results.

### Statistical analysis

Associations found with the allele contrast, codominant, dominant, and recessive models were analyzed. The strengths of the associations between *IL-10* polymorphisms and risk of IBS were estimated using odds ratios (OR) with 95% confidence interval (CI). We assessed the heterogeneity among the studies using the Cochran's *Q*-test. We also calculated the inconsistency index *I*<sup>2</sup> to quantify heterogeneity<sup>[26]</sup>. A fixed effects model (*P* > 0.05) was used to pool the results if a heterogeneity test was not significant, otherwise a random-effects model was

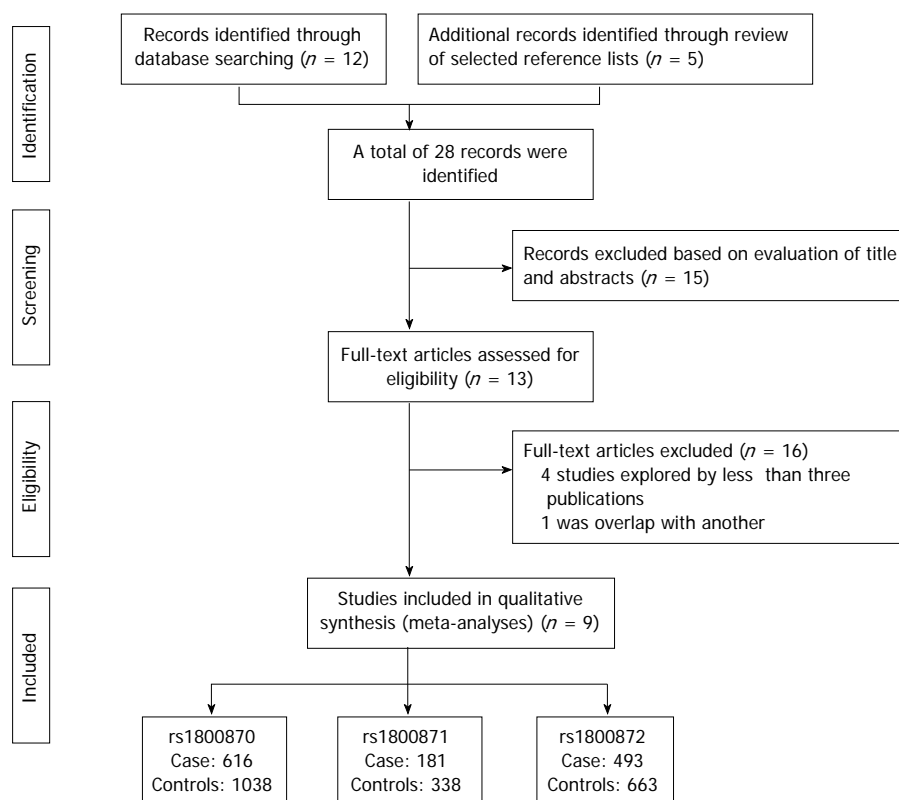


Figure 1 Flow chart of study selection.

used ( $P < 0.05$ )<sup>[27,28]</sup>. Subgroup analyses were performed to investigate narrower subsets of studies.

A Hardy-Weinberg equilibrium (HWE) was applied to the control population to evaluate data quality. The HWE analysis for genotype distribution among control populations was performed using a Chi-squared test. Further, a sensitivity analysis was performed to exclude studies that were not in HWE<sup>[29]</sup>. An asymmetric plot was used to suggest possible publication biases. Publication bias was examined using the Begg's and Egger's tests for each SNP publication<sup>[30,31]</sup>. All statistical tests were two-sided, and a  $P$  value  $< 0.05$  was considered statistically significant. STATA, version 11.2 (Stata, College Station, TX, United States), was used for all statistical analyses.

## RESULTS

### Study selection process

The initial search yielded 28 studies; 15 were excluded because they were review articles, animal studies, or non-case-control design studies. After screening the full text of the remaining 13 studies, an additional 5 studies were excluded; of these, 4 explored SNPs reported by  $< 3$  studies and one<sup>[19]</sup> did not provide sufficient genotype frequency data, even after contacting the original authors. Thus, a total of 8 case-control studies<sup>[18-24,32]</sup>, involving 928 cases and 1363 controls, were included in the present meta-analysis. The studies analyzed 3 *IL-10* SNPs, rs1800870 (1082 A/G), rs1800871 (819 C/T), and

rs1800872 (592 A/C). Figure 1 provides a summary of the selection process.

### Characteristics of included studies

One study selected IBS patients using the Rome I criteria, 3 used the Rome II criteria, and the other 4 studies used the Rome III criteria. Patients from 4 studies<sup>[18-21]</sup> were Caucasians, and 4 studies involved Asians<sup>[22-24,32]</sup>. The characteristics of the 8 studies and the results of the HWE test for the distribution of the genotype in the control population are shown in Table 1.

### *IL-10* rs1800870 and IBS risk

Seven studies<sup>[13,18,20-24]</sup>, involving 616 IBS subjects and 1038 controls, analyzed the association between the *IL-10* rs1800870 polymorphism and IBS risk. The distribution of the controls in 2 studies<sup>[20,21]</sup> deviated from the HWE. Overall, the GG+GA *vs* AA (OR = 0.80, 95%CI: 0.66-0.96,  $P = 0.018$ ) and AA+GA *vs* GG (OR = 0.68, 95%CI: 0.52-0.90,  $P = 0.007$ ) models presented a decreased risk of IBS. Little heterogeneity was found in the AA+GA *vs* GG model ( $I^2 = 0.0\%$ ,  $P = 0.542$ ) by the  $I^2$  test and  $Q$ -test; however, there was significant heterogeneity in the GG+GA *vs* AA ( $I^2 = 79.4\%$ ,  $P = 0.000$ ) model. There were no significant associations between the GG *vs* AA ( $P = 0.523$ ) and G *vs* A ( $P = 0.892$ ) models and IBS risk. Egger's and Begg's tests suggested little publication bias in the 4 models (all,  $P > 0.05$ ) (Table 2, Figure 2A). In the sensitivity analysis, after removing 2 studies<sup>[20,21]</sup> in which the controls deviated from the

**Table 1** Characteristic of individual studies in the meta-analysis

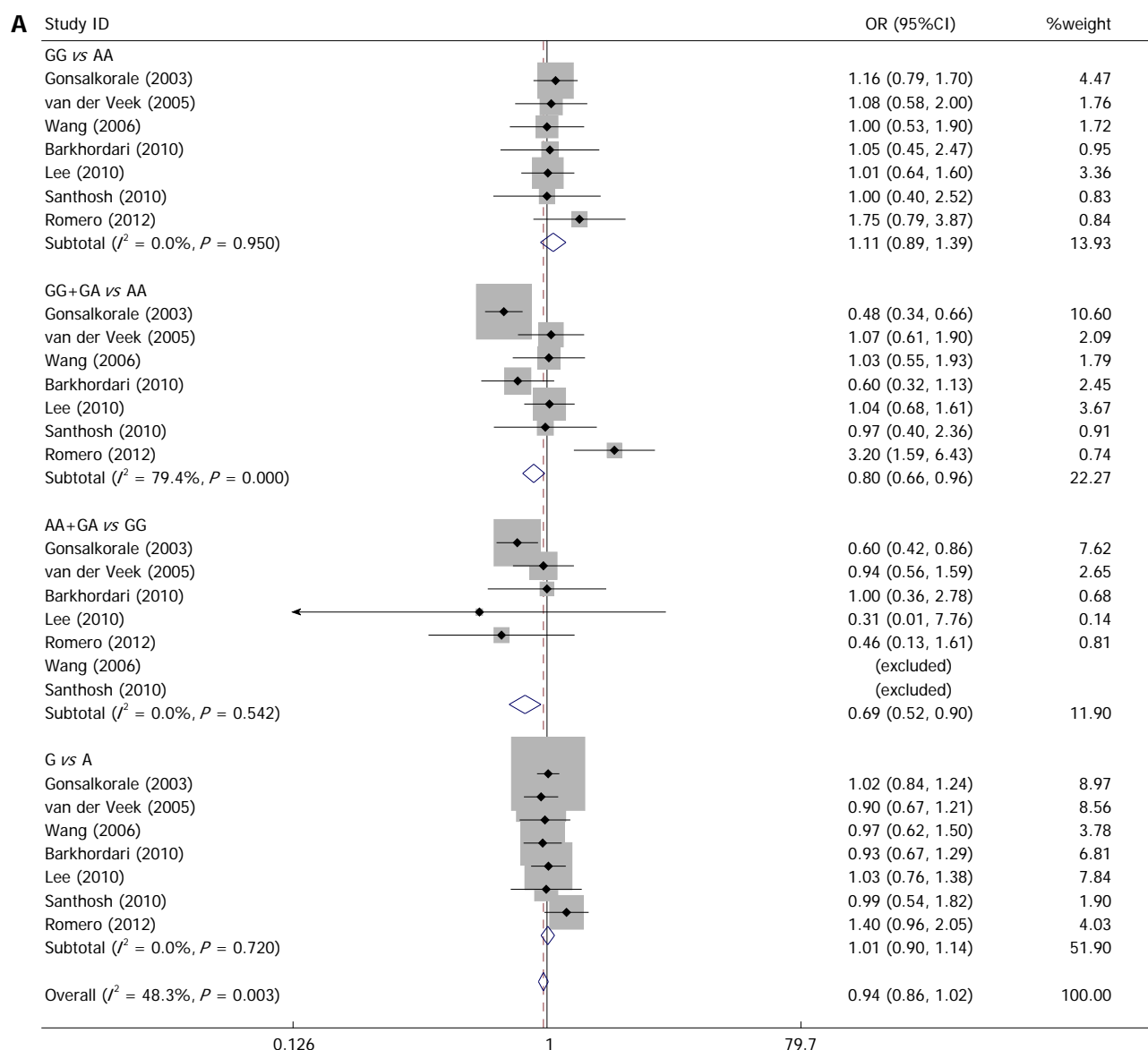
Ref.	Year	Country/ethnicity	SNP of IL-10	IBS/controls	Genotyping methods	Diagnostic criteria	HWE of control
Gonsalkorale <i>et al</i> <sup>[21]</sup>	2003	United Kingdom	rs1800870	230/450	PCR-SSP	Rome I	0.000
van der Veek <i>et al</i> <sup>[13]</sup>	2005	Netherlands	rs1800870	111/128	PCR-RFLP	Rome II	0.707
Wang <i>et al</i> <sup>[24]</sup>	2006	China	rs1800870	43/41	PCR-RFLP	Rome II	0.678
Barkhordari <i>et al</i> <sup>[18]</sup>	2010	Iran	rs1800870	70/140	PCR-SSP	Rome III	0.041
Lee <i>et al</i> <sup>[22]</sup>	2010	South Korea	rs1800870	94/88	PCR-RFLP	Rome III	0.707
Santhosh <i>et al</i> <sup>[23]</sup>	2010	Indian	rs1800870	23/20	PCR-SSP	Rome II	0.717
Romero-Valdovinos <i>et al</i> <sup>[20]</sup>	2012	Mexico	rs1800870	45/173	PCR-RFLP	Rome III	0.000
Wang <i>et al</i> <sup>[24]</sup>	2006	China	rs1800871	43/41	PCR-RFLP	Rome II	0.619
Barkhordari <i>et al</i> <sup>[18]</sup>	2010	Iran	rs1800871	70/140	PCR-SSP	Rome III	0.907
Santhosh <i>et al</i> <sup>[23]</sup>	2010	China	rs1800871	23/20	PCR-SSP	Rome II	0.662
Romero-Valdovinos <i>et al</i> <sup>[20]</sup>	2012	Mexico	rs1800871	45/173	PCR-RFLP	Rome III	0.920
Wang <i>et al</i> <sup>[24]</sup>	2006	China	rs1800872	43/41	ARMS-PCR	Rome II	0.619
Barkhordari <i>et al</i> <sup>[18]</sup>	2010	Iran	rs1800872	70/140	PCR-RFLP	Rome III	0.97
Jiang <i>et al</i> <sup>[52]</sup>	2010	China	rs1800872	312/325	PCR-SSP	Rome III	0.255
Santhosh <i>et al</i> <sup>[23]</sup>	2010	Indian	rs1800872	23/20	PCR-SSP	Rome II	0.438
Romero-Valdovinos <i>et al</i> <sup>[20]</sup>	2012	Mexico	rs1800872	45/173	PCR-RFLP	Rome III	0.018

SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; IBS: Irritable bowel syndrome; PCR: Polymerase chain reaction; SSP: Sequence specific primer; RFLP: Restriction fragment length polymorphism; ARMS: Amplification refractory mutation system.

**Table 2** Summary odds ratios of polymorphisms with irritable bowel syndrome risk

	P value	OR (95%CI)	I <sup>2</sup>	P heterogeneity	Begg' test	Egger test
rs1800870						
GG vs AA	0.338	1.11 (0.89-1.39)	0.0%	0.950	1.000	0.694
Caucasian <sup>[13,18,20,21]</sup>	0.228	1.19 (0.90-1.58)	0.0%	0.776		
Asian <sup>[22-24]</sup>	0.963	1.01 (0.71-1.42)	0.0%	0.999		
GG+GA vs AA	0.018	0.80 (0.66-0.96)	79.4%	0.000	1.000	0.200
Caucasian	0.003	0.70 (0.55-0.89)	88.5%	0.000		
Asian	0.860	1.03 (0.74-1.43)	0.0%	0.990		
AA+GA vs GG	0.007	0.68 (0.52-0.90)	0.0%	0.542	1.000	0.899
Caucasian	0.008	0.69 (0.52-0.91)	0.0%	0.415		
Asian	0.478	0.31 (0.01-7.76)	-	-		
G vs A	0.815	1.01 (0.90-1.14)	0.0%	0.720	0.764	0.773
Caucasian	0.799	1.02 (0.89-1.16)	17.1%	0.306		
Asian	0.976	1.00 (0.80-1.260)	0.0%	0.974		
rs1800871						
AA vs GG	0.698	0.90 (0.53-1.54)	0.0%	0.440	0.308	0.326
Caucasian <sup>[18,20]</sup>	0.205	0.56 (0.23-1.37)	22.6%	0.255		
Asian <sup>[23,24]</sup>	0.530	1.25 (0.62-2.54)	0.0%	0.969		
AA+GA vs GG	0.969	0.99 (0.61-1.60)	32.4%	0.218	0.308	0.146
Caucasian	0.182	0.58 (0.26-1.29)	0.0%	0.365		
Asian	0.252	1.45 (0.77-2.76)	0.0%	0.364		
GG+GA vs AA	0.651	0.92 (0.64-1.33)	0.0%	0.720	0.734	0.080
Caucasian	0.985	1.00 (0.68-1.49)	0.0%	0.906		
Asian	0.219	0.54 (0.20-1.45)	0.0%	0.967		
A vs G	0.496	1.09 (0.85-1.38)	0.0%	0.809	1.000	0.924
Caucasian	0.939	1.01 (0.75-1.37)	0.0%	0.631		
Asian	0.314	1.23 (0.83-1.82)	0.0%	0.671		
rs1800872						
CC vs AA	0.989	1.00 (0.77-1.19)	0.0%	0.456	0.806	0.506
Caucasian <sup>[18,20]</sup>	0.379	0.73 (0.37-1.47)	41.6%	0.191		
Asian <sup>[23,24,32]</sup>	0.730	1.05 (0.80-1.38)	0.0%	0.466		
CC+CA vs AA	0.028	1.29 (1.03-1.62)	0.0%	0.717	0.221	0.196
Caucasian	0.417	1.27 (0.71-2.26)	0.0%	0.573		
Asian	0.042	1.29 (1.01-1.66)	0.0%	0.410		
AA+CA vs CC	0.833	0.97 (0.72-1.31)	60.2%	0.040	0.806	0.384
Caucasian	0.578	1.13 (0.72-1.71)	3.2%	0.310		
Asian	0.382	0.82 (0.53-1.27)	76.0%	0.016		
C vs A	0.112	1.12 (0.97-1.28)	0.0%	0.863	0.086	0.266
Caucasian	0.614	1.12 (0.97-1.28)	0.0%	0.669		
Asian	0.114	1.14 (0.97-1.33)	0.0%	0.618		





HWE, significant associations with the GG+GA *vs* AA model were no longer observed, and the heterogeneity became negligible (data not shown); the associations remained for the AA+GA *vs* GG (OR = 0.69, 95%CI: 0.52-0.91,  $P = 0.008$ ) model. Subgroup analysis revealed associations between the *IL-10* rs1800870 polymorphisms and IBS risk in Caucasians<sup>[18,20,21]</sup> ( $P = 0.003$ ), but not in Asians<sup>[22-24]</sup> ( $P = 0.860$ ) (Table 2).

#### *IL-10* rs1800871 and IBS risk

Four studies<sup>[18,20,23,24]</sup>, involving 493 IBS subjects and 663 controls, analyzed the associations between the *IL-10* rs1800871 polymorphisms and IBS risk; the distribution of controls in the studies fulfilled the HWE. In the meta-analysis, no significant associations were observed for any of the 4 models: AA *vs* GG ( $P = 0.698$ ), AA+AG *vs* GG ( $P = 0.969$ ), GG+AG *vs* AA ( $P = 0.651$ ), and A *vs* G ( $P = 0.496$ ). Either significant heterogeneity or publication bias was found associated with each of the 4 models (all,  $P > 0.05$ ). A sensitivity analysis, after excluding studies in turn, indicated that the null associations remained (data

not shown). Further, subgroup analyses did not find any associations between the *IL-10* rs1800871 polymorphisms and IBS risk, regardless of ethnicity<sup>[18,20,23,24]</sup> (Table 2, Figure 2B).

#### *IL-10* rs1800872 and IBS risk

Five studies<sup>[18,20,23,24,32]</sup>, involving 181 IBS subjects and 338 controls, analyzed the associations between *IL-10* rs1800872 polymorphisms and IBS risk; the distribution of the controls in the studies fulfilled the HWE. The meta-analysis demonstrated that the CC+CA *vs* AA model was associated with susceptibility to IBS (OR = 1.29, 95%CI: 1.03-1.62,  $P = 0.028$ ). However, significant associations were not found between the other 3 models, CC *vs* AA ( $P = 0.989$ ), AA+CA *vs* CC ( $P = 0.833$ ), and C *vs* A ( $P = 0.112$ ), and IBS risk. Either significant heterogeneity or publication bias was found in each of the 4 models (all,  $P > 0.05$ ). A sensitivity analysis, after removing each study sequentially, demonstrated that the results remained similar to the initial results. Subsequent subgroup analyses showed associations between rs1800872 and IBS risk

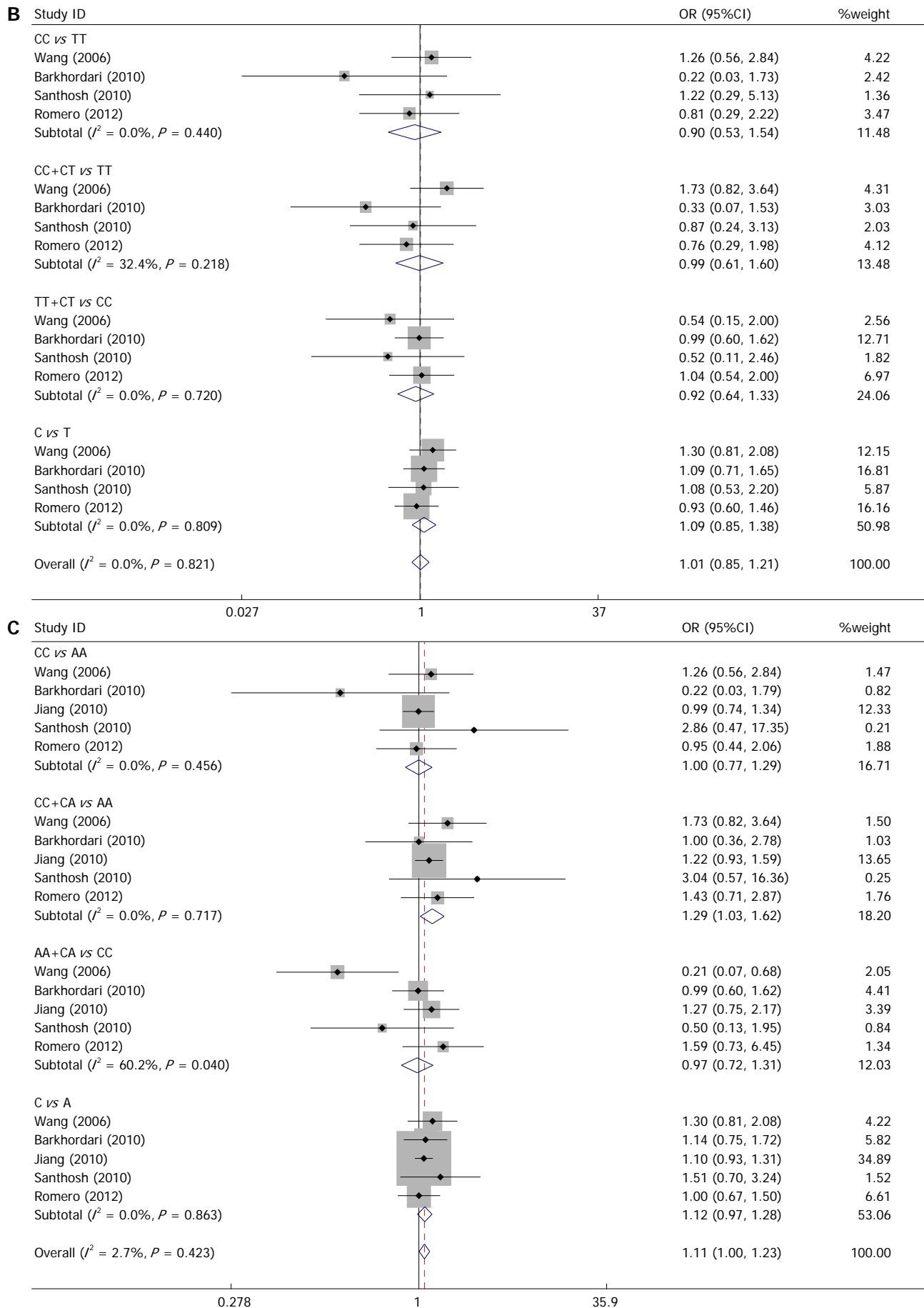


Figure 2 Meta-analysis. A: Interleukin-10 (*IL-10*) *rs1800870* polymorphisms and irritable bowel syndrome risk; B: *IL-10 rs1800871* polymorphisms and IBS risk; C: *IL-10 rs1800872* polymorphisms and IBS risk.

in Asians<sup>[23,24,32]</sup> ( $P = 0.042$ ), but not in Caucasians<sup>[18,20]</sup> ( $P = 0.417$ ) (Table 2, Figure 2C).

## DISCUSSION

The gene encoding IL-10 is located on chromosome 1q31-1q32, and has 3 confirmed biallelic polymorphisms in the promoter region, *i.e.*, rs1800870, rs1800871, and rs1800872. A genetic predisposition for low IL-10 production is associated with the development of IBS<sup>[33-35]</sup>, and previous studies have shown that IL-10 SNPs and some haplotypes are associated with an increased IBS risk<sup>[5,13,18,23]</sup>. Likewise, production of the anti-inflammatory cytokine IL-10 is also associated with SNPs at specific positions.

The A allele of rs1800870 has been reported to be associated with lower production of IL-10 and an accordingly stronger inflammatory response<sup>[36]</sup>. More recently, Bashashati *et al.*<sup>[37]</sup> reported the results of a meta-analysis that showed that the A/G of rs1800870 conferred susceptibility to IBS. However, their study only included 5 studies, and most of the included subjects were Caucasians. When considering the disparity in genetic factors among different racial groups, it is difficult to conclude that rs1800870 was associated with IBS risk, without adjusting for ethnicity and sample size. In the present study, although the overall meta-analysis results showed the presence of significant associations between rs1800870 and IBS risk in the GG+GA *vs* AA ( $P = 0.018$ ) and AA+GA *vs* GG ( $P = 0.007$ ) models, a subgroup analysis revealed that such association existed only for Caucasians, and not for Asians. This disparity between the two ethnicities might be explained by variations in allelic frequencies between the ethnic groups. This was possible since the frequency of the rs1800870 A allele is associated with significantly higher production of IL-10 in Caucasians than in Asians. Moreover, another study showed that the frequency of the high producer *IL-10* genotype is much higher in the Irish population than in Africans or Singaporean Chinese<sup>[38]</sup>. In the present study, 4<sup>[13,18,20,21]</sup> of the 8 included studies analyzed Caucasian subjects, and the frequency of the rs1800870 GG genotype in controls was higher than that observed in Asian subjects. The present meta-analysis results confirmed the association of rs1800870 and IBS risk, but also demonstrated that the association varies according to ethnicity.

The role of rs1800871 in IL-10 production is incompletely understood. Although some studies have reported that rs1800871 is associated with several diseases, such as endometriosis<sup>[39]</sup> and periodontitis<sup>[40]</sup>, the association between rs1800871 and IBS has remained controversial<sup>[11,18,20,23,24]</sup>. In our study, the overall results and the results of the subgroup analysis failed to show a significant association between rs1800871 and IBS risk, regardless of the examined ethnicity. This observation indicates similar distribution of rs1800871 among both IBS patients and controls, supporting the observation that the genetic make-up for IL-10 production levels does not differ between IBS patients and normal subjects<sup>[13,18,24]</sup>. Although

a direct link between rs1800871 and IL-10 production levels has not been established, previous reports have suggested that rs1800871 polymorphisms are in linkage disequilibrium with rs1800870 polymorphisms; that the haplotypes for rs1800870, rs1800871 and rs1800872 are common in Caucasians; and that the GCC/GCC haplotypes are commonly associated with high IL-10 production, whereas the ATA/ATA genotype is associated with low IL-10 production<sup>[41]</sup>. Because only limited data are available in the included studies, further studies are required to perform a haplotype analysis to explore the associations between rs1800871 haplotypes and other SNPs with IBS.

With regard to rs1800872, studies have reported that the presence of rs1800872 confers susceptibility to some diseases such as leprosy<sup>[42]</sup> and hepatocellular carcinoma<sup>[43]</sup>. In studies investigating the association of IL-10 with IBS risk, Santhosh *et al.*<sup>[23]</sup> reported that the C allele was much lower in individuals with IBS than among normal controls (41.3% *vs* 73.5%), in an Indian population. Wang *et al.*<sup>[24]</sup> found similar results in a Chinese population (IBS *vs* controls, 9.3% *vs* 17.1%). However, a Mexican study<sup>[18]</sup> failed to show a significant difference between IBS patients and normal controls with respect to the C allele (72.51% *vs* 71.1%); similar results were reported in 2 other studies<sup>[19,20]</sup>. In the present meta-analysis, we found that CC+CA *vs* AA was associated with an increased IBS risk, and the sensitivity analysis further confirmed this association. However, the subgroup analysis revealed that only Asians demonstrated this association. Although 5 studies were included in the present analysis, the sample size was relatively small; hence, these results should be interpreted with caution.

The present comprehensive meta-analysis demonstrated an association between rs1800871 and rs1800872 polymorphisms and IBS risk, and that the rs1800872 polymorphism conferred susceptibility to IBS. In addition, although Bashashati *et al.*<sup>[37]</sup> reported a meta-analysis demonstrating that rs1800870 conferred susceptibility to IBS, we expanded this finding to show that this association existed in Caucasians but not in Asians, which had not been previously reported. The present study also involved a sensitivity analysis demonstrating that the distribution of controls deviated from the HWE, guaranteeing the reliability of the results. The Begg's and Egger's test results did not show a significant publication bias in the eligible as well as the non-English studies, which also attests to the robustness of the results.

Some limitations to this meta-analysis require careful consideration. First, because only limited data were available, we did not analyze the association between *IL-10* polymorphisms and different types of IBS, *e.g.*, diarrhea or constipation. Thus, the associations between different types of IBS require further investigation. Second, other factors such as genetic and environmental factors, which also may affect susceptibility to IBS, were not adjusted in the present studies. Hence, a well-designed study is warranted to account for potential confounders and to provide a more precise association. Third, also because

of the limited number of available studies, the subgroup analysis of rs1800872 involved comparatively few studies; thus, its association with IBS needs to be confirmed by a study involving a larger number of subjects.

In conclusion, the present meta-analysis suggests that the rs1800870 polymorphism of IL-10 may represent an increased risk of IBS in Caucasians, but not in Asians. Similarly, rs1800872 polymorphisms may represent an increased risk of IBS in Asians, but future studies are necessary to reinforce these findings. The present study failed to find an association between rs1800871 and IBS risk, regardless of ethnicity.

## COMMENTS

### Background

Cytokines are important modulators in the immune responses and inflammatory reaction, which play a central role in intestinal inflammation.

### Research frontiers

Interleukin-10 (IL-10) polymorphisms have been identified as a biomarker causally associated with occurrence of irritable bowel syndrome (IBS) and receives extensive interest. However, its relationship with IBS remains obscure.

### Innovations and breakthroughs

This is the first paper conducting a comprehensive meta-analysis to investigate the association of IL-10 polymorphisms with IBS risk. The authors showed that IL-10 rs1800870 is associated with IBS risk in Caucasian ethnicity, and rs1800872 associate with IBS risk in Asians.

### Applications

This study furthers the understanding of the association of IL-10 polymorphisms with IBS risk.

### Peer review

The study deals with the important topic related to the association between single nucleotide polymorphisms of genes coding for inflammation-linked factors with pathogenesis of chronic inflammatory diseases such as IBS.

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## Endoscopic management of a rare granulation polyp in a colonic diverticulum

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was used to close the diverticulum with an over-the-scope clip. If a granulomatous polyp could arise from a diverticulum, differential diagnosis between a colon neoplasm and a granulomatous polyp would not only be difficult but also necessary for suitable endoscopic treatment.

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**Key words:** Diverticulitis; Endoscopy; Granulation polyp; Mucosal resection; Neoplasm; Recurrence

**Core tip:** The study observed a rare granulation polyp that arose from a diverticulum as a result of repeated episodes of local diverticulitis. The authors successfully resected the polyp using endoscopic mucosal resection. The diverticulum was inverted, and the resected stalk of the polyp was used to close the diverticulum with an over-the-scope clip.

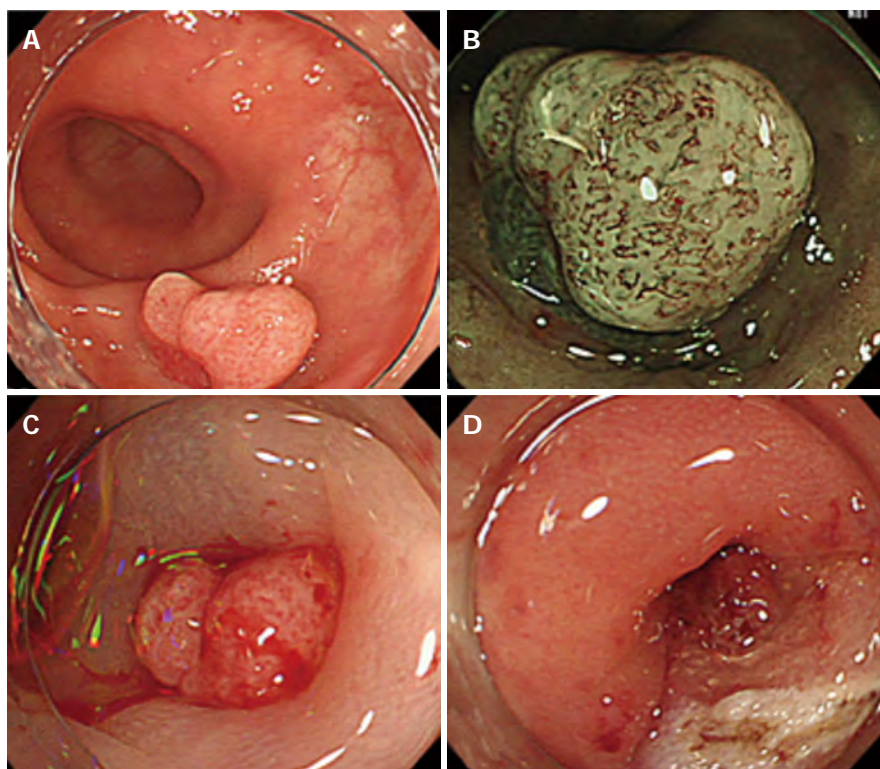
### Abstract

There are many case reports on colon diverticula that cause irritable bowel syndrome, constipation, bleeding, diverticulitis, stricture due to multiple recurrences of diverticulitis, and perforation. However, few articles have examined neoplasms that arise from a diverticulum, such as adenoma and adenocarcinoma, and there have been no reports of granulation polyps that arise from a colon diverticulum after recurrent diverticulitis. We observed a rare granulation polyp that arose from a diverticulum as a result of repeated episodes of local diverticulitis. Narrow band imaging magnified colonoscopy was very useful to diagnose the polyp as a granulation polyp because of the absence of a pit pattern on the surface of the polyp. We successfully resected the polyp using endoscopic mucosal resection. We inverted the diverticulum, and the resected stalk of the polyp

Mori H, Tsushimi T, Kobara H, Nishiyama N, Fujihara S, Matsunaga T, Ayagi M, Yachida T, Masaki T. Endoscopic management of a rare granulation polyp in a colonic diverticulum. *World J Gastroenterol* 2013; 19(48): 9481-9484 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9481.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9481>

### INTRODUCTION

A colon diverticulum is caused by increased intra-colonic pressure or by a weakened colonic wall. Most colon diverticula consist of acquired pseudodiverticula and have been observed in the sigmoid colon of patients in Western countries and in the ascending colon of patients in Japan<sup>[1]</sup>. The most reliable method to identify colon diverticula is a barium enema; however, once a diverticu-



**Figure 1** Endoscopic mucosal dissection of the sigmoid colon polyp. A: A sigmoid colon polyp approximately 25 mm in diameter; B: Narrow band imaging magnified colonoscopy was performed to investigate the polyp in greater detail. Several irregular microvessels were observed on the surface of the polyp, but there was no pit pattern on the surface; C: A local saline injection was administered, and we observed slight elevation of the polyp; D: After the endoscopic mucosal resection procedure and the removal of the polyp, the diverticulum was identified using the resected stalk of the polyp.

lum begins to bleed, colonoscopy is a useful modality to treat the bleeding vessels<sup>[2,3]</sup>. Although the incidence of colonic diverticular bleeding is increasing, treatments have not yet been well established. The risk factors contributing to recurrent hemorrhage after initial improvement in colonic diverticular bleeding are past histories of hypertension or renal deficiency. Follow-up colonoscopy after the initial improvement in colonic diverticular bleeding is needed in patients with hypertension or renal deficiency<sup>[4]</sup>. In addition, local peritonitis due to diverticulitis, and perforation are serious complications<sup>[5]</sup>. Although 85% of patients with colonic diverticulitis will recover with non-surgical treatment, some patients may have complications such as abscesses, fistulas, obstruction and panperitonitis<sup>[6,7]</sup>. On the other hand, few articles have examined neoplasms that arise from the diverticulum, such as adenoma and adenocarcinoma<sup>[8]</sup>.

We describe a rare case of a granulomatous polyp which arose from a colon diverticulum.

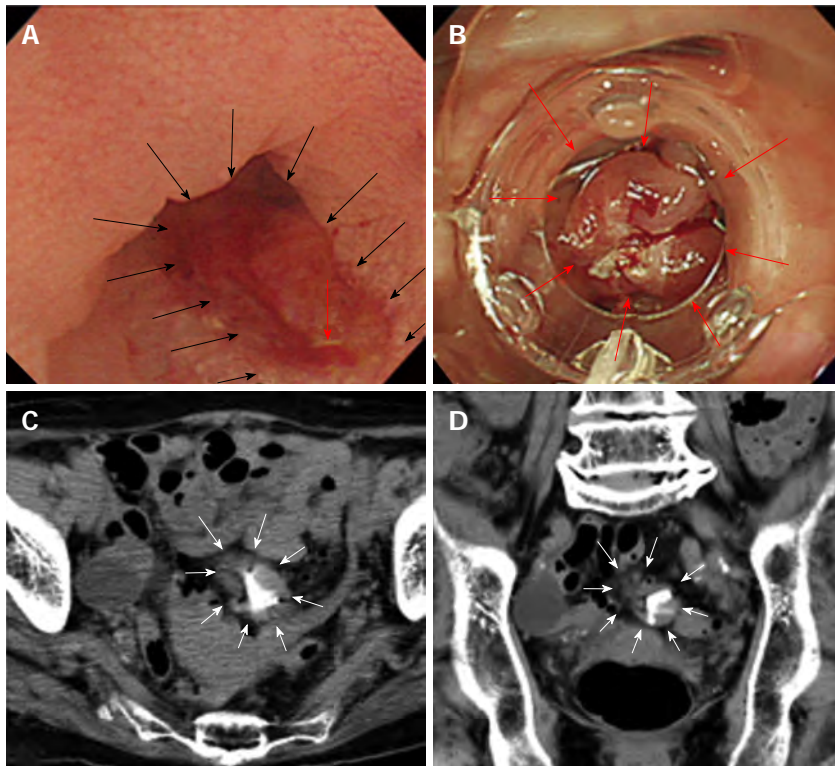
## CASE REPORT

A 62-year-old woman who suffered from repeated left lower abdominal pain and high fever (38 °C) underwent a colonoscopy and was diagnosed with a sigmoid colon polyp that was approximately 25 mm in diameter (Figure 1A). Twice during the previous year, she had suffered from abdominal pain and a high fever, and her blood laboratory data were as follows: a white blood cell count of 12000/ $\mu$ L and a C-reactive protein level of 5.59 mg/mL. After undergoing colonoscopy, her symptoms disappeared. Additionally, narrow band imaging (NBI) magnified colonoscopy was performed to diagnose the polyp in

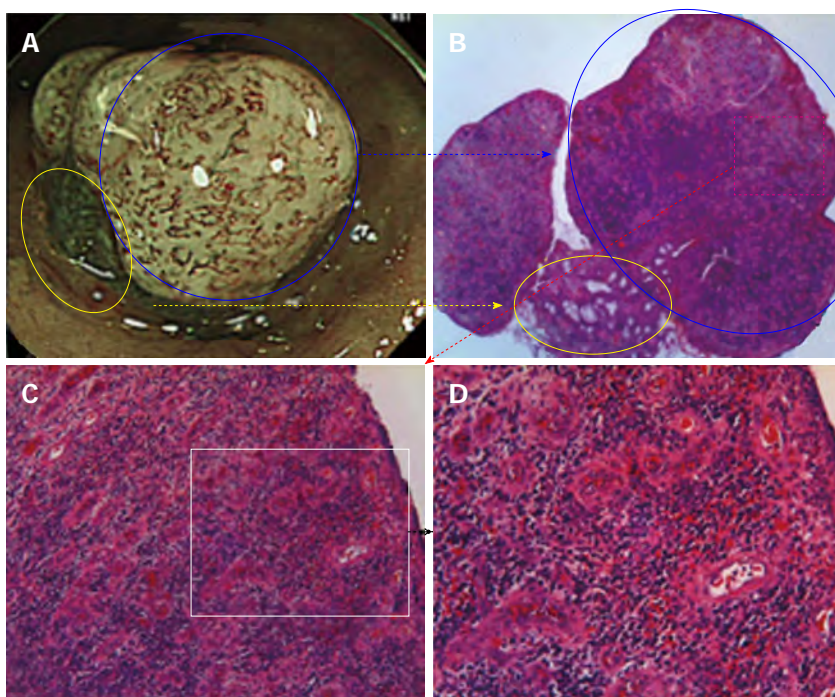
greater detail. Several irregular microvessels were found on the surface of the polyp. However, the pit pattern which is usually observed in neoplasms, such as adenoma and adenocarcinoma, was absent from the surface of the polyp (Figure 1B). The surface was smooth, and we were unable to determine whether the polyp was a neoplasm or an inflammatory polyp. To confirm the qualitative histological diagnosis, we performed endoscopic mucosal resection (EMR) of the polyp. We obtained written informed consent from the patient to perform the EMR procedure for treatment of the polyp. During EMR, a local saline injection was administered, which slightly elevated the polyp (Figure 1C), and allowed resection of the polyp. After removing the polyp, we identified the diverticulum using the resected stalk of the polyp (Figure 1D). A closer view of the resected surface revealed that the cavity of the diverticulum was irregular, and exposed vessels were observed (Figure 2A). The resection of the polyp indicated that it arose from the diverticulum (Figure 2A). To prevent post-EMR bleeding and delayed perforation, we inverted the diverticulum and sutured the inverted diverticulum, including the resected stalk of the polyp, with an over-the-scope clip (OTSC) (Figure 2B). After the EMR procedure, computed tomography was performed to examine the soft tissue density around the OTSC and the increased fat density around the resected site (Figure 2C and D).

According to the clinical course of the patient, which included high fever and repeated left lower abdominal pain, we suspected that post-inflammation granulation tissue arose from the bottom of the diverticulum after repeated episodes of diverticulitis. Seven days after the procedure, the histology of the polyp revealed that it was





**Figure 2** Closure of the diverticulum using the resected stalk of the polyp. A: In a closer view of the resected surface, the cavity of the diverticulum was irregular (the black arrows), and an exposed vessel was identified (red arrow); resection of the polyp indicated that it arose from the diverticulum; B: To prevent bleeding and delayed perforation following the endoscopic mucosal resection (EMR) procedure, we inverted the diverticulum and sutured the inverted diverticulum, including the resected stalk of the polyp, using an over-the-scope clip (red arrows); C, D: After the EMR procedure, computed tomography was performed to examine the soft tissue density around the over-the-scope clip and the increased fat density around the resected site (white arrows).



**Figure 3** Histological findings of the resected polyp. A: A 20 magnified narrow band imaging image of the granulomatous polyp; B: A 20 magnified image with a hematoxylin and eosin (HE) stain, the yellow and blue circles in Panel A corresponding to those in Panel B; C: A 100 magnified image with a HE stain reveals significant infiltration of lymphocytes and plasma cells; D: A 200 magnified image with a HE stain reveals increased outgrowth of microvascular structures and infiltration of lymphocytes, neutrophils and plasma cells, which indicates granulation tissue. There were no atypical cells or structural atypia.

composed only of granulation tissue with no neoplasm (Figure 3). The patient was discharged from our hospital without any complications.

## DISCUSSION

We report the unique case of a granulomatous polyp that arose from a single diverticulum after repeated episodes of local diverticulitis. A colonic diverticulum causes some serious complications such as bleeding, stricture due to

multiple recurrences of diverticulitis, and perforation<sup>[5-7]</sup>. Several case reports have also described neoplasms that arose from a single diverticulum, which was successfully treated with EMR and completely closed with the assistance of laparoscopy<sup>[8]</sup>. However, there have been no previous reports of granulomatous polyps that arose from a single diverticulum after repeated episodes of local diverticulitis, which was treated with EMR and closed with an OTSC. When polyps are diagnosed as neoplastic, magnifying chromoendoscopy and NBI magnifying



image-enhanced endoscopy can be used to detect the pit pattern. A granulomatous polyp should be diagnosed when no pit pattern is observed because a pit pattern reveals the surface characteristics of a neoplasm<sup>[9]</sup>. The resection of a polyp arising from a diverticulum is associated with a risk of perforation or bleeding. A full-thickness resection using a pre-full thickness suture with an OTSC was reported to be the safest method to resect the full-thickness wall of the colon<sup>[10]</sup>. Similarly, after inverting the diverticulum and the polyp into the colon, a full-thickness suture using an OTSC and a full-thickness resection may be safely performed. In this case, we should have initially inverted the diverticulum and the polyp and safely resected the full-thickness of the colon wall after diagnosing the granulomatous polyp that arose from a diverticulum. When many granulomatous polyps arise from many diverticula and the neighboring granuloma fuses, a stricture of the colon may develop; therefore, a differential diagnosis between colon cancer and a granulomatous stricture would be difficult<sup>[11]</sup>.

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**L- Editor:** Cant MR **E- Editor:** Wu HL



## Primary hepatic choriocarcinoma in a 49-year-old man: Report of a case

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biopsy. He died 60 d after initial presentation with no pathological diagnosis. Postmortem studies included histopathological and immunohistological examinations which diagnosed a primary choriocarcinoma of the liver. Primary hepatic choriocarcinoma is very rare but should be considered in the differential diagnosis of a liver tumor in a middle aged man. Establishing this diagnosis may enable treatment of the choriocarcinoma. Liver biopsy and evaluation of serum human chorionic gonadotropin are recommended in these patients.

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**Key words:** Hepatic choriocarcinoma; Male; Human chorionic gonadotropin; Liver biopsy; Fludeoxyglucose-positron emission tomography

**Core tip:** Evaluation of serum human chorionic gonadotropin levels in addition to other liver tumor markers should be performed in middle-aged men with undiagnosed hepatic tumors, to rule-out the possibility of primary hepatic choriocarcinoma. Liver biopsy is important to diagnose this rare and highly malignant tumor.

### Abstract

We report a case of hepatic choriocarcinoma in a man diagnosed at autopsy after a rapid downhill clinical course. The patient was a 49-year-old man who presented with acute right-sided abdominal pain. There were no masses palpable on physical examination. Radiographic findings showed large multi-nodular tumors mainly in the right lobe of the liver. Fludeoxyglucose-positron emission tomography scan showed uptake only in the liver, and no uptake in the testes. We initially planned to perform a liver resection for the presumed diagnosis of intra-hepatic cholangiocarcinoma. However, the tumors grew rapidly and ruptured. Multiple lung metastases rapidly developed resulting in respiratory failure, preventing liver resection or even

Sekine R, Hyodo M, Kojima M, Meguro Y, Suzuki A, Yokoyama T, Lefor AT, Hirota N. Primary hepatic choriocarcinoma in a 49-year-old man: Report of a case. *World J Gastroenterol* 2013; 19(48): 9485-9489 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i48/9485.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i48.9485>

### INTRODUCTION

Choriocarcinoma is a rare, aggressive, malignant germ-cell neoplasm of trophoblastic cells, which are among the first cells to differentiate from the fertilized egg to enable

implantation. Choriocarcinoma is prone to rapid hematogenous metastases, and the first clinical manifestation is often metastatic lesions<sup>[1]</sup>. The characteristic laboratory finding in patients with choriocarcinoma is an elevated serum human chorionic gonadotropin (hCG) level. Choriocarcinoma is less common in men than women, and comprises only 1% of all germ-cell tumors, most often with the primary lesion in the testes<sup>[2]</sup>. There are only seven patients previously reported in the English literature with primary choriocarcinoma of the liver<sup>[3-5]</sup>. These patients have been reported from Asia, including Japan and China. We report here a 49-year-old Japanese male with primary choriocarcinoma of the liver diagnosed at autopsy, who presented initially with acute abdominal symptoms and a rapid downhill clinical course. Establishing the diagnosis early may enable treatment of choriocarcinoma. Consideration of this lesion in a patient with an undiagnosed liver mass is essential, necessitating evaluation of serum hCG level and urgent liver biopsy.

## CASE REPORT

A 49-year-old male presented to the emergency room with acute right-sided abdominal pain and fever. He had a previous history of diabetes mellitus and hepatitis C. Physical examination was positive for abdominal tenderness. Contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) scans revealed a multi-nodular hepatic tumor more than 10 cm in diameter in the right lobe (Figure 1A). Laboratory data showed white blood count (WBC) and liver function tests within normal limits but an elevated C-reactive protein to 8.75 mg/L. Serum carcinoembryonic antigen (CEA) was elevated to 18.5 ng/mL but  $\alpha$ -fetoprotein (AFP) and CA19-9 were within normal limits. We suspected a metastatic liver tumor or intra-hepatic cholangiocarcinoma. Endoscopy found no primary lesion in the gastrointestinal tract and fludeoxyglucose-positron emission tomography (FDG-PET) scan showed abnormal uptake only in the liver (Figure 1B). We planned to perform liver resection with a presumptive diagnosis of intra-hepatic cholangiocarcinoma but avoided performing a liver biopsy due to the risk of dissemination. Before he could undergo liver resection, the tumors grew rapidly and ruptured (Figure 2A). Multiple lung metastases rapidly developed, accompanied by severe respiratory failure (Figure 2B). Due to pulmonary, biopsy or resection of the liver were not possible and the patient died 60 d after initial presentation.

At autopsy, the liver weighed 4080 g with numerous hemorrhagic satellite nodules in the right lobe. There were multiple hemorrhagic lung nodules up to 3 cm in diameter, and microscopic metastases were identified in other viscera, including a para-aortic lymph node, the right adrenal gland, peritoneum, right renal capsule, and spleen. There was no malignant change or scar in the testes. Histological findings of the hepatic tumors showed choriocarcinoma with a biphasic pattern of mononuclear

cytotrophoblasts and giant multi-nucleated syncytiotrophoblast cells (Figure 3A).

Immunohistochemistry was positive for an antibody to hCG subunits  $\alpha$  (Figure 3B) and  $\beta$  (Figure 3C). Control tissue slides of placental chorionic villi stained with the same antibody showed staining limited to the syncytiotrophoblast layer. The syncytiotrophoblast cells in the liver were strongly positive for hCG, as were those in the other organs involved. Serum hCG was evaluated post-mortem, significantly elevated at 53000 IU/mL.

## DISCUSSION

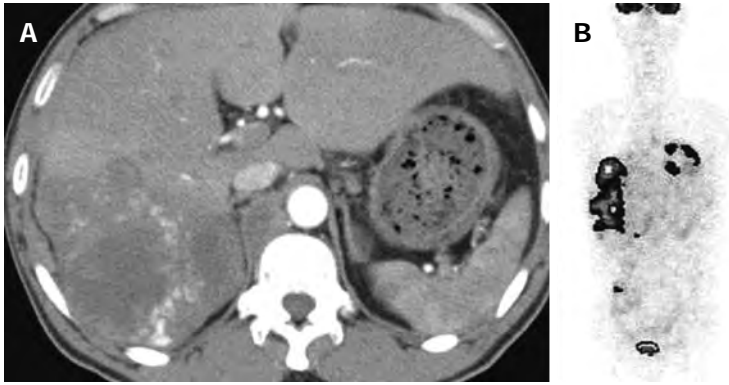
Choriocarcinoma is an uncommon, aggressive trophoblastic malignant neoplasm that is prone to early hematogenous metastases. It typically presents as a primary tumor of the uterus or genital tract in gestational females. In males the primary lesion is usually in the testes, but represents only 1% of all testicular tumors<sup>[2]</sup>. Extra-genital choriocarcinomas are less common, and often exist with other carcinomas, tending to occur in mid-line organs<sup>[1]</sup>. Pure extra-genital non-midline choriocarcinomas are the least common type. Only seven previous male patients with choriocarcinoma of the liver have been reported in the English language literature (Table 1)<sup>[3-5]</sup>. Hepatic choriocarcinoma has been recognized as a primary malignant tumor of the liver since 1992 when first reported by Fernández Alonso *et al*<sup>[3]</sup>. The other patients were reported from Asia (China and Japan), with a majority from China<sup>[4,5]</sup>.

The patient in this report presented with acute abdominal symptoms and a multi-nodular tumor in the right lobe of the liver. Based on radiographic appearance, an elevated serum CEA and the absence of a lesion in the gastrointestinal tract, the leading diagnosis was intra-hepatic cholangiocarcinoma. FDG-PET scan showed no other lesions, including the testes. Based on these findings, a liver resection was planned in this patient. FDG-PET scan has been reported previously in the diagnosis of choriocarcinoma<sup>[6,7]</sup>. Furthermore, FDG-PET scan is also useful to evaluate the efficacy of treatment of liver lesions such as surgery or chemotherapy<sup>[6]</sup>.

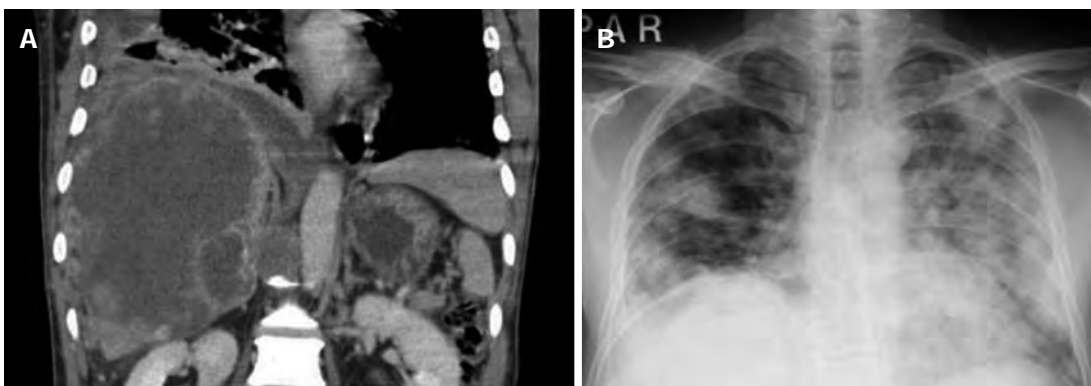
In the differential diagnosis of malignant liver tumors in a patient presenting with an acute abdomen, sarcomatous changes from hepatocellular carcinoma or cholangiocarcinoma must be considered. Both of these tumors can have a rapid clinical course and generally have poor outcomes. Sarcomatous change in primary liver tumors has been reported from Asian countries as well as choriocarcinoma<sup>[8-10]</sup>. Sarcomatous changes are seen in about 2%-4% of patients with resected hepatocellular carcinoma. Patients with sarcomatous changes have a worse prognosis than that in patients with typical hepatic lesions. More than half of the patients with sarcomatous changes died within a year of resection<sup>[8]</sup>.

The characteristic laboratory finding in choriocarcinoma is an elevated hCG level in the blood or urine. This patient had an elevated serum hCG level in a post-

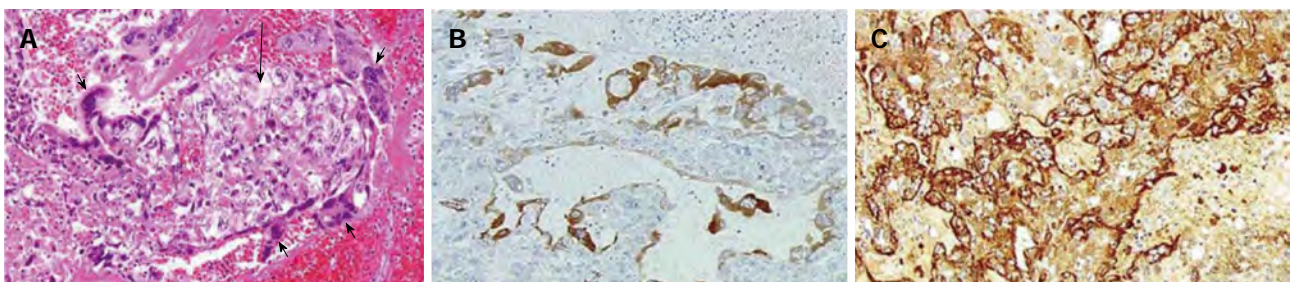




**Figure 1** Enhanced computed tomography of the liver and fludeoxyglucose-positron emission tomography. A: Enhanced computed tomography showed a multi-nodular tumor in the right lobe of the liver; B: Fludeoxyglucose-positron emission tomography scan showed accumulation in the liver with no accumulation in the testes.



**Figure 2** Computed tomography and chest X-ray following ruptured of the tumors with respiratory failure. A: Computed tomography scan following rupture of the tumors showed an enlarged tumor with ascites and a right pleural effusion; B: Chest X-ray showed multiple lung metastases clinically associated with severe respiratory failure.



**Figure 3** Photomicrographs of the liver tumor. A: Ovoid mononuclear cytotrophoblast cells (long arrow) encircled by large amorphous multinuclear syncytiotrophoblast cells (short arrows) among dark-red hemorrhagic tissue (hematoxylin and eosin,  $\times 400$ ); B: Immunohistochemical stain showing  $\alpha$ -human chorionic gonadotropin (hCG) positive cells ( $\times 400$ ); C: Immunohistochemical stain showing  $\beta$ -hCG positive syncytiotrophoblast cells ( $\times 400$ ).

mortem blood sample. In male patients, there are few reasons to evaluate serum hCG levels except in patients with testicular tumors<sup>[11-13]</sup>. When we evaluate a middle aged patient with an aggressive liver tumor, we recommend checking serum hCG as tumor marker in addition to AFP, CEA and CA19-9.

The strategy for choriocarcinoma of the liver is not established because of its rarity and highly malignant behavior. We believe that urgent liver resection before manifestation of distant metastases and chemotherapy may be the best course for prolongation of survival. In

a patient with gastric choriocarcinoma and multiple liver metastases, Waseda *et al*<sup>[14]</sup> reported pathological complete response using etoposide and cisplatin, with a two year disease-free survival after surgical resection. Methotrexate and actinomycin D may also be important agents in the treatment of choriocarcinoma. The use of cyclophosphamide, etoposide and vincristine have also been reported. Cisplatin and 5-FU were used in other reports. Shi *et al*<sup>[5]</sup> reported five patients with hepatic choriocarcinoma. Two of the five patients underwent liver resection with adjuvant chemotherapy, and three of the five patients



**Table 1** Previous reports of men with primary hepatic choriocarcinoma

Ref.	Year	Age, yr	Time to death
Fernández Alonso <i>et al</i> <sup>[3]</sup>	1992	62	1 yr
Arai <i>et al</i> <sup>[4]</sup>	2001	65	45 d
Shi <i>et al</i> <sup>[5]</sup>	2010	39	6 mo
Shi <i>et al</i> <sup>[5]</sup>	2010	45	2 mo
Shi <i>et al</i> <sup>[5]</sup>	2010	48	3 mo
Shi <i>et al</i> <sup>[5]</sup>	2010	36	5 mo
Shi <i>et al</i> <sup>[5]</sup>	2010	40	8 mo
Present case	2011	49	2 mo

with distant metastases were treated with chemotherapy after needle biopsy. The two patients who underwent resection survived only six and eight months respectively, despite having received adjuvant chemotherapy. Of the three other patients reported, two were diagnosed with undifferentiated carcinoma and one with metastatic choriocarcinoma by needle biopsy. These patients underwent chemotherapy including 5-FU and platinum, but all died within five months.

Liver biopsy was not performed in the present patient because the diagnosis of intra-hepatic cholangiocarcinoma was suspected, and biopsy could result in an increased risk of tumor dissemination. His condition rapidly deteriorated due to respiratory failure, which precluded the safe conduct of any invasive procedures. The diagnostic accuracy of needle or aspiration biopsy is not adequate, and may lead to an incorrect diagnosis of poorly differentiated carcinoma because of the similarity to cytrophoblasts. The accuracy of liver biopsy is still controversial in establishing the diagnosis of choriocarcinoma. However, in order to enable rapid treatment of such an aggressive tumor, liver biopsy should be performed without hesitation.

In a middle-aged male patient with an aggressive liver tumor, evaluation of serum hCG levels in addition to other liver tumor markers should be performed. Liver biopsy is important, especially in Chinese and Japanese patients, to detect this rare and highly malignant tumor.

## ACKNOWLEDGMENTS

Written informed consent was obtained from the patient's younger brother for publication of this report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

## COMMENTS

### Case characteristics

Acute abdominal pain and fever in a 49-year-old man.

### Clinical diagnosis

Primary hepatic choriocarcinoma.

### Differential diagnosis

Sarcomatous changes from hepatocellular carcinoma or cholangiocarcinoma.

### Laboratory diagnosis

Elevated hCG in blood or urine is definitive.

### Imaging diagnosis

Multi-nodular liver tumor with uptake by FDG-PET.

## Pathological diagnosis

Liver biopsy with immunohistochemistry is recommended.

## Treatment

Urgent surgical resection and chemotherapy is recommended.

## Term explanation

Hepatic choriocarcinoma in a middle-aged male is the least common.

## Experiences and lessons

In a middle-aged male with an aggressive liver tumor, evaluation of serum hCG levels and liver biopsy should be performed.

## Peer review

The study reported a case of a patient suffering primary hepatic choriocarcinoma, which is a kind of very rare, especially in men, and malignant trophoblastic cancer. The description of this case is very interesting for the detection and differentiation of this type of aggressive tumour among other primary liver cancers and the conclusions are enlightening for the clinical management of these patients.

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**E- Editor:** Zhang DN



## IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis: A case report

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Author contributions: Qu LM, Wen XY, Liu YH, Li YJ performed the majority of the experiments; Liu YH contributed the study design, data collection; Gao RP designed the study and wrote the manuscript; Brigstock DR co-ordinated the study and edited the manuscript; Qu LM and Liu YH contributed equally to the paper.

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### Abstract

Autoimmune pancreatitis (AIP) is a form of chronic pancreatitis that is categorized as type 1 or type 2 according to the clinical profile. Type 1 AIP, which predominantly presents in a few Asian countries, is a hyper-IgG4-related disease. We report a case of IgG4-related AIP overlapping with Mikulicz's disease and lymphadenitis, which is rare and seldom reported in literature. A 63-year male from Northeast China was admitted for abdominal distension lasting for one year. He presented symmetric swelling of the parotid

and submandibular glands with slight dysfunction of salivary secretion for 6 mo. He had a 2-year history of bilateral submandibular lymphadenopathy without pain. He underwent surgical excision of the right submandibular lymph node one year prior to admission. He denied any history of alcohol, tobacco, or illicit drug use. Serological examination revealed high fasting blood sugar level (8.8 mmol/L) and high level of IgG4 (15.2 g/L). Anti-SSA or anti-SSB were negative. Computed tomography of the abdomen showed a diffusely enlarged pancreas with loss of lobulation. Immunohistochemical stain for IgG4 demonstrated diffuse infiltration of IgG4-positive plasma cells in labial salivary gland and lymph node biopsy specimens. The patient received a dose of 30 mg/d of prednisone for three weeks. At this three-week follow-up, the patient reported no discomfort and his swollen salivary glands, neck lymph node and pancreas had returned to normal size. The patient received a maintenance dose of 10 mg/d of prednisone for 6 mo, after which his illness had not recurred.

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**Key words:** IgG4-related disease; Type 1 autoimmune pancreatitis; Mikulicz's disease; Lymphadenitis

**Core tip:** We report a rare case of a 63-year-old North-east Chinese man who suffered from IgG4-related disease (RD) which involved the salivary glands, lymph node and pancreas. The patient responded promptly to prednisone therapy. Further identification and characterization of such cases is required to elucidate the prevalence and clinical features of IgG4-RD in China.

Qu LM, Liu YH, Brigstock DR, Wen XY, Liu YF, Li YJ, Gao RP. IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis: A case report. *World J Gastroenterol* 2013; 19(48): 9490-9494 Available from: URL:

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## INTRODUCTION

Autoimmune pancreatitis (AIP) is an uncommon form of chronic pancreatitis that was first described in Japan in 1995<sup>[1]</sup>. Two subtypes of AIP have been so far recognized<sup>[2,3]</sup>. Type 1 AIP is related to high levels of serum IgG4, dense periductal lymphoplasmacytic infiltration and obliterative venulitis, while type 2 AIP is an IgG4-independent pancreatic disease that is characterized by neutrophilic infiltration into the epithelium of the pancreatic duct<sup>[3,4]</sup>. Although 20%-40% of AIP cases are type 2 in the United States and Europe, most cases of AIP in Japan and Korea are type 1, and type 2 is quite rare<sup>[5]</sup>. The prevalence and clinical features of AIP in China has not been fully clarified so far.

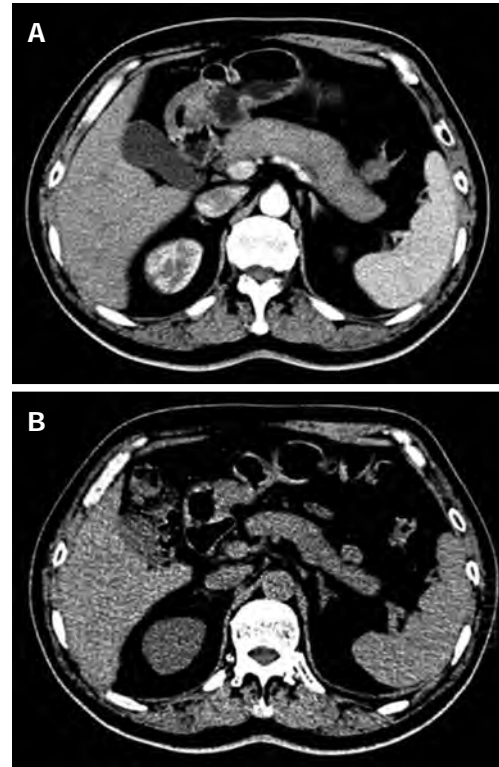
Mikulicz's disease (MD) refers to bilateral and symmetrical swelling of the lacrimal, parotid, and submandibular glands. Based on histological similarities reported by Morgan *et al*<sup>[6]</sup> in 1953, MD was considered a subtype of Sjögren's syndrome (SS). However, several recent reports from Japan have revealed that MD is associated with elevated serum IgG4 levels and prominent infiltration of IgG4-positive plasmacytes<sup>[7,8]</sup>; these findings are distinct from those of SS and have resulted in the recognition of MD as a singular systemic IgG4-related plasmacytic disease<sup>[9]</sup>.

In this report, we describe a case from Northeast China of IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis. This rare clinical condition has seldom been reported in literature.

## CASE REPORT

A 63-year male from Northeast China was admitted for abdominal distension lasting for one year. He presented symmetric swelling of the parotid, and submandibular glands with slight dysfunction of salivary secretion for 6 mo. He had a 2-year history of bilateral submandibular lymphadenopathy without pain. He underwent surgical excision of the swollen lymph node in the right submandibular region one year prior to hospital admission. The patient denied any history of alcohol, tobacco, or illicit drug use. On admission, his blood pressure was 136/88 mmHg, pulse rate was 72/min, and body temperature was 36.7 °C. On examination, he had bilateral swelling of the parotid and submandibular glands as well as left swelling of the submandibular lymph node. His mouth was dry. Abdominal examination revealed mild epigastric tenderness to deep palpation without rebound.

The laboratory test data on admission revealed an elevated neutrophil ratio of 76%, and an elevated fasting blood sugar level of 8.8 mmol/L. Serum amylase was 42 U/L and serum lipase was 65 U/L, both within normal limits. Serological testing for autoimmune function displayed high levels of IgG4 (15.2 g/L) and IgG (18.5 g/L),



**Figure 1** Typical imaging features of type 1 autoimmune pancreatitis. Computed tomography (CT) scan showing diffuse swelling of the pancreas with loss of lobulation (A), and a dramatic decrease in swelling of the pancreas after 3 wk of steroid treatment (B).

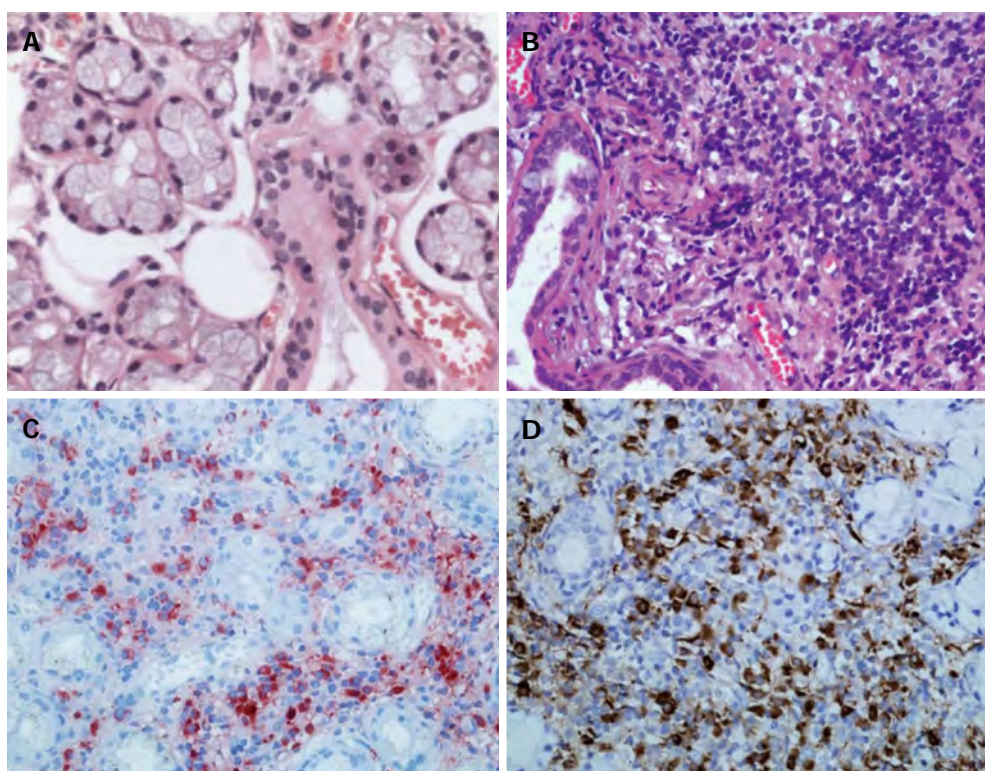
and negative values of anti-SSA and anti-SSB. A computed tomography (CT) scan of the abdomen revealed diffuse enlargement of the pancreas and loss of normal pancreatic lobulation, consistent with autoimmune pancreatitis (Figure 1A).

The patient underwent a minor labial salivary gland biopsy for a possible diagnosis of MD. Labial gland specimens stained with hematoxylin and eosin revealed significant infiltration of lymphocytoma cells in the patient, but no infiltration of these cells in a healthy individual (Figure 2A and B). Immunohistochemical staining showed numerous IgG4-positive plasmacytes in the labial gland of the patient, with a ratio of IgG4/IgG-positive plasmacytes of more than 50% (Figure 2C and D).

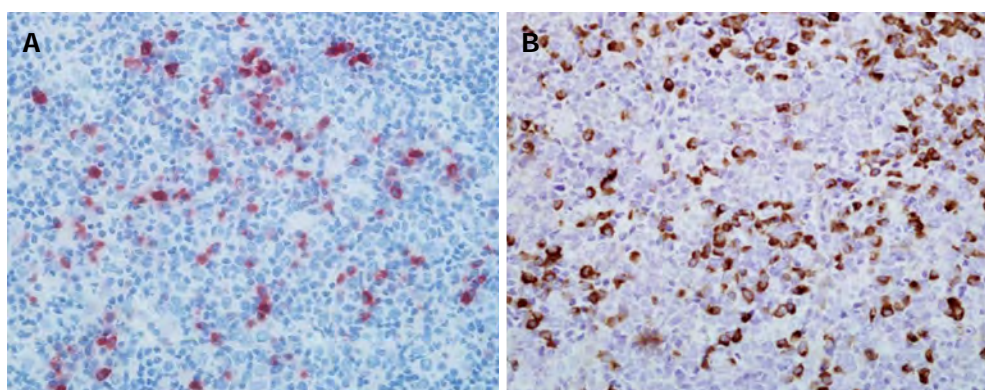
Since both autoimmune pancreatitis and MD meet the criteria for IgG4-related disease, we investigated the IgG4 status of the patient's swollen lymph nodes. Lymph node specimens collected from the patient by excision of the right submandibular lymph node one year prior to admission ago were examined for IgG4 and IgG using immunohistochemistry. As shown in Figure 3, there were diffuse infiltrations of IgG4-positive plasma cells in the patient's lymph node. The ratio of IgG4/IgG-positive cells was greater than 40% thus meeting the diagnostic criteria for IgG4-related lymphadenitis.

On the 8<sup>th</sup> d after admission, the patient was diagnosed with IgG4-related systemic disease. He received 30 mg/d of prednisone for three days without any side effects, and was then discharged with the same steroid





**Figure 2 Histological findings of labial salivary gland specimens.** A: Hematoxylin and eosin stain showing normal labial gland; B: Diffuse infiltration of lympho-plasma cells from the patient; C, D: Immunohistochemical staining for IgG4 (C) or IgG (D) in plasma cells from the patient, consistent with Mikulicz's disease. Original magnification,  $\times 400$ .



**Figure 3 Histological findings of submandibular lymph node specimen.** Immunohistochemical staining showing IgG4-positive plasma cells (A) and IgG-positive plasma cells (B) in lymph node sections of the patient. Original magnification,  $\times 400$ .

dose for the following 3 wk. At three-week follow-up the patient exhibited no signs of either a dry mouth or abdominal distension. His swollen glands including parotid and submandibular glands as well as left submandibular lymph node were no longer palpable. The enlarged pancreas had returned to its normal size (Figure 1B) and elevated morning glucose levels were within the normal range. The patient then received a long-term maintenance dose of 10 mg/d of prednisone after steroid tapering. At six-month follow-up, his illness had not recurred.

## DISCUSSION

Yoshida *et al*<sup>[1]</sup> first proposed the concept of AIP in 1995

based on observations of patients who had hyper- $\gamma$  globulinemia, various autoantibodies, lymphocytic infiltration into pancreatic tissue, and good steroid responsiveness. In 2002 Hamano *et al*<sup>[10]</sup> reported high serum IgG4 concentrations in Japanese AIP patients and abundant IgG4-producing plasma cell infiltration in pancreatic tissue. Some cases of AIP in Europe or America appear to represent an “idiopathic duct-centric chronic pancreatitis”, which are caused by neutrophilic granulocyte infiltration and are not related to IgG4<sup>[11]</sup>. In 2009, Sugumar *et al*<sup>[12]</sup> suggested that IgG4-related AIP should be named as type 1 and neutrophilic granulocyte lesions of AIP as type 2<sup>[12]</sup>. Over the last decade, international consensus diagnostic criteria for AIP were established to be applicable

worldwide and to distinguish between the two types of AIP. The diagnosis of AIP can be usually made based on the presence of at least one of the five cardinal features (*i.e.*, imaging, serology, other organ involvement, histology, and response to steroid therapy)<sup>[3]</sup>. However, sufficient biopsy specimens from the pancreas are difficult to obtain using standard procedures, except laparotomy. The clinical diagnostic criteria to establish type 1 AIP from Japan were the presence of pancreatic swelling together with high serum levels of IgG4 and/or prominent IgG4-producing plasma cell infiltration in pancreas tissue<sup>[11]</sup>. By contrast, in the case reported here, the patient presented a diffuse swelling of the pancreas with loss of lobulation, high serum IgG4 concentration, abundant IgG4-positive plasma cell infiltration into labial or lymph node tissues, and good steroid responsiveness, thus fully meeting multiple type 1 AIP diagnostic criteria<sup>[3,11]</sup>. Additionally, in this case report, three-week steroid treatment caused dramatic improvements in either exocrine insufficiency (abdominal distention) or endocrine insufficiency (elevated fasting blood sugar level) as well as dramatic reduction of the enlarged pancreas, suggesting that both exocrine and endocrine insufficiency might be reversible in IgG4-related type 1 AIP.

The first case of MD was reported by Mikulicz-Radecki in 1888, which was described as bilateral symmetrical enlargement of the salivary and lacrimal glands, and lymphocytic infiltration into lacrimal and salivary gland tissues<sup>[9]</sup>. Since 1953, when Morgan *et al*<sup>[6]</sup> found similarities in histology between MD and SS, MD was considered as a subtype of SS. However, this concept has been modified over the decade in light of compelling Japanese studies showing that MD is associated with elevated serum IgG4 levels and prominent infiltration of IgG4-positive plasmacytes into lacrimal and salivary glands<sup>[7,8]</sup>. Thus, a modern clinical concept of MD is that it is distinct from SS and is instead part of the spectrum of IgG4-RD<sup>[9]</sup>. Diagnostic criteria for IgG4-related MD were approved by the Japanese Sjögren's Syndrome Society in 2008. According to these criteria, IgG4-related MD is defined in the presence of persistent ( $\geq 3$  mo), symmetrical swelling of the lacrimal, parotid and submandibular glands involving at least two pairs, together with either high serum levels of IgG4 ( $\geq 1.35$  g/L) and/or marked IgG4-positive plasmacyte infiltration ( $\geq 50\%$  IgG4-positive/IgG-positive cells in five high power fields) into lacrimal and salivary gland tissues<sup>[9]</sup>. In this study, the patient presented six-month symmetric swelling of the parotid and submandibular glands, elevated serum IgG4 levels, and prominent IgG4-positive plasmacyte infiltration into the labial gland tissue, which fully met the diagnostic criteria for IgG4-related MD<sup>[9]</sup>. Furthermore, both the swollen salivary glands and the dry mouth of the patient promptly recovered in response to prednisone therapy, indicating that IgG4-related MD is a reversible disorder which differs from SS.

Recently, IgG4-RD has been defined as a novel clinical entity with multi-organ involvement and associated

abundant infiltration of IgG4-positive cells<sup>[11,13]</sup>. In this case, the clinical and histopathological features of the patient met diagnostic criteria for IgG4-related type 1 AIP and MD respectively. Additionally, the patient presented abundant infiltration of IgG4-positive plasma cells in lymph node tissue ( $> 40\%$  IgG4/IgG-positive cells) and good steroid responsiveness, which fully met the diagnostic criteria for IgG4-related lymphadenitis<sup>[13]</sup>.

In summary, we report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-RD which involved the salivary glands, lymph node and pancreas. The patient responded promptly to prednisone therapy. Further identification and characterization of such cases is required to elucidate the prevalence and clinical features of IgG4-RD in China and their relationship to similar cases in Japan.

## COMMENTS

### Case characteristics

The patient presented symmetric swelling of the parotid and submandibular glands as well as a diffusely enlarged pancreas with loss of lobulation.

### Clinical diagnosis

The authors report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-related disease which involved the salivary glands, lymph node and pancreas.

### Differential diagnosis

Immunohistochemical staining for IgG4 and IgG is the major method for differential diagnosis between IgG4-related disease and other diseases.

### Laboratory diagnosis

Serological testing for autoimmune function displayed high levels of serum IgG4 and IgG, and negative values of anti-SSA and anti-SSB in the patient.

### Imaging diagnosis

A computed tomography scan of the abdomen revealed diffuse enlargement of the pancreas and loss of normal pancreatic lobulation.

### Pathological diagnosis

Immunohistochemical staining showed numerous IgG4-positive plasmacytes in labial gland and lymph node of the patient, with a ratio of IgG4/IgG-positive plasmacytes of more than 40% in both tissues.

### Treatment

The patient received a dose of 30 mg/d of prednisone for three week, and a long-term maintenance dose of 10 mg/d of prednisone.

### Term explanation

Mikulicz's disease (MD) refers to bilateral and symmetrical swelling of the lacrimal, parotid, and submandibular glands. MD, as a singular systemic IgG4-related plasmacytic disease, was considered a subtype of Sjögren's syndrome. IgG4-related disease (IgG4-RD) has been defined as a novel clinical entity with multi-organ involvement and associated abundant infiltration of IgG4-positive plasmacytes.

### Experiences and lessons

The authors report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-RD which involved the salivary glands, lymph node and pancreas. This rare clinical condition has seldom been reported in literature.

### Peer review

Type 1 autoimmune pancreatitis is related to high levels of serum IgG4, dense periductal lymphoplasmacytic infiltration and obliterative venulitis. In this manuscript, the authors reported a interesting case of IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis. This case is very rare and seldom reported in literature.

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